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CONTROLLED FUNCTIONAL EXPRESSION OF THE CIRCULAR BACTERIOCIN ENTEROCIN NKR-5-3B AND THE LEADERLESS BACTERIOCIN LACTICIN Q

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ABSTRACT

Bacteriocins are antimicrobial peptides that possess a number of desirable properties that can be utilized for application in various industries including the food and pharmaceutical industries. Enterocin NKR-5-3B (Ent53B) and lacticin Q (LnqQ) are well characterized bacteriocins that exhibit exceptional stability and potent antimicrobial activity. Here we report the establishment of a dual-plasmid expression system of these bacteriocins by exploiting the quorum sensing production system of nisin known as the nisin inducible controlled expression (NICE™) system. Using this dual-plasmid expression system, the production of Ent53B and LnqQ can be controlled by the addition of exogenous nisin at sub-lethal concentration, either in pure or crude form, to induce the transcription of the genes responsible for the production of these bacteriocins. Using this system, cheap cultivation media instead of the commonly used complex and expensive cultivation media, can be utilized in the production of these bacteriocins thereby significantly lowering the production cost – one of the major limiting factors for its large-scale application. Additionally, this system provides a robust control of bacteriocin production thereby creating an effective bacteriocin application delivery system.

Keywords: lactic acid bacteria, bacteriocins, controlled bacteriocin expression, dual-plasmid expression

INTRODUCTION

Bacteriocins are antimicrobial peptides produced by diverse bacterial strains as primary metabolites in order for them to gain advantage in their ecological niche (**Perez *et al.*, 2014, Klaenhammer, 1993**). Bacteriocin production among lactic acid bacteria (LAB) is considered a desirable trait especially to those strains involved in food fermentations. LAB bacteriocins are considered food-grade antimicrobials that can be utilized in addressing microbial contamination problems in foods that usually results to food spoilage and food-related infections **(Cotter *et al.*, 2005, Perez *et al.*, 2015**). Aside from being food-grade, LAB bacteriocins are ideal choice as food preservatives based on numerous aspects. Bacteriocins are inherently tolerant to higher thermal stress and are more active at a wider pH range common in many food systems. These bacteriocins are also colorless, odorless, and tasteless thus their application in food does not affect the food's sensory qualities (**Perez et al., 2015**).

It has long been established that bacteriocin production is a result of a coordinated expression of several genes controlled by the so-called quorum sensing-gene expression (**Klaenhammer, 1993, Kuipers *et al.*, 1998**). Quorum sensing-gene expression involves specific molecules that act as signals for the induction of gene expression when a certain threshold concentration of the signal molecule has been reached (**Kuipers *et al.*, 1998**). These specific signal molecules are either the bacteriocin itself or a bacteriocin-like peptide. Thus far, it has been proven that in the case of class I bacteriocins (lantibiotics), the bacteriocin itself acts as the inducing factor regulating the transcription of its own structure gene and the neighboring genes related to its biosynthesis (**Kuipers *et al.*, 1995, Kleerebezem *et al.*, 1997**). In the case of non-lantibiotic bacteriocins (class II bacteriocins) bacteriocin-like peptides have been found to induce the expression of cognate biosynthetic genes (**Kleerebezem *et al.*, 2001, Ishibashi *et al.*, 2014, Ennahar *et al.*, 2000**). The most characterized regulation system of bacteriocin production is the case of nisin biosynthesis. The auto-regulation of nisin biosynthesis is controlled by a two-component system consisting of the response regulator NisR (**van der Meer *et al.*, 1993**) and the sensor protein, histidine kinase NisK (**Engelke *et al.*, 1994**). The sub-inhibitory amounts of extracellular nisin is transduced by autophosphorylation of NisR and subsequent phospho-transfer to NisR, triggering the transcription of the genes under the control of *nisA* and *nisF* promoters (**de Ruyter *et al.*, 1996a**). As application to this quorum sensing expression system of nisin biosynthesis, a nisin-inducible expression system (NICE™ system) has been developed **(de Ruyter *et al.*, 1996b**). The NICE™ system requires three essential elements: (1) a Gram-positive host strain that expresses the *nisRK* genes; (2) nisin or nisin analogs as inducer molecule; and (3) plasmid vector containing the *nisA* or *nisF* promoter fragments.

Meanwhile, there has been a spike in the number of newly reported bacteriocins possessing varying characteristics and bioactivities during the last two or three decades. We recently reported two novel bacteriocins possessing exceptional properties that could be of high industrial relevance, the bacteriocins enterocin NKR-5-3B (Ent53B) and lacticin Q (LnqQ). Ent53B is a novel circular bacteriocin that is ultra-stable and possesses a broad spectrum bioactivity (**Himeno *et al.*, 2015**), whereas LnqQ is novel leaderless bacteriocin with a very potent bioactivity that is higher than that of nisin, through a novel mode of action known as huge-toroidal pore (HTP) mechanism **(Yoneyama *et al.*, 2009**). Moreover, the respective genetic elements responsible for the production of these bacteriocins have already been identified. The gene cluster composed of the genes *enkB1*, *enkB2*, *enkB3*, and *enkB4* encodes the biosynthetic enzymes that works cooperatively to process the Ent53B precursor peptide, encoded by the gene *enkB*, producing its mature form (**Perez *et al.*, 2016**). Whereas the biosynthetic enzymes responsible for the production of LnqQ are encoded in a gene cluster composed of the five genes *lnqBCDEF*. These enzymes provide the secretion and self-immunity mechanisms of the producer strain to synthesize the LnqQ, which is encoded by the gene *lnqQ* (**Iwatani *et al.*, 2012**).

In this present study, we report the establishment of a dual-plasmid heterologous expression system that offers a tight control of the production of Ent53B and LnqQ. Here we employed the NICE™ system that exploits the quorum-sensing regulation system of nisin biosynthesis, utilizing the nisin-inducible promoter to initiate the transcription of the respective structural genes of Ent53B and LnqQ. Here we highlight two main points: first is the capacity of this system to significantly lower bacteriocin production cost as it enables the recombinant strain to produce the bacteriocins Ent53B and LnqQ even in simple cultivation media which are relatively cheaper than the complex cultivation media commonly used in bacteriocin production. One of the main bottleneck hindering

the industrial scale utilization of bacteriocins, particularly in the food industry, is its expensive nature of production (**Cotter et al., 2005**). The second point we highlight in this report is the capacity of this system to offer a tight control of bacteriocin production thereby improving the delivery of these novel bacteriocins. This controlled expression system serves as a virtual switch on-and-off of the bacteriocin production thereby providing control of an effective and timely delivery of Ent53B and LnqQ into the target application environment.

MATERIAL AND METHODS

Bacterial strains and reagents

The strains and plasmids used in establishment of the dual-plasmid expression system are summarized in Table 1. The host expression strain *L. lactis* NZ9000 was cultivated in M17 medium supplemented with 0.5% glucose (GM17) and grown at 30°C. The indicator strains were cultivated in MRS medium (Oxoid, UK) and incubated at their respective optimum incubation conditions. Chloramphenicol (Cm) and erythromycin (Em) were used as antibiotic markers in selective media at a final concentration of 5 µg/ml. All bacteria were stored at −80°C in their respective media with 30% glycerol and cultivated twice before use.

Table 1 Bacterial strains and plasmids used in the dual-plasmid expression system

Strain or plasmid	Description[a]	Reference or source[b]
Strains		
Enterococcus faecium NKR-5-3	Enterocin NKR-5-3B producer strain	**Ishibashi *et al.*, 2012**
Lactococcus lactis QU5	Lacticin Q producer strain	**Fujita *et al*., 2007**
L. lactis NZ9000	Heterologous host strain, *nisRK*	**de Ruyter *et al*., 1996b**
L. lactis NCDO 497	Nisin A producer strain	NCDO
Plasmids		
pMG36c	Cm^r, pWV01-based cloning vector carrying a strong lactococcal based promoter P_{32}, rolling-circle replication	**Perez *et al*., 2016**
pNK-B1234	Cm^r, pMG36c derivative containing *enkB*, *enkB1*, *enkB2*, *enkB3*, and *enkB4*	**Perez *et al*., 2016**
pNKΔB	Cm^r, pNK-B1234 Δ*enkB*	This study
pNZΔB	Cm^r, pNKΔB derivative with nisin-inducible promoter, P_{nis} instead of P_{32}	This study
pLNQ	Cm^r, pMG36c derivative containing *lnqB*, *lnqC*, *lnqD*, *lnqE*, and *lnqF*	**Iwatani *et al*., 2012**
pLNQΔQ	Cm^r, pLNQ Δ*lnqQ*	This study
pLZΔQ	Cm^r, pLNQΔQ derivative with nisin-inducible promoter, P_{nis} instead of P_{32}	This study
pIL-Pnis	Em^r, theta replicating vector, nisin-inducible promoter, P_{nis}	This study
pIL-B	Em^r, pIL-Pnis containing *enkB*	This study
pIL-Q	Em^r, pIL-Pnis containing *lnqQ*	This study

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant.
[b] NCDO, National Collection of Food Bacteria, Reading, UK.

Construction of dual-plasmid expression system

In order to construct a controllable dual-plasmid expression system of Ent53B and LnqQ, their respective structure genes were cloned into a theta-replicating vector, pIL-Pnis, that carry a nisin-inducible promoter, while the genes encoding their cognate biosynthetic enzymes were cloned into a rolling-circle replicating vector, pMG36c, that possess a strong constitutive promoter. These plasmids were introduced to *L. lactis* NZ9000, a *nisRK* expressing strain. To describe briefly, the respective structure genes of these two bacteriocins, *enkB* and *lnqQ*, were PCR amplified using designed primers containing *Nco*I and *Xba*I restriction sites (Table 2) with the genomes of their respective host as template DNA during the PCR reaction. The amplified fragments were then separately digested with *Nco*I and *Xba*I and were individually ligated to the pIL-PnisA plasmid, which was previously digested with the same enzymes. The resulting plasmids were termed pIL-B and pIL-Q respectively. These plasmids were then introduced by electroporation into the host *L. lactis* NZ9000 previously transformed with the respective plasmid carrying the cognate biosynthetic genes, pNKBΔB and pLNQΔQ. Construction of these plasmids was done by inverse PCR of the pNK-B1234 and pLNQ plasmids, respectively. Briefly, outward facing primers were designed (Table 2) to generate a PCR product without the structure genes *enkB* and *lnqQ*, respectively. The resulting PCR products were then phosphorylated using kinase enzyme (T4 polynucleotide kinase; Toyobo, Osaka, Japan) and subsequently self-ligated using Ligation High Ver.2 (Toyobo).

Table 2 List of primers used to construct the dual-plasmid expression system

Primer name	Sequence (5' → 3') [a]	*Tm* (°C)
NcoI-enkB-F	AAACCATGGATTCAGTGGTGGTAGTAACAG	57 [b]
XbaI-enkB-R	AAATCTAGAGACCATGGCCAATTTATTCG	57 [b]
NcoI-lnqQ-F	AAACCATGGGATTGTTGCAAAGAGAAAGCG	61 [b]
XbaI-lnqQ-R	AAATCTAGAATACTAGTGAGATTATTGGC	55 [b]
Inv(XbaI)-pIL-Pnis-F	CCACTAGTTCTAGAGAGCTCCG	55
Inv(NcoI)-pIL-Pnis-R	CATGCCTGCAGTACCCATGGTG	59
Inv-pNKBΔB-F	CAGTCGCTACAATCATTGCT	54
Inv-pNKBΔB-R	CTGTTACTACCACCACTGAATG	53
Inv-pLNQΔQ-F	AGATTGGCTTAATGCAGGTC	54
Inv-pLNQΔQ-R	TAACACGCTTTCTCTTTGC	53
pNis-F	CATTCTTTGCTGTAGATCTAGTC	55
pNis-R	TGCAGTACCCATGGTGAGTGCC	55
ΔP32-F	GCAGAAAAATTCGTAATTCGAGC	58
ΔP32-R	CTAGTCCAAGCTCACAAAAATCC	57

[a] restriction enzyme sites are underlined.
[b] *Tm*, melting temperature at 0.1 nM primer concentration.

Induction of bacteriocin production and quantification of antimicrobial activity

The dual-plasmid expressing *L. lactis* NZ9000 strains were cultivated in 5 ml GM17 liquid medium at 1% inoculation level and incubated at 30°C. Antibiotic markers Cm and Em were added at final concentration of 5 µg/ml each. To induce the bacteriocin production, a nisin solution (either up to 10 ng/ml final concentration of commercial nisin A dissolved in pH 3.0 water or a 500 µl filter-sterilized supernatant of the nisin producer strain *L. lactis* NCDO 497) was added to the culture after 2 hours of incubation and incubation was continued for at least 18 hours.

The antimicrobial activities of the recombinant strains were visualized and quantified using direct-colony overlay assay and spot-on-lawn assay respectively, as described previously (**Perez *et al*., 2016**). To briefly describe the direct-colony overlay assay, recombinant strains were pre-cultivated in 5 ml GM17 medium containing antibiotic markers (Cm and Em) at 5 µg/ml each and incubated at 30°C overnight and sub-cultured into fresh medium containing the same antibiotic markers and kept under the same conditions for 24h. The number of cells of each recombinant strain was normalized to the same optical density

(OD_{600} of 3.0) using sterile distilled water. An aliquot of 1 µl of the normalized cell solutions were then spotted onto a GM17 agar lawn inoculated with 1 % indicator strain. After overnight incubation at 30°C, inhibition zones around the colonies were examined to check for the production of the bacteriocins.

Whereas the spot-on-lawn assay was done by spotting 10 µl of a 2-fold serially diluted cell-free supernatant of the recombinant strains onto a double-layered agar plate, which contained 5 ml of Lactobacilli Agar AOAC medium inoculated with an overnight culture of an indicator strain as the upper layer and 10 ml of the MRS broth supplemented with 1.5% agar as the bottom layer. After an overnight incubation at 30°C, the bacterial lawns were checked and inhibition zones measured. The activity titer expressed as arbitrary activity units (AU) was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strains.

RNA isolation and semi-quantitative reverse transcription PCR (RT-PCR)

Total RNA of each recombinant strain was extracted using the RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions. Isolated RNA was treated with DNase I (Invitrogen, CA) to ensure the removal of residual DNA. The treated RNA was then used as templates for cDNA synthesis using SuperScript VILO™ cDNA synthesis kit (Invitrogen). The synthesized cDNAs were used as template for the RT-PCR to confirm the presence of the respective messenger RNA transcripts of *enkB* and *lnqQ*. Concentration of the cDNAs were measured using NanoDrop™ spectrometer (Thermo Fisher Scientific) and were all normalized to 10 ng/µl by diluting with nuclease-free deionized water prior to the PCR reactions.

Computer analysis of DNA sequence

DNA samples were extracted from each recombinant strain using Exprep Plasmid SV minikit (GeneAll Biotechnology, Seoul, South Korea), submitted for DNA sequencing and were subsequently analyzed using the GENETYX-WIN software, version 8.0.1 (Genetyx, Tokyo, Japan).

RESULTS AND DISCUSSION

Description of the Ent53B and LnqQ Dual-Plasmid Expression System

The dual plasmid system that enables full expression control of Ent53B and LnqQ is based on the aforementioned NICE™ system. In this dual-plasmid expression system, the substrate (bacteriocin precursor peptide) is encoded in a separate plasmid from its catalytic enzymes (cognate biosynthetic enzymes). Here, the respective gene encoding the precursor peptides of Ent53B and LnqQ is cloned upstream the *nisA* promoter region of the theta-replicating plasmid pIL-Pnis while the cognate biosynthetic gene cluster of these bacteriocins are encoded in a rolling-circle plasmid pMG36c carrying a constitutive promoter P_{32} (Fig. 1). The basis of choosing pIL-Pnis and pMG36c plasmids is their compatibility for dual-plasmid expression wherein they do not compete with each other for the replication machinery and do not compromise their plasmid copy numbers. These are critical factors that needs to be considered in developing recombinant protein expression systems **(Rosano & Ceccarelli, 2014)**. A subtle advantage in this dual-plasmid system we describe here is its relative amenability to genetic engineering wherein mutations can be easily introduced to the bacteriocin structure gene, e.g. to enhance the properties of the resulting recombinant bacteriocin, without risking the unnecessary introduction of mutations into the other genes involved in its biosynthesis that is located directly upstream of the original gene locus of the Ent53B (**Perez *et al.*, 2016**) and LnqQ (**Iwatani *et al.*, 2012**) biosynthetic gene clusters.

Controlled Production of Ent53B and LnqQ

The level of *enkB* and *lnqQ* expression and antimicrobial activity of the dual-plasmid recombinant *L. lactis* NZ9000 expressing the pNKBΔB::pIL-B and pLNQΔQ::pIL-Q after exogenous nisin A induction showed in a concentration dependent manner (Fig. 2). The recombinants showed increasing strength of its inhibitory activity against the indicator strain as indicated with the increasing size of the zone of inhibition around the colony of the recombinants with increasing nisin A concentration added for induction. Whereas the same recombinant strains did not show any bioactivity in the absence nisin A (Fig. 2A). Moreover, the level of RNA transcription of the respective bacteriocin structure genes, *enkB* and *lnqQ*, were upregulated with the corresponding increase of nisin A concentration, while no notable change of the level of transcription of the 16S rRNA of the recombinant strains (Fig. 2B). These results clearly show that this dual-plasmid system offers a tight control over the expression of these bacteriocins.

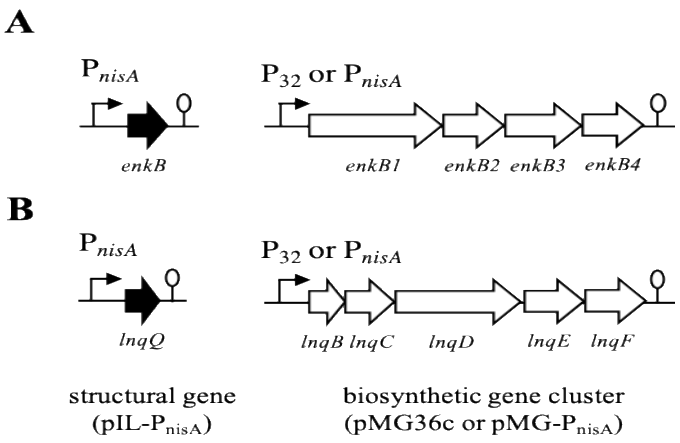

Figure 1 Outline of the biosynthetic machinery of dual-plasmid expression system of enterocin NKR-5-3B and lacticin Q. Open arrows indicate the genes encoding the cognate biosynthetic enzymes of enterocin NKR-5-3B (A) and lacticin Q (B), respectively. These genes are encoded in a rolling-circle replicating vector, pMG36c, that possess a strong constitutive promoter P_{32} or a nisin-inducible promoter, P_{nisA}. Black arrows represent the bacteriocin structural genes of enterocin NKR-5-3B (A) and lacticin Q (B), respectively, and are encoded in a theta-replicating vector, pIL-P_{nisA}. The bent arrows indicate the promoters P_{32} and P_{nisA}. The circles represent putative terminators.

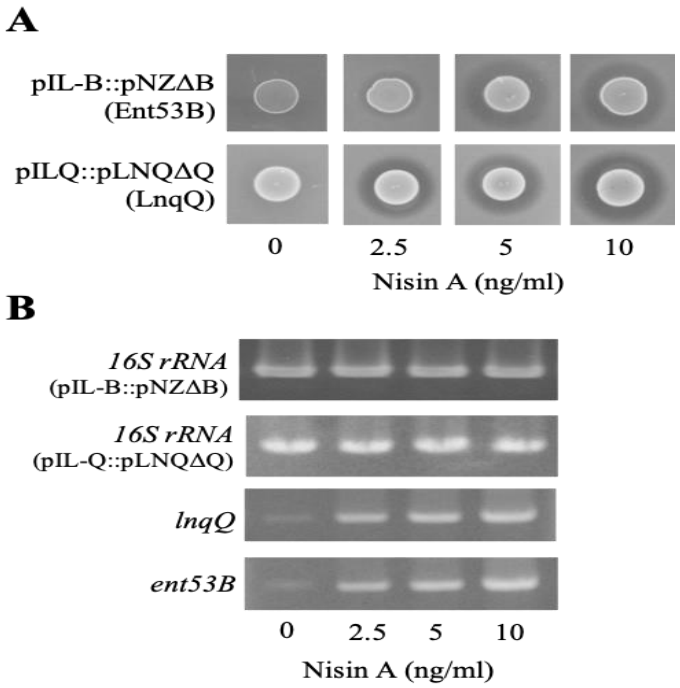

Figure 2. Concentration dependent nisin A-based induction of the expression of the biosynthetic genes of enterocin NKR-5-3B and lacticin Q. Bacteriocin production of *Lactococcus lactis* NZ9000 phenotypes carrying the dual-plasmid expression system, pIL-B::pNZΔB and pIL-Q::pLNQΔQ for the production of enterocin NKR-5-3B and lacticin Q, respectively showing concentration dependent enhancement of their bioactivities (A). The level of gene expression of the bacteriocin structural genes *lnqQ* and *ent53B* these recombinant strains showed concentration-dependent induction monitored using semi-quantitative reverse transcription PCR (RT-PCR) (B). The indicator strain used for the bioassay was *L. lactis* ATCC 19435^T (nisin A minimum inhibitory concentration is 500 nM). The highest nisin A concentration used for the induction 10 ng/ml roughly equates to 3 nM.

To demonstrate the practical implication of this bacteriocin controllable production system, the recombinants were cultivated in a various media including a simple medium that is generally insufficient to support bacteriocin production. Without the addition of the inducing factor, nisin A, the recombinants did not show any bacteriocin production even when cultivated at complex media such as MRS and GM17. Interestingly, the recombinants where able to produce the bacteriocins even when cultivated in a simple media TSBYE in the presence of the inducing factor either in pure form or from a crude culture supernatant of *L. lactis* NCDO 497, a known nisin A producer (Table 3). However, in TSBYE medium less enhancement of bacteriocin bioactivity was observed relative to other complex media MRS and GM17 (Table 3). This is probably because of the

limited induction of the expression of the biosynthetic enzymes in TSBYE which may have acted as the limiting factor in bacteriocin biosynthesis. To circumvent this problem, the promoter region of the vector carrying the biosynthetic enzymes was changed to the nisin-inducible promoter Pnis to produce the pNZΔB and pNZΔQ shuttle vectors. Expectedly, the pIL-B::pNZΔB and pIL-Q::pNZΔQ expressing *L. lactis* NZ9000 strains manifested higher bacteriocin bioactivity even in less complex medium when induced with nisin (Table 3). This result presents a very high practical implication as it can significantly lower the bacteriocin production cost which is known to be a huge limiting factor for the large scale industrial application of bacteriocins (**Cotter *et al.*, 2005**). Most LAB only produce bacteriocins when cultivated in complex cultivation media. It should be interesting to explore viable cheap feedstocks that can support the grow of these recombinants and by simply adding exogenous nisin A, such as that from the supernatant of strain NCDO 497, a potential economically feasible production system of these bacteriocins can be established.

Table 3 Nisin-induced production of Ent53B and LnqQ production in different medium

		Bioactivity Arbitrary Unit (AU/ml)[a]					
		pNKB	pIL-B:: pNKBΔB	pIL-B:: pNZΔB	pLNQ	pIL-Q:: pLNQΔQ	pIL-Q:: pNZΔQ
MRS	non-induced	1600	0	0	3200	0	0
	induced [b]	1600	1600	6400	3200	3200	3200
GM17	non-induced	3200	0	0	3200	0	0
	induced [b]	3200	3200	6400	3200	3200	3200
TSBYE	non-induced	0	0	0	0	0	0
	induced [b]	0	200	3200	0	100	3200
	induced [c]	0	200	3200	0	100	3200

[a] – *L. lactis* ATCC 19435^T as indicator strain
[b] – nisin A solution (Sigma-Aldrich)
[c] – supernatant of nisin A-producer *L. lactis* NCDO 497

Moreover, the virtual switch-on/switch-off capability of bacteriocin production of this dual-plasmid expression system offers a tight control of the delivery of bacteriocin in an actual application system, particularly in food fermentations. Food fermentations normally involves complex microbial flora, each distinctly contributes to the overall sensory and organoleptic characteristics of the food product including the production of compounds that contributes to the flavor and aroma of the food product. Whereas, bacteriocin producing strains has been utilized either as defined starter culture, adjunct, or protective culture in many food fermentations. Using the system we describe here, the recombinants can be introduced into the fermentation environment without affecting the other species, and once the fermentation is completed, bacteriocin production can be triggered by the addition of the inducing factor to ensure the stable quality of the food product. A similar work has been done in the acceleration of the ripening of cheese by utilizing a controlled expression of the lytic genes *lytA* and *lytH* that can trigger the lysis of cheese starter strain *L. lactis* thereby releasing the intracellular enzymes involved in flavor formation (**deRuyter *et al.*, 1997**).

Confirmation of Ent53B and LnqQ from the culture supernatant

To confirm the identity of the heterologously synthesized Ent53B and LnqQ from the dual-plasmid recombinants, we purified the active fraction of the culture supernatants of these recombinants following the early reported purification system of these bacteriocins (**Perez *et al.*, 2016, Fujita *et al.*, 2007**). ESI-TOF/MS analysis of the purified Ent53B and LnqQ showed that both Ent53B and LnqQ showed several ions species with the quadruple charge ions as the most dominant. Computation of the molecular mass of these bacteriocins from the most dominant ion species showed identical to the previously reported molecular mass of the respective bacteriocins thus confirming their identity (Fig. 3).

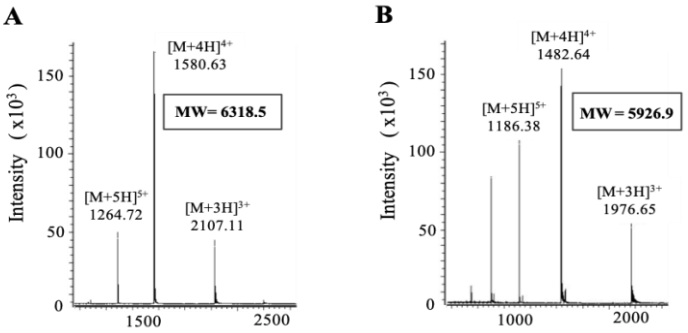

Figure 3 Electrospray ionization time-of-flight mass spectra of Ent53B (A) and LnqQ (B) isolated from culture supernatant of *Lactococcus lactis* NZ9000 phenotypes carrying the dual-plasmid expression system, pIL-B::pNZΔB and pIL-Q::pLNQΔQ respectively. Detected multiple charge ions of Ent53B and LnqQ are indicated. Observed MWs of each bacteriocin is indicated in the inset of each panel. Observed MWs were calculated from the most abundant ion species which is the quadruple charged ions for both bacteriocins.

CONCLUSION

In the present study, we report the establishment of a controllable dual-plasmid expression system of the bacteriocins Ent53B and LnqQ using the NICE™ system. Here we showed that the activation of the bacteriocin genes, *enkB* and *lnqQ*, expression through the induction of exogenous nisin A is highly robust which offers a rigid control in the production and delivery of Ent53B and LnqQ. The highlighted practical implication of this developed system is its capacity to enable the recombinant strain to produce these bacteriocins even in simple and cheap cultivation media thus lowering the production cost of these bacteriocins.

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