

## Isolation and Characterization of Nisin-Resistant *Leuconostoc mesenteroides* for Use in Cabbage Fermentations

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*Leuconostoc mesenteroides* strains that are resistant to high levels of nisin (up to 25,000 IU/ml in broth) were isolated. These nisin-resistant mutants were evaluated to determine their potential use as starter culture strains for cabbage fermentations. We found that some *L. mesenteroides* strains could be adapted to high levels of nisin resistance, while others could not. The nisin resistance trait was found to be stable for at least 35 generations, in the absence of nisin selection, for all mutants tested. The effects of nisin and salt, separately and in combination, on growth kinetics of the nisin-resistant strains were determined. Salt was the most influential factor on the specific growth rates of the mutants, and no synergistic effect between nisin and salt on specific growth rates was observed. The nisin-resistant strains were unimpaired in their ability to rapidly produce normal heterolactic fermentation end products. The use of these *L. mesenteroides* mutants as starter cultures in combination with nisin may extend the heterolactic phase of cabbage fermentations.

Commercial fermentation of cabbage in the United States is commonly carried out in 45- to 150-ton tanks. Cabbage is dry salted and loaded into the tanks, resulting in a brine with about 2% NaCl upon equilibration with water extracted from the cabbage by osmosis. A natural fermentation follows, initiated by the heterofermentative *Leuconostoc mesenteroides* (19, 21). As the fermentation progresses, *L. mesenteroides* is displaced by more acid-tolerant organisms; the homofermentative *Lactobacillus plantarum* typically completes the fermentation. The initial heterolactic phase of the fermentation, during which *L. mesenteroides* is the predominant organism, results in the production of lactic acid, acetic acid, ethanol, mannitol, and other compounds which are important for the flavor of sauerkraut (8, 21).

Bulk storage of sauerkraut for prolonged periods can result in an unpredictable balance of fermentation end products (7). High concentrations of lactic acid (in excess of 200 mM) that result from long-term storage can make the final product too acidic. Dilution of the brine to reduce acidity results in loss of flavor and nutritional value and creates waste disposal problems. It is, therefore, desirable to maximize hexose utilization by heterofermenting organisms before they are displaced by homolactic fermenters. Our research efforts have focused on the use of the bacteriocin nisin to delay, not eliminate, the homolactic (terminal) phase of the sauerkraut fermentation. Nisin is a readily available bacteriocin that has been approved for use in processed cheese in the United States (10) and in a variety of cheese and canned foods in Europe (14).

Previous work in our laboratory led to the proposal of a paired starter culture system for use in sauerkraut production (12). This system involved use of a nisin-resistant *L. mesenteroides* strain and a nisin-producing *Lactococcus lactis* strain to inhibit the growth of *Lactobacillus plantarum* in model sauerkraut fermentations. Harris et al. (12) demonstrated that sufficient nisin could be produced in mixed-culture cabbage juice broth (CJB) fermentations to reduce

the numbers of *Lactobacillus plantarum* below the level of detection and allow the nisin-resistant *L. mesenteroides* strain to reach a maximum cell density of approximately  $10^9$  CFU/ml. The nisin-resistant strains of *L. mesenteroides* isolated from sauerkraut, described by Harris et al. (12), were unable to grow in CJB containing greater than 500 IU of nisin per ml. Because the biological activity of nisin is rapidly lost in brined cabbage (1), we wanted to conduct experiments with initial nisin concentrations greater than 500 IU/ml in cabbage fermentations. We also wanted to determine whether nisin-resistant mutants of various *L. mesenteroides* strains could be isolated in order to identify strains useful in nisin-treated cabbage fermentations. Criteria considered desirable for *L. mesenteroides* cultures to be used in brined cabbage fermentations include (i) unimpeded growth in the presence of relatively high concentrations of nisin (greater than 500 IU/ml), (ii) a resistance mechanism by the culture that does not result in the destruction of nisin, (iii) stability of the genotype conferring nisin resistance, and (iv) rapid production of acid in brined cabbage, giving a typical heterolactic fermentation with the salt and pH conditions normally found in sauerkraut fermentations.

### MATERIALS AND METHODS

**Bacterial strains.** *L. mesenteroides* C33 (25), LA145 (also known as NCK293 [12]), LA108 (ATCC 13146), and LA113 (ATCC 10882) were obtained from the U.S. Food Fermentation Laboratory culture collection (Raleigh, N.C.). Strains LA113-MO and LA113-M3 were nisin-resistant mutants isolated from strain LA113; LA108-M1 and LA108-M2 were nisin-resistant mutants isolated from strain LA108 (this study). *Lactococcus lactis* subsp. *cremoris* LA120 (ATCC 14365) was used as the indicator strain for nisin bioassays.

**Media.** *Lactobacillus* MRS broth (Difco Laboratories, Detroit, Mich.) was prepared according to the supplier's instructions. Agar was added at 1.5% for plating in solid medium. CJB was prepared by the method of Kyung (16). Cabbage (obtained from commercial sources) was quartered and placed in an autoclave, and the temperature was raised

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quickly to 121°C. The steam was then shut off, and the autoclave chamber was allowed to return to atmospheric pressure, requiring about 10 min. The heated cabbage was then homogenized in a Waring blender, and juice was extracted by pressing through cheesecloth. The juice was clarified by centrifugation at  $40,000 \times g$  (Beckman Instruments, Fullerton, Calif.) for 30 min at 5°C and then filtered through a sterile 0.22- $\mu\text{m}$ -pore-size filter (type CA; Costar Corp., Cambridge, Mass.).

**Isolation of nisin-resistant mutants.** Isolated colonies from MRS agar plates of prospective nisin-resistant *L. mesenteroides* strains were used to inoculate MRS broth containing 500 IU of nisin per ml. Broths that became turbid within 48 h were chosen for further processing. Serial transfers of selected strains, using a 1% inoculum at each transfer, were carried out, increasing the nisin concentration by 100 to 1,000 IU/ml with each transfer. Transfers were carried out every 24 or 48 h until MRS broth cultures that became turbid in 24 h with 25,000 IU of nisin per ml were obtained. This required a total of 75 to 100 generations. The cultures were then streaked for isolation onto MRS agar plates to ensure that a pure culture was obtained. Confirmation of the identity of the derivative and parental strains was done by comparative biochemical tests (API CHL kit; API Analytab Products, Plainview, N.Y.), and the nisin-resistant strains were frozen at  $-85^\circ\text{C}$  in MRS broth containing 15% glycerol.

**HPLC analysis of fermentation end products.** Cabbage juice samples were prepared for high-pressure liquid chromatography (HPLC) analysis by filtration through a 0.22- $\mu\text{m}$ -pore-size filter (Costar) to remove particulate matter. Organic acid and sugar concentrations were determined by the method of McFeeters et al. (17), using a Dionex conductivity detector, CDM-II (Dionex Corp., Sunnyvale, Calif.), and a Dionex pulsed amperometric detector to identify organic acids and sugars, respectively. The chromatographic separation was accomplished with an organic acid column (type ROA; Phenomenex, Rancho Palos Verdes, Calif.) held at  $65^\circ\text{C}$ , using 1.6 mM heptafluorobutyric acid (Aldrich Chemical Co., Milwaukee, Wis.) as the solvent. Mannitol and fructose were separated by the method of McFeeters et al. (18), using an Aminex HPX-87P (Bio-Rad Laboratories, Richmond, Calif.) cation-exchange column, and the concentrations were determined with a pulsed amperometric detector (Dionex).

**Nisin concentration and bioassay.** Purified nisin was supplied by Applied Microbiology, Inc., Brooklyn, N.Y. (batch NP28), at a potency of  $50 \times 10^6$  IU/g. The biological activity of nisin was determined by a modification of the method of Tramer and Fowler (26). A 60- $\mu\text{l}$  agar plug was removed from MRS agar plates (1.5% agar). Molten agar, containing the sample to be assayed or purified nisin for a standard curve, was added to the 60- $\mu\text{l}$  well. When the plug had solidified, the plate was overlaid with MRS soft agar (0.75%) containing approximately  $5 \times 10^7$  CFU of *Lactococcus lactis* subsp. *cremoris* ATCC 14365 per ml, and the plates were incubated for 15 h at  $30^\circ\text{C}$ . A standard curve was generated with 0.5 to 500 IU (based on the potency as determined by the supplier) of nisin in the 60- $\mu\text{l}$  agar plug by measuring the diameter of the inhibition zone of ATCC 14365 around the agar plug.

**Growth kinetics, salt tolerance, and nisin resistance.** Specific growth rates and lag times were determined with a microtiter plate reader (model EL312; Bio-Tek Instruments, Winooski, Vt.). All experiments were carried out at  $28^\circ\text{C}$  to maintain a constant temperature above the ambient (room) temperature. Individual growth curves were measured in

300- $\mu\text{l}$  microtiter wells containing 200  $\mu\text{l}$  of growth medium. Growth media included CJB or MRS with or without NaCl or nisin as indicated. The growth medium was overlaid with 75  $\mu\text{l}$  of sterile mineral oil to prevent evaporation. Cells were added to the growth medium by using a 1% dilution of a 15-h culture grown in the same medium, giving an initial population of approximately  $10^7$  CFU/ml. Growth curve data were generated during an incubation period of 36 h. This allowed 96 independent growth curves to be measured simultaneously (using a sterile 96-well microtiter plate). The plate reader was controlled by KinetiCalc software (Bio-Tek), with a protocol that included shaking the plates and measuring the optical density of each well at 1-h intervals. The optical density data were stored on a floppy disk in ASCII text format, consisting of one column of 3,456 optical density readings (96 wells  $\times$  36 readings). This file was read by Regress software (2), a C++ language program that will sort the data into individual growth curves and calculate growth rates and lag times. Specific growth rates ( $\mu$ ; per hour) and lag times (in hours) were calculated as described by Pirt (23). Three or four points on the growth curve were used (corresponding to 3 or 4 h of growth) for the specific growth rate calculations, and in all cases the regression line used for the rate determination had an  $r^2$  value greater than 0.95 or the data were discarded. The set of points used was selected by the Regress program as those points that gave the maximum slope ( $\mu$ ) for a given growth curve. A detailed description of the microtiter method for the determination of growth rate kinetics and the Regress program will be given elsewhere (2). For determination of the relative lag times, the lag values obtained for fermentations with salt and/or nisin were divided by the lag values for a control fermentation in the same medium inoculated with the same cell culture, lacking treatment with salt and/or nisin. Therefore, the relative lag time reflects only the effect of the treatment (salt and/or nisin) on the lag values. Statistical analysis of the data was carried out with PC SAS version 6.03 (SAS Institute, Cary, N.C.). The equation for the response surface analysis was generated from microtiter plate growth data, using the general linear model procedure. Data used to derive the response surface equation were generated with duplicate microtiter wells containing NaCl concentrations of 0, 1, 2, 3, and 4%, in addition to nisin concentrations of 0, 250, 500, 1,000, 2,000, 4,000, and 5,000 IU/ml. The equation used to generate the response surface graph was as follows:  $\mu = 0.389 - 6.63 \times 10^{-2}(s) - 1.78 \times 10^{-5}(n) + 1.29 \times 10^{-6}(s \times n)$ , where  $s$  is the NaCl concentration (percent) and  $n$  is the nisin concentration (international units per milliliter).

**Stability of the nisin resistance phenotype.** Nisin-resistant cultures were transferred daily for 14 days in MRS broth in the absence of nisin, with a 1% inoculum at each transfer (allowing approximately 100 generations in total). Cells were harvested at each transfer and frozen at  $-85^\circ\text{C}$  in MRS broth containing 15% glycerol. After all frozen samples were collected, each sample was inoculated onto MRS agar, and isolated colonies were used to inoculate an overnight MRS broth. The cultures were grown for 15 h and used to inoculate microtiter plate wells with a 1% inoculum to determine the relative lag times, as described above. Cells from the identical culture that had been grown for seven generations in the presence of nisin served as the control for relative lag determinations.

**Nisin retention in CJB.** The nisin-resistant culture was prepared with a 1% inoculum from an overnight culture grown in CJB. Cells from the overnight culture were grown in CJB broth containing 2% NaCl and 5,000 IU of nisin per

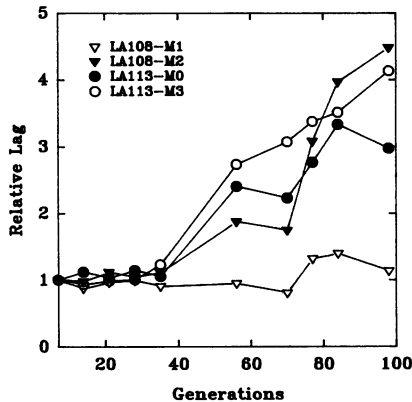


FIG. 1. Stability of the nisin-resistant phenotype for four mutant strains of *L. mesenteroides*. The stability of the resistance phenotype is represented by the relative lag required for growth in 5,000 IU of nisin per ml versus generations in the absence of selection. Each point represents two or more independent determinations in CJB.

ml. The culture was sampled at intervals to determine CFU per milliliter (using a Spiral Systems model D2 spiral plater [Spiral Systems Inc., Cincinnati, Ohio]), nisin concentration by the bioassay procedure as described above, and pH. An uninoculated control broth (CJB with 2% NaCl and 5,000 IU of nisin per ml but no cells) was set up and sampled at the same time as the inoculated broth to determine the nisin concentration and pH.

## RESULTS

**Isolation of nisin-resistant mutants.** *L. mesenteroides* strains from a variety of sources, including strains LA145, C33, LA108, and LA113, were screened for nisin resistance in broth and on MRS agar plate medium containing 50 to 500 IU of nisin per ml. Strains LA108 and LA113 grew on agar plates containing 500 IU of nisin per ml. Interestingly, these strains also grew on agar plates containing much higher levels of nisin, up to 5,000 IU/ml; however, no growth was seen in MRS broth containing greater than 1,250 IU of nisin per ml. This apparent difference in susceptibility to nisin of LA108 and LA113 on MRS agar plates and in MRS broth could be explained by the limited ability of nisin to diffuse in the solid medium. We were unable to produce mutants with a high tolerance to nisin from strains LA145 and C33, which had been described previously for use in cabbage fermentations (12, 24). Two independently isolated mutants each from strains LA108 (LA108-M1 and LA108-M2) and LA113 (LA113-M0 and LA113-M3) were selected for further characterization. These strains were checked for production of bacteriocin by bioassays of the culture supernatant, and no bacteriocidal activity was detected (data not shown).

**Stability of the resistance phenotype.** The ability of the nisin-resistant mutants to retain the resistance phenotype in the absence of nisin selection was determined for two mutants each of LA108 and LA113 (Fig. 1). Because of the difficulty in plating cells to determine nisin resistance (see above), we used relative lag time for growth in MRS broth with 5,000 IU of nisin per ml as an indicator of the stability of the nisin resistance trait. All of the mutants tested retained the nisin resistance phenotype for more than 30 generations, while strain LA108-M1 showed no change in lag time after 100 generations of growth in the absence of nisin.

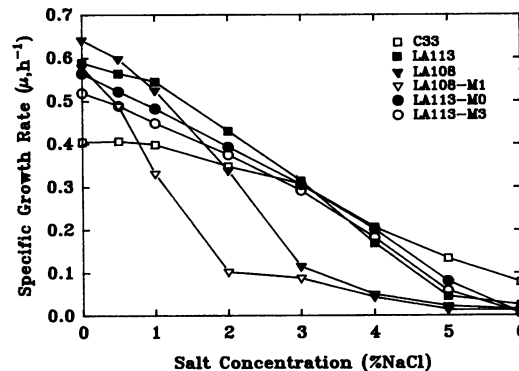


FIG. 2. Effect of salt on specific growth rate of parental and nisin-resistant mutant strains of *L. mesenteroides*. Each point represents two or more independent determinations in CJB.

**Growth kinetics and salt tolerance.** Nisin-resistant strains were tested for salt tolerance in microtiter wells containing 0 to 6% NaCl in CJB. Strain C33, a strain which has been used previously for cabbage fermentations (25), was used for comparison. The growth rates are shown in Fig. 2. On the basis of this test, the nisin-resistant mutants LA113-M0 and LA113-M3 were chosen for further study. Strain LA108-M1, which was found to be the most stable in terms of retention of the resistance phenotype in the absence of selection, was found to be sensitive to salt concentrations commonly found in cabbage fermentations. The parental strain, LA108, demonstrated a similar response to NaCl. The maximum cell density obtained for strains C33, LA113, and LA113-M3 in CJB or MRS with 2% NaCl was approximately  $10^9$  CFU/ml (data not shown).

**Nisin resistance.** We determined the ability of LA113-M3 to grow in the presence of various concentrations of nisin in both CJB and MRS broth (Fig. 3A and B). The specific growth rate decreased more rapidly in CJB than in MRS broth as the nisin concentration was increased from 0 to 5,000 IU/ml. No growth of the mutant strain was observed in CJB with 10,000 IU of nisin per ml after 36 h of incubation, but the same cells, in MRS broth, grew in concentrations of nisin up to 25,000 IU/ml, with a specific growth rate of  $0.267 \text{ h}^{-1}$ . We found the change in specific growth rate of LA113-M3 in MRS broth as a function of nisin concentration (international units per milliliter) to be  $-3.7 \times 10^{-4} \text{ h}^{-1}$  (Fig. 3A) over the range of nisin concentrations tested (0 to 25,000 IU/ml). The lag time for this strain was not greatly affected by nisin (Fig. 3B), and the final optical density achieved by LA113-M3 in MRS broth was constant regardless of nisin concentration (data not shown).

A response surface graph showing the combined effects of nisin concentration and salt concentration on the specific growth rate of LA113-M3 in MRS broth is shown in Fig. 4. The surface was generated from the predicted values as described in Materials and Methods, with an  $r^2$  value for the model of 0.908. Salt concentration accounted for 83.3% of the variance, and nisin concentration accounted for 7.2% of the variance in growth rate. MRS broth was used to generate the data for the response surface graph because of the difficulty in producing cabbage juice that would give consistent growth rate data. The MRS data shown in Fig. 4, however, were similar to the response to NaCl in the CJB data presented in Fig. 2. It is apparent from Fig. 4 that while the salt concentration lowers the overall growth rate of

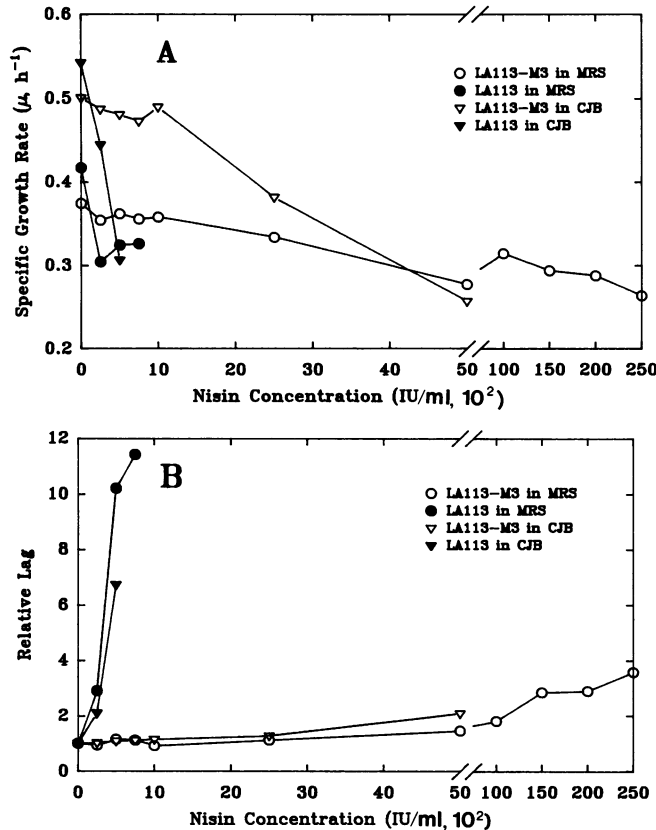


FIG. 3. Effect of nisin concentration on specific growth rates (A) and relative lag times (B) of *L. mesenteroides* cultures grown in MRS and CJB. Each point represents the average of two or three replicates.

LA113-M3, it does not influence the effect of nisin on growth rate in MRS broth.

**Retention of nisin during fermentation.** To determine whether the nisin-resistant cells were inactivating nisin, LA113-M3 was grown in CJB containing 5,000 IU of nisin per ml. Figure 5 shows that the detectable bioactivity of nisin dropped initially in both the inoculated and the uninoculated

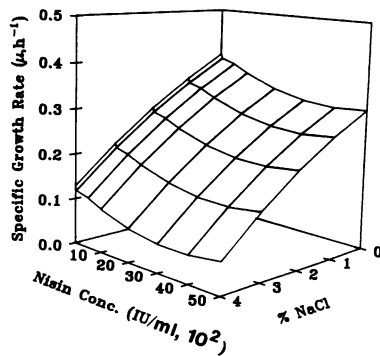


FIG. 4. Response surface graph showing the combined effects of nisin and NaCl concentration on the specific growth rate of LA113-M3 in MRS broth. The surface represents predicted growth rate values. Points of intersection on the lattice represent conditions for which specific growth rate data were obtained from two or more independent fermentations.

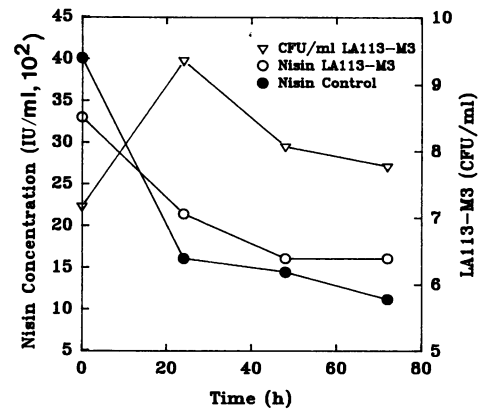


FIG. 5. Nisin recovery from fermented and nonfermented cabbage juice. Growth of LA113-M3 in CJB containing 5,000 IU of nisin per ml and 2% NaCl (∇). The nisin control sample contained no cells. Nisin concentrations for the control broth (●) and the LA113-M3-containing broth (○) were determined by biological assay, as described in Materials and Methods.

broths, ending at roughly 30% of the initial nisin concentration. The pH of the uninoculated CJB was approximately 5.8, within the range in which nisin is stable (14); therefore, we assume that components of the CJB were interacting with nisin and binding or otherwise preventing the detection of biological activity. While the inoculated CJB initially showed less recoverable biological activity of nisin than the uninoculated control, both ended with approximately 1,500 IU/ml remaining at 72 h. Because the residual level (at 72 h) was the same for the inoculated and uninoculated broths and 1,500 IU/ml is sufficient to kill the parental cells in CJB (data not shown), we concluded that the resistance phenotype was not mediated by a mechanism that degraded nisin to sublethal levels.

**Ability of nisin-resistant mutants to ferment cabbage juice.** An HPLC analysis of fermentation end products of nisin-resistant mutants and parental strains is shown in Table 1. The end products of fermentation for the three strains tested were similar. Approximately 80% of the fructose was quantitatively reduced to mannitol by each strain. The fermentation patterns seen were typical of heterolactic fermentations for *L. mesenteroides* (3, 25). The terminal pH for each strain in cabbage juice (as well as the total amount of acid produced) varied with the source of cabbage used for cabbage juice preparation, but all of the *Leuconostoc* strains tested, including C33, LA113, and LA113-M3, demonstrated the ability to lower the pH to 3.7 to 4.1 within 24 h in pure culture fermentations.

DISCUSSION

Bacteriocins have been proposed for use in controlling food spoilage and bacterial fermentations (for reviews, see references 13 and 14). Recently, Foegeding et al. (9) demonstrated control of *Listeria monocytogenes* in dry fermented sausage, using a bacteriocin-producing *Pediococcus acidilactici* strain. Daeschel et al. (6) used nisin and a nisin-resistant *L. oenos* strain to control the malolactic acid fermentation in wine. Harris et al. (12) proposed the use of a paired starter culture system for sauerkraut, employing a nisin-resistant *L. mesenteroides* strain and a nisin-producing *Lactococcus lactis* strain, and demonstrated the effectiveness of this method with mixed-culture CJB fermentations.

TABLE 1. Fermentation products of mutant and parental strains of *L. mesenteroides* in CJB<sup>a</sup>

Strain	Cabbage substrates (mM)			Fermentation products (mM)			
	Glucose	Fructose	Malic acid	Succinic acid	Lactic acid	Acetic acid	Mannitol
After 48 h of incubation							
Uninoculated <sup>b</sup>	136.69	85.95	7.01	0.00	0.00	0.00	0.00
LA113	94.73	17.56	0.00	2.57	45.15	52.39	67.97
LA113-M3	96.39	20.92	0.00	2.65	50.55	51.63	65.66
C33	97.76	19.99	6.79	0.00	36.92	41.60	68.43
After 96 h of incubation							
LA113	93.35	11.34	0.00	2.54	49.39	57.05	74.82
LA113-M3	94.20	15.04	0.00	2.69	53.40	54.07	70.82
C33	97.36	19.80	6.56	0.85	35.67	41.20	68.27

<sup>a</sup> The broth contained 2% NaCl and was incubated at 28°C for 48 or 96 h.

<sup>b</sup> The uninoculated control sample represents components in the unfermented CJB.

These approaches have the advantage of using the combined effects of an added culture and a bacteriocin to suppress indigenous bacteria in a fermentation. To adopt this method for use in cabbage fermentations, we have isolated and characterized nisin-resistant mutants of *L. mesenteroides*.

Low concentrations of nisin (100 IU/g) have been shown to slow the rate of acid production in kimchi (4). The use of nisin-resistant *L. mesenteroides* strains in conjunction with the addition of purified nisin, or production of nisin by a nisin-producing *Lactococcus lactis* strain, in sauerkraut (or kimchi) fermentations offers the potential for controlling the indigenous lactic acid bacterial population. Nisin is ideally suited for preventing the growth of lactic acid bacteria in cabbage fermentations as it specifically attacks gram-positive organisms and is stable and bactericidal at low pH.

Natural isolates of nisin-resistant *Bacillus* species have been reported (15). These isolates do not produce nisin, and the mechanism of nisin resistance was shown to result in inactivation of nisin. Nisin resistance is also associated with nisin production (15), in the form of nisin immunity. Two nisin resistance genes from nisin-producing strains have been cloned recently (11, 27). The mechanism of action of these nisin resistance genes has not yet been reported. The level of nisin resistance reported by von Wright et al. (27) is around 500 IU/ml. Lactic acid bacteria that are resistant to nisin but do not produce the bacteriocin may use mechanisms that do not degrade nisin or inactivate biological activity. Strains of lactic acid bacteria with this phenotype have been identified by Collins-Thompson et al. (5) and Daeschel et al. (6).

Because selection for nisin resistance may have resulted in the isolation of populations that lacked desirable characteristics for cabbage fermentations, nisin-resistant isolates were screened to determine their growth kinetics in CJB and MRS, in both the presence and the absence of nisin. Our results indicated that the nisin resistance mechanism did not greatly alter the growth kinetics or final cell mass (as estimated by optical density) of the nisin-resistant mutant LA113-M3. Therefore, it is likely that the resistance mechanism did not greatly impair the metabolism of the cell. We speculate that the nisin resistance phenotype may be due to blockage or alteration of a nisin receptor on the outside of the cells, preventing nisin from binding.

It is possible that bactericidal compounds present in CJB (16, 22) affect the growth rates of LA113-M3 and act synergistically with nisin. These inhibitory compounds likely resulted in the variability we observed in the growth rates of

*L. mesenteroides* strains in CJB. Other factors that may affect the growth rate of LA113-M3 in CJB include pH and the buffering capacity of the medium. The pH drops more rapidly during fermentation with *Leuconostoc* strains in CJB than in MRS (data not shown), and nisin has been shown to be more active at low pH values (14). We also considered the possibility that growth rate itself influences the sensitivity of strain LA113-M3 to nisin. The decrease in the observed growth rate with increasing nisin concentration for LA113-M3 in CJB may be due to inability of the rapidly dividing cells to survive in the presence of nisin. We are currently investigating these possibilities.

The nisin-resistant *L. mesenteroides* strains described in this report are currently being used in laboratory sauerkraut and kimchi fermentations to delay the heterolactic fermentation. For sauerkraut fermentations, it may be possible to identify nisin concentrations that allow the heterolactic fermentation to be maximized but eventually allow a homo-lactic fermentation to occur. The rapid loss of biological activity of nisin in brined cabbage may work to the advantage of this strategy, allowing the eventual outgrowth of nisin-sensitive homofermentative species (*Lactobacillus plantarum*). In addition, we are using the methods described above to isolate nisin-resistant homofermentative lactic acid bacteria.

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