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Common Occurrence of Plasmid DNA and Vancomycin Resistance in *Leuconostoc* spp.†

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Resistance to vancomycin permitted detection, in a culture of *Streptococcus cremoris* 290PC, of a contaminant gram-positive coccus. Morphological and physiological characteristics indicated that this bacterium was a strain of *Leuconostoc* sp., designated PO184. This strain contained four plasmid species, which were distinct from those harbored by *S. cremoris* 290PC. Antibiotic disk susceptibility tests indicated that *Leuconostoc* sp. strain PO184 was also resistant to sulfathiazole and trimethoprim and susceptible to 17 other antimicrobials. The MIC of vancomycin for this strain was >2,000 μg/ml, and resistance did not depend on drug inactivation. *Leuconostoc* sp. strain PO184 produced a substance which was inhibitory to *S. cremoris* U134, but not to *S. lactis* ATCC 11454. Five other leuconostocs produced substances with antibacterial activity. Of 18 strains of *Leuconostoc* sp., 14 were resistant to at least 500 μg of vancomycin per ml, including four *L. oenos* strains which harbored no plasmid DNA in the 1- to 76-megadalton range. Twelve *Leuconostoc* sp. strains contained at least one plasmid species in this mass range. These findings are discussed from the physiological, taxonomical, and ecological standpoints and with regard to their potential applications.

Leuconostoc is a genus of fastidious, gram-positive bacteria of the family *Streptococcaceae*, found in fermenting vegetables, dairy products, and wine and in sugar refineries (7). Their capacities to produce C_4 compounds (diacetyl and acetoin) and to carry out malo-lactic fermentation are important to the dairy and wine industries, respectively. Leuconostocs are taxonomically and ecologically related to group N streptococci.

Vancomycin, a product of *Streptomyces orientalis*, is a complex glycopeptide antibiotic which acts by interfering with the biosynthesis of the bacterial cell wall peptidoglycan. It is highly potent against gram-positive bacteria, but not against gram-negative organisms. Mycobacteria, molds, and yeasts are unsusceptible to vancomycin (10, 17). Among the susceptible bacterial species, resistant mutants are very rare or nonexistent (4, 13).

This paper reports on the occurrence of high-level resistance to vancomycin as a common trait of *Leuconostoc* sp. strains. We have also applied a recently described, streptococcal plasmid isolation method (16) to these bacteria, to extend the original observation of O'Sullivan and Daly (T. O'Sullivan and C. Daly, Ir. J. Food Sci. Technol. **6**:206, 1982) on the presence of plasmid DNA in *Leuconostoc* spp.

MATERIALS AND METHODS

Bacterial strains. The identity and origin of the strains used in this study are given in Table 1.

Culture media. For *Leuconostoc oenos* strains, culture maintenance, cell propagation before lysis, and MIC determinations were carried out in modified Rogosa medium (18), from which liver extract was omitted and to which fructose (3.0 g/liter) and L-malic acid (2.0 g/liter) were added. For other *Leuconostoc* sp. strains, the medium used was MRS broth (3) containing only 0.4% glucose and in some instances fortified with filtered V-8 juice (Campbell Soup Co.) at 200 ml/liter. (Siegel et al. [20] observed that cells of *Streptococcus* sp. grown in media containing higher glucose concentrations were refractory to lysis.) This modified MRS broth was also used in the determination of vancomycin MICs for lactic streptococci. *Escherichia coli* V517 (14) was propagated in M17 broth (21).

Carbohydrate fermentation test broth contained, in grams per liter: beef extract, 3.0; Difco Proteose Peptone no. 3 (Difco Laboratories), 10.0; yeast extract, 3.0; bromocresol purple, 0.015; $MnSO_4 \cdot 2H_2O$, 0.055; $MgSO_4$, 0.24; and the indicated carbohydrate, 10.0. The pH was 6.65. Acid production was detected as color change to yellow after 24 h of incubation at 24°C (0.1% inoculum). Sucrose indicator agar (15) was fortified with 3.0 g of polypeptone (BBL Microbiology Systems) and 0.12 g of $MgSO_4$ per liter.

Mueller-Hinton agar and brain heart infusion, MRS, Pennassay, and Todd-Hewitt broths were from Difco Laboratories.

Chemicals. Vancomycin hydrochloride was purchased from Sigma Chemical Co. Stock solutions were prepared in sterile distilled water immediately before use.

Determination of antibiotic susceptibility. Disk tests were performed on Mueller-Hinton agar (2), using commercially obtained antibiotic disks (Difco or BBL). MIC determinations in the media indicated above were performed at 24°C. For *L. oenos* strains, 5% inoculum and up to 44 h of incubation were used; for all other strains, these parameters were 1% and 22 h, respectively.

Test for bacteriocin production. The method of Kékessy and Piguet (12) was followed for the bacteriocin production test. The medium used was MRS agar containing V-8 juice, as described above. Each plate was incubated anaerobically for 23 h at 29°C before inoculation of the reverse side of the agar with an indicator strain. Results were read after a second incubation period under the same conditions.

Cell lysis, isolation of plasmid DNA, and agarose gel electrophoresis. A recently described method (16) was used for cell lysis, plasmid DNA isolation, and agarose gel electrophoresis, with the following modifications: 8-ml *Leuconostoc* sp.

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TABLE 1. Bacterial strains

Identity	Source[a]	Vancomycin MIC (μg/ml)[b]	Plasmid profile (Figure-lane)
Escherichia coli V517	E. Lederberg (PRC)	Not tested	1-A, 2-A
Leuconostoc oenos ML34	R. Kunkee (UCD)	>1,000[c]	1-E[d]
L. oenos MLT-kli	A. Gryczka (MLT)	>1,000[c]	—[d]
L. oenos 23	T. Sozzi (NESTEC)	1,000	—[d]
L. oenos Erla	Oregon wine[e]	1,000	—[d]
L. oenos Ey2d	Oregon wine[e]	>1,000	Not examined
L. mesenteroides 98	This laboratory	>1,600	2-E
L. cremoris 91404	This laboratory	1,000	1-F
Leuconostoc sp. strain 16-9	This laboratory	>1,000	2-F
L. cremoris CAF7	This laboratory	>1,000	2-B
Leuconostoc sp. strain P325	This laboratory	1,000	2-J
L. dextranicum LDF	This laboratory	>1,000[c]	2-I
Leuconostoc sp. strain 14AM	This laboratory	<10	2-M
Leuconostoc sp. strain 39	This laboratory	<10	2-L
Leuconostoc sp. strain 29	This laboratory	>1,000	2-H
L. dextranicum 180	This laboratory	>1,000	2-C
L. dextranicum 181	This laboratory	>1,000[c]	2-D
L. cremoris 225	This laboratory	500	Not examined
L. dextranicum 688	This laboratory	<5	1-D, 2-K
Leuconostoc sp. strain PO184	This study	>2,000	1-C, 2-G
Streptococcus lactis MG1363	M. Gasson (NIRD)	<10	Not examined
S. lactis NCDO 1403	NIRD	Not tested	Not examined
S. lactis NCDO 1404	NIRD	<10	Not examined
S. lactis ATCC 11454	D. LeBlanc (NIDR)	Not tested	Not examined
S. lactis ATCC 7962	This laboratory	<10	Not examined
S. lactis C10	This laboratory	Not tested	Not examined
S. cremoris 290PC	This laboratory	<10	1-B
S. cremoris U134	This laboratory	<10	Not examined

[a] PRC, Plasmid Reference Center, Stanford University School of Medicine, Stanford, Calif.; UCD, Department of Viticulture and Enology, University of California, Davis; MLT, Microlife Technics, Sarasota, Fla.; NESTEC, Nestlé Products Technical Assistance Co. Ltd., La Tour du Peilz, Switzerland; NIRD, National Institute for Research in Dairying, Shinfield, Reading, U.K.; NIDR, National Institute for Dental Research, Bethesda, Md.

[b] Concentrations tested were 0, 10, 30, 100, 500, and 1,000 μg/ml, unless otherwise noted.

[c] Growth was impaired, but not completely inhibited, at this concentration.

[d] This strain contained no plasmids in the 1- to 76-Md mass range; see legend to Fig. 1.

[e] Y. S. Izuagbe et al., submitted for publication.

cultures from a 10% inoculum were incubated at 24°C until their absorbance at 660 nm (1.0-cm path) reached at least 0.50 (but not more than 0.70). For most strains, including *L. oenos* Ey2d, 10 h was sufficient; other *L. oenos* strains (23, Erla, ML34, and MLT-kli) required up to 48 h of incubation. Cells were harvested, washed, and frozen overnight at −72°C before lysis. Tris-sucrose buffer (16) was added after 6.5 min of incubation with lysozyme and mutanolysin at 37°C, and the samples were immediately chilled for 2 min before addition of triisopropylnaphthalenesulfonic acid (sodium salt), followed by NaOH. In this protocol, the strains examined lysed to transparency, with the exception of *L. oenos* Ey2d and the new isolate described in this paper (*Leuconostoc* sp. strain PO184). The latter strain nevertheless yielded plasmid DNA in quantities comparable to those obtained from other leuconostocs. After RNase treatment (15 min), the samples were extracted with 200 μl of chloroform-isoamyl alcohol (24:1), and 10 μl of the aqueous phase was used directly in electrophoresis. Molecular masses were estimated from a double-logarithmic plot constructed on the basis of *E. coli* V517 standards (14).

RESULTS

Isolation and characterization of a vancomycin-resistant, gram-positive coccus. During an investigation of antimicrobial resistance in lactic streptococci (submitted for publication), we observed that a culture of *Streptococcus cremoris* 290PC exhibited heterogeneity with regard to susceptibility to vancomycin. In the Bauer-Kirby disk test (2), numerous resistant clones grew inside the zone of inhibition around a disk containing this antibiotic (30 μg). Heterogeneity could not be observed after culture reisolation, with a zone of complete inhibition by vancomycin occurring instead. Whereas the original culture displayed heavy bottom growth and light growth throughout M17 broth (21), after 24 h of incubation at 24°C, only heavy bottom growth was seen with the reisolated culture. In M17 broth containing vancomycin (15 μg/ml), the original culture still displayed light growth throughout the medium, whereas heavy bottom growth was abolished. Cells from such an M17 broth culture, containing 60 μg of vancomycin per ml, were isolated on glucose-M17 agar with the same concentration of the antibiotic.

This vancomycin-resistant isolate displayed the following characteristics: round or slightly elongated gram-positive cocci, mostly in chains of 4 to 10 cells, but also in pairs or small clusters; catalase negative. In MRS broth (3), growth was observed at 7.5 and 30°C, but not at 37°C. At 30°C, this strain grew poorly in brain heart infusion, Pennassay, or Todd-Hewitt broth and only slightly better in M17 broth containing either glucose or lactose. In contrast, abundant growth was observed in MRS and tomato juice broths (1). On blood agar, this isolate produced very small colonies (<1 mm in diameter) and no hemolysis after 48 h of aerobic incubation at 30°C. Large (2.0-mm) mucoid colonies, suggestive of dextran production, were observed after 24 h of anaerobic incubation at 30°C on sucrose indicator agar (15). The results of a series of carbohydrate fermentation and litmus milk tests performed with this strain and, for comparison, with two strains of *Leuconostoc* sp. are shown in Table 2. None of these three cultures fermented dulcitol, erythritol, glycerol, inositol, inulin, rhamnose, or sorbose, in complete agreement with what is accepted for the genus *Leuconostoc* (7). Taken together, these results indicated that the vancomycin-resistant isolate was a strain of *Leuconostoc* sp. This bacterium is here designated *Leuconostoc* sp. strain PO184.

TABLE 2. Carbohydrate fermentation and litmus milk tests

Test	*L. mesenteroides* 98	*L. dextranicum* 688	Vm[r] isolate[a]
Acid from:			
Arabinose	+	+	+
Cellobiose	+	+	−
Dextrin	+	+	−
Glucose	+	+	+
Lactose	−	+	+
Mannitol	−	−	−
Melibiose	+	−	+
Ribose	+	+	−
Salicin	+	+	+
Sucrose	+	+	+
Trehalose	+	+	−
Xylose	+	−	+
Litmus milk[b]			
Clot	−	−	−
Reduction	−	−	−
Gas	+[c]	+[c]	+[c]
Supplemented litmus milk[d]			
Clot	−	+	+
Reduction	+	+	+
Gas	+	+	+

[a] Vm[r], vancomycin resistant.
[b] Results were read after 36 h at 24°C.
[c] Minute amounts.
[d] Glucose (1.0%) and yeast extract (0.5%) added.

Confirmation of nonidentity between *Leuconostoc* sp. strain PO184 and the culture which originally harbored it, *S. cremoris* 290PC, came from the comparison of the plasmid profiles of the two strains, which are obviously different (Fig. 1). Great dissimilarity was also observed upon microscopic examination of the two cultures: *S. cremoris* 290PC formed long chains (>50 cells in length), whereas *Leuconostoc* sp. strain PO184 did not.

Clearly, it was of interest to examine whether *Leuconostoc* sp. strain PO184 exhibited resistance to other antimicrobial agents. Disks containing the following substances were used in the Bauer-Kirby test (2): amikacin, ampicillin, bacitracin, cephalothin, chloramphenicol, chlortetracycline (aureomycin), erythromycin, gentamicin, kanamycin, lincomycin, nafcillin, neomycin, penicillin G, rifampin, streptomycin, sulfathiazole, tetracycline, tobramycin, trimethoprim, and vancomycin. This organism was susceptible to all of these antimicrobials, with the exceptions of sulfathiazole, trimethoprim, and vancomycin, which caused no detectable inhibition. Low susceptibility to sulfathiazole and trimethoprim has also been observed in our studies of the lactic streptococci (submitted).

Vancomycin resistance and plasmid DNA in strains of *Leuconostoc* sp. Eighteen strains of *Leuconostoc* sp. were randomly selected and examined for susceptiblity to vancomycin and for plasmid DNA content. The strains and their respective vancomycin MICs are listed in Table 1, and their plasmid profiles are shown in Fig. 1 and 2. Four strains of *L. oenos* examined contained no plasmid DNA in the range of 1 to 76 megadaltons (Md); lysis of a fifth strain (*L. oenos* Ey2d) was not achieved. All of the other strains did contain plasmid DNA, varying in molecular mass from ca. 1.9 to ca. 76 Md. Fourteen of the strains were resistant to at least 500 μg of vancomycin per ml, whereas three others, as well as five lactic streptococcal strains tested under identical conditions, were inhibited by the smallest concentration tested (10 μg/ml).

Lack of vancomycin inactivation and production of an antibacterial substance by *Leuconostoc* sp. strain PO184. An experiment was performed to examine whether the high-level resistance to vancomycin exhibited by *Leuconostoc* sp. strain PO184 was due to inactivation of the drug in its culture medium. Three tubes were prepared, each containing MRS broth (4 ml). To tubes A and B vancomycin was added, to a final concentration of 1,000 μg/ml; tube C received no antibiotic. Tubes A and C were inoculated with 50 μl of a fresh culture of *Leuconostoc* sp. strain PO184; tube B was not inoculated. All three tubes were incubated at 30°C for 15 h. Cultures A and C were then neutralized with 10% NaOH, to the pH of uninoculated broth (6.4), and the cells were removed by centrifugation followed by filtration through a membrane filter (0.45-μm pore size; Millipore Corp.). Each solution (20 μl of A, B, or C) was spotted onto 6.5-mm sterile filter paper disks (Difco), which were allowed to dry briefly, and used in the Bauer-Kirby susceptibility test (2). (It can easily be calculated that such disks contained no more than 20 μg of vancomycin). Four strains of vancomycin-susceptible lactic streptococci were used as indicator organisms. A commercially obtained disk with 30 μg of vancomycin (Difco) was included as a control. Inhibition zone diameters were measured after 24 h of incubation at 30°C.

The results (Table 3) indicated that this bacterium produced little or no alteration in the concentration of vancomycin in the medium. It was evident that a rather high concentration of active antibiotic remained in the broth after growth of *Leuconostoc* sp. strain PO184. A clear zone of inhibition of *S. cremoris* U134 was produced by the filtrate of a culture to which vancomycin had not been added,

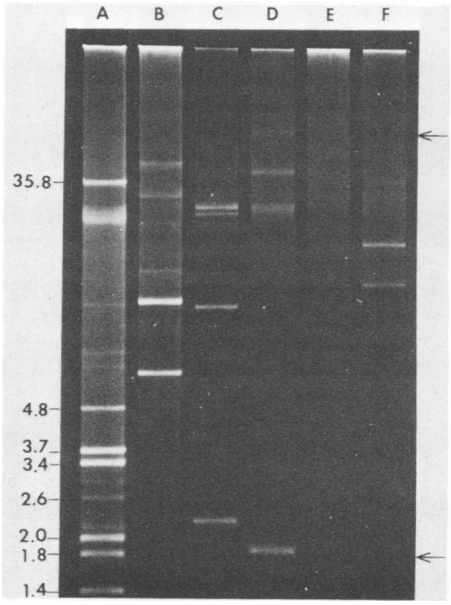

FIG. 1. Bacterial plasmid profiles. Lane A, *E. coli* V517; values on the left denote molecular masses in megadaltons (14). Lane B, *S. cremoris* 290PC (6.5, 11, 33, 49 Md). Lane C, *Leuconostoc* sp. strain PO184 (2.4, 11, 25, 28 Md); this strain was originally harbored, as a contaminant, by the culture shown in lane B. Lane D, *L. dextranicum* 688 (1.9, 47, 76 Md); of the leuconostocs examined, this bacterium carried both the smallest and the largest plasmid species (arrows). Lane E, *L. oenos* ML34, showing absence of plasmid DNA in this size range; similar results were obtained with *L. oenos* strains 23, Er1a, and MLT-k1i. Lane F, *L. cremoris* 91404 (13, 19, 35 Md).

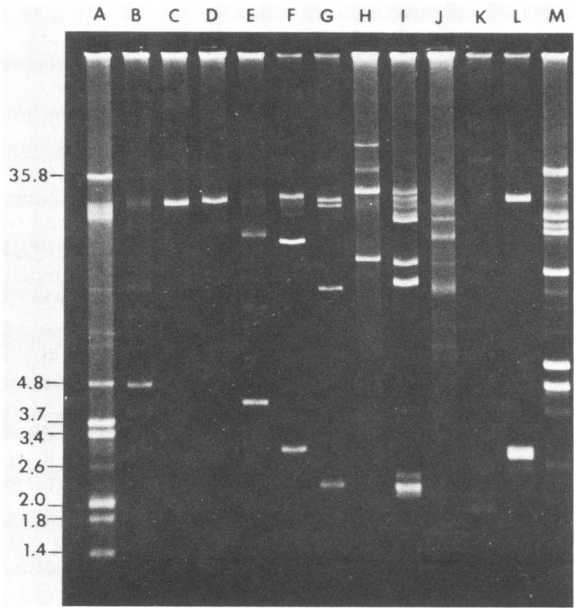

FIG. 2. Bacterial plasmid profiles. Lane A, *E. coli* V517. Lanes B to M, *Leuconostoc* sp. strains identified in Table 1. The apparent molecular masses (megadaltons) of the plasmid species observed are given after the letter which identifies the lane: B (4.8); C (27); D (27); E (4.3, 18); F (2.9, 17, 29); G (2.4, 11, 25, 28); H (14, 32, 40, 65); I (2.2, 2.3, 11, 13, 21, 26, 31); J (6.2, 6.5, 10, 18, 22, 26); K (1.9, 47, 76); L (2.7, 2.9, and 28); and M (4.9, 5.5, 12, 19, 20, 22, 41).

showing that *Leuconostoc* sp. strain PO184 produces a substance or substances with antibacterial activity.

Production of antibacterial substances by strains of *Leuconostoc* sp. With the exception of *L. oenos* strains, all of the remaining leuconostocs listed in Table 1 were used as both producers and indicators in the Kékessy-Piguet (12) test for bacteriocin production. Indicator strains also included *S. lactis* ATCC 11454 and NCDO 1404. The following strains caused inhibition of *Leuconostoc* sp. or *S. lactis* strains: LDF, 29, 39, 180, and 181.

DISCUSSION

In 1974 Reinbold and Reddy (19) observed that two strains of *L. dextranicum* were resistant to vancomycin as determined by a disk susceptibility test. These authors did not examine this matter in further detail.

TABLE 3. Activity of vancomycin (Vm) added to *Leuconostoc* sp. strain PO184 culture medium

Indicator	Diam of inhibition zone (mm)[a]			
		Disk with filtrate (20 μl) from:		
	Standard disk[b]	Culture with 1,000 μg of Vm per ml	Uninoculated control with 1,000 μg of Vm per ml	Culture without Vm
S. lactis C10	21	21	19	7.5[c]
S. lactis ATCC 11454	20	20	20	0
S. lactis NCDO 1403	18	18	18	7.5[c]
S. cremoris U134	22	22	22	10

[a] Two disks from each filtrate and one standard disk were used per indicator strain. Results from filtrates represent averages.
[b] Commercially obtained disk with 30 μg of vancomycin (Difco).
[c] A very narrow zone of inhibition was visible around disk (disk diameter, 6.5 mm).

Typical MICs of vancomycin for gram-positive bacteria such as *Streptococcus* or *Staphylococcus* spp. are of the order of 1.0 μg/ml (10). Approximately 500-fold-higher concentrations of this antibiotic could not inhibit most of the *Leuconostoc* sp. strains examined in this study. To our knowledge, this is the first detailed account of the existence of non-acid-fast, gram-positive bacteria which are naturally resistant to high levels of vancomycin. To a certain extent, it is not surprising that the unsusceptibility of *Leuconostoc* sp. strains to this antibiotic remained undetected for so long after its discovery, given the nonpathogenicity and lack of involvement of this genus in any aspect of medical microbiology. It should prove interesting to investigate whether other medically unimportant bacteria related to *Leuconostoc* sp., such as *Pediococcus* sp., might also be unsusceptible to vancomycin.

The antibacterial action of vancomycin is due to complexing with the pentapeptide precursor of peptidoglycan biosynthesis. Binding of the antibiotic requires that the peptide precursor have five amino acids and that the terminal pair be D-alanyl-D-alanine (4, 6, 17). Our results show that, in the case of one strain at least, resistance to vancomycin does not depend on its inactivation. Although Harney et al. concluded that there was little difference between the chemical compositions of the *Leuconostoc* sp. and *Streptococcus* sp. cell walls (9), our observations suggest that one or more steps in the respective biosynthetic pathways are dissimilar enough to account for an enormous difference in susceptibilities to this antibiotic. That not all of the *Leuconostoc* sp. strains examined were unsusceptible to vancomycin (or, for that matter, equally susceptible to the lysis protocol used) might reflect the findings of Kandler; i.e., that several types of murein exist within this genus (11).

The high susceptibility to vancomycin found in lactic streptococci, which are closely related to *Leuconostoc* sp. from the standpoint of ecology, suggests that the resistance exhibited by the latter genus might be coincidental, rather than a specific adaptation to the possible presence of antibiotic-producing *Streptomyces* spp. in their habitat. An obvious application of our observations would be the use of vancomycin in selective media for the isolation of wild strains of *Leuconostoc* sp. or for the enumeration of these bacteria in mixed-strain, lactic starter cultures. Media containing sodium azide (J. V. Mayeux, W. E. Sandine, and P. R. Elliker, abstr. M47, J. Dairy Sci. **45**:655, 1962), salt (5), or low concentrations of tetracycline (F. E. McDonough, R. E. Hargrove, and R. P. Tittsler, abstr. M48, J. Dairy Sci. **45**:656, 1962) have been used for these purposes.

The production of inhibitory substances by *Leuconostoc* sp. strains, as exemplified by our results, must clearly be taken into consideration when composing mixed-strain fermentation starters.

We have also demonstrated the application of a recently described lactic streptococcal plasmid isolation method (16) to the genus *Leuconostoc*. O'Sullivan and Daly first reported on the presence of plasmid DNA in these bacteria (Ir. J. Food Sci. Technol. **6**:206, 1982). All of 10 strains examined by these authors contained at least one plasmid species, in the 2.5- to 40-Md range; wine leuconostocs were not examined.

In her description of the genus *Leuconostoc*, Garvie (7) emphasizes the clear distinction between *L. oenos* and the other species in this genus. The results obtained with the strains of *L. oenos* examined in this study suggest that the

presence of plasmid DNA may be less frequent in this species than in other leuconostocs; if true, this would be yet another characteristic separating the two groups of microorganisms. On the other hand, their unity as parts of the same genus is supported by the shared trait of resistance to vancomycin.

Garvie has also stated (8) that *L. dextranicum* and *L. cremoris* may be subspecies of *L. mesenteroides* and that further work on the relationship of these bacteria is needed. It seems reasonable to suppose, based on what is known for the lactic streptococci, that some of the traits for which *Leuconostoc* bacteria exhibit a high degree of variability, such as the fermentation of different carbohydrates, might depend on plasmid-carried genes. This hypothesis is supported by the concurrent loss of plasmid DNA and of the capacity to utilize lactose or citrate observed in leuconostocs by O'Sullivan and Daly (Ir. J. Food Sci. Technol. **6**:206, 1982).

The complexity of *Leuconostoc* sp. plasmid profiles makes these bacteria similar to the lactic streptococci in yet another aspect. It is tempting to speculate that future genetic manipulations of cultures used in the dairy and wine industries might involve transfer of genetic material between these two groups of microorganisms.

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