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Characterization of Two Nisin-Producing *Lactococcus lactis* subsp. *lactis* Strains Isolated from a Commercial Sauerkraut Fermentation

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Two *Lactococcus lactis* subsp. *lactis* strains, NCK400 and LJH80, isolated from a commercial sauerkraut fermentation were shown to produce nisin. LJH80 was morphologically unstable and gave rise to two stable, nisin-producing (Nip$^+$) derivatives, NCK318-2 and NCK318-3. NCK400 and derivatives of LJH80 exhibited identical morphological and metabolic characteristics, but could be distinguished on the basis of plasmid profiles and genomic hybridization patterns to a DNA probe specific for the iso-ISS*1* element, IS*946*. NCK318-2 and NCK318-3 harbored two and three plasmids, respectively, which hybridized with IS*946*. Plasmid DNA was not detected in NCK400, and DNA from this strain failed to hybridize with IS*946*. Despite the absence of detectable plasmid DNA in NCK400, nisin-negative derivatives (NCK402 and NCK403) were isolated after repeated transfer in broth at 37°C. Nisin-negative derivatives concurrently lost the ability to ferment sucrose and became sensitive to nisin. A 4-kbp *Hin*dIII fragment containing the structural gene for nisin (*spaN*), cloned from *L. lactis* subsp. *lactis* ATCC 11454, was used to probe genomic DNA of NCK318-2, NCK318-3, NCK400, and NCK402 digested with *Eco*RI or *Hin*dIII. The *spaN* probe hybridized to an 8.8-kbp *Eco*RI fragment and a 10-kbp *Hin*dIII fragment in the Nip$^+$ sauerkraut isolates, but did not hybridize to the Nip$^-$ derivative, NCK402. A different hybridization pattern was observed when the same probe was used against Nip$^+$ *L. lactis* subsp. *lactis* ATCC 11454 and ATCC 7962. These phenotypic and genetic data confirmed that unique Nip$^+$ *L. lactis* subsp. *lactis* strains were isolated from fermenting sauerkraut.

The succession of specific lactic acid bacteria during the natural fermentation of vegetables is dependent on the chemical (substrates, salt concentration, pH) and physical (vegetable type, temperature) environments. Reports of broad-spectrum, bacteriocin-producing lactic acid bacteria isolated from naturally fermented vegetables indicate that antimicrobial proteins also play a role in the ecology of traditionally fermented foods (2, 5, 6, 11, 21). The specific roles and culture interactions of bacteriocin-producing strains in natural ecosystems remain undefined. However, bacteriocin-producing starter cultures capable of growth in vegetable brines may have a competitive advantage that could be exploited in the development of commercial starter cultures for fermented vegetable products.

Nisin, produced by some strains of *Lactococcus lactis* subsp. *lactis*, is a broad-spectrum bacteriocin with enhanced antimicrobial activity and stability at low pH (19). These properties make nisin a good candidate for use as an antimicrobial agent in fermented vegetables. Historically, *L. lactis* subsp. *lactis* has not been associated with vegetable fermentations, although this species can be isolated in small numbers from raw plant material (33, 38). However, much of the early work on the ecology of "lactic streptococci" is of limited value because of difficulty in growing these organisms on selective media (32) and failure to differentiate them from fecal streptococci (16). Lack of isolation of *L. lactis* subsp. *lactis* from fermented vegetables may have been due to a failure to look for these strains.

Large numbers of *L. lactis* subsp. *lactis* organisms were found in the initial stages of a sauerkraut fermentation; however, it was not determined whether these strains produced nisin (42). A nisin-producing (Nip$^+$) *L. lactis* subsp. *lactis* strain was isolated from fermented carrots (2, 3), although it was mistakenly identified as *Lactobacillus plantarum* (3). In the present study, lactic acid bacteria isolated from commercial sauerkraut tanks were screened for antimicrobial properties. Of 153 strains tested, 14 produced bacteriocins that inhibited one to three of the test strains. Two strains, identified as *L. lactis* subsp. *lactis*, produced bacteriocins which inhibited all three of the original test strains and a broad spectrum of other gram-positive organisms. A study was initiated to characterize these bacteriocin-producing strains, identify the bacteriocin, and determine its genetic basis relative to other bacteriocin-producing lactococci. Nisin production was confirmed phenotypically and genetically in both strains. The overall organization of nisin genetic determinants was identical for the two strains, but was distinct from that of previously characterized, nisin-producing *L. lactis* subsp. *lactis* strains.

MATERIALS AND METHODS

Bacteria and culture conditions. A description of lactococci used in this study, their relevant phenotypes, and plasmid profiles are given in Table 1. Reference plasmids were isolated from *Escherichia coli* V517 (26), *E. coli* J53 (pSa; 34.5 kbp [22]), *L. lactis* subsp. *lactis* ML3-C145 (90 and 50 kbp [34]), and *Lactobacillus acidophilus* C-7 (62 kbp [34]). Strains used as indicator organisms for bacteriocin screening were obtained from the culture collection maintained by the Food Fermentation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Raleigh, N.C. All cultures were stored at −70°C in their appropriate growth

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TABLE 1. Lactococcal strains and plasmids

Strain	Description[a]	Plasmid(s) (kb)	Source or reference
L. lactis subsp. *lactis*			
LJH80	Nip^+ Nis^r Suc^+, unstable wild type	Variable	This study
NCK318-2	Nip^+ Nis^r Suc^+, stable LJH80 derivative	38, 55	This study
NCK318-3	Nip^+ Nis^r Suc^+, stable LJH80 derivative	38, 55, 75	This study
NCK400	Nip^+ Nis^r Suc^+, wild type	ND[b]	This study
NCK401	Nip^+ Nis^r Suc^+ Str^r Rif^r, NCK400 derivative	ND	This study
NCK402	Nip^- Nis^s Suc^- Str^r Rif^r, NCK401 derivative	ND	This study
NCK403	Nip^- Nis^s Suc^-, NCK400 derivative	ND	This study
ATCC 11454	Nip^+ Nis^r Suc^+	NA[c]	ATCC[d]
ATCC 7962	Nip^+ Nis^r Suc^+	NA	ATCC
C2	Lac^+ Prt^+	NA	7
L. lactis subsp. *cremoris*			
ATCC 14365	Nisin-sensitive indicator	NA	ATCC

[a] Abbreviations: Nip^+, nisin producing; Nis^-, nisin negative; Nis^r, nisin resistant; Nis^s, nisin sensitive; Suc^+, sucrose fermenting; Suc^-, sucrose negative; Str^r, streptomycin resistant (1,000 μg/ml); Rif^r, rifampin resistant (100 μg/ml); Lac^+, lactose fermenting; Prt^+, proteinase activity.
[b] ND, none detected.
[c] NA, not applicable.
[d] ATCC, American Type Culture Collection, Rockville, Md.

medium with 16% glycerol. Frozen stock cultures were streaked onto agar, and an isolated colony was transferred to M17 broth (Difco Laboratories, Detroit, Mich.) with 0.5% glucose (M17G), MRS (Difco), or Trypticase soy (TS) broth (BBL Microbiology Systems, Cockeysville, Md.) before each experiment. When solid medium was required, 1.5% agar (Difco) was added to the broth. Cultures were incubated at 30°C (lactic acid bacteria) or 37°C (non-lactic acid bacteria), unless otherwise indicated.

Cabbage juice broth. Cabbage (cv. Green Boy) was purchased from the North Carolina State Farmer's Market, Raleigh, N.C. Sterile cabbage juice broth (CJB) was prepared by the method of Stamer et al. (37). Briefly, cabbage heads were stripped of outer leaves, quartered, and passed through a Fitz mill. The resulting pulp was pressed to extract the juice and then filtered through an ultrafiltration unit with a 1×10^6 M_w cartridge (Amicon Corp., Danvers, Mass.). The clear yellow juice was stored at −20°C in glass containers. Before use, CJB was thawed at room temperature and filter sterilized through a 0.2-μm bottle-top filter (Costar, Cambridge, Mass.).

Strain isolation. Lactic acid bacteria were isolated from six sauerkraut-manufacturing plants located in various states across the United States. Brine from tanks of sauerkraut which had been fermenting for 3 to 6 days was used to inoculate modified MRS (MMRS) and APT (Difco) agar slants. MMRS agar was prepared by adding 0.02% sodium azide to MRS agar (33). Growth from MMRS and APT slants was used to streak MMRS or APT agar plates. Duplicate colonies of each morphological type arising on the plates were selected and purified on MRS agar.

Analysis of sauerkraut brine. A 5-ml brine sample, preserved with 1 to 2 drops of 1% Merthiolate (Eli Lilly and Co., Indianapolis, Ind.), was obtained from each sauerkraut tank at the time of bacteriological sampling. The tank temperature was recorded at the sampling time. The titratable acidity, pH, and NaCl concentration were determined as described previously (10).

Identification of bacteriocin-producing strains. Bacteriocin production was determined by a modification of the agar spot test described by Fleming et al. (11). Overnight cultures were spotted (2 μl) onto TS agar plates. The cultures were incubated for 24 h before being overlaid with 3 ml of agar (0.75%) inoculated with 3 μl of an overnight culture of the indicator strain. After further incubation for 24 h, colonies were examined for clear zones of inhibition surrounding them.

Strain characterization. Gas production from glucose was determined by inoculating (0.5% [by volume] of an overnight culture) tubes of MRS broth containing inverted Durham tubes. Tubes were examined for the presence of gas after incubation for 24 h. The production of ammonia from arginine was determined by the method of Niven et al. (35), except that 0.1% (vol/vol) Tween 80 (Fisher Scientific Co., Pittsburgh, Pa.) and 0.005% $MnSO_4 \cdot 7H_2O$ (Fisher) were added to the test medium. Strains were evaluated for their ability to grow in the presence of 4 and 6.5% NaCl. MRS broth with the appropriate NaCl concentration was inoculated (0.5%) and checked for turbidity after 48 h and 1 week. Plain MRS broth was similarly inoculated and incubated at 45°C to determine growth at elevated temperatures. Fermentation patterns were determined by using Rapid CH* strips as specified by the manufacturer (API Analytab Products, Plainview, N.Y.). Fermentation reactions were scored after 24 and 48 h. Strain identification was confirmed by fatty acid analysis, which was performed by Microbial ID, Inc., Newark, Del.

Bacteriocin production, assay, and characterization. Bacteriocin production was assayed by a variation of the critical dilution method (28). Culture supernatant (200 μl) was heated in a boiling-water bath for 1 min and cooled rapidly on ice. Serial twofold dilutions of the heated supernatants were made in 0.02 N HCl, and 10 μl of each dilution was spotted onto fresh, duplicate indicator lawns. Indicator lawns were prepared by overlaying agar plates with 3 ml of 0.75% agar inoculated with 3 μl of an overnight culture of *L. lactis* subsp. *cremoris* ATCC 14365. Cultures were incubated for 24 h. The nisin titer was defined as the reciprocal of the highest dilution which completely inhibited the indicator lawn and was expressed as activity units (AU) per milliliter.

Bacteriocins were characterized with respect to proteinase sensitivity. Stock solutions of trypsin and α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) were dissolved (1 mg/ml) in 0.1 M potassium phosphate buffer (pH 6.5), filter sterilized with a 0.22-μm syringe filter (Millipore Corp., Bedford, Mass.), and stored at −20°C. Enzyme solution (10 μl) was added to 75 μl of bacteriocin preparation, and the

mixture was incubated at 37°C for 1 h. Heat-inactivated enzymes (100°C for 10 min) served as controls.

Nisin production in broth. Nisin-producing lactococci were compared for nisin production in various broths. Overnight cultures grown in MRS broth were pelleted and suspended in sterile saline. CJB, MRS, M17G, and brain heart infusion (BHI; Difco) broths were inoculated (0.1%) and incubated for 48 h. The bacteriocin titer and pH of the supernatant were determined. The pH was measured with a combination electrode (Orion Research, Inc., Cambridge, Mass.) and a pH meter (Fisher).

Antibiotic-resistant and bacteriocin-negative derivatives. Spontaneous streptomycin-resistant (1 mg/ml) and rifampin-resistant (100 μg/ml) derivatives of *L. lactis* subsp. *lactis* NCK400 were isolated by sequential selection on M17G agar containing increasing concentrations of each antibiotic (Sigma). Isogenic sucrose-negative (Suc^-) derivatives were isolated on bromcresol purple-sucrose indicator agar (29) following repeated transfer in M17G broth at 37°C. Sucrose-negative strains were subsequently screened for bacteriocin production by using the agar spot test.

Ability to clot milk. Nip^+ lactococci were tested for their ability to clot milk. Isolated colonies were inoculated into an 11% (wt/vol) solution of powdered skim milk which had been steamed for 1 h and then rapidly cooled in water to prevent overheating. Inoculated tubes were incubated at 22°C and checked daily for evidence of clot formation.

DNA isolation and analysis. Plasmid DNA was extracted by a modified procedure of Anderson and McKay (1). The lysozyme concentration was increased to 15 mg/ml, and the incubation was extended to 30 min at 37°C. Proteins were extracted with a phenol-chloroform (2:1) mixture. When required, plasmid DNA was purified by using cesium chloride-ethidium bromide equilibrium gradients (27). Plasmids were separated by electrophoresis in 0.8% agarose at 3 V/cm for 3 to 4 h (27).

Genomic (total) DNA was isolated essentially as described by Leenhouts et al. (25), except that a 10-ml overnight culture was used and a mixture of 6.7% sucrose, 50 mM Tris (pH 8), and 1 mM EDTA was substituted for the lysis solution. Genomic and purified plasmid DNA were digested with restriction enzymes as recommended by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.) DNA fragments were resolved by electrophoresis in 0.6% agarose gels at 1 V/cm for 12 h and, when necessary, were electroeluted from the gel (27).

Southern hybridization. DNA from agarose gels was transferred by capillary action to GeneScreen Plus nylon membranes (New England Nuclear Corp., Boston, Mass.). A Multiprime DNA-labeling kit (Amersham Corp., Arlington Heights, Ill.) was used with [^{32}P]dCTP to generate radiolabeled probes. Unincorporated nucleotides were removed by using a Sephadex G-50 spin column (Boehringer Mannheim). Hybridizations were carried out at 42°C in a solution of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 1% Denhardt solution, and 50 μg of salmon sperm DNA (Sigma) per ml (27). When required, membranes were stripped of labeled DNA as specified by the manufacturer.

RESULTS

Isolation and identification of bacteriocin-producing strains. From a total of 153 isolates, 14 strains produced antimicrobial compounds which inhibited one or more of the indicator organisms (*Leuconostoc paramesenteroides* NRRL B3471, *Leuconostoc* sp. strain HETA, and *Lactobacillus* sp. strain LA51). Two isolates, NCK400 and LJH80, produced compounds which inhibited all three indicator strains; they were therefore selected for further characterization. NCK400 and LJH80 were isolated from the same commercial sauerkraut tank. The tank was in the early stages (day 5) of fermentation and had a temperature of 20°C, an NaCl concentration of 0.89%, a pH of 4.0, and a titratable acidity of 1.2%, expressed as lactic acid.

TABLE 2. Inhibitory spectra of *L. lactis* subsp. *lactis* NCK400 and LJH80

Indicator strain	Bacteriocin producer[a]	
	NCK400	LJH80
Bacillus cereus B2[b]	+	+
Listeria monocytogenes ATCC 33909[c]	+	+
Staphylococcus aureus B32	+	±
Staphylococcus epidermidis B10	+	+
Streptococcus faecalis ATCC 14508	+	+
Lactobacillus brevis ATCC 14869	+	+
Lactobacillus buchneri ATCC 4005	+	+
Lactobacillus fermentum ATCC 11739	+	+
Lactobacillus plantarum ATCC 14917	+	+
Lactobacillus sp. strain LA51	+	+
Lactobacillus viridescens ATCC 12706	+	+
Lactococcus diacetilactis ATCC 13675	+	+
Lactococcus lactis subsp. *cremoris* ATCC 14365	+	+
Lactococcus lactis subsp. *lactis* LM0230	+	+
Lactococcus lactis subsp. *lactis* ATCC 7962	−	−
Lactococcus lactis subsp. *lactis* ATCC 11454	−	−
Lactococcus lactis subsp. *lactis* LJH80	−	−
Lactococcus lactis subsp. *lactis* NCK400	−	−
Leuconostoc mesenteroides ATCC 8293	+	+
Leuconostoc mesenteroides ATCC 23386	+	+
Leuconostoc paramesenteroides NCDO 803[d]	+	+
Pediococcus acidilactici LA82	+	+
Pediococcus acidilactici LA54	+	+
Pediococcus pentosaceus ATCC 43200	+	+
Pediococcus pentosaceus LA60	+	±

[a] Symbols: +, inhibition; −, no inhibition; ±, variable reaction.
[b] Culture collection, Food Fermentation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Raleigh, N.C.
[c] ATCC, American Type Culture Collection, Rockville, Md.
[d] NCDO, National Collection of Dairy Organisms, Reading, United Kingdom.

NCK400 and LJH80 exhibited similar growth characteristics and fermented *N*-acetylglucosamine, amygdalin, L-arabinose, arbutin, cellobiose, esculin, fructose, galactose, gentibiose, gluconate, glucose, lactose, maltose, mannitol, mannose, ribose, salicin, starch, sucrose, trehalose, and D-xylose. Both strains were able to deaminate arginine and grow in the presence of 4% NaCl, but were unable to grow in 6.5% NaCl or at 45°C. Gas was not produced from glucose. On the basis of these data, NCK400 and LJH80 were tentatively identified as *L. lactis* subsp. *lactis*. This identification was confirmed by fatty acid analysis (data not shown) performed by Microbial ID, Inc.

Bacteriocin characterization. NCK400 and LJH80 produced bacteriocins capable of inhibiting a broad range of gram-positive bacteria (Table 2). Both strains were resistant to nisin concentrations of at least 10 μg/ml, and the bacteriocin they produced did not inhibit Nip^+ *L. lactis* subsp. *lactis* ATCC 11454 or ATCC 7962. Bacteriocin activity was stable to boiling for at least 5 min at pH 2.0, resistant to trypsin digestion, and sensitive to digestion by α-chymotryp-

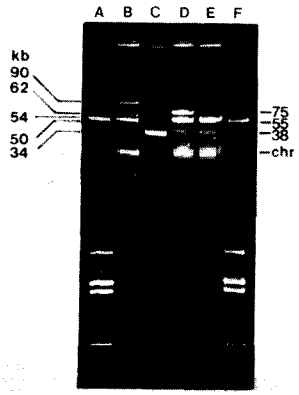

FIG. 1. Plasmid DNA profiles of *L. lactis* subsp. *lactis* NCK318-2 and NCK318-3. Lanes: A and F, *E. coli* V517 (54, 7.3, 5.5, 5.1, 3.1, 2.7, and 2.1 kbp); B, molecular size standards (90, 62, and 50 kbp); C, pSa (34 kbp); D, NCK318-3 (75, 55, and 38 kbp); E, NCK318-2 (55 and 38 kbp).

sin. These characteristics are typical of nisin (19) and indicated that the bacteriocin produced by NCK400 and LJH80 was nisin.

Differentiation of bacteriocin-producing strains. Plasmid DNA was not detected in NCK400, whereas both the plasmid profile and colony morphology of LJH80 were variable. A mixture of small- and large-colony morphologies arose from LJH80 on M17G agar after the culture was grown in M17G broth. Both small- and large-colony types were capable of producing nisin. The large-colony phenotype remained morphologically stable upon subculture in broth. The small-colony phenotype consistently gave rise to both large- and small-colony morphologies when plated on agar media after growth in broth. The large-colony isolates had two distinct plasmid profiles which were stably maintained after initial isolation. One isolate type, designated NCK318-2, carried two plasmids of approximately 38 and 55 kbp (Fig. 1, lane E). The other large-colony type, designated NCK318-3, harbored a 75-kbp plasmid in addition to the 38- and 55-kbp plasmids (Fig. 1, lane D).

Genomic analysis. NCK318-2, NCK318-3, and NCK400 were further differentiated on the basis of their genomic patterns of hybridization to a probe of IS*946*, an iso-ISS*1* element isolated from the conjugative phage resistance plasmid TR2030 (36). Genomic and plasmid DNA, digested with *Eco*RI, was hybridized with ^{32}P-labeled pBluescript::IS*946* (Fig. 2, center panel). pBluescript sequences hybridized to linear low-molecular-weight DNA standards (Fig. 2, center panel, lane A). DNA from NCK400 and its derivatives (see below) did not hybridize with pBluescript::IS*946* (lanes B to D). The genomic hybridization patterns of NCK318-2 (lane E) and NCK318-3 (data not shown) were identical. Plasmid DNA in NCK318-2 (lane H) and NCK318-3 (lane G) correlated with three of the genomic IS*946*-hybridizing bands (9.5, 6, and 3.5 kbp). The additional genomic hybridizing bands (17, 15, and 8 kbp) were identified in the plasmid DNA preparation of NCK318-3 and correlated with the appearance of the 75-kbp plasmid in this strain. All of the genomic IS*946*-hybridizing bands in NCK318-3 are contributed by the plasmids present in this strain.

Ability to clot milk. *L. lactis* subsp. *lactis* Nip$^+$ strains were tested for their ability to clot milk. None of the Nip$^+$ strains were able to form a firm clot in milk. With the exception of NCK400, all strains formed a weak "acid" clot after incubation at 22°C for 3 to 4 days. NCK400 did not curdle the milk even after 1 week of incubation. A typical, firm clot was observed within 36 h with the positive control *L. lactis* subsp. *lactis* C2, a nisin-negative, lactose-positive proteolytic strain. On subculture in milk, *L. lactis* subsp. *lactis* C2 formed a firm clot within 18 h.

Genetic evidence for nisin production. We further characterized NCK318-2, NCK318-3, NCK400, and the streptomycin- and rifampin-resistant NCK400 derivative NCK401 to confirm nisin production. Despite the absence of detectable plasmid DNA in NCK400 and NCK401, Suc$^-$ derivatives which were also Nip$^-$ were isolated after repeated transfer in M17G broth at 37°C. These strains were designated NCK403 (from NCK400) and NCK402 (from NCK401).

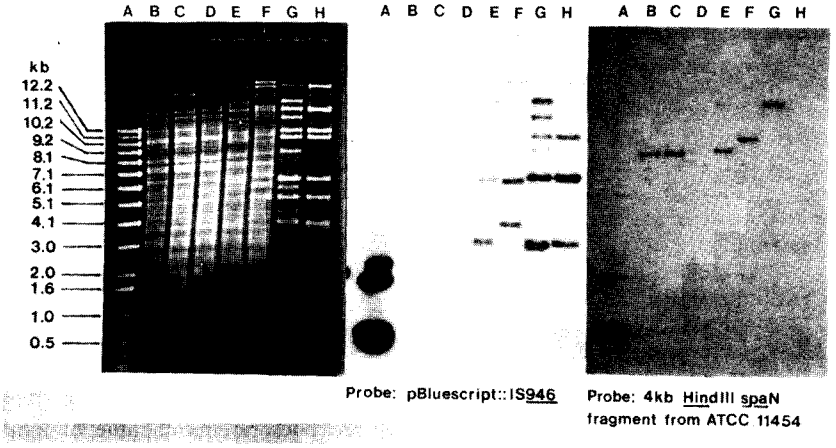

FIG. 2. Hybridization patterns of *Eco*RI-digested DNA from nisin-producing *L. lactis* subsp. *lactis* strains. (Left) Agarose gel of the 1-kbp ladder (12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.0, 2.0, 1.6, 1.0, and 0.5 kbp; Bethesda Research Laboratories) (lane A); digested genomic DNA of NCK400 (Nip$^+$) (lane B), NCK401 (Nip$^+$ Str^r Rif^r) (lane C), NCK402 (Nip$^-$ Str^r Rif^r) (lane D), NCK318-2 (Nip$^+$) (lane E), and ATCC 11454 (Nip$^+$) (lane F); and digested plasmid DNA of NCK318-3 (lane G) and NCK318-2 (lane H). (Center) Autoradiogram prepared after hybridization of a ^{32}P-labeled probe of whole plasmid DNA pBluescript::IS*946*. (Right) Autoradiogram prepared after hybridization of a ^{32}P-labeled probe of a 4-kbp *Hin*dIII fragment from pSNRD-3 containing the precursor structural gene for nisin cloned from ATCC 11454 (4).

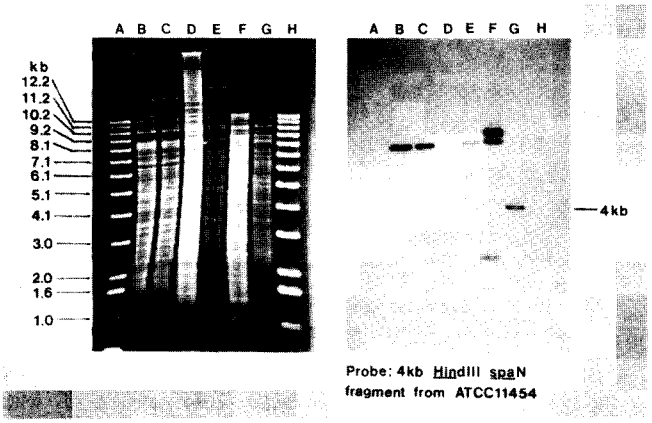

FIG. 3. Hybridization patterns of genomic DNA digested with *Hin*dIII. (Left) Agarose gel of the 1-kbp ladder (12.2, 11.2, 10.2, 9.2, 8.1, 7.1, 6.1, 5.1, 4.1, 3.0, 2.0, 1.6, and 1.0 kbp; Bethesda Research Laboratories) (lanes A and H) and *Hin*dIII-digested genomic DNA from NCK400 (Nip$^+$) (lane B), NCK401 (Nip$^+$ Str^r Rif^r) (lane C), NCK402 (Nip$^-$ Str^r Rif^r) (lane D), NCK318-2 (Nip$^+$) (lane E), ATCC 7962 (Nip$^+$) (lane F), and ATCC 11454 (Nip$^+$) (lane G). (Right) Autoradiogram prepared by hybridization to a ^{32}P-labeled probe of a 4-kbp *Hin*dIII fragment containing *spaN* cloned from *L. lactis* subsp. *lactis* ATCC 11454 (4).

TABLE 3. Inhibitory activity and final pH in broth media[a]

Strain	Activity[b] (AU/ml) and pH in[c]:							
	MRS		CJB		M17G		BHI	
	Activity	pH	Activity	pH	Activity	pH	Activity	pH
NCK318-2	1,600	4.3	500	4.0	<100	5.2	<100	5.8
NCK318-3	1,600	4.3	500	4.0	<100	5.2	<100	5.8
NCK400	1,600	4.3	800	4.0	600	5.2	500	5.9
ATCC 7962	1,600	4.5	1,200	4.1	1,000	5.2	800	5.8
ATCC 11454	1,600	4.5	300	4.1	800	5.2	400	5.9
NCK403	<100	4.3	<100	4.0	<100	5.4	<100	5.9
Uninoculated	<100	6.5	<100	5.7	<100	7.0	<100	7.3

[a] Determined after incubation for 48 h.
[b] Assayed against *L. lactis* subsp. *cremoris* ATCC 14365.
[c] Results are means of duplicate determinations within replicates.

Attempts to isolate nisin-negative strains from NCK318-2 and NCK318-3 by using the same methods were unsuccessful.

pSNRD-3, containing the genetic determinant for pronisin (*spaN*) cloned from *L. lactis* subsp. *lactis* ATCC 11454, was used to confirm nisin production in the sauerkraut isolates. A 4-kbp *Hin*dIII fragment containing the genetic determinant *spaN* was isolated from an agarose gel and was used to probe *Eco*RI (Fig. 2, right panel) and *Hin*dIII (Fig. 3) genomic digests of nisin-producing strains and their derivatives. The *spaN* probe hybridized to a 9.5-kbp *Eco*RI (Fig. 2, right panel, lane F) and a 4-kbp *Hin*dIII (Fig. 3, right panel, lane G) fragment in ATCC 11454. These hybridization patterns correlate with data published by Buchman et al. (4) for this strain. The same probe hybridized to three bands (9.5, 8.5, and 2.2 kbp) present in *Hin*dIII-digested genomic DNA from ATCC 7962 (Fig. 3, right panel, lane F). For Nip$^+$ NCK400, NCK401, and NCK318-2, the *spaN* probe hybridized to an 8-kbp *Eco*RI fragment (Fig. 2, right panel, lanes B, C, and E) and an 8.5-kbp *Hin*dIII fragment (Fig. 3, right panel, lanes B, C, and E), but failed to hybridize to Nip$^-$ NCK402 (Fig. 2, right panel, and 3, right panel, lanes D).

A 17-kbp *Eco*RI fragment which hybridized to the *spaN* probe was also evident in the genomic digests of NCK318-2 (Fig. 2, right panel, lane E) and NCK318-3 (data not shown) and in the plasmid digest of NCK318-3 (Fig. 2, right panel, lane G). We did not determine whether this second hybridizing band was an additional copy of the *spaN* gene or simply a region of homology within the 4 kbp of sequence flanking *spaN* in the probe.

Nisin production in broth. Noting that the gross organization for nisin genetic determinants in NCK400, NCK318-2, and NCK318-3 is similar, we expected that nisin production by these strains would also be similar. All strains grew well in all media, as indicated by the turbidity observed in the tubes after 24 h of incubation. The pH, measured after 48 h, differed among media but did not vary greatly among strains in a particular medium (Table 3). Sauerkraut isolates and *L. lactis* subsp. *lactis* ATCC 11454 and ATCC 7962 produced comparable levels of nisin in MRS broth. Nisin production was lower and more variable in CJB, even though all strains produced detectable quantities of nisin within 48 h. In contrast to other nisin-producing strains, NCK318-2 and NCK318-3 failed to produce detectable levels of nisin in M17G or BHI broth.

DISCUSSION

Two *L. lactis* subsp. *lactis* strains, LJH80 and NCK400, isolated from a commercial sauerkraut fermentation, were shown to produce nisin. LJH80 was morphologically unstable but gave rise to two stable, nisin-producing derivatives. NCK400 and the LJH80 derivatives could be distinguished on the basis of plasmid profiles and patterns of DNA hybridization to a DNA probe of IS*946*.

Despite the absence of detectable plasmid DNA in NCK400, sucrose-negative, nisin-negative, and nisin-sensitive derivatives were isolated after transfer in M17G broth at 37°C. Loss of nisin production and immunity in conjunction with sucrose-fermenting ability has been well-documented (9, 12, 14, 24, 39), suggesting that the genetic basis for nisin production in sauerkraut isolate NCK400 was similar to that in previously studied strains.

The inability to detect plasmid DNA in NCK400 and lack of hybridization of *spaN* to the plasmid fraction apparent in NCK318-2 suggested that *spaN* was chromosomally encoded in these strains. The genetic determinants for nisin were originally thought to be plasmid borne, because of the relative ease with which the nisin-producing ability could be cured or conjugally transferred (12, 14, 23, 24, 39). Recent data indicate that the nisin structural gene is chromosomally mediated (8, 9, 18, 40) by a 70-kbp transposon designated Tn*5301* (18).

The gross genetic organization of *spaN* was similar for the sauerkraut isolates, but was different from that of Nip$^+$ *L. lactis* subsp. *lactis* ATCC 11454 and ATCC 7962. The two sauerkraut strains were isolated from the same sauerkraut sample. A similar overall genetic arrangement of *spaN* in NCK400 and LJH80 derivatives suggested that these strains were related. Differences in plasmid profiles and patterns of hybridization to IS*946* between the strains were eliminated when plasmids from NCK318-3 were ignored. It is possible that NCK400 is a plasmid-free derivative of NCK318-3, but this was not investigated. The hybridization data and plasmid profiles also suggested that the 75-kbp

plasmid in NCK318-3 was integrated into the chromosome of NCK318-2.

Despite the identical gross genetic organizations of *spaN*, nisin production differed between NCK400 and LJH80 derivatives in M17G broth, BHI broth, and CJB. Production parameters for nisin appeared to be different between the strains. Plasmid composition differences between NCK318-2 and NCK318-3 had no effect on nisin production. Differences in inhibitory spectra for different nisin-producing strains have been reported (13, 17), suggesting that minor differences in the amino acid sequence of nisin molecules do occur from strain to strain. *L. lactis* subsp. *lactis* NIZ022186 produces nisin Z, which differs from nisin by a substitution of Asn for His at amino acid residue 27 (31). Confirmation of molecular differences (structural or regulatory) among NCK400, NCK318-2, and NCK318-3 would aid in understanding the structure-function relationships and production of the nisin molecule.

The natural habitat of *L. lactis* subsp. *lactis* has been the subject of much debate. Stark and Sherman (38) believed that the habitat was plant material, whereas others note the probability of recent adaptation to milk (16, 32). Plasmid-linked traits essential to milk fermentation (lactose-fermenting ability, bacteriophage resistance, and proteinase systems) are common in the lactococci (30), further supporting Hirsch's hypothesis (16). Cross-inhibition of nisin and diplococcin produced by *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, respectively, can be seen as a result of active competition in milk (16). However, as in the case of ATCC 11454, ATCC 7962, and the sauerkraut isolates, most Nip^+ *L. lactis* subsp. *lactis* isolates are unable to cause milk to clot or rapidly lose the ability after isolation (15, 20, 41). Given the broad inhibitory spectrum of nisin and the weak correlation between coexpression of proteolytic activity and nisin production, it seems more likely that nisin-producing strains are suited to the greater microbial competition that would be expected in plant ecosystems.

The relative sensitivity of *L. lactis* subsp. *lactis* to acid and salt would suggest that these organisms have a minor role in most vegetable fermentations. From a practical standpoint, nisin-producing starter cultures may be most effective in low-salt fermentations, and, noting their relative acid sensitivity, at the early stages of the fermentation. The sauerkraut tank from which NCK400 and LJH80 were isolated had a relatively low salt level (0.9% NaCl). Further research is needed to determine the parameters under which nisin is produced by these strains.

It is unclear whether nisin-producing lactococci have a role in the succession of microorganisms in vegetable fermentations and whether nisin-negative strains are also involved. It is possible that nisin-producing strains are simply opportunistic saprophytes in fermenting vegetables and normally compete in other plant ecosystems. Enumeration of Nip^+ and Nip^- *L. lactis* subsp. *lactis* organisms in vegetable fermentations by using DNA probes specific for *spaN* or for *L. lactis* may shed some light on the occurrence and actual role of these organisms in natural vegetable fermentations.

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