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A Nisin Bioassay Based on Bioluminescence

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A *Lactococcus lactis* subsp. *lactis* strain that can sense the bacteriocin nisin and transduce the signal into bioluminescence was constructed. By using this strain, a bioassay based on bioluminescence was developed for quantification of nisin, for detection of nisin in milk, and for identification of nisin-producing strains. As little as 0.0125 ng of nisin per ml was detected within 3 h by this bioluminescence assay. This detection limit was lower than in previously described methods.

The lantibiotic nisin (17) produced by some *Lactococcus lactis* strains is a small antibiotic peptide that is active against several gram-positive bacteria, such as *Clostridium*, *Bacillus*, *Listeria*, and *Staphylococcus* species. Because of its nontoxicity for humans and its broad range of antimicrobial activity, it is used as a preservative in the food and dairy industries (4). Two naturally occurring nisin variants, nisin A (3) and nisin Z (16, 26), which differ in a single amino acid residue have been described. The genes encoding proteins involved in nisin biosynthesis, regulation, and self-immunity are arranged in two inducible operons, *nisA/ZBTCIPRK* and *nisFEG* (11, 19, 20, 25, 27, 28, 30, 31, 36). The genes *nisR* and *nisK* encode the response regulator (NisR) and the histidine kinase (NisK) of the two-component regulatory system (11, 20, 36), which is responsible for the autoregulation of nisin biosynthesis. Extracellular nisin acts as the environmental signal to which NisK responds. This signal is transmitted to NisR, which ultimately leads to transcriptional activation of both the *nisA/Z* and *nisF* promoters (5, 24, 27, 28) (Fig. 1A).

Most of the methods developed for quantification of nisin are based on its inhibitory activity to a test organism (8). The agar diffusion bioassay (14, 35) is still the most widely used. Several parameters affect the accuracy and sensitivity of this method (37). In addition, equal concentrations of nisins A and Z produce inhibition zones of different sizes due to the better diffusion properties of nisin Z (7). Immunological detection methods have also been developed. The sandwich-type enzyme-linked immunosorbent assay for nisin A, based on sheep polyclonal antibodies and developed by Falahee et al. (13), has a detection limit of 0.5 ng/ml for pure nisin and 0.2 μg/ml for nisin analyzed from spiked cheese. Other methods for immunodetection of nisin involving competitive direct enzyme-linked immunosorbent assays with polyclonal (32) and monoclonal (33) antibodies from mice have been developed. These assays showed nisin detection limits of 5 to 10 and 10 ng/ml, respectively. A rabbit antiserum against nisin Z was used to develop an immunodot method for detection of nisin, with a detection limit of 3 ng/ml for pure nisin Z and 0.155 μg/ml for nisin analyzed from milk and whey (2). All the immunoassays described are considerably more sensitive than the agar diffusion assay. Still, methods based on antibodies against nisin are not totally reliable due to possible cross-reactions with related compounds (12). The *gusA* gene has been placed under control of the nisin promoters for studies of transcriptional activity (5, 6, 24). Strains with the *nisA* or *nisF* promoter fused to *gusA* could be induced by small amounts of nisin to produce β-glucuronidase activity in a linear dose-response relationship. The specificity of nisin recognition was high, because the other tested bacteriocins, including subtilin, which is the closest structural homolog of nisin, were not functional inducers (24). However, even if these strains could be used to develop a nisin quantification assay, the assay method requires breakage of the cells and takes several hours to perform, making this approach unfavorable. In this communication, we present a fast, simple, and sensitive bioassay based on the autoinducibility of the nisin promoter P_{nisF} and bioluminescence derived from bacterial luciferase genes fused to the nisin promoter (Fig. 1B). This assay detects nisin in milk, water and supernatants from nisin-producing strains.

Construction of the indicator strain. A genomic fragment including the intact *nisR* and *nisK* genes and the promoter of *nisF* originating from the nisin Z-producing strain *L. lactis* subsp. *lactis* N8 was cut from plasmid pLEB190 (20) as an *Eco*RI-*Bam*HI fragment. This fragment was used to replace the *Eco*RI-*Bcl*I fragment containing the P_{45} promoter in plasmid pTC*lux*Hb (21). In the resulting plasmid, pLEB535 (Fig. 2), the bioluminescence genes *luxAB* from *Xenorhabdus luminescens* (named *luxHb* in plasmid pTC*lux*Hb) are placed under control of the P_{nisF} promoter. This construct was electroporated (18) into the non-nisin producer *L. lactis* MG1614 (15) and plated at 30°C on M17 (34) plates containing 0.5% (wt/vol) glucose and 0.5% (wt/vol) sucrose (M17GS) and chloramphenicol (5 μg/ml). The resulting indicator strain was named LAC182.

Nisin bioluminescence assay. The indicator strain LAC182 was grown in M17GS plus chloramphenicol (5 μg/ml) without aeration until an optical density at 600 nm (OD_{600}) of 0.5 was reached. Glycerol (87%) was added to one-fifth of the volume, and the bacteria were stored at −70°C before use. For nisin detection, these precultured cells of LAC182 were diluted 1:100 in M17GS plus 0.1% Tween 80. Nisin (Sigma) standards were made in 0.1% Tween 80. The solution was prepared with distilled water acidified to pH 2.5 with HCl (hereafter referred to as 0.1% Tween 80). Volumes between 5 and 70 μl of the nisin dilutions were mixed with 1 ml of diluted indicator bacteria, which were then grown for 3 h at 30°C without aeration. The 1-ml bacterial sample was added to 40 μl of *n*-decylaldehyde (1% [vol/vol]) in absolute ethanol in a cuvette, and the luciferase activity was determined immediately as relative light units with the Bio-Orbit 1253 luminometer connected to a computer using Lucyfer version 1.1β software (Teemu Teeri,

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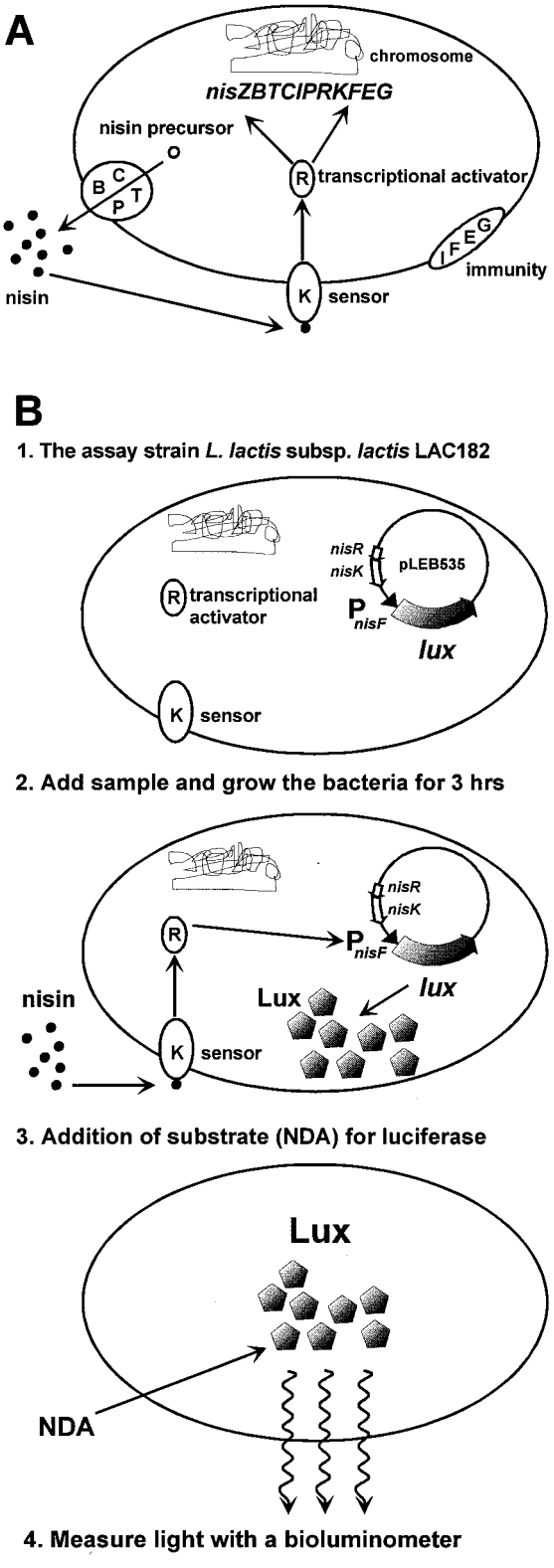

FIG. 1. (A) Schematic presentation of the autoregulation of nisin biosynthesis in *L. lactis* subsp. *lactis* N8 by signal transduction. Nisin is modified and secreted by the biosynthetic machinery (BCTP). Extracellular nisin activates the histidine kinase NisK, which is autophosphorylated. NisK then phosphorylates the response regulator NisR, which activates the transcription from the promoters upstream of *nisZ* and *nisF*. The nisin immunity system (IFEG) is present to protect the nisin producer, by an unknown mechanism, from being killed by nisin. (B) Schematic presentation of the nisin bioluminescence assay based on nisin signal transduction coupled to luciferase production. The sample is added to cells of LAC182, which are grown for 3 h. If the sample contains nisin, it will activate the *nisF* promoter upstream of the *luxHb* gene in plasmid pLEB535, resulting in production of luciferase. Addition of the luciferase substrate *n*-decyl-aldehyde (NDA) to the cells results in emission of light, which is measured by a bioluminometer.

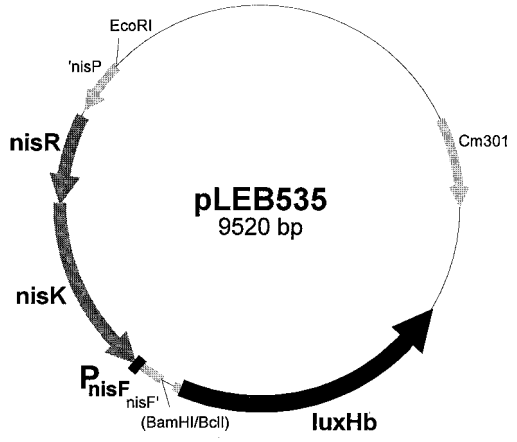

FIG. 2. Physical map of the nisin bioluminescence assay plasmid pLEB535.

University of Helsinki, Finland). Growth was measured as OD_{600} by using an UltrospecII spectrophotometer (Pharmacia LKB).

Background and nisin-induced luciferase activity. Luciferase background activity was detectable 1 h after initiation of growth and increased slowly for the next 4 h. Between 5 and 6 h after initiation of growth (OD_{600} of 0.3 to 0.5), the background luciferase activity increased rapidly. After 6 h, before the bacteria reached the stationary phase, the activity dropped suddenly, so that by 8 h the activity was the same as at the beginning of the growth. The luciferase activity in induced (0.01 IU of nisin/ml, final assay concentration) bacteria showed a steady increase from the beginning of growth, reaching its maximum level at 6 h, after which it dropped. After 3 h of growth, before an OD_{600} of 0.1 was reached, the ratio of induced luciferase activity to uninduced was highest; therefore, this time point was chosen for the nisin bioluminescence assay. However, an increase in luciferase activity of induced cells compared with the control could already be detected after 1 h of growth, showing that this bioassay could be used to determine rapidly whether a sample contains nisin (data not shown).

When luciferase activity was induced with different amounts of nisin, the presence of Tween 80 proved to be crucial. Nisin is an amphipathic molecule with a high tendency to adhere to various surfaces. This may cause a large loss of nisin, especially when low concentrations are being used. The aggregation and adsorption of nisin can be prevented by addition of Tween 80 (22). Without Tween 80, the lowest concentration of nisin that could be detected as an increase in luciferase activity compared with the control was 0.01 IU/ml. When 0.1% Tween 80 was included in the diluent, the detection limit decreased to 0.0005 IU/ml, corresponding to 0.0125 ng/ml. This indicates that 95% of the nisin was lost during dilution without Tween 80. In both cases, the luciferase activity increased linearly with nisin concentration until enough nisin was present to exert its inhibitory effect on the indicator bacteria. In the presence of 0.1% Tween 80, the highest luciferase activity was reached at a nisin concentration of 0.03 to 0.07 IU/ml (0.75 to 1.75 ng/ml) (Fig. 3).

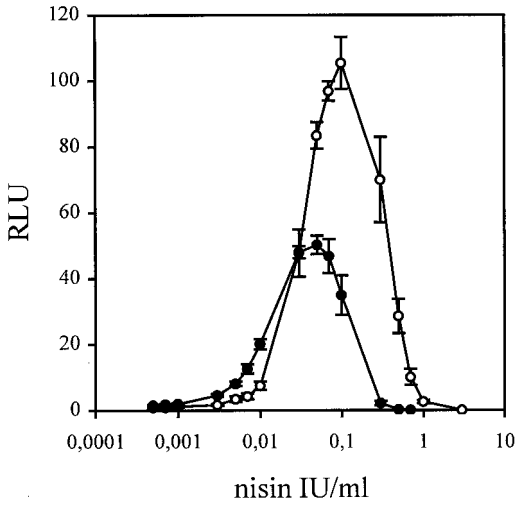

FIG. 3. Luciferase activity recorded as relative light units (RLU) of *L. lactis* subsp. *lactis* LAC182 cells induced for 3 h with different amounts of nisin diluted in 0.1% Tween 80 (●) or milk (○) with or without 0.1% Tween 80. The nisin concentrations are given as final assay concentrations. The result shown is the mean and standard deviation from five experiments.

After the 3-h growth period chosen for the bioassay, the maximum luciferase activity of nisin-induced cells was 40 to 50 times higher than the activity of the control cells. Induction with nisin concentrations higher than 0.3 IU/ml (7.5 ng/ml) gave lower luciferase activity values than those of the control, due to inhibition of the indicator bacteria by nisin.

To measure the induction in response to nisin A compared with nisin Z, we analyzed supernatants from the nisin A producer *L. lactis* ATCC 11454 (American Type Culture Collection, Rockville, Md.) and the nisin Z producer *L. lactis* N8 (16). The same amounts of nisins A and Z, as estimated from the MIC (7) determined in the agar diffusion assay (27), gave approximately the same response in the bioluminescence assay (data not shown). However, nisin A resulted in an approximately 25% higher maximum luciferase activity than nisin Z did. This indicates that in this assay nisin A is either a more efficient inducer or a less efficient killer than nisin Z.

To detect possible cross-reactions of other bacteriocins, we tested the bioassay on supernatants from the subtilin producer *Bacillus subtilis* ATCC 6633 (American Type Culture Collection) the carnocin producer *Carnobacterium piscicola* LMG233 (Valio Ltd., Helsinki, Finland), and the sakacin A producer *Lactobacillus sake* Lb706(pSAK27) (1). None of the tested bacteriocins, including subtilin, the structurally closest homolog of nisin, gave a positive response in the bioluminescence assay (data not shown).

Detection of nisin in milk. The applicability of the bioluminescence assay to the detection of trace levels of nisin in food samples was tested by using milk (homogenized and pasteurized, 1.5% fat). Nisin was diluted in milk with or without 0.1% Tween 80, and different amounts of nisin in 70 μl of milk were mixed with the indicator bacteria; otherwise the samples were processed as described above. We observed that no nisin was lost when milk was used as the diluent. The background luciferase activity of the indicator bacteria was not affected by the presence of milk; instead, larger amounts of nisin could be added without inhibition of the bacteria. The sensitivity was slightly reduced in the presence of milk. The lowest concentration in each experiment that clearly resulted in an increase in luciferase activity was 0.003 IU/ml (0.075 ng/ml) (Fig. 3).

TABLE 1. Identification of nisin-producing strains by the nisin bioluminescence assay

Strain	Luciferase activity[a] at:			Nisin producer
	1:1	1:100	1:10,000	
Lactobacillus sp.	0.2	1.0	1.0	−
Pediococcus sp.	1.0	0.9	1.1	−
Lactococcus sp.	0.9	1.0	1.0	−
Nisin Z producer	60.5	1.7	1.1	+
Nisin A producer	<0.1	16.2	1.2	+
Variant of nisin A	<0.1	15.4	1.0	+
Unknown test microorganism	<0.1	17.6	1.1	+

[a] The values indicate the ratio of the mean activities of the diluted duplicate supernatants compared with that of the uninduced indicator strain LAC182.

Identification of nisin-producing strains by the nisin bioluminescence assay. Screening programs aimed at finding new bacteriocins result in the isolation of bacteria that produce antimicrobial substances. It is of value to rapidly and easily identify previously described bacteriocins in order to be able to concentrate efforts on novel ones. Because nisin is a broad-spectrum bacteriocin and is produced by many strains of *L. lactis*, it has been repeatedly detected. Therefore, a rapid and simple identification test for nisin is needed.

We conducted a test in a blind manner on strains provided by Quest Ltd. (Bussum, The Netherlands). Seven randomized samples were shipped in duplicate as supernatants and stored at 8°C for 14 days before being tested. The samples were diluted 1:100 and 1:10,000 in 0.1% Tween 80. For nisin detection, 5 μl of the supernatants and the dilutions were mixed with indicator bacteria and processed as described above. The nisin producers were identified correctly among the unknown strains (Table 1). Higher luciferase activity than in the control was obtained from the nisin producers only, including the unknown test microorganism. The undiluted supernatant from the *Lactobacillus* strain reduced the luciferase activity, as did three of the supernatants containing nisin. Diluting the *Lactobacillus* supernatant did not cause induction, which proves that the inhibitory substance is not nisin.

In conclusion, the nisin bioluminescence assay can be used to quantitate nisin, to detect nisin in milk, and to identify strains that produce either nisin A or nisin Z. The overall response range (given as the final assay concentration) in the bioassay is 0.0005 to 0.3 IU/ml, corresponding to 0.0125 to 7.5 ng/ml. In milk, the detection range is 0.003 to 1 IU/ml (0.075 to 25 ng/ml), meaning that undiluted milk samples containing 0.04 to 1,000 IU/ml (0.001 to 25 μg/ml) can be assayed without inhibition of the indicator strain. The sensitivity is thus higher than that of the traditional agar diffusion assay and most of the immunoassays described previously (2, 13, 32, 33). Previously described methods for detection of nisin in milk include pretreatment of the milk samples. Still, the sensitivity remained quite low compared with the detection limit for pure nisin (2, 29). The major advantages of the above-described bioluminescence assay for detection of nisin in milk are that it is much less complicated and far more sensitive. The fact that large dilutions are required in some cases before the detection is possible is an advantage in instances where interfering substances might be present. To test undiluted samples containing more than 300 IU of nisin/ml, a more resistant indicator strain is needed. Since the nisin A promoter was inducible by nisin in a linear dose-response relationship in several heterologous hosts (10, 23), transfer of the system into another species could provide a way of overcoming the present limitations. The tra-

ditional agar diffusion assay measures the inhibitory activity of nisin, while this bioluminescence assay measures the inducing activity. These two activities do not necessarily correlate for a given nisin molecule (9, 24). A bioassay based on the induction capacity is therefore an important complement when engineered nisin molecules are being characterized. The simplicity of our method makes it an ideal system for automation of nisin quantification. Because an increase in the luciferase activity is detected after 1 h, it could be used as an on-line method of detection of nisin concentrations in fermentation experiments.

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