Isolation of nisin-producing Lactococcus lactis WNC 20 strain from nham, a traditional Thai fermented sausage

W. Noonpakdee,*, C. Santivarangkna, P. Jumriangrit, K. Sonomoto, S. Panyim

aDepartment of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
bLaboratory of Microbial Science and Technology, Division of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

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Abstract

A total of 14,020 lactic acid bacteria (LAB) were isolated from nham and screened for bacteriocin production. One Lactococcus lactis strain WNC 20 produced a bacteriocin that not only inhibited closely related LAB, but also some food-borne pathogens including Listeria monocytogenes, Clostridium perfringens, Bacillus cereus and Staphylococcus aureus. Biochemical studies revealed that the bacteriocin was heat-stable even at autoclaving temperature (121 °C for 15 min) and was active over a wide pH range (2–10). The bacteriocin was inactivated by α-chymotrypsin and proteinase K but not other proteases. The antimicrobial spectrum and some characteristics of this bacteriocin were nearly identical to that of nisin. The gene encoding this bacteriocin was amplified by polymerase chain reaction (PCR) with nisin gene-specific primer. Sequencing of this gene showed identical sequences to nisin Z as indicated by the substitution of asparagine residue instead of histidine at position 27. The ability of the bacteriocin produced by Lc. lactis WNC 20 may be useful in improving the food safety of the fermented product.

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1. Introduction

Lactic acid bacteria (LAB) are widely used in food fermentation including dairy, meat, vegetable and bakery products (Marrug, 1991). They are known to produce many different antibacterial substances including bacteriocins, which can inhibit the growth of several undesirable Gram-positive bacteria in the genera Bacillus, Enterococcus, Listeria, Clostridium and Staphylococcus (Tagg et al., 1976; Klaenhammer, 1993). Many bacteriocins have been isolated and there is increasing interest in using these bacteriocins as natural food preservatives (Jack et al., 1995). The bacteriocin nisin, which is produced by Lactococcus lactis subsp. lactis, has been extensively studied and used as a food preservative because of its lethal action and wide spectrum of activity (Hurst, 1981; Delves-Broughton et al., 1996). Two nisin variants, which differ only by one amino acid substitution at position...
27, have been studied extensively. Nisin A contains histidine and nisin Z asparagine (Mulders et al., 1991). Usually, nisin producer strains are isolated from dairy products (Rauch et al., 1994), some from vegetables (Cai et al., 1997; Choi et al., 2000; Harris et al., 1992) and very few from fermented meat (Rodriguez et al., 1995).

Nham is a Thai traditional fermented sausage made from ground pork with garlic, pepper, salt and cooked rice. It can be consumed raw or cooked. This fermented meat product has developed to full-scale commercialization, but the technology of fermentation is still mediated by indigenous bacteria rather than added starter cultures. The bacteria found most commonly in Thai fermented meat products are lactobacilli, pediococci and micrococci, but the precise role of these bacteria in the quality of the products is not known (Thiravattanamontri et al., 1998). Developments in this meat fermentation have been focusing on application of defined starter cultures to increase process control and product consistency (Campbell-Platt, 1995). In addition to difficulties in obtaining product consistency, pathogens in raw fermented sausage still cause food safety problems. Listeria monocytogenes, which has been frequently recovered from fermented sausage (Johnson et al., 1990; Aymerich et al., 1998), is of particular concern because it results in a high mortality rate and of its ability to grow at low pH (Gahan et al., 1996).

The use of bacteriocin-producing strains as starter cultures or protective cocultures in the in situ control of food pathogens is one of the possible ways to improve food safety (Kim, 1993; Holzapfel et al., 1995; Stiles, 1996; Caplice and Fitzgerald, 1999; Hugas, 1999). Cultures used in fermentation of meat are mostly Lactobacillus (Sobrino et al., 1991; Garriga et al., 1993; Hugas et al., 1995; Swetwiwatthana et al., 1999) and Pediococcus (Bhunia et al., 1988; Harris et al., 1989). However, the isolation of nisin-producing Lactococcus strains from dry fermented sausage (Rodriguez et al., 1995) indicated the potential use of lactococci in meat fermentation systems. More recently, the use of bacteriogenic Lactococcus strains in salami and sausage production has been successfully developed (Coffey et al., 1998; Scannell et al., 2001). This study reports the detection and isolation of a nisin Z-producing Lc. lactis strain (WNC 20) isolated from a Thai fermented pork sausage (nham).

2. Materials and methods

2.1. Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. All LAB isolates and indicator strains were grown in MRS broth (Merck) at 30 or 37 °C. Listeria, Salmonella, Bacillus were grown in tryptic soy broth (Difco) at 30 °C. Enterococcus and Staphylococcus were grown in nutrient broth (Difco). Escherichia coli was grown in Luria–Bertani (Difco) medium at 37 °C. Clostridium was grown under anaerobic conditions in reconstituted Clostridium medium (E. Merck) at 30 °C. All cultures were maintained as frozen stocks at -80 °C in appropriate broth containing 20% glycerol (wt/vol). Throughout the experiments, strains were subcultured every 2 weeks on agar media and kept at 4 °C. Before use in experiments, cultures were propagated twice in broth overnight. Soft agar media were prepared by adding 0.6% (wt/vol) agar to liquid media. Catalase at a final concentration of 50 U ml (Boehringer Mannheim) was added to soft agar overlays to eliminate the potential inhibitory effect of H₂O₂ produced by colonies.

2.2. Isolation of bacteriocin-producing bacteria and bacteriocin assay

Bacteriocin-producing bacteria were isolated by the direct plating method (Coventry et al., 1997). A 10% food sample in diluent (0.85% NaCl) was homogenized and 10-fold serially diluted. Pour plates of serial dilutions (1-ml aliquots) in media MRS were incubated under anaerobic conditions (anaerobic genera tion kit; Merck) for 48 h at 30 °C. Multiple plates of serial dilution (three to five plates providing a total of approximately 1000 colonies) were overlaid with a set of four indicators and incubated at 30 °C for another 18–24 h. The four indicator strains used were Lactobacillus curvatus ATCC 25601, Lactobacillus plantarum TISTR 850, Pediococcus pentosaceus TISTR 374 and Propionibacterium freudenreichii TISTR 446. Colonies suspected to be producing zones of growth inhibition in the indicator lawn were
randomly selected and removed using a sterile Pasteur pipette. The agar plug (from Pasteur pipette) was inoculated into broth media and incubated anaerobi-
cally for 48 h at 30 °C. Culture supernate was obtained by centrifugation at 12,000 rpm for 20 min at 12 °C and then was adjusted to pH 6.5 with 5 M NaOH and filtered through a 0.45-μm filter. Inhibition was tested by spotting 10 μl of the supernatant onto soft agar lawn (0.6%) seeded with 0.1 ml of an overnight grown indicator strain and incubated overnight. Cultures producing an inhibitor in broth were then purified by streaking from the broth and restreaking for single colony isolates two to three times.

For a semiquantitative assay of the bacteriocins, twofold serial dilutions of the supernatant were tested using Lb. plantarum TISTR 850 as the indicator strain. The activity was expressed in arbitrary units (AU ml⁻¹). One AU was defined as the reciprocal of the highest serial twofold dilution, which did not show inhibition of the indicator strain per milliliter (Schillinger et al., 1993).

2.3. Identification of the bacteriocin-producing isolate

Only one isolate (designated WNC 20) of the 14,020 screened stably produced bacteriocin in broth media. Strain WNC 20 was therefore further characterized and identified on the basis of Gram stain, catalase reaction and other identification tests (Schillinger and Lucke, 1987). Carbohydrate fermentation was determined using the API 50 CHL system (Bio-
Merieux, France). Salt tolerance was tested using 4% and 6.5% NaCl in MRS medium. Total soluble whole cell proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the pattern was compared with that of reference LAB (Elliot et al., 1991).

2.4. Determination of antimicrobial spectrum

Cell-free supernatant was used to determine the antimicrobial spectrum of activity. Cells were grown in MRS broth for 20 h at 30 °C and supernatant was prepared as described above. The spectrum of activity of the supernatant was tested against a wide range of indicator strains comprising LAB and food-borne pathogens as shown in Table 1. The antimicrobial spectrum of the nisin-producing strain Lc. lactis subsp. lactis DL 11 was compared with that of the bacteriocin-producing strain isolated from nham.

### Table 1
Antibacterial spectrum of bacteriocins produced by Lc. lactis subsp. lactis WNC 20 and Lc. lactis subsp. lactis DL 11

<table>
<thead>
<tr>
<th>Indicator strain *</th>
<th>Sensitivity **</th>
<th>DL 11 ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus pumilus TISTR 061</td>
<td>++ ²⁺</td>
<td>++</td>
</tr>
<tr>
<td>Bacillus subtilis A-48</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>B. cereus IFRPD 2037</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>B. cereus ATCC 11778</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lactococcus sp. A28</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Lb. plantarum TISTR 850</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Lactobacillus sake TISTR 911</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Lactobacillus pentosus LP-711</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Lb. curvatus ATCC 25601</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Leuconostoc cremoris ATCC 19254</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Leuconostoc mesenteroides ATCC 10830</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lis. monocytenes IFRPD 2068</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lis. monocytenes DMST 7995</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lis. monocytenes DMST 2871</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Listeria innocua IFRPD 2071</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Micrococcus luteus TISTR 884</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Pediococcus acidilactici ATCC 8081</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>P. pentosaceus TISTR 374</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Pr. freudenreichii TISTR 446</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Staphylococcus aureus TISTR 118</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Staphylococcus aureus IFRPD 2034</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Enterococcus faecalis TISTR 927</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Enterococcus faecium TISTR 928</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Clostridium butyricum A-4</td>
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<td>+</td>
</tr>
<tr>
<td>Clostridium perfringens DMST 11147</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Salmonella sp. TISTR 101</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Salmonella derby A-2</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>Escherichia coli ATCC 25922</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>E. coli ATCC 43895</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection; TISTR, Thailand Institute of Scientific and Technological Research; DMST, Department of Medical Science Thailand; IFRPD, Institute of Food Research and Product Development.

** All indicators were tested for inhibition of growth as determined by a zone of growth inhibition in the indicator lawn culture.

*** DL 11 is Lc. lactis subsp. lactis, a nisin-producing strain, a gift from Dr. A.J. Hillier (Commonwealth Scientific and Industrial Research Organization, Division of Food Science and Technology, Australia).

²⁺ Inhibition zone (mm): +++ , 10 – 15 mm; ++ , 5 – 9 mm; +, 1 – 4 mm; ☐, no inhibition.

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filter-sterilized cell-free supernatant with 20 A of the following enzyme solutions at a final concentration of 1 mg/ml: proteinase K (pH 7.0; Sigma), trypsin (pH 7.0; Sigma), pepsin (pH 3.0; Merck), a-chymotrypsin (pH 7.0; Sigma), papain (pH 6.0; Sigma), a-amylase (pH 7.0; Sigma) and lipase (pH 7.0; Sigma). After 2 h of incubation at 37 °C, enzyme activity was terminated by heating at 100 °C for 5 min. Untreated samples were used as control. The residual bacteriocin activity was assayed against indicator strain Lb. plantarum TISTR 850. The sensitivity of the active substances to different pH values was estimated by adjusting the pH of the supernatant samples between 2 and 10 using 5 M NaOH or 5 M HCl. After 2 h of incubation at room temperature, the residual activity was assayed. Samples treated with proteinase K before pH adjustment were used as control to correct inhibition due to pH. To evaluate the effect of heat and pH on bacteriocin activity, the cell-free supernatant samples were heated at 100 °C for 30 min or 121 °C for 15 min under different pH conditions. The residual activity was then assayed against the indicator strain.

2.6. Production studies

The kinetics of bacteriocin production was determined by inoculating strain WNC 20 at 1% vol/vol solution into 200 ml of M17 broth (Oxoid) and incubating at 30 and 37 °C under uncontrolled pH conditions. The initial pH of the culture broth was 6.8. At appropriate intervals, 10-ml samples were removed from the cultures and analyzed for cell growth and bacteriocin activity. The growth of cells was followed by measuring the optical density (OD) at 600 nm.

2.7. DNA extraction, polymerase chain reaction (PCR) and DNA sequencing

Genomic DNA was extracted by a modification of the method of Engelke et al. (1992). The polymerase chain reaction analysis followed procedure described by Horn et al. (1991) with some modifications. The PCR amplification was carried out in a 50-A mixture in a DNA thermo cycler (Perkin Elmer Cetus Model TCI). The conditions consisted of 30 cycles of 90 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The primers were designed from nisin A structural gene, which were complementary to regions 17 bp upstream and 2 bp downstream of the coding region (Dodd et al., 1990; Mulders et al., 1991). The restriction sites EcoRI and KpnI were added at 5'end, respectively, for cloning purpose in other studies elsewhere. Forward and reverse primers were as followed:

primer 1: 5Vprimer 5GCCG GAA TTC ATA AGG AGG CAC TCA AAA TG 3V
primer 2: 3Vprimer 5CGG GGT ACC TAC TAT CCT TTG ATT TGG TT 3V

The amplified PCR products was purified from low melting point agarose and the nucleotide sequences were determined using a Dye Terminator Sequencing kit (Perkin-Elmer) and ABI PRISM 377 DNA sequencer (Perkin Elmer) as described by the instructions of the manufacturer.

3. Results

3.1. Isolation and identification of bacteriocin-producing strains

A total of 14,020 colonies from 12 nham samples were examined for potential inhibitory substance
against the four indicator strains. A total of 28 colonies were recorded as positive, producing inhibition clear zones on agar media (detection rate 0.2%). Of these colonies, only one designated WNC 20 stably secreted inhibitory substance into culture broth. This bacteriocin-producing strain WNC 20 was a Gram-positive, catalase-negative coccus, nonmotile and produced no gas from glucose. The strain hydrolyzed arginine grew at 10, 15 and 40 °C at pH 4 and 9 and in media containing NaCl at 4% but not at 6.5%. Based on these characteristics and the analysis of the carbohydrate fermentation pattern by the API CHL kit, WNC 20 was potentially identified as Lc. lactis subsp. lactis. This identification was confirmed by comparing whole cell protein pattern of strain WNC 20 on SDS-PAGE with that of reference strain Lc. lactis subsp. lactis DL 11. Both had the identical protein patterns (data not shown).

3.2. Characterization of the bacteriocin

The inhibitory spectrum of the bacteriocin produced by Lc. lactis WNC 20 is presented in Table 1. The bacteriocin exhibited inhibitory activity against a broad range of closely related bacteria in the genera Lactobacillus, Pediococcus, Leuconostoc and some patho-

<table>
<thead>
<tr>
<th>pH</th>
<th>Residual bacteriocin activity (AU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No heat (control)</td>
</tr>
<tr>
<td>3.0</td>
<td>12,800</td>
</tr>
<tr>
<td>5.0</td>
<td>12,800</td>
</tr>
<tr>
<td>7.0</td>
<td>12,800</td>
</tr>
</tbody>
</table>

Fig. 1. Growth (•) and bacteriocin production (▲) of Lc. lactis subsp. lactis WNC 20 in MRS broth with uncontrolled pH condition at 30 °C.

Fig. 2. Agarose gel electrophoresis of PCR products with nisin gene-specific primer. Genomic DNA used were from: lane 1: Lc. lactis subsp. lactis DL 11 (positive control); lane 2: Lc. lactis subsp. lactis WNC 20; lane 3: P. pentosaceus TISTR 374 (nonnisin-producing strain); lane 4: water as a negative control. Lane M was loaded with 100-bp ladder DNA markers (Gibco).
genic strains of Bacillus, Staphylococcus, Listeria and Clostridium. The bacteriocin was not effective against Gram-negative bacteria. Nisin A produced by Lc. lactis DL 11 was used as an experimental control and showed an inhibitory spectrum identical to that of Lc. lactis WNC 20. The degree of inhibition of bacteriocin produced, however, showed slight differences. Inhibition zones obtained from WNC 20 strain in agar diffusion assay were larger than those obtained from Lc. lactis DL 11 especially towards Bacillus cereus IFRPD 2037 and Lis. monocytogenes DMST 2871.

The effect of enzyme and pH on the activity of the bacteriocin produced by strain WNC 20 is presented in Table 2. Protease sensitivity assays demonstrated that the antimicrobial substance produced by WNC 20 strain was a bacteriocin since its inhibitory activity was completely eliminated by treatment with enzyme proteinase K and \( \alpha \)-chymotrypsin. The activity was, however, not inactivated by other proteases including trypsin, papain, pepsin and nonprotease enzyme including \( \alpha \)-amylase and lipase. These characteristics were similar to those of nisin. The bacteriocin was active over a wide pH range between 2 and 10, but the activity was much lower in basic pH.

The effect of heat and pH on the bacteriocin activity is shown in Table 3. Inhibitory activity was not destroyed by exposure to elevated temperature at pH 3 even at autoclaving temperature (121 \( \degree \)C for 15 min). The inhibitory activity was, however, destroyed if heated at higher pH value, a characteristic similar to nisin. Based on these results, it appeared that Lc. lactis WNC 20 produced a nisin-like bacteriocin.

Cell growth and bacteriocin production properties of Lc. lactis WNC 20 were examined in flask cultures at 30 and 37 \( \degree \)C in the noncontrolled pH condition. The production of bacteriocin was higher at 30 \( \degree \)C (data not shown). The bacteriocin was produced maximally in the early stationary phase, and the activity declined during the late stationary phase as shown in Fig. 1.

3.3. Confirmation that WNC 20 is a nisin-producing strain

To prove that the bacteriocin produced by Lc. lactis WNC 20 was nisin, PCR analysis using the published sequences of the nisin structural gene (Dodd et al., 2000).

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the nisZ gene isolated from Lc. lactis subsp. lactis WNC 20. The amino acid sequence is shown below the coding sequence. The nucleotide in the nisZ sequence that differs with that in the nisA gene sequence is in bold and italic. Primers are in bold and underline. Stop codon is shown by asterisk.
1990) was performed. Two primers complementary to sequences occurring proximal to the 3\textsuperscript{V} and 5\textsuperscript{V} ends of the nisin A structural gene were used to amplify nisin gene from the genomic DNA of Lc. lactis DL 11 and Lc. lactis WNC 20. The result is shown in Fig. 2. A 227-bp fragment was amplified from the genomic DNA of Lc. lactis WNC 20 (lane 2), which was identical to that amplified from a nisin-producing strain of Lc. lactis DL 11 (lane 1). No DNA fragment was amplified using genomic DNA extracted from P. pentosaceus TISTR 374, which did not produce bacteriocin (lane 3), or no DNA was present (lane 4). The amplified PCR product of Lc. lactis WNC 20 was subsequently sequenced as shown in Fig. 3. Results indicated that sequences were 100% identical to that of nisin A except for a C-to-A transversion at position 148. This resulted in an asparagine (AAT) residue at position 27 of the nisin peptide, instead of histidine (CAT). This indicates that the bacteriocin produced by Lc. lactis WNC 20 is nisin Z.

4. Discussion

The aim of this current study was to isolate and characterize bacteriocin-producing LAB from nham, a traditionally fermented pork sausage which is usually consumed without cooking in various parts of Thailand. This fermented meat product is typically produced on a small scale or at household levels and is sometimes associated with problems such as product short shelf-life and poor hygiene. The use of starter cultures is not a common practice, resulting in product failure and inconsistency. Moreover, the presence of food spoilage and pathogenic bacteria in nham sometimes poses problems (Swetwivatthan, et al., 1999). The bacteriocin-producing Lc. lactis WNC 20 could prove to be useful as a starter culture or protective culture in fermented meat and other fermented food products as well.

Few strains of Lc. lactis have been reported to be isolated from meat substrates even though meat environments have been considered adequate for the growth of lactococci (Garver and Muriana, 1993; Rodriguez et al., 1995; Barakat et al., 2000). Garver and Muriana (1993) isolated Lc. lactis FS92 from meat products and Rodriguez et al. (1995) isolated two nisin-producing Lc. lactis strains from dry fermented sausages from different regions of Spain. Recently, Barakat et al. (2000) isolated one strain of Lc. lactis from cooked, modified atmosphere-packaged, refrigerated, poultry meat. Our isolation of nisin-producing Lc. lactis WNC 20 and those two strains from Rodriguez et al. (1995) suggests that nisin-producing Lc. lactis strains may be more widespread in meat products than previously reported. The detection rate of bacteriocin-producing strains from total number of colonies tested in this study was about 0.2%, which was comparable with previous report by Rodriguez et al. (1995) where less than 0.5% of 4608 LAB examined was a bacteriocin producer and identified as Lc. lactis. Both results indicate that in meat substrates, this species may only be present in very low number.

The antimicrobial substance produced by Lc. lactis WNC 20 strain had similar enzymatic pH sensitivity and heat insensitivity pattern to nisin. The identical antimicrobial spectrum and larger inhibition zones in agar diffusion assay of bacteriocin obtained from WNC 20 as compared with those from DL 11, a nisin A-producing strain, suggested that the bacteriocin produced by WNC 20 was nisin Z (De Vos et al., 1993). This was confirmed by sequence analysis of nisin structural gene of WNC 20 (Fig. 3), which contained an asparagine at position 27 instead of histidine as in nisin A (Mulders et al., 1991). This finding that WNC 20 strain produced nisin Z is different from the previous report by Rodriguez et al. (1995) where both Lactococcus strains isolated from dry fermented sausages produced nisin A.

Many studies have shown that the properties of fermenting sucrose and nisin production were closely linked and the genes involved on these traits might be located on the conjugative transposon on the chromosome (Horn et al., 1991; Rauch and De Vos, 1992; Olasupo et al., 1999). The capacity of strain WNC 20 to ferment sucrose suggested that this nisin Z gene might be located on a conjugative transposon on the chromosome.

The relative sensitivity of Lc. lactis to acid would suggest that these organisms have a minor role in meat fermentation. However, they could be used at early stages of meat fermentation or together with other more acid-tolerant, nisin-resistant meat starter cultures. Moreover, Lc. lactis WNC 20 could become...
more acid-tolerant as a result of a pH adaptation process similar to those reported for enteric bacteria (Foster and Hall, 1990). At present, studies are in progress to determine the properties of this WNC 20 strain as a starter culture in meat fermentation system.

Acknowledgements

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