INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, July 1996, p. 664–668 Vol. 46, No. 3
0020-7713/96/$04.00+0

Phenotypic and Genotypic Characterization of Atypical *Lactococcus garvieae* Strains Isolated from Water Buffalos with Subclinical Mastitis and Confirmation of *L. garvieae* as a Senior Subjective Synonym of *Enterococcus seriolicida*

LÚCIA M. TEIXEIRA,[1,2]* VÂNIA LÚCIA C. MERQUIOR,[1,3] MARIA DA CONCEIÇÃO E. VIANNI,[1,4] MARIA DA GLÓRIA S. CARVALHO,[1] SÉRGIO E. L. FRACALANZZA,[1] ARNOLD G. STEIGERWALT,[2] DON J. BRENNER,[2] AND RICHARD R. FACKLAM[2]

Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941,[1] Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro 20551,[3] and Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica 23851,[4] Brazil, and Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[2]

During a survey of bacterial agents that cause subclinical mastitis in water buffalos, we isolated several strains of gram-positive cocci that appeared to be enterococci except that they grew very slowly at 45°C and grew slowly in broth containing 6.5% NaCl. On the basis of the results of conventional physiologic tests, these strains were identified as *Enterococcus durans*. However, none of the strains reacted with the AccuProbe *Enterococcus* genetic probe. The whole-cell protein profiles of these organisms were compared with the profiles of *Enterococcus* and *Lactococcus* reference strains. Apart from minor quantitative differences, the mastitis isolates had indistinguishable protein profiles that were similar to the profiles of the *Lactococcus garvieae* and *Enterococcus seriolicida* type strains. The results of DNA relatedness studies performed by using the hydroxyapatite method at 55 and 70°C indicated that all of the mastitis isolates were related to the type strain of *L. garvieae* at the species level, despite the fact that they exhibited several uncommon phenotypic characteristics (growth at 45°C, growth in broth containing 6.5% NaCl, and failure to produce acid from mannitol and sucrose). The high levels of DNA relatedness between strains of *L. garvieae* and *E. seriolicida* demonstrated that these taxa are members of a single species. Since *L. garvieae* is a senior synonym of *E. seriolicida*, *L. garvieae* should be retained as the species name and strain ATCC 43921 should remain the type strain of this species.

The genus *Lactococcus* was established as a separate genus (distinct from the genus *Streptococcus*) in 1985 (12). The members of this genus, which formerly were known as the lactic acid group of streptococci, are not usually considered significant pathogens of humans and animals because they are infrequently isolated clinically and are probably associated with opportunistic infections (1, 7, 11). However, the role of these microorganisms as infectious agents remains unclear since it is difficult to differentiate them from members of physiologically similar genera and to identify them to the species level. It is believed that because they resemble other better-known clinical isolates, especially enterococci, the lactococci may be misidentified or overlooked in clinical laboratories, which contributes to the paucity of reports concerning their clinical significance.

Data for cultures received at the Centers for Disease Control and Prevention indicate that lactococci may be involved in a variety of infections in humans and that the genus *Lactococcus* should be considered whenever a bacterial isolate resembles an *Enterococcus* sp. in all but a few characteristics. *Lactococcus garvieae* is the species that is most frequently isolated from such infections, followed by *Lactococcus lactis* (5, 7). On the other hand, although information concerning animal infections is even more scarce, it should be noted that *L. garvieae* (originally named *Streptococcus garvieae* in 1983) was described as an organism isolated from an animal with bovine mastitis (3).

In 1991, a new enterococcal species, *Enterococcus seriolicida*, was described as a fish pathogen (9) on the basis of low levels of DNA relatedness to the type strains of previously described enterococcal species. In 1993, *E. seriolicida* was found to be similar to *L. garvieae* as determined by 16S rRNA sequencing (4), but DNA hybridization studies were not done.

Recently, during a survey of bacterial agents that cause subclinical mastitis in Brazilian water buffalos raised for milk production, we isolated a group of gram-positive cocci that had phenotypic characteristics similar to those of either enterococci or lactococci. These strains accounted for the majority of the bacterial agents isolated in the survey (13a).

The purpose of this study was to determine the identity of the water buffalo mastitis isolates and to determine if *L. garvieae* and *E. seriolicida* are a single species or distinct species.

MATERIALS AND METHODS

Bacterial strains. A total of 21 strains isolated from water buffalos with subclinical mastitis were studied. The infected animals were members of herds located in the state of Rio de Janeiro, Brazil. Seven type and reference strains (obtained from the culture collection of the Centers for Disease Control and Prevention or from the American Type Culture Collection) representing the species of gram-positive cocci that were physiologically most similar to the clinical isolates were also included in the study (Table 1).

Characterization of strains. Phenotypic characteristics were determined by performing conventional physiologic tests (6, 8). These tests included tests for production of pyrrolidonyl arylamidase, leucine aminopeptidase, and gas from glucose in Mann-Rogosa-Sharpe *Lactobacillus* broth; hydrolysis of esculin in the

* Corresponding author. Mailing address: Childhood and Respiratory Diseases Branch, MS-CO2, Centers for Disease Control and Prevention, Atlanta, GA 30333. Phone: (404) 639-1379. Fax: (404) 639-3123.

TABLE 1. Characteristics of the strains included in this study

Characteristic	*Lactococcus garvieae* ATCC 43921Ta	*Lactococcus garvieae* SS-1290	*Enterococcus seriolicida* ATCC 49156^{T}	*Lactococcus lactis* ATCC 19435^{T}	*Enterococcus durans* ATCC 11576	*Enterococcus durans* ATCC 19432^{T}	*Enterococcus hirae* ATCC 8043^{T}	Clinical isolates (n = 21)^{b}
Gas production in Mann-Rogosa-Sharpe broth	−	−	−	−	−	−	−	−
Pyrrolidonyl arylamide activity	+	+	+	−	+	+	+	+
Leucine aminopeptidase activity	+	+	+	+	+	+	+	+
Hydrolysis of esculin in the presence of bile	+	+	+	+	+	+	+	+
Growth in the presence of 6.5% NaCl	+^{c}	+^{c}	+	−	+	+	+	+^{c}
Growth at 10°C	+	+	+	+^{c}	+	+	+	+
Growth at 45°C	+^{c}	+^{c}	+	+^{c}	+	+	+	+^{c}
Vancomycin susceptibility	+	+	+	+	+	+	+	+
Arginine hydrolysis	+	+	+	+	+	+	+	+
Hippurate hydrolysis	−	−	−	−	+	−	−	−
Pyruvate utilization	−	−	−	−	−	−	−	−
Tellurite tolerance	−	−	+	−	−	−	−	V
Motility	−	−	−	−	−	−	−	−
Pigment production	−	−	−	−	−	−	−	−
Voges-Proskauer reaction	+	+	+	+	+	+	+	+
Acid production from:								
Arabinose	−	−	−	−	−	−	−	−
Glycerol	−	−	−	−	+	−	−	−
Inulin	−	−	−	−	−	−	−	−
Lactose	+	−	−	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	−	−	−	−	−
Melibiose	−	−	−	−	−	−	+	−
Raffinose	−	−	−	−	−	−	+	−
Ribose	+	+	+	+	+	+	+	+
Sorbitol	−	−	−	−	−	−	−	−
Sorbose	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	+	−
Trehalose	+	+	+	+	+	−	+	+
Serogroup	−	−	−	N	D	D	D	−
Enterococcal probe reaction	−	−	−	−	+	+	+	−

[a] T = type strain.
[b] +, all strains were positive; −, all strains were negative; V, 40% of the strains were positive.
[c] Delayed on weak reaction.

presence of bile; hydrolysis of arginine; hydrolysis of hippurate; growth in broth containing 6.5% NaCl; growth at 10 and 45°C; susceptibility to vancomycin; motility; pigment production; pyruvate utilization; tellurite tolerance; the Voges-Proskauer reaction; and acid production from L-arabinose, glycerol, inulin, lactose, maltose, D-mannitol, melibiose, raffinose, ribose, D-sorbitol, sorbose, sucrose, and trehalose. Serogroups were determined by the Lancefield hot-acid extraction procedure and by capillary precipitation or agar diffusion tests performed with group D and group N antisera. Strains were also tested for reactivity with the AccuProbe *Enterococcus* culture confirmation test (Gen-Probe, Inc., San Diego, Calif.) as directed by the manufacturer.

Analysis of whole-cell protein profiles by SDS-PAGE. Strains were grown on brain heart infusion-sheep blood plates for 24 h at 37°C. Preparation of extracts and analysis of whole-cell protein profiles by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were performed as described by Merquior et al. (10). Briefly, bacterial cells were removed from the surfaces of the blood agar plates and suspended in 0.5-ml portions of an aqueous lysozyme solution (10 mg/ml). The resulting suspensions were incubated in a 37°C water bath for 2 h. Whole-cell extracts were obtained by mixing 1 volume of each sample with 1 volume of 0.5 M Tris-HCl (pH 6.8) containing 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol and boiling the preparation for 5 min. Soluble samples were applied to wells in a 4% acrylamide stacking gel over a 10% acrylamide separating gel. SDS-PAGE was performed with a Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.) at a constant current of 20 mA. The whole-cell protein bands were stained with Coomassie brilliant blue R-250.

Protein profiles were compared by using the correlation coefficients to form a similarity matrix and were clustered by the unweighted pair group method with averages by using the Whole Band Analyzer software of the Bio Image Electrophoresis Analyzer System (Bio Image/Millipore Corp., Ann Arbor, Mich.).

DNA relatedness studies. Twelve clinical isolates, as well as the seven enterococcal and lactococcal type and reference strains, were used in DNA relatedness experiments. Each strain was grown in 2 liters of Todd-Hewitt broth at 37°C for 18 to 20 h with gentle shaking. Harvesting and lysis of the bacterial cells were performed as recommended by Teixeira et al. (13). The procedures used to extract and purify the DNA and to determine DNA relatedness by the hydroxyapatite hybridization method were essentially the procedures recommended by Brenner et al. (2). The DNAs were labeled enzymatically with [^{32}P]dCTP by using a nick translation reagent kit (Gibco BRL Life Technologies, Inc., Gaithersburg, Md.), as recommended by the manufacturer. DNA hybridization experiments were performed at 55°C for optimal DNA reassociation. Levels of DNA reassociation were also determined at a stringent temperature (70°C). Levels of divergence for related sequences were determined by assuming that each 1°C of heteroduplex instability, compared with the melting temperature of the homologous duplex, was caused by approximately 1% unpaired bases. Levels of divergence were calculated to the nearest 0.5%.

RESULTS AND DISCUSSION

The physiologic characteristics of the strains which we studied are shown in Table 1. The isolates obtained from water buffalos with mastitis had similar physiologic profiles. They were positive for pyrrolidonyl arylamidase activity, leucine aminopeptidase activity, hydrolysis of esculin in the presence of bile, hydrolysis of arginine, and the Voges-Proskauer test; growth occurred at 10°C and, although the growth was weak and slow, at 45°C and in broth containing 6.5% NaCl. All of the strains produced acid from lactose, maltose, ribose, and trehalose, and none of the strains produced acid from arabinose, glycerol, inulin, mannitol, melibiose, raffinose, sorbitol, sor-

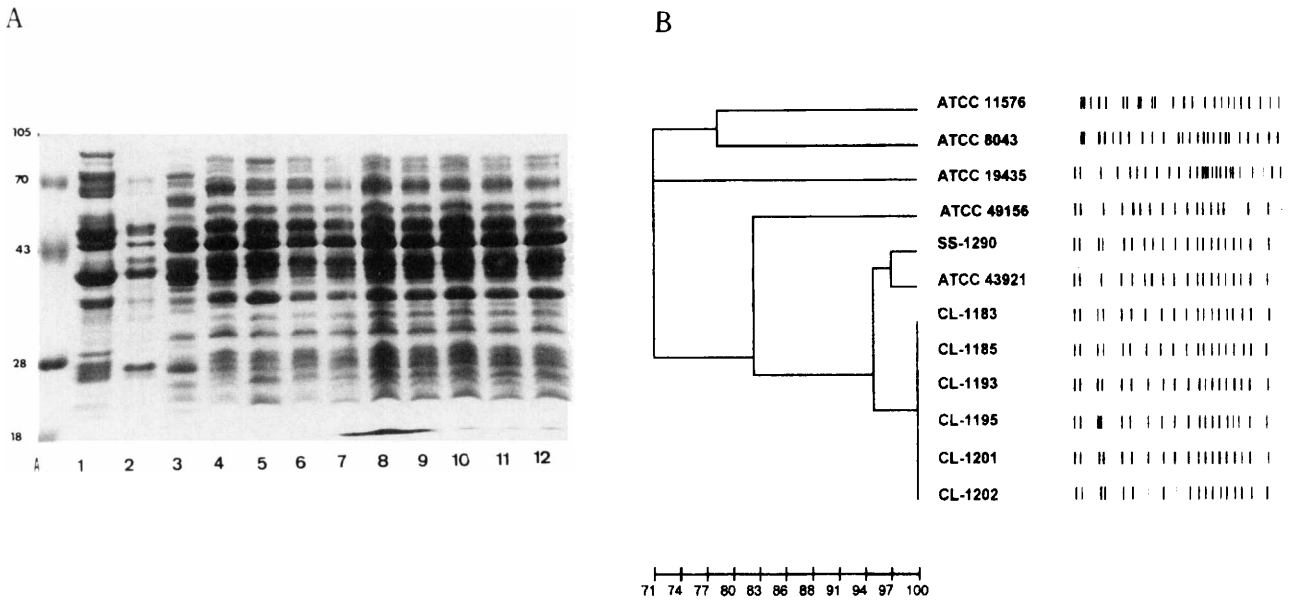

FIG. 1. (A) SDS-PAGE patterns of whole-cell protein extracts of representative strains used in this study. Lane A, molecular mass markers; lane 1, *Enterococcus durans* ATCC 11576; lane 2, *Enterococcus hirae* ATCC 8043^T (T = type strain); lane 3, *Lactococcus lactis* ATCC 19435^T; lane 4, *Enterococcus seriolicida* ATCC 49156^T; lane 5, *Lactococcus garvieae* SS-1290; lane 6, *Lactococcus garvieae* ATCC 43921^T; lane 7, strain CL-1183; lane 8, strain CL-1185; lane 9, strain CL-1193; lane 10, strain CL-1195; lane 11, strain CL-1201; lane 12, strain CL-1202. The numbers on the left indicate the positions of molecular mass markers (in kilodaltons). (B) Dendrogram resulting from a computer-assisted analysis of the protein profiles shown in panel A.

bose, and sucrose. All of the strains were susceptible to vancomycin, nonmotile, and nonpigmented and were negative for production of gas, hippurate hydrolysis, and pyruvate utilization. No strain reacted with either "streptococcal" group D or group N antisera.

On the basis of the results of the conventional physiologic tests, the most likely identity of these microorganisms was *Enterococcus durans*, as they were similar to enterococci except that they grew slowly at 45°C and in broth containing 6.5% NaCl. However, none of the strains reacted with the Accu-Probe *Enterococcus* genetic probe. Therefore, we also explored the possibility that these strains were biochemically atypical lactococci, although growth at 45°C and growth in the presence of 6.5% NaCl, especially in conjunction with a failure to produce acid from mannitol and sucrose, are uncommon characteristics for lactococci isolated from humans, which account for most of the data available (7).

An analysis of the electrophoretic whole-cell protein profiles of the water buffalo isolates was performed, and the profiles obtained were compared with the profiles obtained for type and reference strains of the species that are phenotypically most similar to the clinical isolates. Our analysis of whole-cell protein profiles revealed that the water buffalo mastitis strains had virtually indistinguishable profiles that were not similar to the profile of any enterococcal species and, thus, were not likely to belong to the genus *Enterococcus*. Additional tests showed that, apart from minor quantitative differences, the protein profiles of the water buffalo mastitis isolates were very similar to the profiles of the *L. garvieae* and *E. seriolicida* type strains (Fig. 1).

The results of the DNA relatedness experiments were consistent with the results of the protein profile analysis (Table 2). All of the mastitis isolates exhibited species level relatedness to the type strain of *L. garvieae* and low levels of relatedness to the enterococcal strains. All of the mastitis strains conformed to at least two of the following three species level DNA-DNA relatedness criteria (14): a level of relatedness of 70% or more under optimal conditions (in this study, the levels of relatedness ranged from 63 to 75%), a level of divergence of less than 5% for related sequences (range in this study, 0.5 to 2.5%), and a level of relatedness of 60% or more under stringent conditions (range in this study, 63 to 78%). Because the relative binding ratios under the optimal conditions were close to the lowest values recommended for members of a species, we decided to label one of the water buffalo isolates (CL-1202) and determine its levels of relatedness to all other strains. The strains were found to be closely related to each other; the relative binding ratios with CL-1202 ranged from 86 to 100% (average, 98%). The results of the DNA relatedness studies, in conjunction with the results of the biochemical tests, indicate that the water buffalo isolates may constitute a unique *L. garvieae* biogroup.

Careful interpretation of tests to determine growth at 45°C and growth in broth containing 6.5% NaCl is recommended to differentiate between the enterococci, which usually grow well under these conditions, and the lactococci, which usually grow poorly or not at all (7). Serogrouping can also be used to differentiate the enterococci from the lactococci on the basis of the presence of group D and group N antigens, respectively. However, the water buffalo lactococcal isolates analyzed in this study had very similar physiologic profiles with an unusual combination of characteristics (slow growth at 45°C and in broth containing 6.5% NaCl and failure to produce acid from mannitol and sucrose) that made them more difficult to properly differentiate and identify. They were phenotypically indistinguishable from the enterococcal species *E. durans* and had characteristics that are not common among human isolates of *L. garvieae*. In addition, no group D or N antigens were detected.

These findings indicate that biochemical variation is possible among strains isolated from sources other than human sources. The original phenotypic description of *L. garvieae* should be broadened to include the variants that grow at 45°C. Our data also show that it is necessary to use additional methods for

TABLE 2. Levels of DNA relatedness of physiologically atypical *L. garvieae* strains isolated from buffalos with subclinical mastitis

Source of unlabeled DNA[a]	Labeled DNA from *Lactococcus garvieae* ATCC 43921[T][b]			
	Relative binding ratio at 55°C	% Divergence	Relative binding ratio at 70°C	Relative binding ratio at 55°C with labeled DNA from strain CL-1202
Reference strains				
Lactococcus garvieae ATCC 43921[T] (= NCDO 2155[T] = SS-1270[T])	100	0.0	100	77
Lactococcus garvieae SS-1290	73	1.0	75	84
Enterococcus seriolicida ATCC 49156[T] (= SS-1311[T])	77	1.0	77	77
Lactococcus lactis ATCC 19435[T] (= NTCC 6681[T] = SS-668[T])	14	15.0		
Enterococcus durans ATCC 19432[T] (= SS-661[T])	3	16.0		
Enterococcus durans ATCC 11576 (= SS-1225)	3	16.0		
Enterococcus hirae ATCC 8043[T] (= NCDO 1258[T] = SS-1227[T])	4	12.0		
Clinical strains				
CL-1183	69	1.0	69	95
CL-1185	72	0.5	63	100
CL-1192	63	1.5	65	86
CL-1193	75	1.5	71	96
CL-1195	72	2.5	63	100
CL-1197	72	1.0	64	100
CL-1201	72	1.0	63	99
CL-1202	71	1.0	65	100
CL-1203	75	1.5	63	99
CL-1206	75	1.5	78	100
CL-1207	74	1.5	64	100
CL-1208	71	1.5	63	97

[a] ATCC, American Type Culture Collection; SS, standard strain from the culture collection of the Centers for Disease Control and Prevention; NCDO, National Collection of Dairy Organisms; NTCC, National Type Culture Collection.
[b] T = type strain.

precise identification of such isolates. Enterococcal probe reactions were found to be a useful tool for including or excluding strains from the enterococcal group. Therefore, we recommend that this test be used when bacterial isolates resemble enterococci or lactococci in all but a few characteristics. In addition, electrophoretic whole-cell protein profile analysis was shown to be a reliable and relatively simple method for identifying atypical *L. garvieae* strains.

The high levels of DNA relatedness between strains of *L. garvieae* and *E. seriolicida* (77% under either optimum or stringent conditions, with 1% divergence) demonstrate that these organisms belong to a single species, as suggested previously on the basis of the results of an analysis of biochemical and protein profiles, as well as 16S rRNA sequencing (4). Since *L. garvieae* was described first, *L. garvieae* is a senior subjective synonym of *E. seriolicida*. Therefore, the name *L. garvieae* should be retained, strain ATCC 49156 should be renamed *L. garvieae*, and the type strain of *L. garvieae* remains ATCC 43921 (= NCDO 2155).

ACKNOWLEDGMENTS

This study was supported in part by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Financiadora de Estudos e Projetos (FINEP), Brazil.

We thank Susan Hunter and Bala Swaminathan of the Centers for Disease Control and Prevention for providing facilities for our computer-assisted analysis and Carlos Ausberto B. de Souza of the Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, for technical assistance.

REFERENCES

1. **Aguirre, M., and M. D. Collins.** 1993. Lactic acid bacteria and human clinical infection. J. Appl. Bacteriol. **75:**95–107.
2. **Brenner, D. J., A. C. McWhorter, J. K. Leete Knutson, and A. G. Steigerwalt.** 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. J. Clin. Microbiol. **15:**1133–1140.
3. **Collins, M. D., J. A. E. Farrow, B. A. Phillips, and O. Kandler.** 1983. *Streptococcus garvieae* sp. nov. and *Streptococcus plantarum* sp. nov. J. Gen. Microbiol. **129:**3427–3431.
4. **Domenech, A., J. Prieta, J. F. Fernandez-Garayzabal, M. D. Collins, D. Jones, and L. Dominguez.** 1993. Phenotypic and phylogenetic evidence for a close relationship between *Lactococcus garvieae* and *Enterococcus seriolicida*. Microbiol. Sem. **9:**63–68.
5. **Elliot, J. A., M. D. Collins, N. E. Pigott, and R. R. Facklam.** 1991. Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole-cell protein patterns. J. Clin. Microbiol. **29:**2731–2734.
6. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. J. Clin. Microbiol. **27:**731–734.
7. **Facklam, R. R., and J. A. Elliot.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. Clin. Microbiol. Rev. **8:**479–495.
8. **Facklam, R. R., and D. F. Sahm.** 1995. *Enterococcus*, p. 308–314. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
9. **Kusuda, R., K. Kawai, F. Salati, C. R. Banner, and J. L. Fryer.** 1991. *Enterococcus seriolicida* sp. nov., a fish pathogen. Int. J. Syst. Bacteriol. **41:**406–409.
10. **Merquior, V. L. C., J. M. Peralta, R. R. Facklam, and L. M. Teixeira.** 1994. Analysis of electrophoretic whole-cell protein profiles as a tool for characterization of *Enterococcus* species. Curr. Microbiol. **28:**149–153.
11. **Ruoff, K. L.** 1995. *Leuconostoc*, *Pediococcus*, *Stomatococcus*, and miscellaneous gram-positive cocci that grow aerobically, p. 315–323. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
12. **Schleifer, K. H., J. Klaus, C. Dvorak, R. Kilpper-Balz, M. D. Collins, and W.**

Fischer. 1985. Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. Syst. Appl. Microbiol. **6**:183–195.

13. **Teixeira, L. M., R. R. Facklam, A. G. Steigerwalt, N. E. Pigott, V. L. C. Merquior, and D. J. Brenner.** 1995. Correlation between phenotypic characteristics and DNA relatedness within *Enterococcus faecium* strains. J. Clin. Microbiol. **33**:1520–1523.

13a. **Vianni, M. C. E., et al.** Unpublished data.

14. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper.** 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int. J. Syst. Bacteriol. **37**:463–464.