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Bioluminescence-based identification of nisin producers — A rapid and simple screening method for nisinogenic bacteria in food samples

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ABSTRACT

We present a simple and rapid method for screening nisin producers that directly identifies nisinogenic bacteria by induction of bioluminescence within the *Lactococcus lactis* NZ9800lux biosensor strain (Immonen and Karp, 2007, *Biosensors and Bioelectronics* 22, 1982–7). An overlay of putative nisinogenic colonies with the biosensor strain gives identification results within 1 h. Functionality and specificity of the method were verified by screening nisin producers among 144 raw milk colonies and a panel of 91 lactococcal strains. Studies performed on strains and colonies that did not induce bioluminescence but inhibited growth of the biosensor demonstrated that only nisinogenic bacteria can cause induction. Bacteria known to produce bacteriocins other than nisin failed to induce bioluminescence, further verifying the specificity of the assay. We discovered a non-inducing but inhibitory lactococcal strain harboring a modified nisin Z gene, and demonstrated that the source of the inhibitory action is not a non-inducing variant of nisin, but a bacteriocin of lower molecular weight. The concentration of nisin producers in a raw milk sample was 1.3×10^2 CFU/ml. We identified from raw milk a total of seven nisin Z producing *L. lactis* subsp. *lactis* colonies, which were shown by genetic fingerprinting to belong to three different groups. Among the panel of 91 lactococci, four strains were nisin A producers, and one strain harbored the modified nisin Z gene. The method presented here is robust, cost-effective and simple to perform, and avoids the pitfalls of traditional screening methods by directly specifying the identity of the inhibitory substance.

1. Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria. Gram-positive bacteriocins are divided into three classes based on structure and presence of post-translational modification (Rea et al., 2011). Lantibiotics are a major group of post-translationally modified bacteriocins whose name stems from lanthionines, unusual amino acids in their structure (Willey and van der Donk, 2007). Lantibiotics exhibit an antagonistic activity often more potent than classical antibiotics, and are generally effective against gram-positive organisms including many antibiotic-resistant pathogens (Field et al., 2010). Numerous lantibiotics, many of them produced by lactic acid bacteria (LAB), are naturally present in food products, and are generally regarded as safe. This, combined with their outstanding antimicrobial potency, has led to many applications of lantibiotics in both food protection and medicine (Field et al., 2010; Gálvez et al., 2007).

Nisin, discovered in 1927, is the prototype lantibiotic (Willey and van der Donk, 2007). It is licensed as a food preservative (E234), and can be added in selected foodstuffs as a concentrated preparation or be produced by bacteria in situ (Delves-Broughton et al., 1996; Gálvez et al., 2007). Therefore, nisinogenic strains are of interest as a source of nisin and as protective cultures in production of fermented foodstuffs (O'Sullivan et al., 2002). Other applications of nisin include treatment of ulcers, bacterial mastitis as well as staphylococcal (including MRSA) and enterococcal infections (Delves-Broughton et al., 1996; Field et al., 2010). Nisin is also used as a preservative in hygiene products (Gálvez et al., 2007).

A recent review reports more than 60 different lantibiotic peptides (Field et al., 2010). In addition, a wealth of novel lantibiotic genes awaits finding as the genome data currently being generated is subjected to closer scrutiny. As an example of this, a genome mining software tool BAGEL2 identified seven putative lantibiotic genes within three analyzed bacterial genomes (de Jong et al., 2010). Together with the tools for identifying lantibiotic genes within bacteria, methods for detecting antimicrobial activity are necessary for recognizing actual lantibiotic producers. The presence of lantibiotic structural genes does not ensure their expression: several studies report presence of nisin genes in non-nisinogenic strains (Moschetti et al., 1996; Vuyst, 1994). Therefore, false positive results may arise if methods based on recognition of nucleic acid sequences alone are used. Screening methods based on antimicrobial activity usually cannot determine the molecule producing the antagonistic activity, and

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therefore additional studies are required for identification (Alegría et al., 2010; Noonpakdee et al., 2003; Ortolani et al., 2010; Perin et al., 2012). A screening method combining detection of the presence of antimicrobial activity as well as direct identification of the antagonistic molecule would therefore be ideal for recognizing novel producers of lantibiotics.

This study describes application of the bioluminescent whole-cell nisin biosensor *Lactococcus lactis* subsp. *cremoris* NZ9800lux (Immonen and Karp, 2006, 2007) in screening nisin producers in raw milk and among a panel of 91 *Lactococcus* strains. The whole-cell biosensor is based on the nisin-controlled gene expression system (NICE) which facilitates efficient over-expression of heterologous genes (Mierau and Kleerebezem, 2005). Nisin-induced expression of the bacterial luciferase operon *luxABCDE* within the biosensor bacteria results in bioluminescence, facilitating specific and sensitive detection of nisin presence and concentration. Bioluminescence production occurs without addition of an exogenous substrate, making use of the sensor straightforward. Detection of bioluminescence with an in vivo imaging system facilitates real-time detection of bioluminescence development. Due to specificity of the sensor, bioluminescence is induced only around a colony producing nisin, instantly distinguishing it from colonies producing other antagonistic activities.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

The nisin biosensor *L. lactis* subsp. *cremoris* strain NZ9800lux (Immonen and Karp, 2006, 2007) was cultured at 30 °C without agitation in M17 broth (Merck, contains 0.5% w/v lactose) supplemented with 0.5% w/v glucose (M17G) and 25 μg/ml chloramphenicol (M17GCm). Solid medium was prepared by addition of 1.5% w/v agar. Two control strains were used in the study: nisin non-producer *L. lactis* subsp. *cremoris* NZ9000 was a kind gift from Dr. O. Kuipers, NIZO, the Netherlands, and nisin producer *L. lactis* subsp. *lactis* DSM 20729 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). A panel of 91 lactococcal strains was obtained from the collections of Department of Food Science and Microbiology (Di.S.T.A.M.) at University of Milan, Italy. The panel included 31 *L. lactis* subsp. *cremoris*, 44 *L. lactis* subsp. *lactis*, and 16 *L. lactis* subsp. *lactis* bv. *diacetilactis* strains. All strains were of dairy product or starter origin except one isolated from cow meat. The strains were cultured in M17 broth or on agar supplemented with 1% w/v glucose and 0.5% w/v lactose (M17GL). Incubation was performed at 30 °C for 24 h without agitation. Bacteriocin-producing strains of genera *Carnobacterium*, *Pediococcus* and *Lactobacillus* were from the collections of Di.S.T.A.M. Carnobacteria were cultured on TSB agar (Becton Dickinson) supplemented with 2% w/v glucose, lactobacilli and *Pediococcus acidilactici* on MRS agar (Lab M Ltd.), and *Streptococcus salivarius* on M17GL agar at 30 °C for 24 h. All strains were maintained as 20% v/v glycerol preparations at −80 °C.

2.2. Screening nisin producers

A tenfold serial dilution of raw cow milk obtained from Valio Ltd, Tampere plant, Finland was prepared in peptone water and plated on M17GL agar. The plates incubated at 30 °C for 48 h were overlaid with the nisin biosensor as follows: 10 ml of semisolid M17G (agar content 0.8% w/v) tempered to 50 °C was seeded with 200 μl of an overnight culture of NZ9800lux, and a thin layer was pipetted on the plates. The plates were imaged with Xenogen IVIS Lumina II in vivo imaging system (Caliper Life Sciences) using an exposure time of 30 s. The development of bioluminescence was followed for 2 h. In between measurements, the plates were incubated at 30 °C. The nisin producing colonies were collected for subculturing from under the agar overlay using the narrow end of a sterile Pasteur pipette to extract a small agar plug containing the nisinogenic colony, which was then spread on an M17G plate. All selected colonies were characterized by PCR amplification and sequencing of the nisin and 16S rRNA genes.

The 91 *Lactococcus* strains were inoculated on M17GL plates divided in a grid of 2 cm × 2 cm squares. After an overnight incubation at 30 °C, the plates were overlaid with nisin biosensor, imaged as described above, and incubated overnight at 30 °C. The colonies producing nisin-induced bioluminescence or a zone of inhibition in the biosensor overlay were characterized by PCR amplification and sequencing of the nisin gene.

2.3. Validation of screening assay specificity

To confirm the screening method detects all nisin producers within a sample but does not react to other antimicrobials, 144 randomly picked and isolated colonies from raw milk were tested in the screening assay on square plates as described above. Thereafter, the plates were incubated overnight at 30 °C for the development of inhibition zones to reveal all colonies with antagonistic activity. The colonies producing nisin-induced bioluminescence or a zone of inhibition in the biosensor overlay were characterized by PCR amplification and sequencing of the nisin gene. The colonies harboring the nisin gene were further characterized by sequencing of the 16S rRNA gene.

Specificity of the screening assay was tested using eleven strains producing bacteriocins other than nisin. The strains used were *P. acidilactici* PAC-1.0 and *Lactobacillus plantarum* VHE92 (producers of pediocin PA-1), *S. salivarius* K12 (salivaricin A, B and 9), *Carnobacterium divergens* NCFB 2855 (divergicin A), and *Lactobacillus reuteri* (reuterin i.e. β-hydroxypropionaldehyde, not a bacteriocin). In addition, two *C. divergens* strains (1.1 and 2.5) and four *Carnobacterium maltaromaticum* strains (9.4, LMG 11393, ML1-97, F46-1) that produce uncharacterized bacteriocins were tested.

2.4. PCR amplification of the nisin and 16S rRNA genes and the histidine operon

Genomic DNA was isolated from antagonistic strains isolated from raw milk using InstaGene Matrix (Bio-Rad Laboratories) according to the manufacturer's instructions. For amplification of the nisin gene, forward primer Nisin frw (5′-AATTCTGAAGTTTGTTAG-3′) and reverse primer Nisin rev (5′-TATAAAAATAGGATAGTATC-3′) were designed. They amplify a 304 bp fragment containing the prenisin structural gene (174 bp) of nisin A, Z and Q. The amplification performed with DyNAzyme II polymerase (Finnzymes) consisted of an initial denaturation step of 4 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 49 °C and 1 min at 72 °C, and a final extension for 2 min at 72 °C. PCR products were sent to Macrogen Inc. (South Korea) for sequencing with Nisin frw and rev primers.

The 16S rRNA genes were amplified with forward primer P0 (5′-GAGAGTTTGATCCTGGCTCAG -3′) and reverse primer P6 (5′-CTACGGCTACCTTGTTACGA-3′) (Grifoni et al., 1995). The amplification performed with DyNAzyme II polymerase consisted of an initial denaturation step of 4 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, and a final extension for 10 min at 72 °C. PCR products were sent to Macrogen Inc. (South Korea) for sequencing with primer P0 and internal primer 1100R (5′-GGGTTGCGCTCGTTG-3′) (Lane, 1991).

To differentiate between *L. lactis* subsp. *lactis* and *cremoris*, PCR amplification of part of the histidine biosynthesis operon was performed as reported by Corroler et al. (1999). In short, primers His frw (5′-CTTCGTTATGATTTTACA-3′) and His rev (5′-CAATATCAACAATTCCAT-3′) were used for PCR amplification. The result is a 933 bp product from *L. lactis* subsp. *lactis* and approximately 200 bp longer (933 bp + n × 59 bp) product from *L. lactis* subsp. *cremoris* due to presence of a number of 59 bp repeats.

2.5. Genetic fingerprinting of bacterial isolates

The bacterial isolates were genetically characterized at intra-species level by means of BOX-PCR (primer used: BoxA1, 5′-CTACGGCAAGG CGACGCTGACG-3′) and random amplified polymorphic DNA (RAPD, primers used: M13, 5′-GTAAAACGACGGCCAGT-3′ and PedAF, 5′-ATACTACGGTAATGGGGT-3′), according to the protocols previously described (Guglielmetti et al., 2008, 2010).

2.6. Bacteriocin analysis by SDS-PAGE

To characterize the factor in strain *L. lactis* subsp. *lactis* SL149 that caused growth inhibition of the NZ9800lux biosensor strain, an SDS-PAGE was run to determine the size of the inhibitory molecule. SL149 and nisin producer I-12 were grown overnight in M17GL. The culture was diluted 1:1 in 0.1% w/v Tween 80 acidified to pH 2.5 with HCl, and cells as well as any precipitated material were removed by centrifugation. The supernatant pH was adjusted to 5.2–5.5 with NaOH and concentrated to approximately one fifth of the original volume in a vacuum centrifuge. The concentrated preparation was analyzed by SDS-PAGE. A gel containing 10% acrylamide stacking and 17% acrylamide resolving gel in a Laemmli buffer system was prepared according to instructions of the Mini-PROTEAN® 3 Cell system (Bio-Rad). The samples were loaded on the gel as duplicates, and Precision Plus Protein Dual Xtra standard (Bio-Rad) was included for size determination. Electrophoresis was run at 20 mA for 110 min and 30 mA for 30 min at room temperature. After the run the gel was cut in half. One half was stained overnight with Coomassie brilliant blue. The other half was washed with deionized water for 3 h, and then placed on a prepoured plate of semisolid M17GL + 1% v/v Tween 80 (agar content 0.75% w/v). The gel was overlaid with semisolid agar seeded with 1% v/v of overnight culture of NZ9800lux. The plate was imaged after 1 h incubation at 30 °C with Xenogen IVIS Lumina II in vivo imaging system (Caliper Life Sciences) using an exposure time of 30 s. To detect growth inhibition zones, the plate was incubated overnight at 30 °C.

3. Results

3.1. Nisin producer screening concept

Two colonies of the control strains DSM 20729 and NZ9000 were cultured on a Petri dish and overlaid with the nisin biosensor for testing of the screening assay concept. Imaging results showed induction of bioluminescence during 1 h incubation only around nisin producing DSM 20729 colonies (Fig. 1). After overnight incubation, inhibition zones caused by nisin were visible around DSM 20729 colonies only. The screening test is simple to perform and provides results rapidly, within 1 h of application of the sensor layer on the cultivation plate.

3.2. Isolation of nisin producers from raw milk

Screening nisin producers was performed among raw milk lactic streptococci cultured on M17GL agar. Dilution factor of raw milk was adjusted so that plating resulted in 30–40 colonies per plate. Performing a nisin biosensor overlay of three plates resulted in the identification of four bioluminescence-inducing colonies (Fig. 2A). These colonies named RM-I, RM-II, RM-III1 and RM-III2 were confirmed as nisin Z producers by detection of the nisin structural gene through PCR amplification (Table 1). All four colonies were identified as *L. lactis* subsp. *lactis* by sequence of the 16S rRNA gene and length of the histidine operon PCR product. Fingerprinting experiments through BOX-PCR and RAPD failed to evidence genotypic differences suggesting that RM-I, RM-II, RM-III1 and RM-III2 could be the same multi-isolated strain (Supplementary Fig. 1A and B). The total lactic streptococcal concentration of raw milk determined by M17GL plating was 3.03×10^3 CFU/ml, and concentration of nisin producers among them was 1.30×10^2 CFU/ml, representing more than 4% of total.

3.3. Identification of nisin producers among a panel of lactococci

We employed the screening method developed in this study to characterize a panel of 91 lactococcal strains for nisin production. No bioluminescence-inducing nisin producers were detected among the 31 *L. lactis* subsp. *cremoris* strains screened. However, 9 strains out of them showed antagonistic activity i.e. produced inhibition zones after overnight incubation. Furthermore, we found 2 bioluminescence-inducing nisin producers and 5 otherwise antagonistic strains among the 44 *L. lactis* subsp. *lactis* strains, as well as 2 bioluminescence-inducing nisinogenic strains among the 16 *L. lactis* subsp. *lactis* bv. *diacetilactis* strains. PCR amplification revealed the presence of nisin A structural gene in the four bioluminescence-inducing strains (SD12, SD14, SL28 and SL29) (Table 1). Interestingly, a modified nisin Z gene (Fig. 3) was detected in *L. lactis* subsp. *lactis* strain SL149 that showed

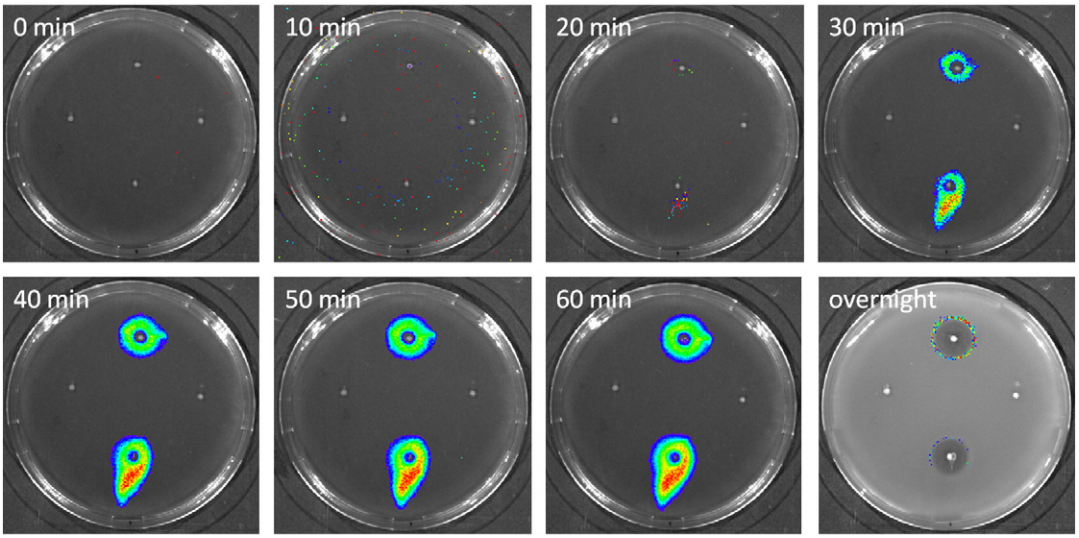

Fig. 1. Bioluminescence imaging of nisin producers. The sequence of images follows bioluminescence induction by nisin producer DSM 20729 (middle two colonies) and nisin non-producer NZ9000 (colonies on left and right). Only the nisin-producing strain showed induction of bioluminescence and development of inhibition zones in the biosensor layer after an overnight incubation. The maximum counts increased from 8 at 0 min to 70 at 30 min and 225 at 60 min.

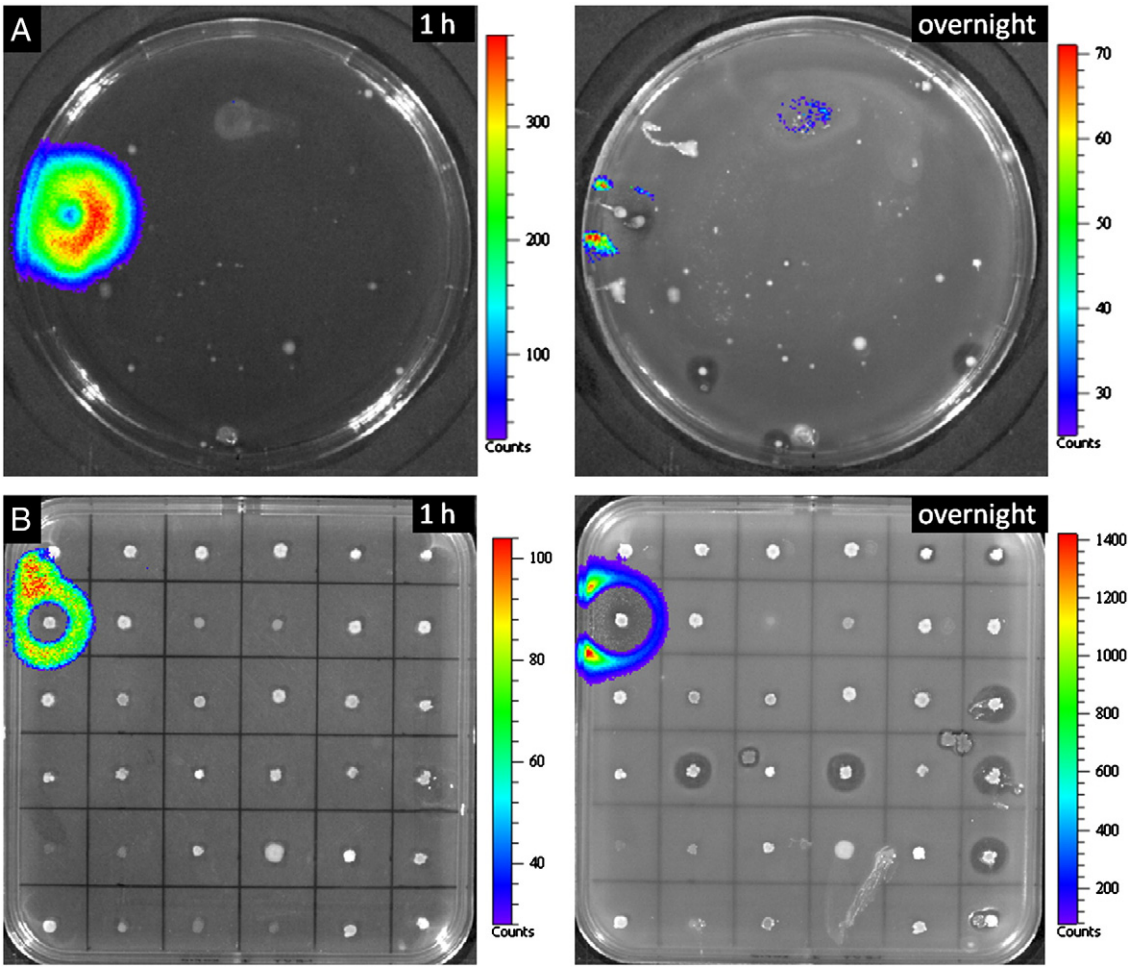

Fig. 2. Bioluminescence-based screening for nisin producers. A. Nisin producer screening among a mixed culture of lactic streptococci originating from raw milk. Bioluminescence developed within 1 h around a nisin producing colony, but after overnight incubation, inhibition zones were visible around other colonies as well. A total of four nisin producing colonies were isolated from three mixed culture plates. B. Specificity verification by screening among colonies isolated from raw milk. A total of 144 colonies on four plates were screened in this manner, resulting in the identification of three nisin producing colonies. Results from one plate are presented here. After overnight incubation, inhibition zones were visible around other colonies than the nisin producer. PCR experiments showed none of these harbored the nisin gene, and therefore produced other antagonistic substances than nisin. Screening of a panel of 91 lactococcal strains was performed in the manner presented in B., resulting in the identification of four more nisin producers.

antagonistic activity (i.e. produced an inhibition zone) but did not induce bioluminescence (Table 1).

3.4. Screening method specificity

The screening method was used to efficiently identify nisin producers among dozens of colonies on a single plate. However, after an overnight incubation at 30 °C, inhibition zones were detected around colonies other than the bioluminescence-inducing ones (Fig. 2A). Therefore, we determined the specificity of the method by randomly picking and isolating 144 colonies from the raw milk plates. Screening of these isolates revealed three bioluminescence-inducing colonies and 28 colonies that had an inhibition zone surrounding them after an overnight incubation (Fig. 2B). As expected, none of the colonies that produced an inhibition zone but did not induce bioluminescence, harbored the nisin structural gene. The three bioluminescence-inducing colonies named I-12, III-32 and IV-17 were confirmed as nisin Z producers and were shown to be *L. lactis* subsp. *lactis* by sequence of the 16S

Table 1
Nisin producing or nisin gene harboring strains identified in this study.

Source	Group	Isolate	Strain	Species	Nisin Z/A producer	Homology—% (bp/16S gene)
Raw milk	1	I-12	–	*Lactococcus lactis* subsp. *lactis*	Z	99.0% (1060/1071)
Raw milk	2	III-32, IV-17	–	*Lactococcus lactis* subsp. *lactis*	Z	99.3–99.5% (1063–6/1071)
Raw milk (mixed culture)	3	RM-I, RM-II, RM-III1, RM-III2	–	*Lactococcus lactis* subsp. *lactis*	Z	99.3–99.7% (1064–8/1071)
DiSTAM[a]	–	–	SD12	*Lactococcus lactis* subsp. *lactis* bv. *diacetilactis*	A	–
DiSTAM	–	–	SD14	*Lactococcus lactis* subsp. *lactis* bv. *diacetilactis*	A	–
DiSTAM	–	–	SL28	*Lactococcus lactis* subsp. *lactis*	A	–
DiSTAM	–	–	SL29	*Lactococcus lactis* subsp. *lactis*	A	–
DiSTAM	–	–	SL149	*Lactococcus lactis* subsp. *lactis*	Harbors *nisZ*	–

[a] DiSTAM = Department of Food Science and Microbiology, University of Milan, Italy.

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Leader_Z   MSTKDFNLDLVSVSKKDSGASPR 23
Leader_A   MSTKDFNLDLVSVSKKDSGASPR 23
SL_149     MSTKDFNLDLVSVSKKNSGASPR 23

Nisin_Z    ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK 34
Nisin_A    ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK 34
SL_149     ITSISLCTPGCKTGAVMGCNMKTATCNCSIHVSK 34
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Fig. 3. ClustalW2 sequence alignments of nisin Z and nisin A leader peptides (23 amino acids) and propeptides (34 amino acids). Compared to nisin Z, the product of SL149 nisin gene would have two modifications in the amino acid sequence, D-7N in the leader sequence and L16V in the antimicrobial peptide.

rRNA gene and length of histidine operon PCR product (Table 1.; Supplementary Fig. 1C). Furthermore, BOX-PCR and RAPD experiments showed that the genetic fingerprints of isolates I-12, III-32 and IV-17 were different from that of the four RM multi-isolates (Supplementary Fig. 1A and B). The screening assay was also tested with 11 strains producing reuterin or bacteriocins other than nisin. None of the strains induced bioluminescence, indicating nisin specificity of the assay (Supplementary Fig. 2). The screening method was therefore verified as a specific method for identification of nisin producers.

3.5. The source of inhibitory activity in strain SL149

An SDS-PAGE analysis was performed to determine the size of the SL149-produced inhibitory molecule (Fig. 4). The Coomassie stain (Fig. 4a) showed a rather pure preparation of the two inhibitory molecules, nisin in I-12 culture medium and a smaller molecular weight compound in SL149 culture medium. The samples caused growth inhibition of NZ9800lux (Fig. 4b) in the same region of the gel where the Coomassie stained proteins were present. The inhibition zone of sample SL149 again resided lower than that of I-12 in the gel, thus confirming that it was caused by a molecule of lower molecular weight than nisin, and that the inhibitory molecule in question is not a non-inducing variant of nisin. Bioluminescence imaging (Fig. 4c) showed light induction on a much larger area than growth inhibition. Therefore, induction occurred at lower concentrations than growth inhibition, suggesting that bioluminescence imaging can be a much more sensitive tool for nisin detection than growth inhibition assays.

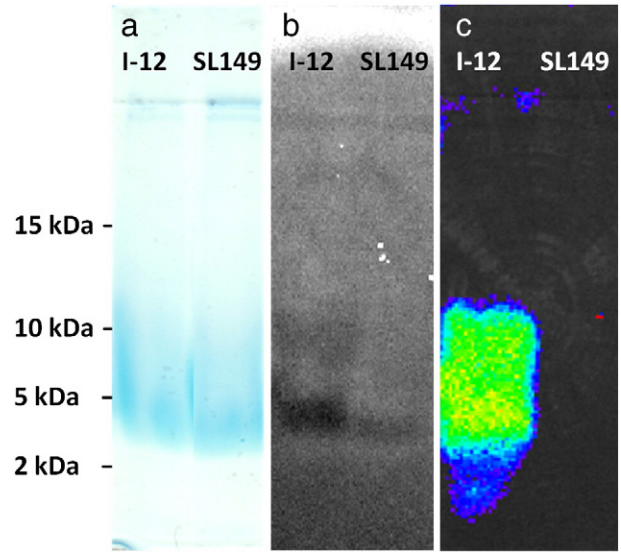

Fig. 4. SDS-PAGE analysis of growth media of I-12 and SL149 cultures. a) Coomassie stain, b) growth inhibition zone, and c) bioluminescence imaging results.

4. Discussion

The ability to express and secrete nisin is given by four operons, *nisABTCIPRK*, *nisI*, *nisRK* and *nisFEG* located on the conjugative transposon *Tn5276* (Lubelski et al., 2008). The products of the genes enable nisin immunity, production, posttranslational modification into mature peptide, as well as regulation of these processes. In this study, a non-functional nisin structural gene was discovered in *L. lactis* subsp. *lactis* strain SL149. The presence of non-functional nisin genes can be due to a dysfunction or lack of any of the genes related to nisin expression or modification, or regulation of these processes (Moschetti et al., 1996; Vuyst, 1994). Studies conducted on producers of the bacteriocin colicin indicate that losing the ability to produce the bacteriocin but maintaining resistance will lead to an increase in fitness and give the bacterium a competitive edge (Riley, 2011). This may also explain the occurrence of non-functional nisin genes.

The product of SL149 nisin gene would have two amino acid sequence modifications in comparison to nisin Z, D-7N in the leader sequence and L16V in the nisin propeptide. These differences constitute a new variant of nisin, as they are not similar to any of the known natural variants nisin A (Gross and Morell, 1971), nisin Z (Mulders et al., 1991), nisin Q (Zendo et al., 2003), nisin U and nisin U2 (Wirawan et al., 2006), or nisin F (de Kwaadsteniet et al., 2008). The mutations are in locations that should not affect expression or posttranslational modification of nisin. Mutation D-7A in the leader sequence had no effect on nisin secretion (van der Meer et al., 1994), and nisin Z mutant L16P was produced, secreted and active (Kuipers et al., 1996). Because mutation of residue L16 does not affect auto-induction of nisin production, induction of bioluminescence in the nisin biosensor should occur as well. However, the substance produced by strain SL149 does not induce bioluminescence. Therefore, nisin expression from strain SL149 is likely hindered by a dysfunction or lack of nisin expression or modification genes, and the mutations may have accumulated over time in the non-functional gene.

The antimicrobial agent produced by SL149 is not nisin because the substance does not induce bioluminescence in the biosensor and has lower mass than the 3.33 kDa nisin Z. It has proteinacious nature and can withstand acidification and heating during preparation of SDS-PAGE samples without losing its activity, which is characteristic of lantibiotics. Based on a search for lactococcal bacteriocins smaller than nisin in bacteriocin databases BACTIBASE (Hammami et al., 2010) and BAGEL2 (de Jong et al., 2010), likely candidates are lacticin 481 (Piard et al., 1992), and bacteriocin J46 (Huot et al., 1996). Another possible source of antagonistic activity is lacticin 3147, a two-peptide lantibiotic, whose peptides A1 (3.31 kDa) and A2 (2.85 kDa) interact synergistically to produce the active form (Wiedemann et al., 2006). On an SDS-PAGE gel, the inhibition zone of lacticin 3147 would be located between these two peptides, and therefore would appear below the nisin band.

The expression of genes *nisABTCIPRK* and *nisFEG* under their respective promoters *nisA* and *nisF* is controlled by nisin itself via the two-component system NisRK (Lubelski et al., 2008). The membrane kinase NisK autophosphorylates upon interaction with extracellular nisin, and phosphorylates the activator NisR, which then induces transcription from P*nisA* and P*nisF*. The nisin-controlled gene expression system (NICE) exploits this nisin mediated auto-induction (Mierau and Kleerebezem, 2005). NICE provides a linear dose–response relationship, and has been utilized in construction of the nisin biosensor strain used in this study (Immonen and Karp, 2006, 2007). Hu et al. (2009) reported the use of another biosensor based on the NICE system for identifying nisin producers. The method involved using culture supernatants of potential nisin producers for induction of Nuc protein expression in the biosensor, and detection of the presence of Nuc by SDS-PAGE. This method is more labor-intensive and time-consuming than the assay we present here. Our direct method includes no separate assay for the reporter gene product, and does not require pure cultures as sample material. Instead, nisinogenic colonies can be isolated on basis of

nisin production indicated by bioluminescence. A fluorescent nisin biosensor has been reported (Hakovirta et al., 2006) that could be utilized in a similar direct assay. However, the slow expression and maturation of the reporter green fluorescent protein requires overnight induction, which would make the assay less rapid than the one presented here.

We tested the specificity of the nisin producer assay by screening nisinogenic strains among 144 raw milk colonies as well as a panel of 91 lactococcal strains. None of the colonies other than those later verified as nisin producers induced bioluminescence in the biosensor strain. In addition, we tested 11 strains that are producers of known or uncharacterized bacteriocins, and found that none of them induced bioluminescence. The ability of lantibiotics subtilin, carnocin and sakacin A to induce bioluminescence in a nisin biosensor based on the NisRK control system has been tested by Wahlström and Saris (1999), who reported no detectable induction even by subtilin, the structurally closest homolog of nisin. Kuipers et al. (1995) reported that lantibiotics subtilin, lacticin 481, and Pep5 as well as the antimicrobial peptide lactococcin A were all ineffective as inducers of transcription of *nisA* gene via the NisRK control system. Our observation that the strain SL149 produces a non-inducing lantibiotic such as lacticin 481 is in good agreement with these findings.

We have established in this study bioluminescence induction in biosensor NZ9800lux by nisin A and Z. Of the other natural nisin variants, nisin U has been determined to induce nisin A production (Wirawan et al., 2006), and nisin F and Q can be produced by engineering the nisin structural gene in a nisin A-producing strain (Piper et al., 2011). Therefore these variants can also utilize the nisin autoinduction system present in the nisin biosensor. The screening method presented here should thus be able to recognize all known natural nisin variants.

The biosensor strain NZ9800lux has previously been used in an assay determining nisin concentrations in food samples (Immonen and Karp, 2006, 2007), and can therefore be used to determine nisin production levels of the identified nisinogenic strains. Due to high signal strength in the screening assay, bioluminescence detection can be performed with a simple CCD camera to improve cost-effectiveness. The identification of nisin producing bacteria by conventional methods typically entails exhaustive studies involving characterization of antimicrobial spectrum and enzymatic inhibition, as well as genetic fingerprinting and PCR sequencing to identify the producing species and nisin structural gene (Alegría et al., 2010; Noonpakdee et al., 2003; Ortolani et al., 2010; Perin et al., 2012). Consequently, there is a need for more simple molecular and phenotypic methodologies to characterize bacteriocinogenic bacteria. Unlike methods based on nucleic acid detection, our method directly identifies bacteria capable of nisin production, avoiding false positives arising from non-functional nisin genes. It also gives direct information on the nature of the inhibitory molecule, which growth inhibition methods fail to do. In addition to identifying nisin producers, the method could be utilized in monitoring population dynamics of nisin-producing organisms e.g. during a fermentation process. The method could also be extended to other bacteriocins by utilizing auto-inducible regulation of their biosynthetic gene clusters. Construction of biosensors able to recognize other bacteriocins would ease the task of identifying the source of antagonistic activity and ruling out candidate molecules.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2012.07.007.

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