JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1990, p. 1728–1733
0095-1137/90/081728-06$02.00/0
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Vol. 28, No. 8

Characterization of *Leuconostoc lactis* Strains from Human Sources

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Received 13 December 1989/Accepted 24 April 1990

An examination of 23 vancomycin-resistant, streptococcuslike isolates of clinical origin revealed a group of 14 to be related to *Leuconostoc lactis* (type strain, CIP 102422) on the basis of chemotaxonomic studies. These isolates were initially shown to be atypical by classical biochemical tests. However, they were characterized in particular by their polar lipid patterns by thin-layer chromatography and, additionally, by whole-cell protein patterns by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It is feasible, therefore, that common biochemical tests may continue to serve the purpose of routine identification, even though such isolates were formerly thought to be only of dairy origin.

The isolation of *Leuconostoc* spp. from human sources has repeatedly been related to serious infections (6, 7, 17, 18, 23, 25, 30). Thus, it was of particular interest when the Collection de l'Institut Pasteur (CIP), Paris, France, received, between 1984 and 1987, 23 vancomycin-resistant, streptococcuslike strains, 14 of which were determined to be *Leuconostoc lactis* (Table 1). The others were *Pediococcus pentosaceus* (two strains), *Pediococcus acidilactici* (three strains), *Leuconostoc mesenteroides* subsp. *mesenteroides*, and *Leuconostoc paramesenteroides* (one strain). Very recently, two more clinical strains, closely related genetically to the type strain of *L. lactis*, were also reported (11).

We examined the CIP isolates in relation to *Leuconostoc* type strains (Table 2). Certain simple chemotaxonomic characters related these 14 isolates to *L. lactis*, thus enabling the use of common biochemical tests for their identification, despite the fact that initially there were differences from the characters described in the literature (13).

The standard reference source indicates that *L. lactis* is mostly of dairy origin (13). Therefore, it was of importance to clearly establish the biochemical patterns of *L. lactis* isolated from clinical sources.

MATERIALS AND METHODS

Growth conditions and biochemical tests. Growth of all organisms and all biochemical tests were carried out at 22°C under aerobic conditions with inocula prepared from 28-h cultures. MRS broth (8) was used as the basal medium; unless otherwise stated, all media were obtained from Diagnostics Pasteur, Garches, France.

The following tests were used: colony development on Columbia agar slants; growth temperature range and halotolerance in MRS broth; gas production in MRS broth covered by a petrolatum wax seal (27); Voges-Proskauer test (14, method 2) in Clark-Lubs broth; dextran formation (15) on tryptic soy agar with sucrose (50 g/liter) incubated at 15°C; nitrate and nitrite reduction (14) in heart infusion with potassium nitrate (1 g/liter) and potassium nitrite (3 g/liter), respectively; urease production in urea-indole medium (1); production of arginine, lysine, and ornithine decarboxylases (15) in LDC-ODC-ADH media; and ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and para-nitrophenyl-β-D-xylopyranoside (PNPX) hydrolysis with ONPG and PNPX solutions (4 g/liter; Sigma Chemical Co., St. Louis, Mo.), respectively (1). Plate methods with spot inoculations were used for exoenzyme testing (15). Acid production tests were performed with the API CH50 system (API System S.A., La Balme les Grottes, France) as previously described (1). All results were recorded after 1, 4, and 10 days.

For chemotaxonomic purposes, a cell mass was prepared from 48-h cultures grown in 300 ml of MRS broth. After a purity check, cells were killed by the addition of 2% Formalin (vol/vol) (E. Merck AG, Darmstadt, Federal Republic of Germany), washed twice, taken up in distilled water, and freeze-dried.

Amino acid analysis of peptidoglycan. Cell wall material was prepared by a modification of the method of Keddie and Cure (21). Freeze-dried bacteria (5 mg) were suspended in 2 ml of a 5% potassium hydroxide solution and heated at 98°C. The turbidity was recorded with a model 21 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) at 390 nm until a steady optical density was attained; then, 10 ml of distilled water was added. The preparation was subsequently centrifuged at 10,000 × *g* for 10 min (Centrikon H-401; Kontron & Hermle KG, Gosheim, Switzerland), washed, and hydrolyzed with 2 ml of 6 M hydrochloric acid at 100°C for 18 h. The hydrolysate was filtered on a 0.22-μm-pore cellulose membrane (Millex; Millipore Corp., Bedford, Mass.) and evaporated under an air stream at 90°C, and the residue was dissolved with 30 μl of 10% 1-propanol in distilled water. Volumes of 2 to 5 μl were spotted on high-performance thin-layer chromatography cellulose plates (10 by 10 cm; Merck). The plates were developed by two-dimensional chromatography as described by Brenner et al. (4): first dimension, 1-propanol–50% acetic acid (20:10); second dimension, methanol–pyridine–10% HCl (20:2.5:5.5). The amino acids were revealed by spraying the plates with freshly prepared 0.2% ninhydrin in ethanol-collidine (95:5 [vol/vol]), followed by heating at 90°C (4).

Sugar analysis of whole-cell extracts. Freeze-dried bacteria (5 mg) were hydrolyzed in 1 ml of M sulfuric acid at 100°C for 2 h. The hydrolysate was mixed with 220 mg of barium hydroxyde and neutralized with a saturated barium hydroxide solution. The resulting precipitate was centrifuged at 8,000 × *g* for 20 min, and the supernatant was filtered on a 0.22-μm-pore cellulose membrane (Millex) and evaporated under an air stream at 60°C. The extract was taken up with 50 μl of a 10% 1-propanol solution, and 5 μl was applied to high-performance thin-layer chromatography–cellulose plates (10 by 20 cm; Merck) to form a 12-mm band. A mixture of pure sugars at 0.5% in 10% 1-propanol was used

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TABLE 1. Strains of *L. lactis* studied

Strain[a]	Group	Source
CIP 102422	Type strain	DSM 20202 (= ATCC 19256 and NCDO 533)
CIP 100915	H1	Blood, 1984, Paris
CIP 101238	H1	Blood, 1984, Paris
CIP 101240[b]	H1	Blood, 1984, Paris
CIP 101268	H1	Blood, 1984, Paris
CIP 101269	H1	Blood, 1984, Paris
CIP 102167	H1	Blood, 1986, Clermont-Ferrand
CIP 102869	H1	Blood, 1987, Poissy
CIP 102870	H1	Blood, 1987, Paris
CIP 102574	H2	Blood, 1986, Clermont-Ferrand
CIP 101549	H3	Blood, 1985, Paris
CIP 101878	H3	Nourishing pump, 1985, Paris
CIP 101991	H3	Blood, 1985, Suresnes
CIP 102307	H3	Saliva, 1986, Rennes
CIP 102827	H3	Blood, 1986, Paris

[a] All the clinical strains were isolated in French laboratories and are available under their CIP accession numbers.
[b] Strain CIP 101240 has atypical whole-cell protein patterns (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

as a standard. The components were separated by three developments with ethyl acetate-pyridine-water (100:35:25) as described by Schaal (26) and revealed by spraying the plates with aniline phthalate reagent (phthalic acid, 1.5%; aniline, 1%; water-saturated butanol, 100 ml), followed by heating at 90°C (26).

Polar lipid analysis. Free lipids were extracted by the method of Bligh and Dyer (2). Freeze-dried cells (100 mg) were stirred for 4 h with 3.8 ml of a chloroform-methanol-water solution (20:10:8) and centrifuged at 10,000 × *g* for 20 min, and the extraction was repeated. The two supernatants were pooled, and 2 ml of chloroform and 2 ml of water were added. The layers were separated by centrifugation at 10,000 × *g* for 10 min. The lower phase was filtered through a 1PS-Separator Phase Filter (Whatman, Maidstone, United Kingdom) and evaporated under a nitrogen stream. The dried preparation was dissolved in 0.2 ml of chloroform-methanol (vol/vol). The extract (5 μl) was applied to Kieselgel G 60 plates (10 by 20 cm; Merck) to form a 12-mm band. As described by Mangold (24), the components were separated by a single development with chloroform-methanol-acetic acid-water (60:25:0.8:4) and visualized by spraying the plates with the following reagents: molybdenum blue reagent (9) for phosphate esters, α-naphthol reagent (19) for glycolipids, ninhydrin reagent (see above-described amino acid

TABLE 2. Characteristics in which the 14 clinical *L. lactis* strains differed from the *Leuconostoc* type strains

Characteristic	Reaction[a] of:					
	L. lactis CIP 102422[T]	Clinical strains	*L. mesenteroides* subsp. *cremoris* CIP 103009[T]	*L. mesenteroides* subsp. *dextranicum* CIP 102823[T]	*L. mesenteroides* subsp. *mesenteroides* CIP 102305[T]	*L. paramesenteroides* CIP 102821[T]
Growth with NaCl at:						
6%	w	w	−	w	w	+
8%	−	−	−	−	−	+
10%	−	−	−	−	−	w
Growth at:						
10°C	w	+	w	+	+	+
41°C	+	+	−	−	−	−
Gas from gluconate	−	−	−	−	−	+
Dextran formation	−	(+)	−	+	+	−
ONPG hydrolysis	+	d	−	−	+	−
Esculin hydrolysis	−	(−)	−	−	+	−
Acid from:						
Amygdalin	−	−	−	−	w	−
L-Arabinose	w	d	−	−	+	+
Arbutin	−	(−)	−	−	w	−
Cellobiose	−	(−)	−	−	w	−
Fructose	−	+	−	+	+	+
Galactose	+	+	+	−	+	+
Gentobiose	−	−	−	−	w	w
Gluconate	−	w	−	+	+	+
Lactose	+	d	−	w	w	−
Maltose	+	+	−	+	+	+
Mannitol	−	w	−	−	+	+
Mannose	−	+	−	+	+	+
Melibiose	−	+	−	−	+	w
Raffinose	−	+	−	−	+	w
Ribose	−	−	−	−	+	+
Salicin	−	(−)	−	−	w	−
Trehalose	−	−	−	+	+	+
Turanose	−	−	−	−	+	+
D-Xylose	−	d	−	−	w	−
α-Methyl-glucoside	−	−	−	−	+	+
Rhamnose in whole-cell extracts	+	+	−	+	−	−
Glycolipid G3 (see Fig. 2)	+	+	−	−	−	−

[a] +, All strains positive; −, all strains negative; d, different reactions; w, delayed reactions; (+) and (−), some exceptions excluded.

TABLE 3. Characteristics differentiating vancomycin-resistant cocci from clinical sources

Characteristic	Reaction[a] of:			
	L. lactis	*L. mesenteroides*	*L. paramesenteroides*	*Pediococcus* spp.
Gas from glucose	+	+	+	−
ADH	−	−	−	+
Growth at:				
45°C	−	−	−	+
41°C	+	−	−	+
Growth with 8% NaCl	−	−	+	+
Acid from trehalose	−	+	+	d
Acid from ribose	−	d	+	+

[a] +, All strains positive; −, all strains negative; d, different reactions.

analysis) for free amino groups, and vanillin-sulfuric acid (5) for total visualization.

Polyacrylamide gel electrophoresis of soluble proteins. Cells from a 10-ml culture sample were harvested by centrifugation at 6,000 × *g* for 10 min and washed with 10 ml of distilled water. Fifty microliters of the cell mass was suspended in 1 ml of a lysozyme solution (Sigma) in distilled water (1 g/liter) and incubated at 37°C for 3 h. Preparations were washed with 1 ml of distilled water and heated at 100°C for 5 min in 0.5 ml of 0.35 M Tris hydrochloride buffer (pH 6.8) containing sodium dodecyl sulfate (2%), glycerol (10%), and 1,4-dithiothreitol (1.6%); the extract was centrifuged at 10,000 × *g* for 2 min, and the supernatant was stored at −20°C until needed. Discontinuous gels were prepared as described by Laemmli (22) with a Protean II cell (Bio-Rad Laboratories, Richmond, Calif.) and 10% separation plate gels (120 by 150 by 1.5 mm). Protein samples of 20 μl were loaded into 7-mm-wide wells. Electrophoresis was performed at a constant current of 45 mA, and gels were stained with Coomassie blue.

RESULTS

Biochemical tests. All the *Leuconostoc* strains studied were nonmotile coccobacilli occurring commonly in pairs and chains; they produced small colonies without pigment on Columbia agar, while good growth occurred in MRS broth between 20 and 30°C. Their metabolism was consistent with that of facultative anaerobes; they produced acid and gas from glucose. They were all Voges-Proskauer test negative. The bacteria were able to grow at 10°C and with a sodium chloride concentration of 0 to 40 g/liter but failed to grow at 43°C or with a sodium chloride concentration of 100 g/liter. Catalase, urease, and alkali from arginine (ADH), lysine (LDC), or ornithine (ODC) were not produced; nitrate and nitrite were not reduced; and PNPX, extracellular DNA, starch, gelatin, casein, and Tween 80 were not hydrolyzed. Acid was always formed from *N*-acetylglucosamine and from sucrose; no acid was formed from adonitol, D-arabinose, arabitol, dulcitol, erythritol, fucose, glycerol, glycogen, inositol, inulin, lyxose, melizitose, rhamnose, sorbitol, sorbose, starch, tagatose, xylitol, L-xylose, and β-methylxyloside.

Characteristics differentiating the *L. lactis* clinical strains from the *Leuconostoc* type strains are listed in Table 2. For orientation purposes, the common biochemical tests are shown in Table 3. These were found to be highly reliable. On the basis of numerical analysis, the *L. lactis* clinical strains seemed to be differentiated into three groups (Fig. 1 and

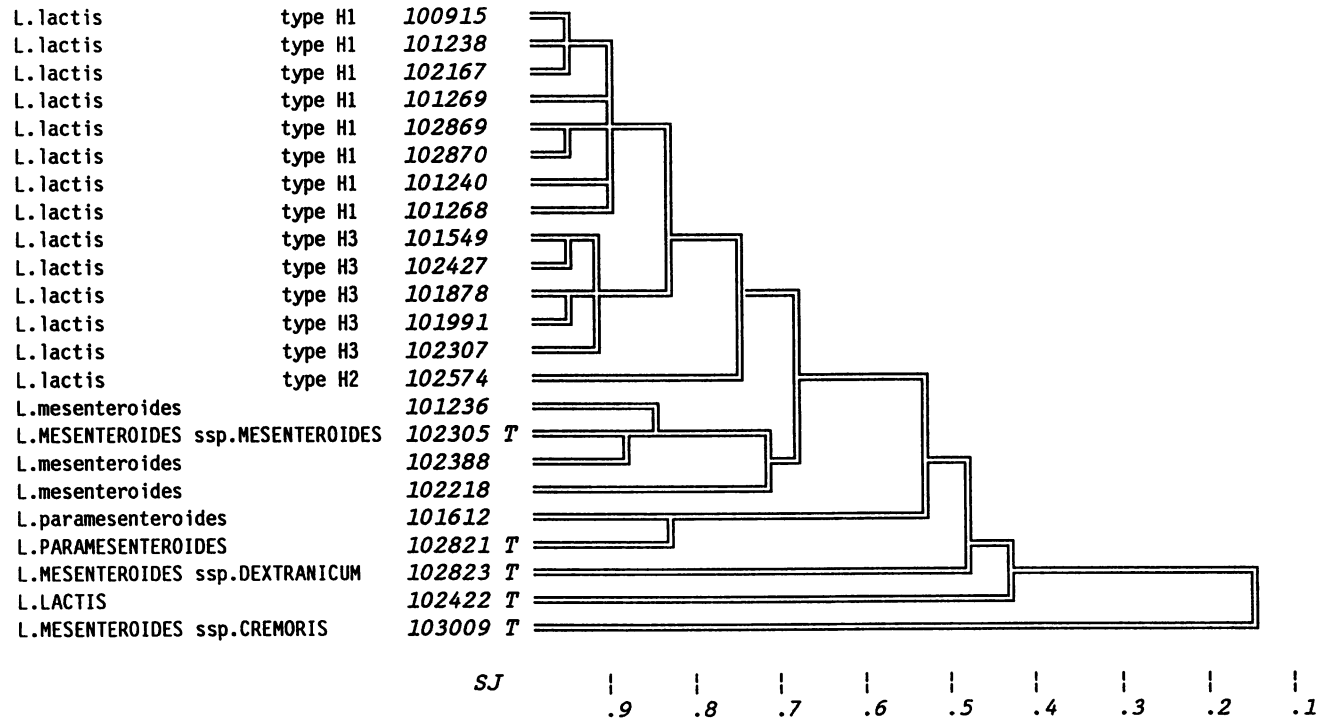

FIG. 1. Dendrogram of a numerical analysis of 86 phenotypic features of *Leuconostoc* strains with the SJ coefficient and the single-linkage method. ssp., Subspecies. T, Type strain.

TABLE 4. Characteristics in which the 14 clinical strains of *L. lactis* differed

Characteristic	Reaction[a] found by Bercovier et al. (1)	Reaction[a] of strains of the following group (*n*):		
		H1 (5)	H2 (1)	H3 (8)
Dextran formation	−	3/5 +	+	+
ONPG hydrolysis	ND	+	+	−
Acid from:				
L-Arabinose	−	−	+	6/8 +
D-Xylose	−	−	+	7/8 +
Mannitol	−	+	+	+
Melibiose or raffinose	d	+	+	+
Cellobiose or arbutin	−	−	+	−
Esculin hydrolysis	−	−	+	−

[a] +, All strains positive; −, all strains negative; ND, not determined; d, different reactions.

Table 4). These groupings were independent of the calculation procedure used (SJ or SM coefficient, single-linkage or unweighted-average-linkage method). On the basis of the biochemical test results, the clinical strains were related neither to the type strain nor to the species description (13).

Chemotaxonomic data. The peptidoglycan from all *Leuconostoc* strains contained lysine, serine, and alanine, in accordance with classical data (20). Analysis of whole-cell extracts revealed the presence of galactose without exception, whereas rhamnose was found in the *L. lactis* strains (Table 2).

Polar lipids identical to phosphatidylglycerol and phosphatidylethanolamine were common to all *Leuconostoc* strains. However, *L. lactis*, both type and clinical strains, was differentiated from *L. mesenteroides* (all subspecies included) and *L. paramesenteroides* mainly by the presence of a glycolipid labeled G3 (Fig. 2).

The whole-cell protein patterns obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Fig. 3. Only strain CIP 101240 showed some differences from the type strain (CIP 102422), whereas it was not distinguishable from the other clinical strains of group H1 (Fig. 1) on the basis of biochemical and chemotaxonomic features.

DISCUSSION

Growth and biochemical characteristics aside, the presence of glycolipid G3 seems to be an essential feature of *L. lactis* strains. It differentiates them especially from *L. mesenteroides* subsp. *mesenteroides* strains, the most closely related strains on the basis of the biochemical test results (Table 2 and Fig. 3). Also, it is not found (unpublished data) in *Lactobacillus confusus* (type strain, CIP 103172) or *Lactobacillus viridescens* (type strain, CIP 102810), frequently mistaken for *Leuconostoc* species by their physiological properties (13), or in the recently designated species of clinical interest, *Leuconostoc citreum* (type strain, CIP 103315) and *L. pseudomesenteroides* (type strain, CIP 103316). This conclusion is substantiated by known genomic data for moles G+C percent (11, 13) and DNA-DNA hybridization (11, 12, 16). Moreover, *L. citreum* is unable to grow even at 40°C; however, it produces acid from α-methyl-glucoside but not from raffinose, and it hydrolyzes esculin (11). *L. pseudomesenteroides* is known to be ribose and, in most cases, α-methyl-glucoside positive (11).

By analogy, the phenotypic divergences between the clinical and type strains of *L. lactis* present no more severe a problem than those of the genetically related but biochemically differentiated species of lactic acid bacteria, i.e., *Streptococcus thermophilus* and *Streptococcus salivarius* (10); *Lactobacillus bulgaricus*, *Lactobacillus delbrueckii*, and *Lactobacillus lactis* (29); and *Leuconostoc mesenteroides* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *dextranicum*, and *L. mesenteroides* subsp. *mesenteroides* (12). Nevertheless, molecular methods are needed to provide accurate species identification.

Our *L. lactis* strains possibly represent a variant from those of a less versatile nature found in dairy products. Their

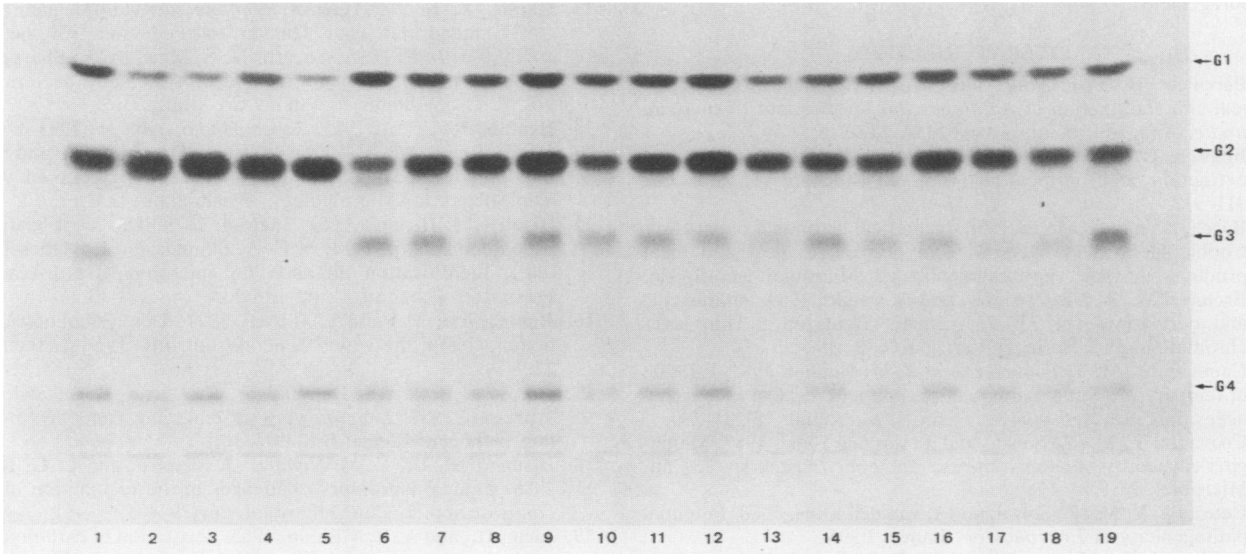

FIG. 2. Glycolipid patterns of the clinical *L. lactis* strains versus the *Leuconostoc* type strains as revealed by the α-naphthol reagent. Lanes: 1, *L. lactis* CIP 102822^T; 2, *L. mesenteroides* subsp. *cremoris* CIP 103009^T; 3, *L. mesenteroides* subsp. *dextranicum* CIP 102823^T; 4, *L. mesenteroides* subsp. *mesenteroides* CIP 102305^T; 5, *L. paramesenteroides* CIP 102821^T; 6 to 19, *L. lactis* clinical strains CIP 100915, CIP 101238, CIP 102427, CIP 101268, CIP 101549, CIP 101878, CIP 101991, CIP 102167, CIP 101240, CIP 102307, CIP 102574, CIP 102869, CIP 102870, and CIP 101269, respectively.

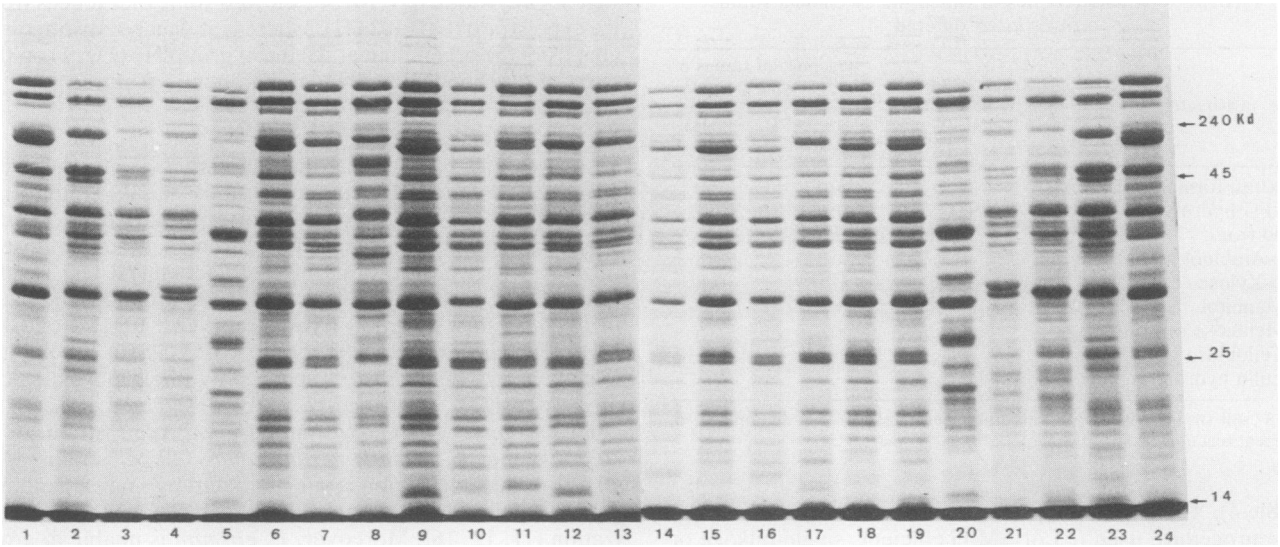

FIG. 3. Whole-cell protein patterns (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of the *L. lactis* clinical strains versus the *Leuconostoc* type strains. Lanes: 1 to 6, as in Fig. 2; 6 to 19, *L. lactis* clinical strains CIP 101991, CIP 102167, CIP 101240, CIP 102307, CIP 102574, CIP 102869, CIP 102870, CIP 101269, CIP 101878, CIP 101549, CIP 101268, CIP 102427, CIP 101238, and CIP 100915, respectively; 20, *L. paramesenteroides* CIP 102821^T; 21, *L. mesenteroides* subsp. *mesenteroides* CIP 102305^T; 22, *L. mesentroides* subsp. *dextranicum* CIP 102823^T; 23, *L. mesenteroides* subsp. *cremoris* CIP 103009^T; 24, *L. lactis* CIP 102822^T. Molecular masses (in kilodaltons [Kd]) are shown on the right.

low incidence in clinical material indicates that they are probably not normal inhabitants of humans, while in general the common isolation of *Leuconostoc* strains from meat products (3, 28), coupled with the divergent characteristics of our strains, indicates that the distribution of *L. lactis* outside dairy environments cannot be overestimated.

ACKNOWLEDGMENTS

We thank F. Fleur, T. Rivera, and N. Rouillon for technical assistance and M. Sebald and M. Goldner for comments and encouragement.

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