Ligand determinants of nisin for its induction activity

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ABSTRACT

Nisin has been widely used in the food industry as a safe and natural preservative and has the potential to be used as a biomedicine. Improving nisin production is important for its enormous applications. Nisin A is produced in *Lactococcus lactis* and its biosynthesis is induced through the regulation of the 2-component system NisKR. In this study, alanine-scanning mutagenesis was applied to study the key structure or AA in nisin for inducing the 2-component system NisKR to regulate downstream gene expression. Assay of β -galactosidase activity revealed that either ring A or ring B was necessary for nisin to induce *lacZ* reporter gene expression. A substituted first ring formed by Thr2 and Cys7 in S3A instead of ring A (formed by Ser3 and Cys7) fully retained nisin induction activity. Mutation of cationic AA and addition of cationic ions hardly affected nisin induction activity. These results demonstrated that the N-terminal ring structures in nisin were involved in activating NisKR to act as an inducing molecule, whereas the electrostatic force might not contribute to this process. In addition, 2 specific residues were revealed to have potential for improving both nisin induction and antimicrobial activity, which might be used for increasing nisin production.

Key words: nisin, lantibiotics, induction activity, alanine-scanning mutants

INTRODUCTION

Lactococcus lactis strains are usually found in milk-based fermentation products and used as starter cultures for dairy foods. Certain strains of *L. lactis* are known to produce nisin, a 34-AA lantibiotic with several unusual dehydro residues and 5 lanthionine ring structures (Chatterjee et al., 2005; Zhang et al., 2012). Because of its broad antimicrobial spectrum, nisin has been widely

used as a safe preservative in the food industry (Ghar-sallaoui et al., 2016a,b). Nisin could also potentially be used for therapeutic purposes (Field et al., 2015). For example, nisin has been applied for the treatment of clinical bovine mastitis in the veterinary field due to its antimicrobial activity against corresponding gram-positive pathogens (Cao et al., 2007; Wu et al., 2007). A recent study also reported that nisin was effective in the inhibition of head and neck cancer tumorigenesis (Kamarajan et al., 2015).

Because of its many applications, the need to optimize nisin production is increasing. It is well known that nisin is not only an antimicrobial agent but also a peptide pheromone to induce gene expressions for its own biosynthesis. The gene cluster related to nisin production, *nisABTCIPRKFEG*, includes 11 genes. The *nisA* gene encodes nisin precursor in which serine and threonine residues are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb) by NisB and cyclized with cysteines by NisC to form thioether bridges. Nisin precursors are exported across the cell membrane by NisT, and the leader peptide is cleaved by protease NisP, releasing mature nisin. The *nisI*, *nisF*, *nisE*, and *nisG* genes are involved in cell immunity against nisin, whereas *nisR* and *nisK* encoded proteins regulate nisin biosynthesis (Engelke et al., 1994). Histidine kinase NisK and response regulator NisR belong to the family of bacterial 2-component signal transduction systems. Nisin acts as an auto-inducer to activate NisK, which is a nisin receptor to be auto-phosphorylated. The phosphate group is then transferred to NisR to promote the expression of *nisA* and other *nis* genes to regulate nisin biosynthesis. In this circular pathway, the induction activity of nisin plays an important role to regulate its own production. Knowing the structural features of nisin needed for auto-induction is a prerequisite for improving nisin production and genetic engineering.

Many other lantibiotics such as subtilin (Stein et al., 2002), salivaricin (Upton et al., 2001), mersacidin (Schmitz et al., 2006), bovicin HJ50 (Ni et al., 2011), and suicin (Wang et al., 2014) also work as inducing molecules to initiate *lan* gene expression and their own

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biosynthesis through their corresponding 2-component regulatory systems, LanKR, named by their association with lantibiotic biosynthesis (Chatterjee et al., 2005). Lantibiotics nearly activate other unrelated histidine kinases to keep their biosynthesis to be regulated specifically. However, some derivatives of lantibiotics show similar inducing activity and specificity. Salivaricin A biosynthesis is supposed to be regulated by the SalKR 2-component system activated by itself as well as the closely related lantibiotics salivaricin A1, A2, A4, and A5 (Wescombe et al., 2006). It has been shown that derivatives of nisin, such as nisin A or nisin Z, could induce the expression of *nisA*, whereas unmodified precursor peptide of nisin and several other antimicrobial peptides, for example, subtilin, which have similar ring structures to nisin, could not induce *nisA* transcription through NisKR (Kuipers et al., 1995; Qiao et al., 1996; Spieß et al., 2015). Several reports show the structural features or some specific AA of nisin needed for auto-induction (Kuipers et al., 1995; Van Kraaij et al., 1997; Spieß et al., 2015). For example, it has been shown that nisin fragments containing rings BC or DE were not able to activate NisKR to regulate gene expression, and N-terminal rings AB showed only 2% inducing capacity compared with the nisin wild type. This means that the lack of either N-terminal or C-terminal of nisin severely affected nisin-inducing activity, whereas the former 11 residues including ring A are a minimal requirement (Kuipers et al., 1995). Substitution of the first residue tryptophan in subtilin with isoleucine, which is the first residue in nisin, showed about 40% inducing activity to NisK compared with nisin (Spieß et al., 2015).

Previous research mainly focused on the truncated fragments or several specific sites of nisin for its induction activity. Here we applied alanine-scanning mutagenesis of nisin with a biosynthetic system constructed in *Escherichia coli*, and describe that as a whole induc-

ing molecule, the N-terminal 2 rings of nisin A are necessary for activating NisKR and regulate the reporter gene expression. Besides, several mutants with higher induction and antimicrobial abilities were obtained, and might be used for improving nisin production.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Strains and plasmids related to this study are listed in Table 1. *Lactococcus lactis* CV56 (Gao et al., 2011), *Lactococcus lactis* NZ9000 (de Ruyter et al., 1996), as well as their derivatives were routinely cultured in GM17 medium [M17 medium added with 0.5% (wt/vol) glucose] at 30°C with 5 µg/mL erythromycin added if necessary. *Escherichia coli* DH5α, *E. coli* BL21 (DE3), and *E. coli* MC1061 (Casadaban and Cohen, 1980) were maintained in Luria-Bertani broth at 37°C with 10 µg/mL of chloramphenicol, 100 µg/mL of erythromycin, or 100 µg/mL of ampicillin added if necessary. *Escherichia coli* DH5α and MC1061 were used as hosts for transformation of pETDuet and pMG36e (van de Guchte et al., 1989), respectively. *Escherichia coli* BL21 (DE3) was used for protein expression.

Plasmid Constructions

The *nisA*, *nisB*, *nisC*, and *P_{nisA}* were amplified from the genome of *Lactococcus lactis* CV56, and the *P_{nisA}-lacZ* DNA fragments were obtained by fusion PCR. Plasmids, PCR products, or DNA fragments were purified by Axygen kits (Axygen, Union City, CA). The *nisA* was amplified with primers containing recognition sites of *Bam*HI and *Hind*III and *nisB* was amplified with primers containing recognition sites of *Nde*I and *Xho*I; they were inserted into plasmid pETDuet-1 to

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>Lactococcus lactis</i> CV56	Nisin A production strain	Gao et al. (2011)
<i>L. lactis</i> NZ9000	MG1363 derivative; <i>nisRK::pep</i>	de Ruyter et al. (1996)
<i>L. lactis</i> NZ9000 NlacZ	<i>L. lactis</i> NZ9000 pMG36e-NlacZ	This work
<i>Escherichia coli</i> BL21 (DE3)	Protein purification strain	Promega
<i>E. coli</i> BL21 ABC	<i>E. coli</i> BL21 pETDuet-AB	This work
<i>E. coli</i> MC1061	Cloning host	Casadaban and Cohen (1980)
Plasmids		
pMG36e	<i>E. coli</i> - <i>L. lactis</i> shuttle vector, Em ^r	van de Guchte et al. (1989)
pMG36e-NlacZ	pMG36e <i>P_{nisA}-lacZ</i>	This work
pETDuet-1	T7 promoter-controlled expression vector, Amp ^r	Novagen
pETDuet-AB	pETDuet-1 with <i>nisA</i> and <i>nisB</i> inserted	This work
pACYCDuet-1	T7 promoter-controlled expression vector, Cm ^r	Novagen
pACYCDuet-C	pACYCDuet-1 with <i>nisC</i> inserted into it	This work
pET28a(+)	T7 promoter-controlled expression vector, Kan ^r	Novagen

obtain pETDuet-AB. The *nisC* was amplified with primers containing recognition sites of *XhoI* and *BglII*, and was inserted into plasmid pACYCDuet-1 getting pACYCDuet-C. These 2 plasmids were co-transformed to *E. coli* BL21 (DE3) to obtain strain *Escherichia coli* BL21 ABC. Site-directed mutagenesis of *nisA* were performed according to the protocols reported (Chiu et al., 2004), generating different pETDuet-AB derivatives. P_{*nisA*}-*lacZ* was amplified with primers containing recognition sites of *PstI* and *HindIII* and was inserted into pMG36e, getting plasmid pMG36e-NlacZ, which was transformed to *Lactococcus lactis* NZ9000, generating strain *L. lactis* NZ9000 NlacZ. Site-directed mutagenesis of *nisA* was performed according to the protocols reported (Chiu et al., 2004), generating different pETDuet-AB derivatives. The restriction enzymes and Phusion DNA polymerase applied in PCR procedures were purchased from Thermo Fisher (Waltham, MA).

Protein Expression and Purification

Escherichia coli BL21 ABC strain was incubated in Luria-Bertani broth (500 mL) at 37°C, 200 rpm. Isopropyl β-D-1-thiogalactopyranoside (Amresco, Solon, OH) was added to a final concentration of 0.5 mM when optical density at 600 nm (OD₆₀₀) of cell cultures reached 0.6 to 0.7. After induced for 20 h at 16°C, cells were harvested and nisin prepeptide were purified by ion-metal affinity chromatography with Ni²⁺ column (GE Healthcare, Little Chalfont, UK). After purified to homogeneity by reverse-phase HPLC (Shimadzu, Kyoto, Japan) with a C18 column, the prepeptide solution was lyophilized and finally dissolved in AB buffer (25 mM NaAc, 5 mM Tris base, and 5 mM CaCl₂, pH 7.0). The nisin prepeptide was digested by trypsin (0.2 mg/mL) at 37°C for 2 h to cleave the leader peptide. The mature nisin molecule were purified by C18 reverse-phase HPLC and identified by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) using a model 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Foster City, CA). The alanine-scanning mutants and other mutants of nisin were purified and identified by the similar way.

Peptide Modification and MS Analysis

Nisin mutant S3A was subjected to chemical modification with *N*-ethyl-maleimide (NEM). Successful modification would lead to a 125-Da increase per free thiol (Lin et al., 2011). The NEM reaction was performed by incubation of purified sample with 5 mM DL-dithiothreitol (DTT) and 20 mM NEM (pH 7.5) in an ice bath for 30 min. After the reaction, the samples

were purified by C18 Zip Tips and detected by MALDI-TOF MS. The DTT and NEM were purchased from Sigma-Aldrich (St. Louis, MO).

Assay of β-Galactosidase Activity

Lactococcus lactis NZ9000 NlacZ were incubated in GM17 medium overnight at 30°C and the cell cultures were transferred to a fresh medium with 1% inoculant. The cells were induced by nisin with a concentration of 9 ng/mL (2.68 nM) for 2 h when the OD₆₀₀ of these strains reached 0.6 to 0.7. Cells were collected in 2 tubes with 1 mL of inoculant in each tube and washed once with Z buffer (0.06 M Na₂HPO₄·7H₂O, 0.04 M NaH₂PO₄·H₂O, 0.01 M KCl, 0.001 M MgSO₄, 0.05 M β-mercaptoethanol, pH 7.0). One sample was resuspended in 1 mL of Z buffer to measure OD₆₀₀, whereas the other one was dissolved in 0.5 mL of Z buffer with 10 mg/mL of lysozyme at 37°C for 60 min to decompose the cell wall. Another 0.5 mL of Z buffer with 0.1% SDS was added to the latter sample to dissolve cell membrane at 37°C for 15 min. The samples were centrifuged at 4°C with 100 × *g* and the supernatant was collected for β-galactosidase activity assay. Forty microliters of ONPG was loaded into 200 μL of supernatant or diluted supernatant to start the reaction at 25°C. When the mixtures were yellow enough, 100 μL of Na₂CO₃ (1 M) was added to stop the reaction. The reaction time was recorded precisely. The values at OD₄₂₀ and OD₅₅₀ of each sample were measured to calculate the activity of the enzyme using the following equation: Miller units = 1,000 × [OD₄₂₀ − 1.75 × OD₅₅₀]/(T × V × OD₆₀₀). The OD₄₂₀ and OD₅₅₀ were read from the reaction mixture. The OD₆₀₀ reflects cell density in the washed cell suspension, T = time of the reaction (min), and V = volume of culture used in the assay (mL).

RESULTS AND DISCUSSION

Biosynthesis of Nisin Alanine-Scanning Mutants in *E. coli*

To find the key AA residues through the whole nisin molecule (Figure 1A) for its induction activity, the prepeptides of nisin A alanine-scanning mutants were produced in *E. coli* and digested by trypsin. Mature nisin mutant peptides were released and their molecular weights were confirmed by MALDI-TOF MS (Table 2). The modification of nisin mutants was identified by comparison of their molecular weights with wild type nisin (3,352 Da). For example, the molecular weight of C7A was 3,320 Da, 32 Da less compared with nisin wild type, corresponding to the cysteine (121 Da) sub-

Figure 1. The structure of nisin and ring-disrupted mutants. The structure of nisin wild type (A) and ring-disrupted mutants including C7A (B), T8A (C), C19A (D), C26A (E), and T25A (F) in which ring A, ring B, ring C, ring D, and ring E were disrupted, respectively.

stitution of alanine (89 Da) and serine (105 Da) with OH^- (17 Da) dehydrated positioned at 3 changing to Dha (98 Da). As a result, ring A was disrupted in C7A mutant (Figure 1B). Similarly, in T8A, C19A, C26A, and T25A, rings B, C, D, and E were disrupted, respectively (Figure 1C–1F).

Inducing Activities of Nisin Alanine-Scanning Mutants

To test the inducing abilities of nisin mutants, reporter gene *lacZ* was fused with *nisA* promoter P_{nisA} and inserted into vector pMG36e. The obtained plasmid pMG36e-NlacZ was transformed into *L. lactis* NZ9000, which contained constitutive expressed *nisK* and *nisR* without other nisin biosynthesis related genes, forming

reporter strain *L. lactis* NZ9000 NlacZ (Figure 2A). The reporter strain was induced by nisin alanine-scanning mutants and β -galactosidase assays were performed to determine the inducing abilities of these mutants. The reliability of inducing abilities of nisin alanine-scanning mutants was confirmed by mutants ISL456VFG (Rink et al., 2007) and M17W (Kuipers et al., 1995), which have been reported to have lower and higher induction activity, respectively. Figure 2B showed that the inducing abilities of mutants C7A and T8A were significantly reduced compared with nisin wild type, indicating that either ring A or ring B was very important for nisin to act as an inducing molecule. This result was supported by the report that the minimal requirement for retaining induction capacity of nisin A (2%) was the presence of residues 1 to 11 comprising the first 2 rings (Kuipers

Figure 2. Induction activity analyses of nisin alanine-scanning mutants. (A) Schematic drawing of the reporter strain *Lactococcus lactis* NZ9000 NlacZ for testing the inducing abilities of nisin mutants. The *nisR* and *nisK* genes were constitutively expressed in the genome, and *lacZ* expression was controlled by *nisA* promoter (P_{nisA}). (B) β -galactosidase activities of *L. lactis* NZ9000 NlacZ induced by nisin mutants, each at the final concentration of 9 ng/mL. The last bar (–) indicates that the reporter strain was not induced by nisin or any nisin mutant. Values and standard deviation are calculated from at least 3 independent experiments performed in triplicate. Significance ($*P \leq 0.05$; $**P \leq 0.01$) was determined by Student's *t*-test. MU = Miller units; $MU = 1,000 \times (OD_{420} - 1.75 \times OD_{550}) / (T \times V \times OD_{600})$, where OD = optical density, T = reaction time (min), and V = culture volume (mL).

et al., 1995). We further demonstrated that either ring A or ring B of nisin was necessary for its induction activity, and to retain the full inducing ability, the C-terminal part of nisin was also necessary. L6A retained about 65% inducing ability, indicating that leucine at position 6 also contributed to activate NisKR. Meanwhile, L16A and M21A showed increased induction activities by 24 and 32%, respectively. Other mutants did not show obvious differences in their induction activity

compared with nisin, demonstrating that as a whole molecule, ring A and ring B might interact with NisK to initiate the following signal transduction.

Novel Ring Formed in the N-Terminal of Nisin Retained Nisin-Inducing Ability

Interestingly, mutant S3A, which could not form ring A because of the lack of serine at position 3, still pos-

Table 2. The calculated molecular weight (MW) and the molecular mass (MS) of nisin A and nisin A mutants

Name	MW (Da)	MS (Da)	Name	MW (Da)	MS (Da)
Nisin	3,351.6	3,352.4	N20A	3,308	3,309
I1A	3,310	3,309	M21A	3,291.63	3,292.56
S3A	3,353	3,352.5	K22A	3,294.57	3,294.3
I4A	3,309	3,310	T25A	3,339	3,340
S5A	3,353.6	3,354.5	C26A	3,319	3,320
C7A	3,319	3,320	H27A	3,386	3,386
T8A	3,339	3,340	C28A	3,320	3,320
P9A	3,325	3,326	S29A	3,335.63	3,336
G10A	3,365	3,384	I30A	3,309	3,310
K12A	3,294.57	3,295.4	H31A	3,285.6	3,286.4
G14A	3,365	3,366	V32A	3,323.6	3,324.5
L16A	3,309.6	3,310	S33A	3,353	3,354
M17A	3,291	3,292	IS1456VFG	3,360	3,360
G18A	3,365	3,366	M17W	3,406	3,407
C19A	3,319	3,320			

sessed similar inducing ability with nisin wild type. The molecular weight of mutant S3A was 3,352.5 Da, corresponding to residue substitution of Ser to Ala without changing H_2O dehydration. Thus we deduced that another ring might be formed instead of ring A (Figure 3A). Thus, NEM modification and tandem MS analysis were carried out to determine the ring structure of mutant S3A. The NEM (125 Da) could modify free thiol in protein and the molecular weight of the modified molecule would be increased by 125 Da per free thiol. If free thiol were present in S3A, the molecular weight would be 3,477 Da after the mutant was treated with DTT (free thiol predicted in Cys7) and modified with NEM. The molecular weight of S3A treated by DTT and NEM was 3,352 Da (Figure 3B), corresponding to

S3A with no NEM modification. This meant that no free thiol was present in nisin S3A, and an additional ring might be formed. The tandem MS analysis of S3A exhibited a mass peak of 909 Da, which was the theoretical mass from Thr2 to Cys11 with $3H_2O$ (54 Da) lost (Figure 3C). This result indicated that an additional ring A-like structure was formed between Thr2 and Cys7 in S3A, and this novel ring also played a crucial role in retaining the inducing ability of this mutant. These results demonstrated that the formation of the first and second rings in the N-terminal were necessary for nisin to act as an inducing molecule.

Sequence alignment showed that nisin and some nisin-like peptides such as subtilin, entianin, ericin, salivaricin, and so on possess a conserved hydrophobic

Figure 3. Structure determination of nisin mutant S3A. (A) The predicted structure of S3A. The Abu and A in the ring structures indicate Thr and Cys residues, respectively, which were posttranslationally modified including dehydration and cyclization. Two bars showed the peptide segment corresponding to the tandem mass spectrometry (MS) peak of 909.3 Da. (B) Molecular weight (MW) of nisin S3A measured by matrix-assisted laser desorption/ionization time-of-flight MS. (C) The tandem MS analysis of nisin S3A. The mass peak of 909.3 Da corresponded to the fragment of Thr2 to Cys11 with $3H_2O$ (54 Da) lost.

zone (SxS/FxCTPGC) in their N-terminal ring A and ring B regions (Figure 4), hinting that the N-terminal ring structures formed by ring A and ring B in these lantipeptides might also be crucial for recognizing their cognate histidine kinases (LanK) and activate the downstream cell responses. Hydrophobicity analysis of nisin AA sequence showed that the N-terminal of nisin including ring A and ring B possessed higher hydrophobicity, and as the ring formation could enhance its hydrophobicity, we supposed that the hydrophobicity of ring A and ring B in nisin was involved in activating NisK. This phenomenon was also found in other peptide molecules for inducing the activity of their sensor histidine kinases, such as lantibiotic bovicin HJ50, AIP, IP-C11, CSP, and so on (Johnsborg et al., 2004; Lyon and Novick, 2004; Teng et al., 2014). Nisin is thought to adsorb to the cell membrane surface to act as a quorum sensing signal (Hilmi et al., 2006), and our results further indicated that the hydrophobicity of nisin is needed for activation of NisK.

Cation Is Not Very Necessary for the Induction Activity of Nisin

Nisin was characterized by cationic and hydrophobic properties, and it has been reported that electrostatic forces play an important part in histidine kinase PhoQ interacting with antimicrobial peptides or divalent cations (Bader et al., 2005). Nisin has 5 cationic residues Lys12, Lys22, His27, His31, and Lys34. To test whether these cationic AA in nisin contributed to its induction activity, they were substituted with alanine, producing single mutants K12A, K22A, H27A, H31A and multi-mutants K12A/K22A, K22A/H27A, H27A/

H31A, K12A/K22A/H27A, and K12A/K22A/H27A/H31A. Unfortunately, we did not obtain the mutant K34A because of the failure of its prepeptide purification from *E. coli*. The above mutants were used to induce the report strain *L. lactis* NZ9000 NlacZ to test their inducing abilities. Single mutants, double mutants, and triple mutants of each cationic residue showed similar inducing abilities with nisin wild type. The mutant K12A/K22A/H27A/H31A with 4 cationic residues substituted by alanine also retained about 21% inducing ability (Figure 5A). These results indicated that AA with positive charges in nisin might not be necessary for identifying NisK. Further, cationic ions Mg²⁺ or Ca²⁺ under final concentrations of 0, 0.01, 0.1, 1, and 2 mM were added to the inducing system to compete with cationic nisin. The β-galactosidase activity results showed no difference between groups under different concentration of bivalent cations (Figure 5B). These results indicated that nisin might identify NisK not through an electrostatic force.

Mutants With Higher Induction and Antimicrobial Activities Were Obtained

Nisin mutants L16A and M21A possessed higher induction activities. Further, these 2 residues were substituted by several other AA with different characteristics producing mutants L16D, L16H, L16M, L16T, L16V, L16W, M21D, M21H, M21L, M21T, M21N, and M21W, and the β-galactosidase activities of the reporter strain induced by them were tested. Figure 6A showed that 6 substitutions at position 16 all exhibited increased induction activities by 15 to 35%, and most substitutions at position 21 also showed increased induction activities

Figure 4. Protein sequences alignment of nisin and some nisin-like prepeptides. The conserved hydrophobic AA (SxS/FxCTPGC) in ring A and ring B region of nisin-like prepeptides were indicated in bold. Ring A and ring B are also indicated.

Figure 5. Inducing activity analyses of cationic residue substitution mutants and competition test between nisin and bivalent cations. (A) β -galactosidase activities of *Lactococcus lactis* NZ9000 NlacZ induced by nisin single and multiple mutants (9 ng/mL) in which the cationic residues were substituted with alanine. The last bar (–) indicates that the reporter strain was not induced by nisin or any nisin mutant. (B) β -galactosidase activities of *L. lactis* NZ9000 NlacZ induced by 9 ng/mL of nisin competed with cationic ions Mg^{2+} or Ca^{2+} with final concentration of 0, 0.01, 0.1, 1, and 2 mM. Values and standard deviation are calculated from at least 3 independent experiments performed in triplicate. Significance (** $P \leq 0.01$) was determined by Student's *t*-test. MU = Miller units; MU = $1,000 \times (OD_{420} - 1.75 \times OD_{550}) / (T \times V \times OD_{600})$, where OD = optical density, T = reaction time (min), and V = culture volume (mL).

by 11 to 17% except M21H with reduced induction activity of 29% and M21W with induction activity of 94% compared with nisin (Figure 6B). These results told us that Leu16 and Met21 might be potential residues to be substituted or modified further to enhance nisin induction activity. The antimicrobial activities of these mutants were also detected and mutants L16A, L16H, L16V, M21A, M21D, and M21N, which show higher

induction abilities, also exhibit increased antimicrobial activities by about 11 to 13%. We supposed that these mutants have potential to be used in bioengineering to improve nisin production. Recently, a novel nisin variant with leucine at position 16 replaced by alanine (L16A) was produced by *L. lactis* isolated from goat milk (Perin and Nero, 2014); however, the production of this variant has not been assayed. With increased

Figure 6. Induction and antimicrobial activity analyses of nisin mutants with substitutions at position 16 and 21. β -Galactosidase activities of *Lactococcus lactis* NZ9000 NlacZ induced by nisin mutants (9 ng/mL) with substitutions at position 16 (A) and 21 (B) were shown. The last bar (–) indicates that the reporter strain was not induced by nisin or any nisin mutant. The diameters of antimicrobial halos produced by nisin mutants were also shown (C, D). Values and standard deviations are calculated from at least 3 independent experiments performed in triplicate. Significance (** $P \leq 0.01$) was determined by Student's *t*-test. MU = Miller units; MU = $1,000 \times (\text{OD}_{420} - 1.75 \times \text{OD}_{550}) / (T \times V \times \text{OD}_{600})$, where OD = optical density, T = reaction time (min), and V = culture volume (mL)

induction and antimicrobial ability, we supposed that the production of this variant would be higher than nisin A.

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