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Cloning and Optimization of a Nisin Biosynthesis Pathway for Bacteriocin Harvest

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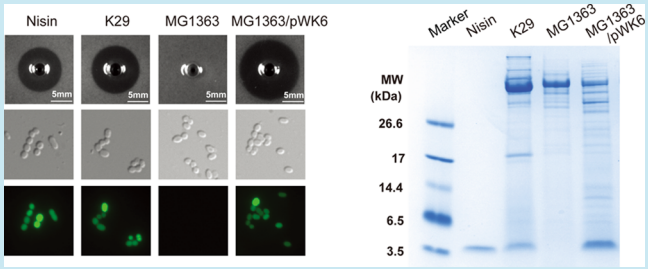
Supporting Information

ABSTRACT: Nisin is an important antimicrobial peptide that has enormous applications in biotechnology. Despite many encouraging efforts, its overproduction has been a long-standing challenge due to the complexity of the underlying pathway and the difficulty in genetic modification of lactic acid bacteria. Here, we cloned an entire nisin biosynthesis pathway from a nisin-producing strain (*Lactococcus lactis* K29) into a plasmid and transplanted the plasmid into a nisin deficient strain *Lactococcus lactis* MG1363, resulting in successful heterologous expression of bioactive recombinant nisin. To increase nisin harvest, we also overexpressed nisA, a gene responsible for nisin precursor production, with a set of constitutive promoters. To further optimize nisin yield, we minimized the metabolic cost of the engineered strains by integrating nisA overexpression cassettes and the recombinant pathway into a single circuit. With our rational construction and optimization, our engineered optimized strain is able to produce bioactive nisin with a yield of 1098 IU/mL, which is more than six times higher than that of the original strain.



KEYWORDS: *nisin biosynthesis, pathway engineering, optimization, lactic acid bacteria*

Nisin is a 34-amino-acid-long antimicrobial polycyclic peptide that is widely used in the food industry.[1−3] It acts effectively against a broad spectrum of Gram-positive organisms, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, lactic acid bacteria (LAB), and others.[4,5] Nisin also serves as the chemical inducer of the NIsin Controlled gene Expression (NICE) system, one of the most versatile and popular expression systems used in modern biotechnological applications.[6−9] Recently, nisin has been further exploited for therapeutic purposes.[10−14] For instance, it has been revealed as an attractive alternative to antibiotics for the treatment of infectious diseases[15,16] and a promising agent to slow and even stop the growth of squamous cell head and neck cancers.[17]

Because of nisin's enormous industrial and medical applications, the optimization of its manufacture has been an important problem in biotechnological researches. As nisin is synthesized autonomously by a subset of wild-type lactic acid bacteria, such as *Lactoccus lactis* subsp. *lactis* (*L. lactis* subsp. *lactis*), various efforts have been attempted to screen for better strains with higher nisin yields.[18,19] In parallel, optimization of culture media and fermentation protocols have also been actively pursued and shown successful.[20−25]

Compared to the above efforts, a more rational approach to increase nisin production is to directly reengineer the pathway responsible for nisin biosynthesis. Recent studies along that line include direct transfer of a nisin pathway, consisting of 11 genes and 4 operons (14.5 kb long in total),[26] into other organisms, such as *Bacillus subtilis* and *Enterococcus sp.* strains, through conjugation or direct cloning.[27,28] However, no autonomous nisin production has been observed. It has also been attempted to overexpress a single gene of the pathway to improve nisin production.[29] Additionally, researchers have tried to separate the biosynthesis into different steps including *in vivo* precursor synthesis and *in vitro* modification at the post-translational level.[26,30] Despite these encouraging efforts, direct engineering and optimization of the nisin pathway has been missing, primarily due to the complexity of the pathway and the lack of efficient engineering tools for LAB modification.

Recently, synthetic biology has emerged as an exciting interdisciplinary field for the engineering of novel cellular capabilities[31−33] and has shown tremendous potential for advancing many research and application areas.[34−36] Inspired by the concepts emerged in synthetic biology; here, we rationally engineered a LAB strain for the overproduction of the bacteriocin nisin.

Due to the lack of efficient genetic tools for LAB and the relative large size (14.5 kb) of the nisin gene cluster, advances in increasing nisin overproduction have been limited over the decades. To enable a systematic optimization of nisin production, we employed a LAB pathway engineering vehicle (pCCAMβ1) that we recently developed.[37] This shuttle system

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consists of a pathway insertion site (NotI), a selection marker (EmR), and two compatible origins of replication, O.pCC1BAC and O.pAMβ1.[38,39] O.pCC1BAC is a copy-controlled bacterial artificial chromosome origin that replicates only in *E. coli* while O.pAMβ1 is a replication origin that only functions in LAB. With this shuttle, a recombinant pathway can be constructed in *E. coli* and phenotyped in LAB. This system hence combines the feasibility of circuit manipulation in *E. coli* and the necessity of phenotypic validation in LAB. With this shuttle system, we transplanted the entire gene cluster from the chromosome of a nisin producing strain *L. lactis* K29 by cloning the cluster through extra-long thermal polymerase chain reactions (PCR) and constructed it into the pCCAMβ1 system with Gibson assembly.[40] The resulting plasmid is named pWK6. Here, the *E. coli* strain DH10B was used for pathway cloning and *L. lactis* subsp. *cremoris* MG1363, a LAB strain deficient in nisin biosynthesis, was adopted as our pathway host strain for nisin overexpression.[41] We named the MG1363 strain that harbors the pathway-containing pCCAMβ1 as MG1363/pWK6.

To examine whether the engineered strain is capable of nisin biosynthesis, we performed a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiment using the cell-free supernatant of the culture of the engineered strain (MG1363/pWK6). In parallel, the supernatants of the pathway source strain (K29) and the wild-type host strain (MG1363) were also assayed for comparison. As shown Figure 1, the engineered strain indeed produces peptides (Lane d)

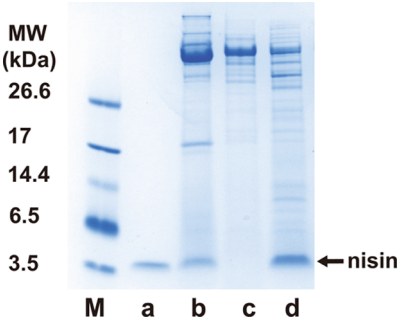

Figure 1. Detection of nisin peptide by Tricine-SDS-PAGE. M, peptide standards; a, commercial nisin; b, supernatant of wild type nisin producing strain *L. lactis* K29; c, supernatant of *L. lactis* MG1363; d, supernatant of *L. lactis* MG1363/pWK6.

whose size is identical to that of the commercial nisin (Sigma N5764) (Lane a) and those in the supernatant of the pathway source strain K29 (Lane b). In contrast, no peptides with a size equivalent to nisin were observed in the supernatant of the wild-type host strain (MG1363) (Lane c), showing that the peptides observed in the recombinant strain (MG1363/pWK6) are due to the transplanted pathway but not the host strain.

Nisin is characterized by its dual roles as an antibacterial agent to kill Gram-positive bacteria and as a signaling molecule to transmit intercellular signals through the NisR/K two-component system.[42] To confirm that the secreted peptides are recombinant nisin and also to validate its bioactivity, we conducted two assays corresponding to the nisin's dual features. We first performed an agar diffusion assay to evaluate its bacteriocin feature.[43] In this experiment, a nisin-sensitive strain, *L. lactis* 117,[43] was used as an indicator strain. In addition, the vector pCCAMβ1 (Em^R) was transformed into the indicator strain *L. lactis* 117 before use in order to eliminate potential inhibition of the antibiotics (erythromycin) present in the supernatants. Figure 2A shows that the supernatant from the

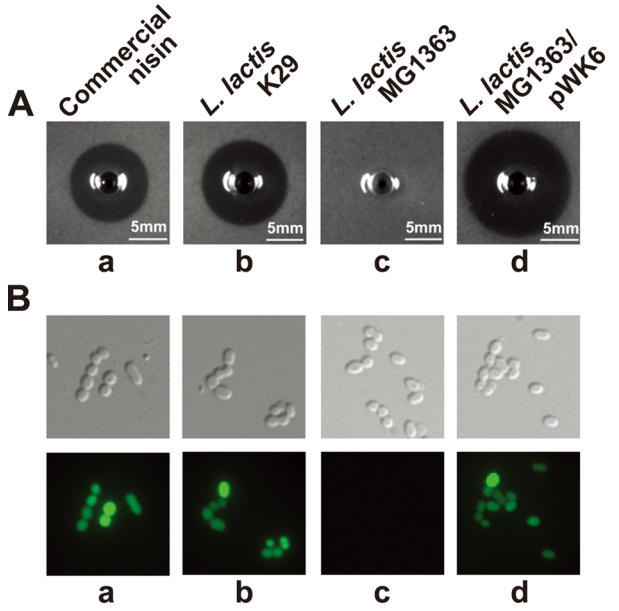

Figure 2. Validation of recombinant nisin's dual bioactivity: lantibiotic and quorum sensing. (A) Agar diffusion assay. Culture of the recombinant strain MG1363/pWK6 (d) inhibits the growth of the nisin indicator strain (an inhibition zone formed), same as commercial nisin (a) and that from *L. lactis* K29 (b). (B) Signaling assay. The same culture of the recombinant strain (d) is able to induce signal transduction of a NICE system (*L. lactis* NZ9000/pLeiss-gfp) as commercial nisin (a). The cells were observed under a fluorescence microscope after 2 h of induction.

engineered strain (M1363/pWK6) (panel d) inhibits the growth of the nisin-indicator strain, same as commercial nisin (panel a) and the supernatant of the nisin producing, pathway source strain (K29) (panel b). However, the supernatant of the wild-type host strain (MG1363) does not suppress the growth of the indicator cells (panel c).

In parallel, we examined the signaling feature of the recombinant peptides. As illustrated in Figure 2B, the supernatant of the engineered strain (panel d) induces the expression of green fluorescence proteins (GFP) of a nisin-responsive strain (*L. lactis* NZ9000/pLeiss-gfp). Here, the GFP response is enabled by loading a nisin-inducible, GFP expressing plasmid pLeiss-gfp into the LAB strain *L. lactis* NZ9000 that contains the nisin inducible NisR/K two-component signaling cassette. For comparison, the supernatant of the host strain itself (panel c) does not activate signal transduction.

These two assays collectively demonstrated the full bioactivity of the recombinant nisin, confirming our successful transplantation of the pathway and production of bioactive recombinant nisin.

We further quantified the nisin yield of the engineered strain using the agar diffusion method,[43] where the diameter of an inhibition zone correlates with the nisin concentration of the corresponding supernatant. Our experiment showed that the engineered strain MG1363/pWK6 (Figure 3, the white bar) produces nisin at a yield of 605 International Units per milliliter (IU/mL), which is 3.4 times higher than that of the wild-type

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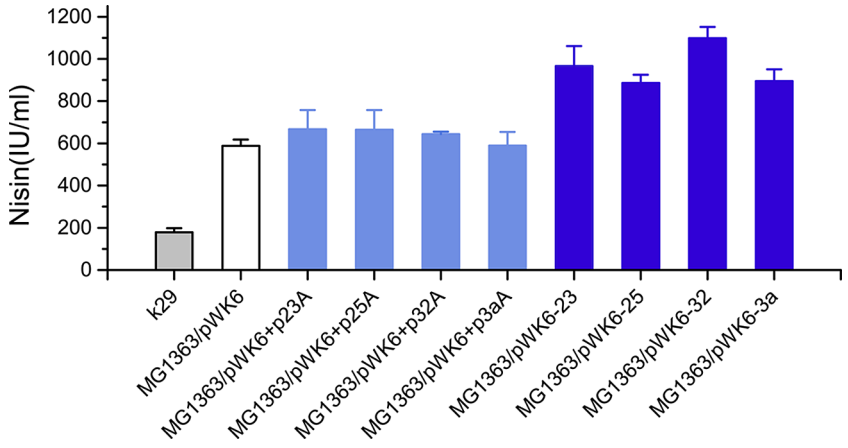

Figure 3. Maximal nisin production of the wild-type and engineered nisin producing strains. Nisin yields were measured using early stationary phase culture of different strains by the agar diffusion assay. Data were acquired from triplicate assays with the means and standard deviations illustrated in the figure.

pathway source strain K29 (Figure 3, the gray bar). To the best of our knowledge, this is the first report on cloning of an entire nisin gene cluster into a plasmid for heterologous expression.

In addition to the direct increase in nisin biosynthesis, transplanting the pathway into pCCAMβ1 also facilitates genetic modification of the pathway for an additional phase of optimization. This is because the feasibility of pathway optimization conferred by the vehicle pCCAMβ1 enables us to quickly alter the gene expression of the entire pathway. The gene *nisA* is responsible for nisin precursor synthesis;[44] therefore, its overexpression holds the potential for additional increase of the overall nisin production. We thus proposed to choose *nisA* as our engineering target.

We implemented our idea by constructing precursor overexpression circuits by placing a copy of *nisA* paired with a constitutive promoter into a high-copy vector pLeiss:Nuc. Here, four well characterized LAB promoters were employed, including the previous characterized synthetic promoters p23, p25, p32, and p3a.[45−47] To screen for maximal nisin production, the four resulting plasmids, named p23A, p25A, p32A, and p3aA, were cotransformed with the recombinant nisin pathway pWK6 into *L. lactis* MG1363. The nisin yields of the four strains, MG1363/pWK6+p23A, MG1363/pWK6+p25A, MG1363/pWK6+p32A, and MG1363/pWK6+p3aA, were then measured using the agar diffusion method. As shown in Figure 3 (the light blue bars), the above two-plasmid strains have a yield of 667, 665, 638, and 590 IU/mL, accordingly, three of which are higher than that of the strain containing the pathway alone (MG1363/pWK6, 605 IU/mL). This result thus demonstrates that overproduction of the precursor can further increase nisin biosynthesis.

On the other hand, the amounts of increase in nisin production of the above two-plasmid strains are not significant—667 IU/mL of the best optimized strains compared with 605 IU/mL prior to optimization. As introducing additional plasmids increase metabolic burden of cells, we continued our optimization by integrating the precursor overproduction cassette (*nisA* driven by a constitutive promoter) into the plasmid pWK6 that contains the pathway. We achieved our goal through RED/ET based recombineering[48] over the plasmid pWK6, which brings four precursor-overexpressing, pathway-containing plasmids, pWK6-23, pWK6-25, pWK6-32, and pWK-3a. The strains harboring the above plasmids were again assayed using the agar diffusion method. As illustrated in Figure 3 (blue bars), their yields were all significantly increased, with the two best strains, MG1363/pWK6-32 and MG1363/pWK6-23, having a yield of 1098 and 966 IU/mL, respectively. Through our multistep engineering, the optimized strains were indeed able to produce nisin that is 6.2 times higher that of the original source strain (K29).

To further reveal the kinetics of nisin biosynthesis of our synthetic strains, we performed a series of optical density (OD) measurements and well diffusion assays for the cultures of the engineered strains over a course of 18 h. Figure 4A shows the temporal profiles of the OD_{600} of the cultures of the wild-type pathway source strain (K29), the pathway-containing engineered strain (MG1363/pWK6), and the two optimized strains (MG1363/pWK6-23 and MG1363/pWK6-32), corresponding to the green square, blue circle, purple square, and orange circle lines, respectively. Accordingly, Figure 4B shows the corresponding nisin level of the cultures over the time course. The highest nisin production levels were achieved at late log phase or early stationary phase in all tested strains.

It is worth notice that the engineered strains (MG1363/pWK6, MG1363/pWK6-23 and MG1363/pWK6-32) have a slower growth rate and a lower final cell density compared with the wild-type pathway source strain (K29): As shown in Figure 4A, it took about 10 h for the engineered strains to reach their saturation densities (OD_{600} of 2.3). In contrast, the wild-type strain reached its saturation density (OD_{600} of 3.3) in 6 h. Meanwhile, the nisin overproduction profiles of the four strains are in opposite to the OD_{600} profiles: The engineered strains reach their peak nisin levels (1098, 966, and 606 IU) between hour 10 to 12 while the wild-type strain K29 has its maximal extracellular nisin level of 178 IU/mL at hour 6. The anticorrelation of the nisin yield and cell density profiles of the strains is likely attributed to the fact that nisin overproduction shunts cellular resource for growth to heterologous nisin biosynthesis. This result therefore further illustrates the importance of global consideration of pathway gene expression and cellular metabolism for pathway optimization.

Nisin production can be affected by several external factors such as producer strain, nutrition, pH, temperature, agitation and aeration.[49] Previous studies on nisin production have indeed exhibited a large variability with maximal reported yields varying from few hundreds to thousands IU/ml.[20−22,24,25,50]

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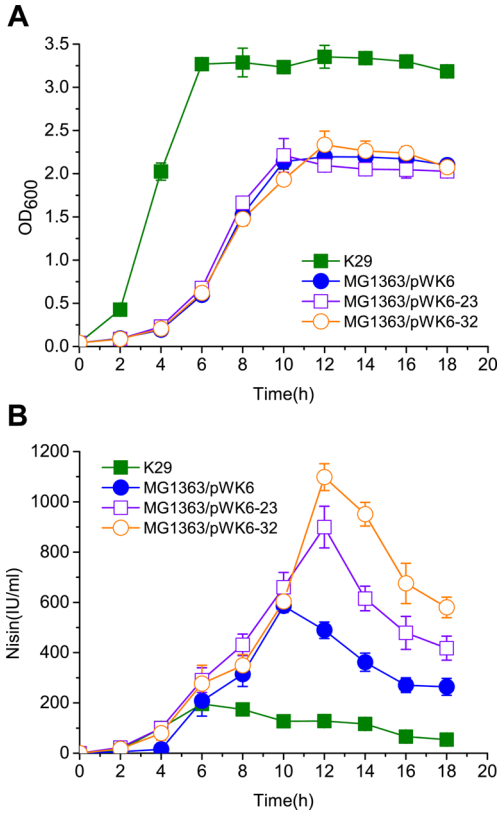

Figure 4. Nisin overproduction kinetics of the wild-type and engineered strains. The temporal profiles of the growth (A) and nisin production (B) of the strains *L. lactis* K29, MG1363/pWK6, MG1363/pWK6-23 and MG1363/pWK6-32 were measured. Data were acquired from triplicate assays with the means and standard deviations illustrated in the figure.

We believe that this is primarily due to the study-to-study differences of experimental settings, culture conditions as well as nonuniform and inaccurate nisin detection methods. It is also important to note that most of previous studies have focused on the optimization of culture medium and fermentation condition, instead of systematic engineering of nisin-producing strains on which our paper focuses. In our study, a fixed set of culturing and measurement conditions were used for all of the strains, ensuring a consistent and valid conclusion as our study has focused primarily on pathway engineering. To enable quantitative measurement, we have further conducted a set of fluorescence intensity assays for our engineered strains. Briefly speaking, we leveraged the signaling feature of nisin and used a nisin-inducible GFP-expressing strain paired with spectrophotometry for quantitative measurement of nisin concentrations of fermentation culture (detailed in the Methods section). We found that the measured nisin yields of the four engineered strains (K29, MG1363/pWK6, MG1363/pWK6-23, and MG1363/pWK6-32) are all consistent with the data in Figure 4 from the agar diffusion assay (Data is shown in Figure S1 in Supporting Information).

Additional increase in nisin yield is possible by optimizing cultural factors with our synthetic strains, and it needs further investigations in the future. In addition, it is worthy notice that there are decrease in nisin production level after reaching the peak value (Figure 4B), which is due to proteolytic degradation and/or adsorption of nisin by producer cells as reported in previous studies[22,24,50]

In summary, we have successfully cloned, constructed, and optimized a nisin pathway and significantly improved LAB's nisin overproduction. This work thus holds the potential to reduce the cost of nisin manufacture for the food industry. In addition, the multistep optimization procedure implemented in this work illustrates the power of rational optimization of biosynthesis pathways for the increase of production of valuable

Table 1. Strains and Plasmids Used in This Study

strain or plasmid	relevant properties	source or reference
	Bacterial Strains	
E. coli DH10B	host for cloning and counter selection, Str^R	
E. coli EPI300	host for copy control pCC1BAC vectors	epicenter
L. lactis subsp. *lactis* K29	wild type nisin producing strain	this work
L. lactis subsp. *cremoris* MG1363	host for heterologous expression of nisin	41
L. lactis subsp. *cremoris* NZ9000	host for nisin controlled gene expression; nisRK integrated into chromosome.	42
L. lactis 117	CNRZ 117; indicator strain for agar diffusion assay	43
	Plasmids	
pCCAMβ1	*E. coli* and *L. lactis* shuttle cloning vector; pCC1BAC origin and pAMbeta1 origin, Em^R	(W. Kong and T. Lu, unpublished data)
pLeiss:Nuc	*E. coli* and *L. lactis* shuttle vector; pSH71 origin, Cm^R	51
pLeiss-gfp	Gfpuv fused to start codon downstream of PnisA promoter, Cm^R	this work
pWK6	14.5 kb nisin gene cluster from *L. lactis* cloned into pCCAMβ1; nisin production plasmid	this work
p23A	*P*23 promoter/nisA cloned into pLeiss-Nuc; Constitutive expression of nisA	this work
p25A	P25 promoter/nisA cloned into pLeiss-Nuc; Constitutive expression of nisA	this work
p32A	P32 promoter/nisA cloned into pLeiss-Nuc; Constitutive expression of nisA	this work
p3aA	P3a promoter/nisA cloned into pLeiss-Nuc; Constitutive expression of nisA	this work
pWK6-23	*P*23 promoter/nisA cloned into pWK6; nisin production and overexpression of nisA plasmid	this work
pWK6-25	P25 promoter/nisA cloned into pWK6; nisin production and overexpression of nisA plasmid	this work
pWK6-32	P32 promoter/nisA cloned into pWK6; nisin production and overexpression of nisA plasmid	this work
pWK6-3a	P3a promoter/nisA cloned into pWK6; nisin production and overexpression of nisA plasmid	this work

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products. In principle, the approach illustrated here can be extended to optimizing other functional pathways in lactic acid bacteria. It thereby offers new opportunities for understanding LAB and new potentials for further synthetic biology applications.

METHODS

Bacterial Strains and Culture Conditions. Bacterial strains and plasmids used in this study are described in Table 1. *L. lactis* strains were grown at 30 °C in M17 broth containing 0.5% (w/v) glucose (GM17). *E. coli* were propagated aerobically in Luria−Bertani broth at 37 °C. When necessary, erythromycin was used at a final concentration of 5 μg/mL in *L. lactis* and 250 μg/mL in *E. coli*. Chloramphenicol was used at 5 μg/mL in *L. lactis* and 10 μg/mL in *E. coli*.

Cloning and Plasmid Construction. In order to clone nisin gene cluster, upstream and downstream sequences of the nisin gene clusters from Genbank were aligned and a pair of primers nisin159 and nisin14703 were designed (see oligonucleotide sequences in Supporting Information Table 1). The nisin gene cluster (14.5 kb) was amplified from genomic DNA of *L. lactis* K29 using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The backbone of pCCAMβ1 vector was amplified using primers pCCF and pCCR that contain overlaps with the gene cluster. The vector pCCAMβ1 and the PCR-amplified nisin gene cluster were assembled using Gibson assembly to generate the plasmid pWK6. To generate nisin induced GFP expression vector pLeiss-gfp, gfpuv and pLeiss:Nuc[51] were amplified and assembled by Gibson assembly. Gfpuv was drived by PnisA promoter through fusing with start codon of nisA.

To generate the single nisA precursor overexpression plasmid, constitutive promoters *P*23, P32, CP25, and P3a were amplified or designed in primers (list in Table S1) as described previously.[45−47] *NisA* gene was amplified from pWK6 and plasmid backbone (pSH71 origin plus Cm^R) was amplified from pLeiss:Nuc. The fragments of promoters, *nisA* and plasmid backbone were then purified and assembled by Gibson assembly, generating plasmids p23A, p25A, p32A, and p3aA.

To combine nisin gene cluster with *nisA* overexpression cassette into a single plasmid, counter selection BAC modification kit (Gene Bridges) was used to insert *nisA* overexpression cassette into plasmid pWK6 downstream of the nisin gene cluster. Briefly, pWK6 was transformed into *E. coli* DH10B/pRed that could express lambda red recombinase under induction by arabinose. The streptomycin^S/kanamycin^R fragment was amplified from the template in the kit using primers 25FSmKn/25RSmKn flanking by homology regions with pWK6. The resulting fragment was then transformed into arabinose induced DH10B/pWK6/pRed. After screening on kanamycin plates, DH10B/pWK6-strep^S-kan^R/pRed was selected. Counter selection was subsequently implemented by first transforming PCR amplified p23-nisA, p25-nisA, p32-nisA, and p3a-nisA fragments (same homology arms with streptomycin^S/kanamycin^R fragment) into DH10B/pWK6-str^S-kan^R/pRed, respectively. After counter selection on streptomycin plates, str^S-kan^R fragment in the plasmid pWK6-str^S-kan^R was replaced by *nisA* overexpression cassette (p23-nisA, p25-nisA, p32-nisA, and p3a-nisA). The resulting plasmids were named pWK6-23, pWK6-25, pWK6-32, and pWK6-3a.

Unless otherwise indicated, all plasmid constructions were first established in *E. coli* and then transferred to *L. lactis* by electroporation as previously described.[52] For pCCAMβ1 derived plasmids, they were first transformed into *E. coli* EPI300 for induction of high copy number. Then plasmids were prepared and transformed into *L. lactis*.

Tricine-SDS-PAGE. Two milliliters of overnight cultures of nisin producing cells were centrifuged at 10 000*g* for 10 min. The supernatants were filtered through a 0.22 μm filter membrane (Millipore) and then were added 10^{-1} volume of 100% trichloroacetic acid (TCA). After incubation on ice for half an hour, the supernatants were centrifuged at 18 000*g* at 4 °C for 15 min. The resulting protein pellets were then washed with 200 μL acetone, air-dried and resuspended in 20 μL of tricine sample buffer (Bio-Rad). After boiling at 100 °C for 5 min, the samples were loaded and run on a 16.5% tricine-tris-gel and stained with Coomassie Blue G-250.[53]

Nisin Induction Experiment. A nisin inducible GFP-expressing plasmid, pLeiss-gfp, was transformed into *L. lactis* NZ9000, the host for NIsin Controlled gene Expression (NICE) system.[42] A single colony of *L. lactis* NZ9000/pLeiss-gfp was grown overnight in GM17 broth and subsequently inoculated into a fresh 5 mL of GM17 broth. When the OD_{600} reached 0.6, the culture was added with 10 μL of supernatant of overnight cultures of nisin producing cells. Commercial nisin was used at a final concentration of 10 ng/mL for calibration. Upon 2 h of induction, GFP expression was measured using spectrophotometry.

Nisin Bioassay. Nisin activity was determined by a double layer agar diffusion method as described previously with some modifications.[43] The bottom agar was prepared by pouring 25 mL of molten media agar (cooled to 50 °C) premixed with 50 μL of overnight culture of indicator strain *L. lactis* 117 to 150 mm plate. The seeded bottom agar was allowed to solidify at room temperature for half an hour. The bottom agar was then overlaid with an additional 25 mL of cooled molten agar. At the same time, a sterile 96-well PCR plate was placed in the molten agar upper layer to make wells. After sufficient solidification, the PCR plate was removed, and 15 μL of nisin standard or testing samples were added into wells. After incubation at 30 °C for 10 h, a standard curve of nisin inhibition zones versus units of commercial nisin was drawn by measuring the diameter of inhibition zone produced by standard nisin and, from this curve, the nisin concentrations of tested samples were estimated.

Kinetics of Nisin Production. Overnight cultures of nisin producing cells were inoculated (1% v/v) into 25 mL of fresh GM17 broth. Samples were taken every 2 h. OD_{600} of cultures were measured and nisin yields in the supernatants were calculated by using the bioassay mentioned above.

ASSOCIATED CONTENT

Supporting Information

Table S1: All oligonucleotide sequences used in this work. Figure S1: Fluorescence intensity based measurements of nisin productivity. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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