JOURNAL OF CLINICAL MICROBIOLOGY, May 1993, p. 1030–1033
0095-1137/93/051030-04$02.00/0

Vol. 31, No. 5

Identification of *Leuconostoc* spp. by Analysis of Soluble Whole-Cell Protein Patterns

JOHN A. ELLIOTT* AND RICHARD R. FACKLAM

Respiratory Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Received 18 November 1992/Accepted 25 January 1993

***Leuconostoc* spp. share several physiologic characteristics, which sometimes makes it difficult to identify these organisms to the species level. We developed a system, based on the patterns of soluble whole-cell proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, that was able to discriminate between the six *Leuconostoc* spp. that are capable of growth at 37°C. Nine unidentified *Leuconostoc*-like bacterial isolates that were included in the study did not fit any of the protein profiles of the type strains and may represent new *Leuconostoc* spp.**

Leuconostoc spp. are gram-positive cocci commonly found on vegetable matter, dairy products, and meat (11, 12, 20, 21). In the past, these bacteria, because of their ubiquitous nature and their reluctance to grow at 37°C (12), were considered to be contaminants when they were isolated from human infections. Recently, however, several investigators have reported that these bacteria can cause severe infections in humans, especially in debilitated persons (1–3, 13–16, 18, 19).

Characteristics that differentiate the leuconostocs from other members of the catalase-negative, gram-positive lactic acid cocci include resistance to vancomycin and the production of carbon dioxide during the fermentation of glucose. Individual *Leuconostoc* spp. have been established by DNA-DNA homology and by partial rRNA sequencing (10, 17, 21). On the basis of genetic information, biochemical tests that can be used to distinguish each of the species have been reported (8, 9, 21).

Although physiologic reactions can generally be used to determine the species of the bacteria, inconsistencies in the test results can make identification difficult. For example, the discrimination between *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* is based on the ability of *L. mesenteroides* to grow in the presence of 6.5% NaCl (8, 9). Tolerance to 6.5% NaCl is sometimes difficult to determine and difficult to reproduce. For *L. mesenteroides*, it may take up to 7 days before even a small amount of growth is observed, and then growth may be so weak that no change in the color indicator (bromcresol purple) will occur. In addition, a small percentage (11%) of the *L. mesenteroides* isolates that we have identified will not grow in 6.5% NaCl even after 14 days (Table 1). Taken together, these factors can sometimes make distinction between these two species problematic when identification is based solely on the salt tolerance test.

The differentiation between *L. mesenteroides* and *Leuconostoc paramesenteroides* causes further difficulties. The physiologic reactions of these species are similar, with slime production on 5% sucrose agar by *L. mesenteroides* used to differentiate between them (9, 21). However, 25% of the isolates (Table 1) that we have identified as *L. mesenteroides* do not produce this slime, which may lead to their identification as *L. paramesenteroides*. When this occurs, the overall physiologic pattern is used, but these reactions are also variable.

These examples illustrating the difficulties encountered with the identification of *Leuconostoc* spp. reveal the need for additional identification procedures that can be used in a reference laboratory. Recently, a technique that uses the unique patterns of soluble whole-cell proteins of bacteria to define individual species has been described (5, 6, 22). We evaluated this technique, which is based on the separation of the proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for its ability to identify the recognized *Leuconostoc* spp. and used it to compare the protein patterns of *Leuconostoc*-like bacteria that could not be assigned to a species on the basis of physiologic reactions alone.

MATERIALS AND METHODS

Bacteria. *Leuconostoc* spp., including unidentified *Leuconostoc*-like bacteria, were isolated from human infections and were stored frozen (−70°C) in defibrinated sheep blood. In previous reports, physiologic reactions and DNA-DNA hybridization results were correlated to define the individual *Leuconostoc* spp. (8–10). This information was used to assign the clinical isolates to a species on the basis of their physiologic reactions (9). Clinical isolates of *L. mesenteroides* (21 from blood, 2 from two wounds, 1 from an abscess, 1 from urine, 1 from cerebrospinal fluid, and 1 from an unknown source), *L. pseudomesenteroides* (13 from blood and 1 from a wound), *Leuconostoc citreum* (17 from blood, 1 from cerebrospinal fluid, 1 from a wound, 1 from an abscess, 1 from peritoneal fluid, and 2 from unknown sources), and *Leuconostoc lactis* (17 from blood and 1 from a wound) were retrieved from our culture collection. Only the type strains of *L. paramesenteroides* and *Leuconostoc dextranicum* were used because we have not identified isolates of these species from any clinical samples that were submitted. The type strains for each of the species (Table 2) were obtained from the American Type Culture Collection (Rockville, Md.) or from Matthew D. Collins or Brian Phillips (Shinfield, Reading, United Kingdom). Table 2 also lists the *Leuconostoc*-like bacteria that were used and their sources of isolation.

Identification. Physiologic characteristics were determined

* Corresponding author.

TABLE 1. Biochemical reactions of *Leuconostoc* spp.

Species (no. of isolates tested)	Results for the indicated test[a]							
	Esc	LM	Raf	Mel	NaCl	Ara	Tre	5% Suc
L. mesenteroides (27)	100	68	89	100	89	96	96	75
L. pseudomesenteroides (14)	100	79	71	100	0	64	96	57
L. citreum (23)	100	17	100	13	61	87	91	78
L. lactis (18)	11	91	89	100	50	50	50	17
L. paramesenteroides (1)	+	−	+	+	+	+	+	−
L. dextranicum (1)	+	−	+	+	−	−	+	−

[a] Numbers indicate percentages of isolates positive for each test. +, positive reaction; −, negative reaction. Abbreviations: Esc, hydrolysis of esculin; LM, acid production in litmus milk; Raf, Mel, Ara, and Tre, acid production from raffinose, melibiose, arabinose, and trehalose, respectively; NaCl, growth in 6.5% NaCl broth; 5% Suc, extracellular slime production on 5% sucrose agar.

as previously described, except that the final results were recorded after 14 days instead of 7 (9).

SDS-PAGE. Soluble whole-cell proteins were prepared essentially as previously described (6, 7). Briefly, bacteria were grown for 16 h in 10 ml of MRS broth (4) at 37°C, pelleted at 2,000 × *g* for 20 min, frozen at −20°C, thawed, suspended in deionized water, and disrupted by using glass beads and a vortex mixer. The soluble whole-cell proteins were separated by SDS-PAGE by previously described methods (6, 7).

RESULTS

The biochemical reactions of the nine unknown *Leuconostoc*-like bacterial isolates are listed in Table 3. Reactions were read after 14 days' incubation at 35°C. All unknown bacteria were pyrrolidonyl arylamidase and leucine aminopeptidase negative, were resistant to vancomycin, and produced gas from glucose in MRS broth. Unknown isolates 2325-87, 2850-87, 3072-90, 3100-90, and 170-91 had biochemical reactions similar to those of the type strain for *L. paramesenteroides* (Table 1). Isolate 983-84 had reactions similar to those listed in Table 1 for *L. lactis*; the other three isolates had reactions that were different from those of any of the type strains used.

TABLE 2. SDS-PAGE lane numbers, sources, and identification of type strains of *Leuconostoc* spp. and *Leuconostoc*-like bacteria

SDS-PAGE lane no.[a]	CDC no.	Source	Species
1	SS1238	ATCC 8293[b]	*L. mesenteroides*
2	SS1292	NCDO 768[b]	*L. pseudomesenteroides*
3	SS1248	ATCC 19256[b]	*L. lactis*
4	SS1291	NCDO 1837[b]	*L. citreum*
5	SS1250	ATCC 33313[b]	*L. paramesenteroides*
6	SS1237	ATCC 19255[b]	*L. dextranicum*
7	983-84	Blood	Unknown
8	2325-87	Blood	Unknown
9	2850-87	Peritoneal fluid	Unknown
10	3100-90	Blood	Unknown
11	2693-90	Blood	Unknown
12	3072-90	Blood	Unknown
13	780-86	Lung	Unknown
14	170-91	Blood	Unknown
15	1882-86	Blood	Unknown

[a] Numbers correspond to the lane numbers in Fig. 1.
[b] Type strain.

TABLE 3. Physiologic reactions of *Leuconostoc*-like bacteria

CDC no.	Result for the indicated test							
	Esc[a]	LM	Raf	Mel	NaCl	Ara	Tre	5% Suc
983-84	−	+	+	+	−	−	−	−
2325-87	+	+	+	+	+w	+	+	−
2850-87	+	+	+	+	+w	+	+	−
3100-90	−	−	+	+	+	+	+	−
2693-90	−	−	−	−	+w	+	+	−
3072-90	+	−	+	+	+	+	+	−
780-86	−	+	+	+	+	+	+	−
170-91	+	−	+	+	−	+	+w	−
1882-86	+	−	−	+	−	−	+	−

[a] Abbreviations: Esc, hydrolysis of esculin; LM, acid and clot in litmus milk; Raf, Mel, Ara, and Tre, acid production from raffinose, melibiose, arabinose, and trehalose, respectively; NaCl, growth in 6.5% NaCl broth; 5% Suc, extracellular polysaccharide (slime) production on 5% sucrose agar; +w, weak reaction.

The protein patterns of the type strains and *Leuconostoc*-like bacteria are shown in Fig. 1. The majority of the differences in protein bands between species were in the 27,000- to 55,000-Da range. All isolates that had previously been assigned to a species on the basis of physiologic tests and nucleic acid analysis had protein patterns in this range identical to those of their respective type strains (data not shown). None of the unknown isolates had whole-cell protein profiles that were identical to those of the type strains.

Isolates 2325-87 (Fig. 1, lane 8) and 2850-87 (Fig. 1, lane 9) had similar profiles; isolate 3100-90 (Fig. 1, lane 10) differed from these two only by lacking a major protein band with a molecular mass of 82,900 Da. Isolates 2693-90 (Fig. 1, lane 11) and 3072-90 (Fig. 1, lane 12) had very similar whole-cell protein patterns; the remaining four unknown isolates had singular patterns.

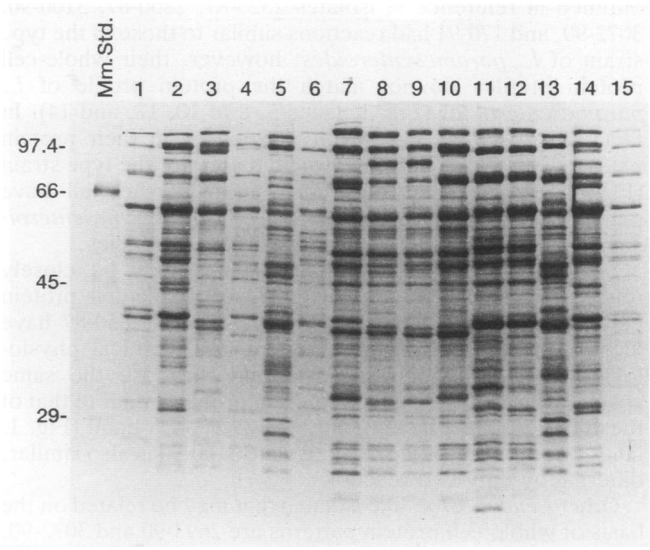

FIG. 1. SDS-polyacrylamide gel of whole-cell proteins of *Leuconostoc* spp. Mm. Std., molecular mass standards (in kilodaltons): phosphorylase *b* (97.4), bovine serum albumin (66), ovalbumin (45), and carbonic anhydrase (29). Lanes are identified in Table 2.

DISCUSSION

A gram-positive, catalase-negative, lactic acid-producing coccus that is resistant to vancomycin, produces gas from glucose, is arginine negative, fails to grow at 45°C, and is pyrrolidonyl arylamidase and leucine aminopeptidase negative can be presumptively identified as a *Leuconostoc* sp. (9). Conventional biochemical reactions that conform to the DNA homology and rRNA sequencing data (10, 17) can be used to determine the species of the majority of leuconostocs isolated in the clinical laboratory. A total of 91 bacterial samples submitted to our laboratory from human sources have been presumptively identified as *Leuconostoc* spp., and according to our phenotypic criteria (9), 82 (90%) were assigned to a particular species. Only nine (10%) of the bacterial samples presumptively identified as *Leuconostoc* spp. could not be definitely assigned to a species on the basis of biochemical reactions.

All bacteria that were assigned to a *Leuconostoc* sp. and the *Leuconostoc*-like bacteria were associated with human infections and were isolated in clinical microbiology laboratories. Presumably, the clinical laboratory will receive only *Leuconostoc* spp. that are capable of multiplying at 37°C. This should eliminate the necessity for identifying *Leuconostoc cremoris* and the recently described species *Leuconostoc carnosum* and *Leuconostoc gelidum* (21) in a clinical laboratory, since these bacteria will not grow at 35 to 37°C in MRS or Todd-Hewitt broth or on sheep blood agar plates (personal observation). Because of this, these species were not included in this study.

Of the *Leuconostoc* spp. that will grow at 35 to 37°C, only *L. lactis* is esculin negative (9). Four of the unknown *Leuconostoc*-like bacterial isolates were esculin negative (Table 3, strains 983-84, 3100-90, 2693-90, and 780-86), but only isolate 983-84 had the same physiologic reactions as those listed in Table 1 for *L. lactis*. None of these isolates, including isolate 983-84, had the same whole-cell protein profile as that of *L. lactis* (Fig. 1, lanes 3, 7, 10, 11, and 13), indicating that they are probably separate species.

Solely on the basis of the basic physiologic reactions as outlined in reference 9, isolates 2325-87, 2850-87, 3100-90, 3072-90, and 170-91 had reactions similar to those of the type strain of *L. paramesenteroides*; however, their whole-cell protein profiles do not match the protein profile of *L. paramesenteroides* (Fig. 1, lanes 5, 8 to 10, 12, and 14). In fact, these isolates had major differences in their protein patterns compared with the protein pattern of the type strain (Fig. 1, lane 5), indicating that although they all have physiologic reactions similar to those of *L. paramesenteroides*, they are probably not members of that species.

Several of these unidentified isolates may be closely related to each other on the basis of whole-cell protein patterns. For example, isolates 2325-87 and 2850-87 have identical whole-cell protein patterns and identical physiologic reactions, which suggests that they are the same species. Isolate 3100-90 has a protein profile similar to that of these two isolates, lacking only the 82,900-Da band (Fig. 1, lanes 8 to 10). Physiologically, isolate 3100-90 is also similar, differing by only two reactions.

Other *Leuconostoc*-like isolates that may be related on the basis of whole-cell protein patterns are 2693-90 and 3072-90. Although they have different biochemical reactions (Table 3), they have identical whole-cell protein patterns (Fig. 1, lanes 11 and 12). As with the three previous unidentified *Leuconostoc*-like bacterial isolates, additional tests will be needed to determine whether these two isolates represent a single species.

Analyses of soluble whole-cell protein patterns were consistent with the physiologic results, with members of each species having similar protein patterns after SDS-PAGE. This procedure will not be useful in the initial identification of *Leuconostoc* spp. in a clinical microbiology laboratory, where biochemical reactions that can identify as many as 90% of the bacteria are more appropriate, but it will be useful in a reference laboratory in the event that biochemical reactions are ambiguous because of inconsistent results. When this occurs, the comparison of the soluble whole-cell protein pattern of the unidentified bacterium and the patterns from the type strains can be used to clarify ambiguous physiologic results and determine whether the *Leuconostoc*-like bacterium belongs to an accepted species. In this study, nine *Leuconostoc*-like bacterial isolates had biochemical reactions nearly identical to those of recognized *Leuconostoc* spp. Their protein patterns indicate, however, that they may represent several new species of this genus.

REFERENCES

1. **Bernaldo de Quirós, J. C. L., P. Muñoz, E. Cercenado, T. Hernandez Sampelayo, S. Moreno, and E. Bouza.** 1991. *Leuconostoc* species as a cause of bacteremia: two case reports and a literature review. Eur. J. Clin. Microbiol. Infect. Dis. **10**:505–509.
2. **Buu-Hoï, A., C. Branger, and J. F. Acar.** 1985. Vancomycin-resistant streptococci or *Leuconostoc* sp. Antimicrob. Agents Chemother. **28**:458–460.
3. **Coovadia, Y. M., Z. Solwa, and J. van den Ende.** 1987. Meningitis caused by vancomycin-resistant *Leuconostoc* sp. J. Clin. Microbiol. **25**:1784–1785.
4. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130–135.
5. **Dicks, L. M. T., and H. J. J. van Vuuren.** 1987. Relatedness of heterofermentative *Lactobacillus* species revealed by numerical analysis of total soluble cell protein patterns. Int. J. Syst. Bacteriol. **37**:437–440.
6. **Elliott, 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.
7. **Elliott, J. A., R. R. Facklam, and C. B. Richter.** 1990. Whole-cell protein patterns of nonhemolytic group B, type Ib, streptococci isolated from humans, mice, cattle, frogs, and fish. J. Clin. Microbiol. **28**:628–630.
8. **Facklam, R., D. Hollis, and M. D. Collins.** 1989. Identification of gram-positive coccal and coccobacillary vancomycin-resistant bacteria. J. Clin. Microbiol. **27**:724–730.
9. **Facklam, R. R., and J. A. Washington II.** 1991. *Streptococcus* and related catalase-negative gram-positive cocci, p. 238–257. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
10. **Farrow, J. A. E., R. R. Facklam, and M. D. Collins.** 1989. Nucleic acid homologies of some vancomycin-resistant leuconostocs and description of *Leuconostoc citreum* sp. nov. and *Leuconostoc pseudomesenteroides* sp. nov. Int. J. Syst. Bacteriol. **39**:279–283.
11. **Garvie, E. I.** 1984. Separation of the species of the genus *Leuconostoc* and differentiation of the leuconostocs from other lactic acid bacteria, p. 147–178. *In* T. Bergan (ed.), Methods in microbiology. Academic Press, Inc., New York.
12. **Garvie, E. I.** 1986. *Leuconostoc*, p. 1071–1075. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams and Wilkins Co., Baltimore.
13. **Golledge, C. L.** 1989. Bacteremia due to *Leuconostoc* species. Clin. Microbiol. Newsl. **11**:20–30.

14. **Handwerger, S., H. Horowitz, K. Coburn, A. Kolokathis, and G. P. Wormser.** 1990. Infection due to *Leuconostoc* species: six cases and review. Rev. Infect. Dis. **12:**602–610.
15. **Horowitz, H. W., S. Handwerger, K. G. Van Horn, and G. P. Wormser.** 1987. *Leuconostoc*: an emerging vancomycin-resistant pathogen. Lancet **ii:**329–330.
16. **Lütticken, R., and G. Kunstmann.** 1988. Vancomycin-resistant *Streptococcaceae* from clinical material. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **267:**379–382.
17. **Martinez-Murcia, A. J., and M. D. Collins.** 1990. A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. **70:**73–84.
18. **Rubin, L. G., E. Vellozzi, J. Shapiro, and H. D. Isenberg.** 1988. Infection with vancomycin-resistant "streptococci" due to *Leuconostoc* species. J. Infect. Dis. **157:**216.
19. **Ruoff, K. L., D. R. Kuritzkes, J. S. Wolfson, and M. J. Ferraro.** 1988. Vancomycin-resistant gram-positive bacteria isolated from human sources. J. Clin. Microbiol. **26:**2064–2068.
20. **Savell, J. W., M. O. Hanna, C. Vanderzant, and G. C. Smith.** 1981. An incident of predominance of *Leuconostoc* sp. in vacuum-packaged beef strip loins—sensory and microbial profile of steaks stored in O_2-CO_2-N_2 atmospheres. J. Food Prot. **44:**742–745.
21. **Shaw, B. G., and C. D. Harding.** 1989. *Leuconostoc gelidum* sp. nov. and *Leuconostoc carnosum* sp. nov. from chill-stored meats. Int. J. Syst. Bacteriol. **39:**217–223.
22. **Vandamme, P., B. Pot, and K. Kersters.** 1991. Differentiation of campylobacters and *Campylobacter*-like organisms by numerical analysis of one-dimensional electrophoretic protein patterns. Syst. Appl. Microbiol. **14:**57–66.