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Differentiation of *Lactococcus lactis* and *Lactococcus garvieae* from Humans by Comparison of Whole-Cell Protein Patterns

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We tested 12 reference and 24 clinical strains of lactococci for physiologic characteristics using a conventional test system, the Gen-Probe *Enterococcus* 2 chemiluminescence assay (Gen-Probe Inc., San Diego, Calif.), the Rapid Strep identification system (Analytab Products, Plainview, N.Y.), and whole-cell protein analysis. The Gen-Probe *Enterococcus* 2 chemiluminescence assay for *Enterococcus* identification was negative with all strains. Neither the conventional test nor the Rapid Strep identification system could differentiate between the two *Lactococcus* spp. most commonly isolated from humans. A simple procedure, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was developed for comparing the whole-cell protein patterns of *Lactococcus* spp. *L. lactis* and *L. garvieae* were differentiated by unique protein patterns.

One of the several changes in the taxonomy of the *Streptococcus* genus that has occurred in the past few years is the establishment of the *Lactococcus* genus (17, 22). These bacteria, formerly known as the lactic group of streptococci, consisted of *Streptococcus lactis*, *Streptococcus cremoris*, and in some texts, *Streptococcus diacetylactis*. When the *Lactococcus* genus was established, additional species were added to the genus and some strains were made subspecies (21, 22). One of the new species added to the genus was *Lactococcus garvieae*. This species was originally described as *Streptococcus garvieae* and was isolated from bovine sources, primarily from cases of mastitis (4).

During our studies of methods of identifying and classifying the enterococci, we discovered several strains of gram-positive cocci that appeared to be enterococci but that could not be identified to the species level (11, 12). DNA homology and partial rRNA sequencing studies clearly identified several of these strains as *Lactococcus lactis* subsp. *lactis* and *L. garvieae* (10). Our studies indicated that despite the overlap of some physiologic characteristics with those of the enterococci, the lactococci could be differentiated from the enterococci by (i) growth at 10°C but not at 45°C (enterococci grow at both temperatures), (ii) failure to react with the Gen-Probe *Enterococcus* 2 chemiluminescence assay, and (iii) failure to have physiologic or serologic characteristics identical to those of *Enterococcus* spp.

The two *Lactococcus* spp. most frequently found in human infections, *L. lactis* subsp. *lactis* and *L. garvieae*, have identical biochemical utilization patterns (21) and can be distinguished from each other in a clinical microbiology laboratory only on the basis of the pyrrolidonylarylamidase (PYRase) enzyme (4). In a recent study with a larger number of isolates, all the *L. garvieae* tested were PYRase positive; however, one third of the *L. lactis* tested were also PYRase positive, making this test unreliable (10). At present, the only dependable way to identify these species is by DNA homology or partial rRNA sequencing (2). The purpose of this study was to find a useful way of differentiating between *L. lactis* and *L. garvieae*, because physiologic tests are unable to do so.

MATERIALS AND METHODS

Bacteria. All clinical strains were taken from the culture collection of the Respiratory Diseases Reference Bacteriology Laboratory, Centers for Disease Control. Reference strains were received from L. McKay, University of Minnesota; M. D. Collins, Reading, England; and the late R. C. Lancefield, Rockefeller University. Bacteria were stored frozen (−65°C) in sheep blood. Initial recovery from frozen stocks and purity checks were done on Trypticase soy agar with 5% sheep blood. All strains used in this study are listed in Table 1.

Identification. The physiologic characteristics of the strains were determined by conventional tube tests and by the API Rapid Strep system (Analytab Products, Plainview, N.Y.) (9, 11).

Serologic characteristics of the strains were determined by Lancefield extraction and capillary precipitin testing by using CDC group D and Burroughs Wellcome (Research Triangle Park, N.C.) group N antiserum (11).

DNA homology and partial rRNA sequencing studies were performed as described previously (1, 6, 13).

All strains were tested for reactivity with the Gen-Probe *Enterococcus* 2 chemiluminescence assay (Gen-Probe Inc., San Diego, Calif.) (9).

SDS-PAGE. Bacteria were incubated overnight at 37°C in 10 ml of Mann, Rogosa, and Sharpe broth (MRS broth), centrifuged at 10,000 × *g* for 30 min, frozen and thawed three times, and resuspended in 1 ml of distilled water. One-half gram of glass beads (diameter, 0.12 to 0.18 mm; Thomas Scientific, Swedesboro, N.J.) was added to the tubes, and the bacteria were vortexed for 1 min at the maximum setting of a Vortex Genie (Scientific Industries, Bohemia, N.Y.). After the glass beads settled, samples were removed for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using previously described methods (8).

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TABLE 1. SDS-PAGE lane number, source of bacteria, and identification of lactococci

SDS-PAGE lane no.	Isolate no.	Source	Identification[a]
1	SS1270	NCDO 2155	*L. garvieae* type strain
2	SS668	ATCC 19435	*L. lactis* type strain
3	SS1176	NCDO 617	*L. raffinolactis* type strain
4	SS1239	ATCC 19257	*L. cremoris* type strain
5	SS1253	ATCC 43199	*L. plantarum* type strain
6	SS1298	Dairy[b]	*L. lactis* biovar diacetylactis
7	SS1299	Dairy[b]	*L. lactis* biovar diacetylactis DRC3
8	SS1300	Dairy[b]	*L. lactis* subsp. *cremoris* AM2
9	SS1302	Dairy[b]	*L. lactis* subsp. *lactis* 11955a
10	SS816	Colindale[c]	*L. lactis*
11	SS834	N/C 559, RCL[d]	*L. lactis*
12	SS854	H201[e]	*L. lactis*
13	868-78	Urine	*L. lactis*
14	810-85	Eye	*L. lactis*
15	1384-85	Blood	*L. lactis*
16	1385-85	Blood	*L. lactis*
17	1585-85	Blood	*L. lactis*
18	2742-86	Blood	*L. lactis*
19	2885-86	Wound	*L. lactis*
20	2211-89	Unknown	*L. lactis*
21	588-80	Blood	*L. garvieae*
22	673-80	Skin	*L. garvieae*
23	2182-81	Blood	*L. garvieae*
24	1108-86	Blood	*L. garvieae*
25	240-88	Urine	*L. garvieae*
26	364-88	Blood	*L. garvieae*
27	66-90	Blood	*L. garvieae*
28	306-79	Urine	*L. garvieae*
29	1042-80	Blood	*L. garvieae*
30	2025-80	Urine	*L. garvieae*
31	1678-84	Blood	*L. garvieae*
32	1688-84	Blood	*L. garvieae*
33	2486-87	Blood	*L. garvieae*
34	2276-89	Blood	*L. lactis*
35	E319	Blood	*L. garvieae*

[a] Isolates in lanes 13 to 35 were identified by DNA homology or partial rRNA sequencing.
[b] Received from Larry McKay, University of Minnesota.
[c] Strain Rice, Colindale, England.
[d] RCL, Rebecca C. Lancefield.
[e] Statens Serum institut, Copenhagen, Denmark.

RESULTS

None of the 24 clinical or 12 reference strains produced gas from glucose in MRS broth, all strains were susceptible to vancomycin, and all were leucine aminopeptidase positive. Of the clinical strains, all strains were bile-esculin (BE) positive, and 80% of the strains grew in broth containing 6.5% NaCl. Seventy-six percent of the clinical strains were positive in the PYRase test. All strains grew at 10°C, whereas 72% failed to grow at 45°C; the remaining 28% grew very slowly at 45°C (growth was not apparent until 48 to 72 h of incubation).

The results of conventional tests with the 15 *L. garvieae* and 9 *L. lactis* clinical isolates are as follows. All strains hydrolyzed esculin, deaminated arginine, and formed acid in mannitol, trehalose, and maltose broth. None of the strains hydrolyzed starch or hippurate, reduced tellurite, produced glucan, utilized pyruvate, formed acid in inulin or sorbose broth, or were pigmented or motile. One strain of *L. garvieae* formed acid in melibiose and raffinose broth, while all other clinical isolates did not. One strain of *L. lactis* formed acid in arabinose broth and another strain formed acid in sorbitol broth, while all other clinical isolates did not. All nine strains of *L. lactis* formed acid in lactose broth, but only 40% of the *L. garvieae* strains did so. All strains of both species except one strain of *L. garvieae* formed acid in salicin broth. Variable results (22 to 89% positive) were observed with the reduction of tetrazolium and acid formation in sucrose and glycerol broths with both species.

Voges-Proskauer and PYRase tests have been reported to be useful in differentiating between *L. garvieae* and *L. lactis* (10, 22). In this study, 93 and 100% of the *L. garvieae* clinical strains were positive in the Voges-Proskauer and PYRase tests, respectively, while 89 and 55% of the *L. lactis* strains were positive in the Voges-Proskauer and PYRase tests, respectively.

Lancefield extracts of 60% of the clinical strains reacted with streptococcal group N antiserum; none reacted with streptococcal group D antiserum.

None of the 36 strains reacted positively in the Gen-Probe *Enterococcus* 2 assay.

One of the 24 clinical strains was identified as an *Enterococcus faecium* (a confirmed *L. lactis* species) by the Rapid Strep identification system. Seven of the other eight *L. lactis* strains were identified as *S. lactis*, and the remaining *L. lactis* strain was identified as *L. lactis*/*Enterococcus faecalis*, low discrimination level of identification. Four of the 15 strains of *L. garvieae* were identified as *S. lactis*, and the remaining 11 strains were identified as either *E. faecalis*/*L. lactis* or *L. lactis*/*E. faecalis*, low discrimination level of identification. In all cases of low discrimination level of identification, instructions were given to perform growth at 10 and 45°C to correctly determine the species. This resulted in all 15 strains of *L. garvieae* being identified as *S. lactis*.

Of the 24 clinical isolates, 15 were identified as *L. garvieae* and 9 were identified as *L. lactis* by DNA hybridization and partial rRNA sequencing (2).

The whole-cell protein patterns of the bacteria listed in Table 1 are shown in Fig. 1 and 2. The isolates identified as *L. lactis* subsp. *lactis* could clearly be distinguished from *L. garvieae* by several differences in proteins bands in the 29,000- to 66,000-Da range. *L. garvieae* had a major protein band of approximately 43,000 Da (Fig. 1, top arrow) which was not found with *L. lactis*. The most distinguishing protein band of *L. lactis* subsp. *lactis* had a molecular mass of approximately 40,200 Da (Fig. 1, bottom arrow). This protein was not found in *L. garvieae*. There were three unique proteins which were found only with *Lactococcus raffinolactis*. These proteins have approximate molecular masses of 71,000, 46,000, and 39,000 Da. The type species of *Lactococcus plantarum* could be identified by proteins with molecular masses of 46,500 and 41,800 Da. Closely related subspecies of *L. lactis* (Fig. 1, lanes 4 and 6 to 9) could not be separated by their whole-cell protein profiles by the conditions that were used.

DISCUSSION

Twenty-four clinical isolates of gram-positive cocci fulfilled the criteria described for the identification of lactococci (12, 15, 19). Prior to this study, only a few cases of lactococci isolated from human infections had been reported (7, 18, 20, 23). Only one *S. lactis* (*L. lactis*) strain was identified from two large studies of patients with streptococcal systemic infections in England (7, 20). The clinical significance of this

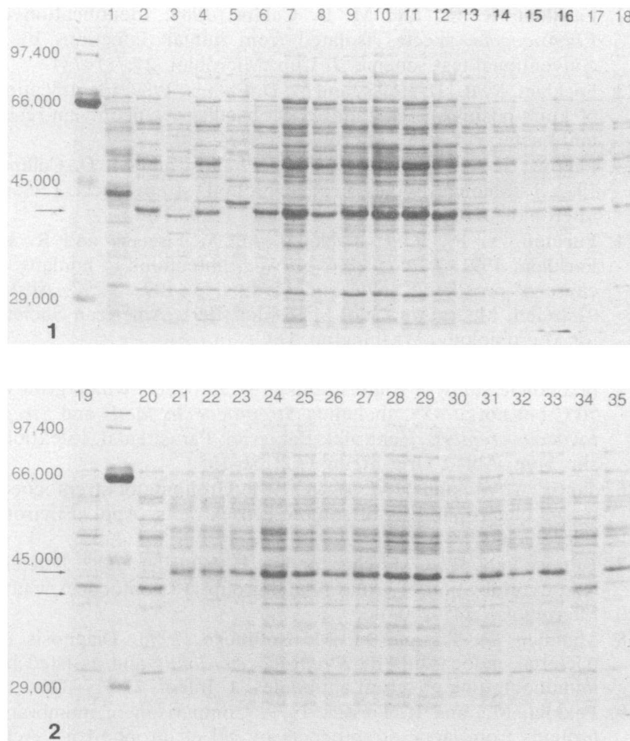

FIG. 1 and 2. SDS-polyacrylamide gels of the whole-cell proteins of *Lactococcus* spp. Fig. 1, lane 1, and Fig. 2, lane 2, molecular mass standards phosphorylase *b* (97,400 Da), bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), and carbonic anhydrase (29,000 Da). Identification of lane numbers is given in Table 1. Arrows indicate the positions of major protein differences between *L. lactis* and *L. garvieae*.

strain was not discussed. In the current study, 18 of the strains were isolated from blood cultures, 4 were isolated from urinary tract infections, and 1 each was isolated from the eye, a wound, and an unknown infection. Three patients with positive blood cultures for *L. garvieae* were diagnosed with prosthetic valve bacterial endocarditis (14). The significance of the other blood culture isolates was less clear, as was the significance of isolates from other sources. Also, the significance of erroneously identifying a *Lactococcus* strain as an *Enterococcus* species is unknown at this time, because we have limited data on the antimicrobial susceptibilities of the *Lactococcus* strains.

The identification of the lactococci by conventional tests is difficult because of the similarity between the lactococci and the enterococci (12). In all probability, most strains of lactococci are identified as enterococci on the basis of positive BE tests, growth in broth containing 6.5% NaCl, and positive PYRase tests. Of the 24 clinical strains, 16 strains were positive in all three tests (BE, 6.5% NaCl, and PYRase), 4 strains were positive in BE and 6.5% NaCl but negative in PYRase, 3 strains were positive in BE and PYRase but negative in 6.5% NaCl, and 1 strain was positive only in the BE test. Therefore, 23 of the 24 strains could possibly be presumptively identified as enterococci. Serogrouping of strains is helpful but not definitive; only 60% of the strains reacted with group N antisera, and most of these reactions were quite weak (30 min of incubation for the capillary test to become positive). Results of tests for vancomycin susceptibility, production of gas from MRS broth, and leucine aminopeptidase reactions were similar for enterococci and lactococci. Only growth at 45°C could be used to aid in the differentiation of enterococci (positive growth) from the lactococci (no growth at 24 or 48 h). Some strains of lactococci may grow after extended incubation (>48 h). The percentage of lactococci showing growth at 45°C after 24 h, 48 h, or extended incubation periods is not known, but it is likely that a high percentage (more than 95%) of strains show growth after 48 h of incubation at 45°C.

The results of testing with the Rapid Strep system indicate that this system is useful in identifying clinical isolates to the genus level but not to the species level. Only one strain was misidentified as an *Enterococcus* sp. if the test was used appropriately. Supplementary tests, growth at 10 and 45°C, were necessary for correct identification to the genus level. Since the data base does not contain information on the identification of *L. garvieae*, it is not surprising that the system does not identify the species.

Attempts to identify the clinical isolates of lactococci as species of enterococci (on the basis of positive presumptive test results for enterococci, BE, 6.5% NaCl, or PYRase) led to the identification of atypical *Enterococcus* spp. Positive results for arginine deamination and acid formation in mannitol broth and negative reactions in tests for acid formation in sorbitol and sorbose broth place the strains in division II of the enterococcal identification scheme (12). The possible identifications are *Enterococcus solitarius*, sorbitol- and pyruvate-negative variants; *E. faecalis*, sorbitol-, pyruvate-, and tellurite-negative variants; and *E. faecium*, arabinose-negative variants. These strains are not motile or pigmented and thus could not be *Enterococcus gallinarum*, *Enterococcus casseliflavus*, or *Enterococcus mundtii*. Prior to 1988 we identified some of these strains as variants of *Enterococcus durans* (3). This was based on reports in the literature of identification of mannitol-positive variants of this species (5). All of these strains were identified as *L. garvieae* in this study.

We suggest that laboratory technicians who attempt to identify the enterococci to the species level be aware that unless measures are taken to positively identify the lactococci, some misidentifications can occur. We suggest that BE-positive, nonhemolytic strains of gram-positive cocci be tested for growth at 10 and 45°C. The Gen-Probe *Enterococcus* assay (all lactococci are negative and most enterococci are positive [9]) is also useful, as is the Rapid Strep system if it is used correctly, as described above.

Until now, the only methods that could accurately identify *L. lactis* and *L. garvieae* were DNA-DNA homology and partial rRNA sequencing (1, 2, 13). Although they are effective, these methods are complicated and are not amenable to routine use. A more basic procedure, soluble whole-cell protein profiles, has been reported to differentiate between several species of lactococci used in the dairy industry (16, 19). We found that the whole-cell protein profiles were able to differentiate between the type strains of *Lactococcus* and were able to distinguish between all clinical isolates of *L. lactis* subsp. *lactis* and *L. garvieae* that had previously been identified by DNA-DNA homology or partial rRNA sequencing results. Although the various subspecies of *L. lactis* (Fig. 1, lanes 4 and 6 to 9) have nearly identical protein patterns and could not easily be distinguished from each other, this problem may not be important, because only *L. lactis* subsp. *lactis* has been implicated in human disease.

One advantage over conventional physiologic tests is that once the bacteria are isolated and identified to the genus

level, the proteins can be prepared and SDS-PAGE results can be determined in 1 day. In contrast, the physiologic tests required for species identification can require an additional incubation of at least 7 days. Analysis of soluble whole-cell proteins, therefore, can be used to determine the species of the most commonly isolated lactococci from human infections, and it is simple to perform compared with genetic analysis.

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