J. Microbiol. Biotechnol. (2005), **15**(5), 1152–1157

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

The Effect of Carbon Sources on Nisin Z Biosynthesis in *Lactococcus lactis* subsp. *lactis* A164

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Received: February 18, 2005
Accepted: April 27, 2005

Abstract The effect of carbon sources on nisin Z biosynthesis in *Lactococcus lactis* subsp. *lactis* A164 was studied in batch culture using M17 broth containing different carbon sources. Among the eleven carbon sources tested, glucose, sucrose, and lactose were suitable carbon sources for cell growth of *L. lactis* A164. In particular, cells grown on lactose produced at least 3-fold greater amount of nisin Z than those on other carbon sources. Galactose resulted in less amount of cell mass than did sucrose or glucose, but gave a higher level of nisin Z activity. Northern blot analysis revealed that lactose increased the transcription of the *nisZ* pre-peptide gene. Although galactose was less efficient than lactose, it increased the transcription of *nisZ* along with a higher level of nisin Z than did sucrose and glucose. These results suggest that the increased nisin Z production is correlated with the induction of *nisZ* by lactose and galactose. Among all the carbon sources tested, no remarkable differences were observed in *nisRK* and *nisFEG* transcripts, indicating that the lactose- or galactose-mediated induction is unique to the *nisZ* promoter.

Key words: Carbon source, *Lactococcus lactis*, nisin Z, nisin biosynthesis, Northern hybridization

Many lactic acid bacteria (LAB) and other Gram-positive bacteria produce small antimicrobial and proteinaceous compounds, named bacteriocin, that typically kill or inhibit the growth of closely related bacteria [18]. Nisin is a bacteriocin, which belongs to a group of lantibiotics and is produced by several strains of *Lactococcus lactis* [16, 23, 25]. It is of special interest because of its widespread use as a natural food preservative [14, 32]. Two natural variants of nisin, nisin A and Z, have been described [24]. They have a similar structure and antimicrobial spectrum, but they differ in a single amino acid residue at position 27: histidine in nisin A and asparagine in nisin Z.

A cluster of 11 genes has been shown to be involved in the complex biosynthesis of nisin and proposed to be transcriptionally arranged as *nisABTCIP*, *nisRK*, and *nisFEG*. Of these genes, the *nisA/Z* gene encodes nisin A/Z pre-peptide consisting of 57 amino acids, *nisFEG* encodes a putative ABC exporter involved in nisin extrusion [11, 34], and *nisR* and *nisK* encode a response regulator [37] and a sensor of the histidine protein kinase family [9, 15, 34], respectively, and these genes belong to a class of two-component regulatory systems [36]. It is well known that nisin-mediated induction occurs via the NisRK two-component regulatory system. Small amounts of fully modified nisin activate the induction of *nisA* and *nisF* promoters by the two-component system, whereas the *nisR* promoter is constitutive [8, 13, 19, 20, 22, 31, 37].

In a previous study, we reported the effects of growth parameters on the fermentative production of nisin Z by *L. lactis* A164 and achieved a more efficient nisin Z production/purification process [3, 4]. We also found that nisin Z production in *L. lactis* A164 was significantly improved by increasing the copy number of *nisRK* or *nisFEG* involved in nisin Z biosynthesis [5].

In this study, we investigated the influence of various carbon sources on nisin Z production in *L. lactis* subsp. *lactis* A164 isolated from kimchi. The induction of *nisZ*, *nisR*, or *nisF* by carbon sources such as lactose, glucose, galactose, and sucrose was also studied by Northern blot analysis.

The nisin Z-producing strain used in this study was *Lactococcus lactis* subsp. *lactis* A164 isolated from kimchi [6]. *L. lactis* A164 was grown in M17 medium (Difco)

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supplemented with 0.5% carbon source (w/v) at 30°C without aeration and pH control. *Weissella paramesenteroides* ATCC33313 and *Micrococcus luteus* ATCC10240 (*Micrococcus flavus* DSM1790) were used as the bacteriocin sensitivity indicator strains. *W. paramesenteroides* and *M. luteus* were grown in Lactobacilli MRS medium (Difco) and BHI medium (Difco) at 30°C, respectively. Nisin Z activity was assayed by a modification of the critical dilution micro-method [3, 7]. The correspondence between arbitrary units (AU ml^{-1}) and international units (IU ml^{-1}) was determined by using Nisaplin (Aplin & Barrett Ltd., Beaminster, U.K.) as described [3]. Based on a calibration curve between AU ml^{-1} and IU ml^{-1}, 1.92 AU was found to correspond to 1 IU (40 IU=1 μg of pure nisin A).

Optimal Carbon Source on Cell Growth and Nisin Z Production

Eleven different carbon sources (xylose, arabinose, glucose, fructose, galactose, mannose, sucrose, maltose, lactose, raffinose, and starch) were compared for the cell growth and nisin Z production by *L. lactis* A164 (Fig. 1), and each carbon source was added to M17 broth at the 0.5% level (w/v). After 12-h of incubation, glucose, sucrose, and lactose were found to be the most suitable carbon sources for cell growth of *L. lactis* A164; however, lactose produced about 3–4-fold greater amount of nisin Z than glucose and sucrose. Galactose resulted in less amount of cell mass than sucrose or glucose, but higher levels of nisin Z activities. Four carbon sources including lactose, sucrose, glucose, and galactose were selected and further tested for the cell growth and nisin Z production during the course of cultivation as well as through transcriptional analysis by Northern hybridization.

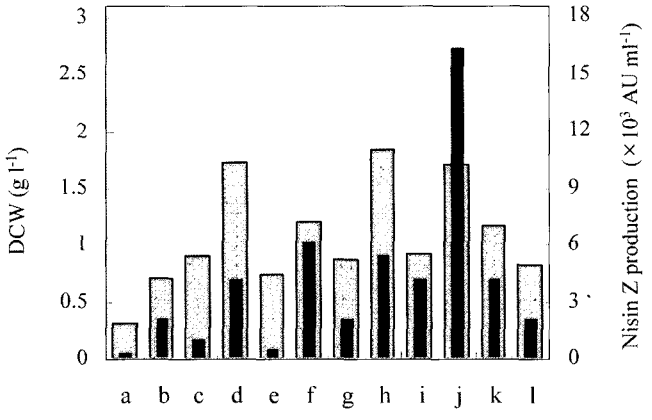

Fig. 1. Influence of various carbon sources on the cell growth (☒) and nisin Z production (■) of *L. lactis* A164 in static flask cultures after 12 h of incubation at 30°C.

a, Control (M17 broth without carbon source); b, xylose; c, arabinose; d, glucose; e, fructose; f, galactose; g, mannose; h, sucrose; I, maltose; j, lactose; k, raffinose; l, starch. Data bars are means of triplicates. Standard errors were less than 5.0% of the means.

Effect of Different Carbon Sources on Nisin Z Biosynthesis

The effects of four different carbon sources on the cell growth and nisin Z production were evaluated using M17 broth supplemented with 0.5% sucrose, lactose, glucose, or galactose. Figure 2(a) shows the growth profile of cells grown on four different carbon sources. Of the four carbon sources, the highest cell mass (1.84 g l^{-1}) was observed in cells grown on sucrose. Cells grown on glucose (1.73 g l^{-1}) or lactose (1.72 g l^{-1}) showed a similar growth profile, yielding

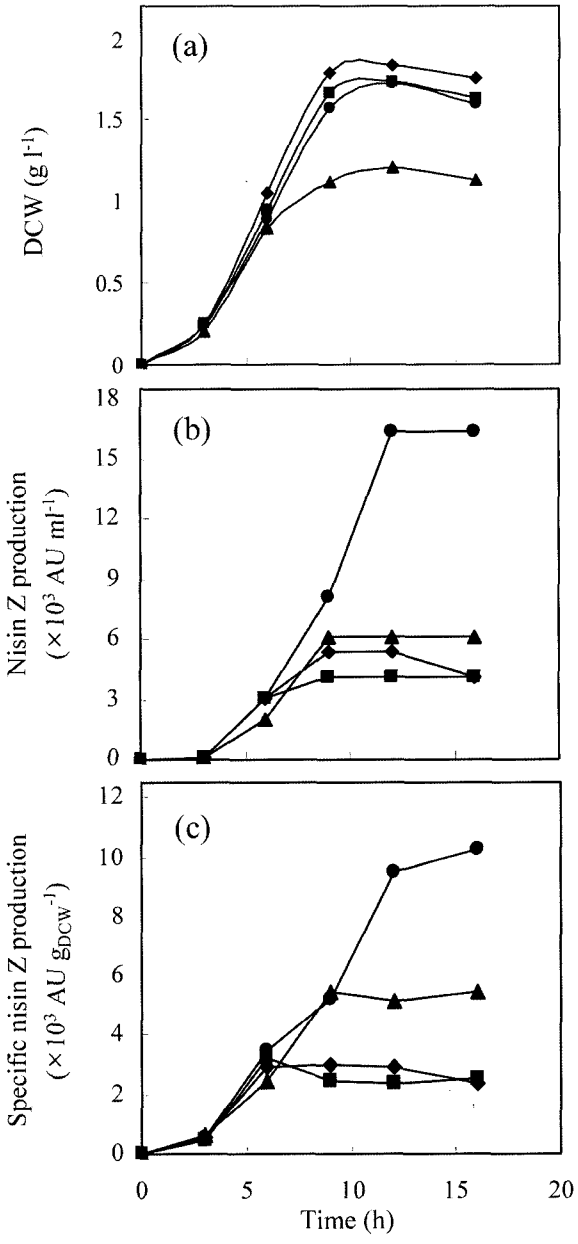

Fig. 2. Influence of sucrose, lactose, glucose, and galactose on the cell growth (a), nisin Z production (b), and specific nisin Z production (c) by *L. lactis* A164 in static flask cultures at 30°C.

◆, Sucrose; ■, glucose; ●, lactose; ▲, galactose. Data points are means of triplicates. Standard errors were less than 5.0% of the means.

slightly less cell mass than those on sucrose. However, galactose (1.20 g l^{-1}) resulted in a significant decrease in cell densities during the course of cultivation. In all cells, maximum cell densities were reached after 9–11 h of cultivation time. The growth profiles showed a slight decrease in cell mass during the stationary growth phase (12 to 16 h).

As shown in Fig. 2(a), sucrose resulted in the highest cell mass; however, lactose produced about 3–4-fold greater amount (16,384 AU ml^{-1}) of nisin Z than sucrose (5,402 AU ml^{-1}) and glucose (4,096 AU ml^{-1}) (Fig. 2b). Although cells grown on galactose yielded the lowest cell mass, they produced slightly higher amounts (6,146 AU ml^{-1}) of nisin Z than those grown on sucrose or glucose. In the case of specific nisin Z production, lactose and galactose produced about 3–4-fold and 2-fold greater amounts of nisin Z per cell mass, respectively, than sucrose and glucose (Fig. 2c). Consistent with previous studies [3, 26, 27], nisin Z production by strain A164 was growth-associated: 1) the activity of nisin Z was detectable from the beginning of growth; 2) during the exponential growth phase, there was a strong increase in nisin Z production; 3) the highest activity was reached at the end of the growth phase where the maximal biomass was observed; and 4) nisin Z production completely stopped when cells entered the stationary phase. It has been previously suggested that nisin production is linked to cell growth, and an increase of growth rate is expected to increase the rate of nisin production [10, 17]. However, in our study, nisin production was not directly proportional to the biomass formation, suggesting that nisin production could rather be linked to the expression level of genes involved in nisin biosynthesis. Our data indicate that lactose is the most suitable carbon source for nisin Z production. It seems likely that lactose somehow increases the transcription of *nisZ* and probably other gene(s) involved in nisin Z expression.

Transcriptional Analysis of the Genes Involved in Nisin Biosynthesis

To determine whether carbon sources affect the induction of genes involved in nisin biosynthesis, total RNA from sucrose-, lactose-, glucose-, and galactose-grown *L. lactis* A164 cells was analyzed by Northern hybridization (Fig. 3).

L. lactis cultures were grown in M17 broth supplemented with 0.5% different carbon sources (sucrose, lactose, glucose, or galactose) to the late exponential growth phase and harvested by centrifugation (5 min, 5,000 ×*g*, 4°C). For isolation of *L. lactis* total RNA, the RNeasy mini kit (Qiagen Inc., Valencia, CA, U.S.A.) was used according to the manufacturer's procedure. For Northern hybridization, about 20 mg of total RNA per sample was denatured by treatment with formamide and size fractionated on a 1% agarose gel containing 2.2 M formaldehyde, according to standard procedures [33]. The gel was transferred by capillary blotting

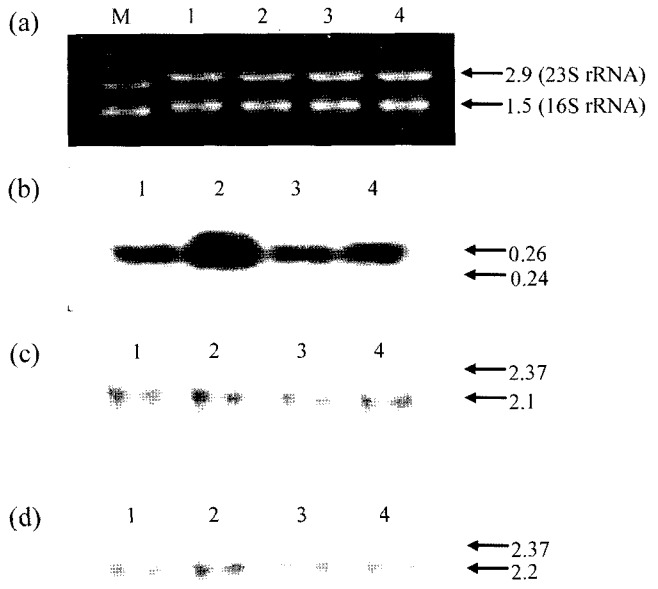

Fig. 3. Formaldehyde agarose gel electrophoresis (a) and Northern blot analysis using total RNA from sucrose-, lactose-, glucose-, and galactose-grown *L. lactis* A164 cells with the *nisZ* (b), *nisR* (c), and *nisF* (d) specific probe.
As a molecular weight marker, the 0.24–9.5-kb RNA ladder (Life Technologies; a, lane M) was used. (a–d) Lanes 1, sucrose-grown A164; 2, lactose-grown A164; 3, glucose-grown A164; 4, galactose-grown A164. Molecular sizes (in kilobases, kb) and positions of the 23S and 16S rRNAs are indicated on the right. The experiments were done in triplicate with freshly isolated RNA. Only minor variation in the results was obtained.

to a Hybond N+ membrane (Amersham Biosciences, U.K.). Labeling of the probes, hybridization, and signal detection were performed according to the manufacturer's recommendations with the ECL nucleic acid detection kit (Amersham Biosciences, U.K.). The probes (160 bp for *nisZ*, 400 bp for *nisR*, and 460 bp for *nisF*) used in Northern hybridizations were made by PCR with primer pairs NISZ-UP/NISZ-DO, NISR-UP/NISR-DO, and NISF-UP/NISF-DO, respectively (Table 1).

Hybridization with a *nisZ* gene probe generated clear *nisZ* transcripts of approximately 0.26-kb, corresponding well with the one described previously [21]. The most intense transcript was detected in the lactose-grown culture (Fig. 3b). Although galactose produced less intense transcript than lactose, it resulted in more intense transcript than sucrose and glucose. Hybridization with the *nisRK*- and *nisFEG*-specific probes revealed one weak transcript of approximately 2.1-kb and 2.2-kb size, respectively (Figs. 3c, 3d), which agreed with the expected sizes of a *nisRK* and a *nisFEG* transcript [31]. No remarkable differences were observed in *nisRK* and *nisFEG* transcripts among the four different carbon sources. Northern blot analysis indicated that lactose and galactose intensified induction of the *nisZ* promoter, thereby contributing to increase of nisin production.

Table 1. Oligonucleotide primers used in this study.

Primer	Sequence (5'→3')*	Application
NISZ-UP	AAGATTTTAACTTGGATTTGGTATC	Amplification of *nisZ* probe for Northern hybridization
NISZ-DO	GCTTACGTGAATACTACAATTACAAG	Amplification of *nisZ* probe for Northern hybridization
NISR-UP	GGATTCGCAGAGAAATATCAACTC	Amplification of *nisR* probe for Northern hybridization
NISR-DO	ATACTCCGAGATTGACCGAAAAAG	Amplification of *nisR* probe for Northern hybridization
NISF-UP	CTGGAACAGTCTGTGGTTTATTAGG	Amplification of *nisF* probe for Northern hybridization
NISF-DO	GACTAGAAATCAAGATTGTCACACC	Amplification of *nisF* probe for Northern hybridization

In the current study, lactose was found to greatly activate the transcription of the *nisZ* gene, whereas galactose was somewhat less efficient than lactose, providing evidence of induction of the *nisZ* promoter by lactose and galactose. Cells grown in sucrose and glucose generated greater cell mass than those in galactose, but yielded lower levels of nisin Z activity along with weaker *nisZ* transcripts, suggesting that sucrose or glucose could not induce the *nisZ* promoter.

The increase of *nisZ* gene transcription by lactose can occur by activation of *nisZ* or *nisRK* promoters. Northern blot analysis showed no remarkable differences in *nisRK* and *nisFEG* transcripts among the various carbon sources. This indicates that the lactose- or galactose-mediated induction is unique to the *nisZ* promoter. Therefore, the most intense pre-nisin Z transcript with the highest level of nisin Z activity observed in lactose-grown cells could be due to an efficient induction of the *nisZ* promoter by lactose, along with a constitutive NisRK-dependent induction.

Recent studies [1, 2] with a transcription fusion of the *nisA* promoter and a *lacZ* reporter gene showed that the *nisA* promoter was induced by galactose and lactose in *L. lactis* LM0230, even in the absence of *nisRK*-mediated signal transduction. The above authors suggested that galactose could be a primary inducer, because a higher level of induction was observed in the presence of galactose than lactose. They also proposed that glucose or a glucose metabolite formed by lactose metabolism might hamper the full induction capacity of galactose. However, in our study, pre-nisin Z transcripts were produced to a greater level by lactose than galactose, suggesting that lactose itself or its metabolite could induce the *nisZ* promoter more efficiently than galactose. This difference could be explained by the different strains used in each study. The strain used in this study is *L. lactis* A164 isolated from kimchi, which contains a complete cluster of 11 genes required for biosynthesis of a mature nisin in its chromosome, whereas *L. lactis* LM0230, a nisin-sensitive strain, was used in Chandrapati and O'Sullivan's studies [1, 2]. To date, the nature of the transcription regulator involved in lactose- or galactose-mediated induction is not yet clear. We are currently investigating to unravel the complexity of this regulatory mechanism that links carbohydrate utilization to nisin biosynthesis.

Recently, we found that the cells containing multicopies of *nisRK* raised the level of nisin Z by 50% in comparison with the control strain [5]. Results from Northern blot analysis indicated that overexpression of NisK and NisR proteins contributed to promote the transcription of the *nisZ* gene. In the current study, the induction of *nisZ* by lactose resulted in at least 3-fold greater amount of nisin Z than other carbon sources, suggesting that an increased induction of nisin pre-peptide gene would lead to significant improvements in the level of nisin production. Our studies provide further insight into *nisZ* promoter regulation, and the approaches can successfully be used to improve the economics of nisin production.

In this study, lactose was found to be the most suitable carbon source for nisin Z production. The production profiles of nisin Z and Northern blot analysis demonstrated that lactose intensified induction of the *nisZ* pre-peptide gene, thereby contributing to increase the level of nisin production. Furthermore, these results indicate that nisin production is associated with an ability to induce transcription of the pre-nisin gene as well as the growth rate.

Acknowledgment

This work was supported by a grant (NO. R01-2003-000-10860-0) from the Basic Research Program of the Korea Science & Engineering Foundation (KOSEF).

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