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Identification, Classification, and Clinical Relevance of Catalase-Negative, Gram-Positive Cocci, Excluding the Streptococci and Enterococci

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INTRODUCTION

The 1985 edition of *Bergey's Manual of Systematic Bacteriology* listed only seven genera of facultatively anaerobic gram-positive cocci (137). Just before the publication of the manual, the *Streptococcus* genus was split into three genera, *Streptococcus*, *Enterococcus*, and *Lactococcus* (138, 139). Since 1985, six additional genera have been described. The new genera and references are listed in Table 1. The application of molecular techniques, DNA-DNA homology, and 16S rRNA sequence analysis led to the redescription of the *Streptococcus* genus (139) and the proposals for the other genera listed in Table 1. At about this time, vancomycin-resistant bacteria began to be isolated from human infections (20, 38, 39, 86, 88, 101, 131, 134, 150). This necessitated that clinical microbiologists adopt procedures to give them the capacity to identify not only the new genera but also the *Leuconostoc* and *Pediococcus* species that had not been previously associated with human infections (4).

Table 1 lists the facultatively anaerobic gram-positive coccal genera, as well as the aerobic genus *Alloiococcus*, that have been proposed to date. The first three genera will not be discussed in this review, but they are listed because of their common growth and Gram stain characteristics. The staphylococci and micrococci can easily be differentiated from the remaining genera by the catalase test. Although the alloiococci usually give a positive catalase test, the growth of these bacteria is much slower than the growth of the other catalase-positive bacteria. The stomatococci give very weak to negative catalase reactions. The stomatococci contain the cytochrome enzymes but not catalase and can be identified by detecting the presence of cytochrome enzymes by the porphyrin test (158). Some strains of aerococci and alloiococci may give a positive reaction in the catalase test; however, these bacteria do not contain catalase. The hydrolysis of hydrogen peroxide is thought to be due to the presence of a peroxidase enzyme that produces much less effervescence (bubbling) than that seen with the staphylococci. The aerococci and alloiococci do not contain cytochrome enzymes (23a).

The Gram stain is used to aid in differentiating the gram-positive cocci. All bacteria listed in Table 1 are gram positive. The arrangement of the cells helps to differentiate the genera. Some bacteria divide on random planes and form grapelike clusters of cells, an arrangement commonly observed with staphylococci. Bacteria that divide in one plane form pairs and eventually form chains if the cells remain attached to each other. This type of cellular morphology is observed with the *Streptococcus*, *Globicatella*, *Enterococcus*, *Lactococcus*, *Vagococcus*, and *Leuconostoc* spp. Bacteria that divide in two planes at right angles form packets of fours, or tetrads. This type of cellular arrangement is observed with the *Aerococcus*, *Alloiococcus*, *Gemella*, *Pediococcus*, *Tetragenococcus*, and *Helcococcus* spp.

One of the most difficult tasks of a microbiologist is to determine whether the bacteria are actually cocci or short rods. Since many of the lactobacilli are gram-positive short rods, sometimes in chains, they are occasionally confused with the streptococci. The clinical sources and colonial morphology on blood agar plates of the lactobacilli are also similar to those of the streptococci, especially those members of the viridans streptococci. When the Gram stain is read, it must be realized that the cellular arrangement is never 100% chains, pairs, tetrads, or clusters. The reader must determine the most common cellular arrangement. For example, some pairs and short chains, as well as tetrads, might be observed with a *Gemella* species. If tetrads are observed in most fields under observation, then the strain is dividing on two planes.

Although the Gram stain can be performed with bacteria grown on any type of medium, for this group of bacteria it is best to use thioglycolate broth. When a Gram stain is done on growth from thioglycolate broth, the staining procedure is modified. The growth cannot be fixed to the slide with heat but must be fixed with methanol.

Once it has been determined that the bacterium in question is a gram-positive, catalase-negative coccus, the next step is to determine the genus of the strain. This can be accomplished by determining the physiologic characteristics listed in Table 2. Table 2 lists the cocci in chains. Later in this review the cocci that are arranged in pairs and tetrads will be discussed; there is some overlap in the final test results between the cocci in chains and those that do not form chains; however, each genus has a unique set of phenotypic characteristics.

This review will not include discussions on the *Enterococcus* or *Streptococcus* genera. The data included in Table 2 for these genera are presented for comparative purposes to aid in the discussions on the identification of the other gram-positive genera. Descriptions of all of the tests described in this docu-

TABLE 1. Cytochrome and catalase content and cellular arrangement of facultatively anaerobic gram-positive cocci

Genus	Cytochrome/catalase[a]	Cellular arrangement	Reference
Staphylococcus	+/+	Clusters	137
Micrococcus	+/+	Clusters	137
Stomatococcus	+/−	Clusters	137
Streptococcus	−/−	Chains	140
Globicatella	−/−	Chains	30
Enterococcus	−/−	Chains	138
Lactococcus	−/−	Chains	140
Vagococcus	−/−	Chains	31
Leuconostoc	−/−	Chains	70
Aerococcus	−/+w	Pairs or tetrads	53
Alloiococcus	−/+w	Pairs or tetrads	1
Gemella	−/−	Pairs or tetrads	128
Pediococcus	−/−	Pairs or tetrads	71
Tetragenococcus	−/−	Pairs or tetrads	35
Helcococcus	−/−	Pairs or tetrads	32

[a] +, positive reaction; −, negative reaction; +/w, weak reaction. See text.

TABLE 2. Percent positive reactions of catalase-negative, gram-positive coccal genera arranged in chains and pairs

Bacterium	No. of strains tested	Phenotypic characteristic (% positive)									
		Vancomycin susceptibility	Gas[a]	PYR	LAP	NaCl[b]	Bile-esculin reaction	Growth at:		Motility	Alpha hemolysis[c]
								10°C	45°C		
Streptococcus spp.[d]											
Viridans	282	100	0	0	98	0	20	1	19	0	70
S. bovis	10	100	0	0	100	0	100	0	100	0	
NVS	19	100	0	77	76	0	0	0	0	0	80
Gemella-like	11	100	0	91	100	0	0	0	0	0	55
Enterococcus spp.											
Vancomycin susceptible	98	100	0	99	90	100	100	96	96	15	80
Vancomycin resistant	62	0	0	100	100	100	100	100	100	0	80
Lactococcus spp.	39	100	0	83	95	70	98	90	43	0	40
Vagococcus spp.	2	100	0	100	100	100	100	100	0	100	50
Leuconostoc spp.	101	0	100	0	0	57	74	71	26	0	47
Globicatella spp.	7	100	0	100	0	71	57	0	29	0	100

[a] Gas, gas from glucose in MRS broth.
[b] NaCl, growth in broth containing 6.5% NaCl.
[c] Alpha hemolysis on Trypticase soy agar containing 5% sheep blood.
[d] Beta-hemolytic streptococci not included in this analysis.

ment and their performance are given in the section on laboratory procedures.

THE *LACTOCOCCUS* GENUS

Members of the *Lactococcus* genus (*lactis*, milk; milk cocci) are facultatively anaerobic, catalase-negative, gram-positive cocci that occur singly, in pairs, or in chains. Several species and subspecies have been described.

Taxonomy and Identification of Genus

Before 1985, catalase-negative, facultatively anaerobic, serogroup N cocci that produced primarily lactic acid from the fermentation of carbohydrates were included in the *Streptococcus* genus. These bacteria are physiologically similar to the serogroup D fecal streptococci (Table 2) and sometimes have been identified as physiologic variants of enterococcal species. However, genetic evidence, based on DNA-DNA and DNA-RNA relatedness, clearly indicated that the lactic acid streptococci were separate species (90, 94). Further genetic analysis, using DNA-DNA relatedness and 16S rRNA sequencing data, placed the fecal streptococci in the *Enterococcus* genus (138) and the lactic acid streptococci in the genus *Lactococcus* (140).

The tests listed in Table 2 can be used to differentiate the lactococci from the other gram-positive cocci that grow in chains. Careful interpretation of the growth at 45°C is required to differentiate between the enterococci, which grow very well at 45°C, and the lactococci, which usually grow poorly or not at all at 45°C. In our studies, about 45% of the lactococcal strains showed some growth in our 45°C temperature tolerance test and, therefore, could not be differentiated from the enterococci with this test. Unlike the type of growth seen at 37°C, that seen at 45°C may not result in production of enough acid to change the color of the pH indicator of the medium. An increase in the turbidity of the medium is a positive indicator of growth and signifies that the bacterium is probably an *Enterococcus* sp. In addition, the enterococci and lactococci can usually be differentiated by the presence of the serogroup D or serogroup N antigen, respectively. However, the serogroup D antigen can be found in only about 80% of the enterococci (58), and only about 60% of the lactococci react with serogroup N antiserum in the capillary precipitin test (55). Some isolates of serogroup-negative, gram-positive cocci that grow in chains, however, will have unusual combinations of the pyrrolidonylarylamidase (PYR), 6.5% NaCl, and 45°C tests (Table 2) that can confuse the assignment of these bacteria to either the *Enterococcus* or the *Lactococcus* genus. These bacteria can be probed with the AccuProbe Enterococcus Culture Confirmation test (Gen-Probe, San Diego, Calif.). All lactococci fail to react with this genetic probe, whereas all known enterococci will react.

In addition to these tests, the tests listed in Table 3 will aid in differentiating lactococci from enterococci. As stated above, the lactococci are most often confused with *Enterococcus* spp., especially *Enterococcus faecalis*, *E. faecium*, *E. durans*, and *E. hirae*. *E. faecalis* is tolerant to tellurite and utilizes pyruvate and produces acid from sorbitol, whereas lactococci have the opposite reactions. None of the *Lactococcus* strains but all of the *E. faecium* strains produce acid in arabinose broth. All clinical isolates of lactococci have formed acid in mannitol broth, which differentiates them from *E. durans* and *E. hirae*.

Identification of Species

There are seven recognized *Lactococcus* species and subspecies that can be identified by physiologic actions (33, 67, 69, 72, 73, 84, 137, 140) (Table 4). These bacteria are very common in the environment and, until recently, were considered nonpathogenic for humans. In the past few years, however, several reports have described infections in humans that were attributed to *Lactococcus* sp. (49, 103, 159). The majority of the

TABLE 3. Differentiation of *Lactococcus* species from *E. faecalis*, *E. faecium*, *E. durans*, and *E. hirae*

Species	Test result[a]					
	MAN	SBL	ARA	RAF	TEL	PYU
E. faecalis	+	+	−	−	+	+
Lactococcus sp.	+	−	−	v	−	−
E. faecium	+	v	+	v	−	−
E. durans	−	−	−	−	−	−
E. hirae	−	−	−	+	−	+

[a] MAN, mannitol; SBL, sorbitol; ARA, arabinose; RAF, raffinose; TEL, 0.04% tellurite; PYU, pyruvate. +, >90% positive; −, <10% positive; v, variable reactions (10 to 90% of strains positive).

TABLE 4. Positive reactions of CDC strains of *L. lactis* and *L. garveiae* and differentiation of *Lactococcus* species

Species	% Positive or result in given test[a]										
	LAC	MAL	MAN	RAF	SUC	SBL	TRE	ARG	HIP	PYR	VP
L. lactis subsp. *lactis*	91	100	100	0	82	0	100	91	0	55[b]	82[b]
L. garveiae	61	95	100	0	50	0	100	100	0	100	100
L. lactis subsp. *cremoris*	+	−	−	−	−	−	−	−	+	−	+
L. lactis subsp. *hordniae*	−	−	−	−	+	−	+	+	−	−	+
L. plantarum	−	+	+	−	+	+	+	−	−	−	+
L. raffinolactis	+	+	v	+	−	−	−	−	−	−	+
L. xyloses	−	+	+	−	+	−	+	+	−	−	+

[a] Acid formation in: LAC, lactose; MAL, maltose; MAN, mannitol; RAF, raffinose; SUC, sucrose; SBL, sorbitol; TRE, trehalose. ARG, deamination of arginine; HIP, hydrolysis of hippurate; VP, Voges-Proskauer. +, positive; −, negative; v, variable.
[b] *L. lactis* is reported to be negative in PYR and Voges-Proskauer tests.

isolates were either *Lactococcus lactis* subsp. *lactis* or *L. garvieae* (49, 103, 159). As noted in Table 4, these two species have very similar reaction patterns, making it difficult to distinguish between them solely on the characteristics that are listed. Additional physiologic tests, such as those obtained from the API 50CH panel (64), and molecular comparisons, such as whole-cell protein analysis (49, 67) and DNA or RNA analysis (8, 16, 23, 96, 97, 118, 127, 130, 135, 147), have been used to help in their identification. However, these additional tests are more labor-intensive than the tests listed in Table 4. The value of determining the exact *Lactococcus* sp. in a clinical laboratory with these additional tests is unknown because it has not been determined if there are species-related differences in the susceptibility to antimicrobial agents. There are little data on the susceptibility of lactococci to the antimicrobial agents generally used for the treatment of infections with gram-positive cocci (120). Identification of most of the *Lactococcus* spp. listed in Table 4 is of interest primarily to the food industry, which uses these bacteria for the preservation of food and for the production of flavors, making it important for them to maintain pure cultures of the desired bacteria.

Before the physiologic tests that described the species were published, these bacteria were variously identified as abnormal enterococcal species, including *E. faecium*, *E. faecalis*, *E. durans*, and *E. hirae*, or were reported as unidentified gram-positive cocci. After the physiologic tests given in Tables 3 and 4 were established, along with whole-cell protein analysis and 16S rRNA sequencing, all clinical isolates that we have received that could be assigned to a species have been either *L. lactis*, subsp. *lactis* (11 strains) or *L. garvieae* (19 strains); the remaining 9 strains could not be assigned definitively to a species. Without these molecular techniques, the definitive identification of these bacteria remains difficult. Several isolates that we initially identified as either *L. garvieae* or *L. lactis* were later reclassified as the opposite species. Because our laboratory receives only isolates that are difficult to identify, most of our recent isolates could be identified only to the genus level.

Clinical Significance and the Experience of the Centers for Disease Control and Prevention (CDC)

Wood et al. (159) were the first to report the isolation of a *Lactococcus* strain from a case of subacute bacterial endocarditis. We have received approximately 39 lactococci from human infections and two isolates from animal infections (one porcine fetus and one rat); they were isolated primarily from blood cultures (25 isolates). Other sources included urine (four isolates), wound (two isolates), and pleural fluid, brain abscess, bile fluid, eye, and skin (one isolate each). The sources of the remaining isolates were not given. Initial clinical diagnosis was available for 24 isolates and included septicemia (13 cases), pneumonia or acute respiratory disease syndrome or chronic obstructive disease (3 cases), endocarditis (3 cases), and cystitis, sudden infant death syndrome, ruptured appendix, amputation wound, and brain abscess (1 case each). Lactococci are probably opportunistic pathogens of debilitated persons; however, we do not have sufficient information about associated illnesses to support that conclusion. Apparently, these bacteria can cause a variety of infections and should be considered whenever an isolated bacterium resembles an *Enterococcus* or nonhemolytic *Streptococcus* sp. in all but a few characteristics.

THE *VAGOCOCCUS* GENUS

Species of *Vagococcus* (wandering coccus) have ovoid cells that occur in singles, pairs, and chains (31). They are catalase negative and facultatively anaerobic. Two species of *Vagococcus* have been described; *Vagococcus fluvialis* (31) and *V. salmoninarum* (152).

Taxonomy and Identification of Genus and Species

The *Vagococcus* genus was created from bacteria that were previously known as motile lactococci (140). The motility characteristic of the vagococci differentiates them from the lactococci (Table 2). Both lactococci and vagococci are susceptible to vancomycin and fail to form gas in Mann, Rogosa, and Sharpe (MRS) broth (45); the majority give positive reactions for PYR and leucine aminopeptidase (LAP), grow in broth containing 6.5% NaCl, and grow at 10°C but poorly if at all at 45°C. In addition, all *Vagococcus* strains we have examined to date have reacted positively with the AccuProbe *Enterococcus* test. This differentiates these strains from the lactococci, which are AccuProbe *Enterococcus* test negative.

The similarity of these strains to the enterococci complicates their accurate identification. Although the description of the species indicates that the vagococci will not grow in broth containing 6.5% NaCl, all strains we have tested grow in the formulation used at the CDC (56). Both clinical isolates from human sources were initially classified as "unidentified enterococci." The tests listed in Table 5 can be used to identify and differentiate the *Vagococcus* strains from phenotypically similar enterococcal strains. The enterococcal strains listed in Table 5 are all arginine deaminase-negative variants of typical enterococcal species.

Clinical Significance and the CDC Experience

We have identified only two strains of *V. fluvialis* from human sources. One strain is a blood culture isolate from a

TABLE 5. Identification of *Vagococcus* species and differentiation from atypical *Enterococcus* species

Species	Test result[a]											
	MAN	SBL	SOR	ARG	ARA	RAF	TEL	MOT	PIG	SUC	PYU	RIB
E. faecalis	+	+	−	−	−	−	+	−	−	+	+	+
Vagococcus sp.	+	+	−	−	−	−	−	+	−	v	+	+
E. gallinarum	+	−	−	−	+	+	−	+	−	+	−	+
E. faecium	+	−	−	−	+	v	−	−	−	+	−	+
E. casseliflavus	+	−	−	−	+	+	−	+	+	+	v	+

[a] Acid formation in: MAN, mannitol; SBL, sorbitol; SOR, sorbose; ARG, arginine; ARA, arabinose; RAF, raffinose; SUC, sucrose; RIB, ribose; TEL, 0.04% tellurite. MOT, motility; PIG, pigmented; PYU, pyruvate. +, >90% positive; −, <10% positive, v, variable (60 to 90% of strains positive).

patient with bacteremia; the other is a peritoneal fluid isolate from a patient undergoing dialysis for kidney disease. Because we have received few isolates, it is not possible to comment further on the clinical significance of the vagococci. As refined procedures are incorporated into the identification schemes for the gram-positive cocci, proper identification of the vagococci may led to more information about the clinical significance of these bacteria.

THE *GLOBICATELLA* GENUS

Globicatella (short chain made up of spheres) are ovoid and gram positive and occur in pairs and short chains. They are catalase negative and facultatively anaerobic (30). One species of *Globicatella*, *Globicatella sanguis*, has been described.

Taxonomy and Identification of Genus and Species

This new genus of gram-positive cocci was only recently described. It originated from a collection of viridans-like streptococci that shared phenotypic characteristics with the aerococci and *Streptococcus uberis*. What makes *Globicatella* distinct from the viridans streptococci is that all *Globicatella* strains are PYR positive and LAP negative and grow in broth containing 6.5% NaCl, while all viridans species are PYR negative and LAP positive and fail to grow in 6.5% NaCl. *Globicatella* strains differ from the aerococci by their cellular arrangement in the Gram stain. Aerococci form tetrads and pairs, whereas globicatellas form short chains. The colonial morphologies of the two genera are also different. The aerococci form much larger colonies on blood agar plates, similar to enterococci, while the globicatella form small viridans streptococcus-like colonies. All *G. sanguis* strains identified to date have been susceptible to vancomycin, PYR positive, and LAP negative, grow in 6.5% NaCl broth, do not grow at 10 or 45°C, are nonmotile, and are alpha-hemolytic. These phenotypic characteristics are identical to those of the aerococci; thus, the Gram stain must be carefully examined to determine the cellular arrangement for correct identification.

Clinical Significance and the CDC Experience

There is very little clinical information on the type of infections caused by *G. sanguis*. We have identified 14 strains that have phenotypic characteristics identical to the type strains (30). Eight strains were isolated from blood cultures; two patients had sepsis, one had meningitis, and the remaining five patients had bacteremia. Four strains have been isolated from the urine of patients with urinary tract infections. One strain was isolated from cerebrospinal fluid (CSF) during an autopsy, and the remaining strain was isolated from a wound infection. Studies to gather more clinical information are under way.

THE *LEUCONOSTOC* GENUS

The members of the *Leuconostoc* genes (*leucus*, clear, light; *nostoc*, algal generic name; *leuconostoc*, colorless nostoc) are facultatively anaerobic, catalase-negative, gram-positive cocci arranged in pairs and chains (70).

Taxonomy and Identification of Genus

All species of *Leuconostoc* are vancomycin resistant and produce CO_2 from glucose. They may appear to be morphologically similar to the heterofermentative vancomycin-resistant lactobacilli but tend to be more coccoidlike than the lactobacilli. Careful interpretation of the Gram stain from the growth of these bacteria in thioglycolate broth is important for the separation of the leuconostocs from the gas-forming lactobacilli.

On the surface of a 5% sheep blood agar plate, the *Leuconostoc* spp. appear very similar to the alpha-hemolytic or nonhemolytic *Enterococcus* or *Lactococcus* spp. Before the recognition that *Leuconostoc* spp. were associated with human diseases (6, 7, 15, 39, 40, 47, 50, 58, 66, 74–77, 81–83, 86–88, 99, 101, 102, 117, 131, 132, 134, 153), these bacteria were generally identified as variants of established human pathogens such as viridans streptococci or enterococci or, more frequently, were reported as unidentified gram-positive cocci. Because up to 31% of the *Leuconostoc* spp. react with the group D antiserum, they were reported as unidentified *Enterococcus* spp. The nonserogroup D bacteria had characteristics that led to their identification as *Streptococcus uberis*, *S. anginosis* group, *S. bovis*, *S. sanguis*, or *S. equinus* or *Aerococcus*-like or *Lactobacillus*-like bacteria. With the recognition that *Leuconostoc* spp. are human pathogens, such mistakes in identification now seldom occur because of the unique results of leuconostocs in the tests listed in Table 2. These bacteria are the only catalase-negative cocci that are vancomycin resistant and PYR and LAP negative and that produce CO_2 from glucose.

Identification of Species

The *Leuconostoc* spp. are environmental organisms that are generally found on vegetable matter and in milk products and other chilled food products (10, 34, 46, 48, 141). A few species have been isolated from human infections, especially after the introduction of vancomycin. Table 6 lists the physiological reactions that are useful for identifying the five *Leuconostoc* spp. that have been isolated from human infections. The identification of *Leuconostoc* species that fail to grow in the laboratory at 35 to 37°C is not included in Table 6 under the assumption that these bacteria would also fail to cause disease in humans. Identification of *Leuconostoc cremoris*, *L. carnosum*, *L. oenos*, *L. fallax*, *L. argentinum*, and *L. gelidum* can be found elsewhere (46, 105, 136, 137, 141, 145).

TABLE 6. Positive reactions of CDC clinical isolates of *Leuconostoc* spp.

Species	% Positive in given test[a]							
	ESC	LM	RAF	MEL	NaCl	ARA	TRE	SUCA
L. mesenteroides	100	11	94	100	89	97	97	74
L. pseudomesenteroides	100	54	77	100	0	62	92	67
L. paramesenteroides	100	50	100	10	100	100	100	0
L. citreum	100	4	8	0	63	92	100	83
L. lactis	4	37	96	100	48	52	48	15

[a] ESC, esculin hydrolysis; LM, acid and clot production in litmus milk. NaCl, growth in broth containing 6.5% NaCl; SUCA, dextran production on sucrose agar. Acid formation from: RAF, raffinose; MEL, melibiose; ARA, arabinose; TRE, trehalose.

Several *Leuconostoc* species are difficult to distinguish from each other. *L. mesenteroides* and *L. pseudomesenteroides* have very similar physiologic reactions and are sometimes confused with each other. In fact, *L. pseudomesenteroides* is most frequently identified as *L. mesenteroides* if the NaCl tolerance test is difficult to interpret. Prolonged incubation of the NaCl broth may be necessary to correctly differentiate between the 6.5% NaCl tolerant *L. mesenteroides* and the 6.5% NaCl intolerant *L. pseudomesenteroides*. Any increase in the number of bacteria in the NaCl broth signifies that the bacterium is *L. mesenteroides*.

At this time, *L. paramesenteroides* is still considered a *Leuconostoc* sp. with physiologic characteristics similar to those of *L. mesenteroides*. Dextran production by *L. mesenteroides* is used to differentiate the two species. Recent phylogenetic analysis has indicated, however, that *L. paramesenteroides* is more closely related to the lactobacilli then to the *Leuconostoc* genus (104, 106, 161), and there has been an informal proposal to reclassify this and related species into a new genus, *Weissella* (34). Morphologically, these bacteria appear as cocci in chains, which is similar to the other *Leuconostoc* spp. and differentiates them from the *Lactobacillus* spp.

L. lactis is the only clinical isolate of *Leuconostoc* sp. that does not hydrolyze esculin (Table 6). As with *L. paramesenteroides*, this species also does not produce dextran from glucose. These characteristics clearly differentiate this species from the other *Leuconostoc* species.

L. citreum is also clearly distinguishable from the other clinically significant species of *Leuconostoc* on the basis that this species is the only one that does not ferment either raffinose or melibiose. As its name implies, this bacterium also produces a characteristic yellow pigment that can be seen with bacteria grown in MRS broth (62).

Table 6 lists the results of the physiologic reactions of the *Leuconostoc* spp. that we have received. In addition, all of the isolates were PYR and Voges-Proskauer negative, produced gas from glucose in MRS broth (45), and were resistant to vancomycin. All isolates that were tested were LAP negative. Susceptibility to bacitracin varied among the species, with 60% of the *L. mesenteroides*, 50% of the *L. pseudomesenteroides*, 13% of the *L. citreum*, and none of the *L. lactis* strains being susceptible. Serogroup D antigen was detected with 14% of the *L. mesenteroides*, 31% of the *L. pseudomesenteroides*, and 13% of the *L. citreum* isolates and with none of the *L. lactis* or *L. paramesenteroides* isolates. Alpha hemolysis on Trypticase soy agar with 5% sheep blood (BBL, Cockeysville, Md.) was varied, being observed with 60% of the *L. mesenteroides*, 54% of the *L. pseudomesenteroides*, 14% of the *L. citreum*, 83% of the *L. lactis*, and 50% of the *L. paramesenteroides* strains.

Clinical Significance and Disease

The *Leuconostoc* spp. have been isolated from a wide variety of infections in humans, including meningitis (39, 66), odontogenic infection (153), catheter-associated infection (83), and bacteremia or septicemia (15, 50, 76, 99, 124). Frequently, bacteria are associated with a polymicrobial infection and are isolated after the patient has been treated with vancomycin to eliminate the suspected pathogen (15, 82, 88, 131). The clinical significance of an infection with these bacteria in nonimmunocompromised patients is not clear. For example, in one study the bacteria were eliminated by several of the patients without the use of antimicrobial agents (99). Infections in immunocompromised patients, however, can be severe, and the bacterium should be identified as a *Leuconostoc* sp. to ensure that an appropriate antimicrobial agent is used to treat the patient. Treatment with high-dose penicillin or clindamycin (15, 39, 82, 88) or with tobramycin (88) has been reported to be successful. Swenson et al. found that none of the isolates were resistant to imipenem, minocycline, chloramphenicol, gentamicin, or daptomycin (144).

The CDC Experience

The CDC has received approximately 101 bacterial isolates that we have identified as one of the five species of *Leuconostoc* implicated as human pathogens. The majority of the bacteria have been *L. mesenteroides* (35 isolates). We have also received *L. lactis* (27 isolates), *L. citreum* (24 isolates), *L. pseudomesenteroides* (13 isolates), and *L. paramesenteroides* (2 isolates).

The sources of 99 isolates were listed by the submitter and included blood (85 isolates), wound (3 isolates), CSF (3 isolates), abscess (2 isolates), catheter or feeding tube (2 isolates), and food, gastric fluid, peritoneal fluid, and pleural fluid (1 isolate each). We have received an additional 16 bacteria that were resistant to vancomycin and produced CO_2 in MRS broth, but their Gram stain morphology or their physiologic reactions precluded their identification as one of the *Leuconostoc* spp. None of these bacteria appeared to be lactobacilli, but this possibility has not been eliminated. Until further tests can be identified for differentiating between the *Leuconostoc* and *Lactobacillus* spp., they are being classified as probable *Leuconostoc* sp.

The initial clinical diagnosis that led to the isolation of the *Leuconostoc* spp. was varied and included bacteremia or sepsis (28 cases), meningitis (4 cases), subacute bacterial endocarditis (3 cases), pneumonia (3 cases), AIDS (2 cases), and liver abscess, urinary tract infection, metabolic acidosis, multiple myeloma, prostate cancer, surgical wound infection, and peritonitis (1 case each). The lack of information for the majority of the isolates about underlying disease in the patients limits

TABLE 7. Positive reactions of gram-positive coccal genera arranged in pairs, tetrads, and clusters[a]

Genus	No. of strains tested	% Positive reactions in given test							
		Vancomycin susceptibility	PYR	LAP	Bile-esculin reaction	NaCl[b]	Growth at:		Alpha hemolysis[c]
							10°C	45°C	
Aerococcus	28	100	100	0	82	100	0	5	100
ALO	7	100	0	100	0	100	0	71	84
Helcococcus	7	100	100	0	0	100	0	0	0
Alloiococcus	23	100	100	100	0	100	0	0	0
Gemella	34	100	100	94	0	0	0	6	0
Pediococcus	47	0	0	100	94	62	5	76	34
Tetragenococcus	1[d]	100	0	100	100	100	0	100	100

[a] Gas from glucose in MRS broth resulted in no positive reactions.
[b] NaCl, growth in broth containing 6.5% NaCl.
[c] Alpha hemolysis on Trypticase soy agar containing 5% sheep blood.
[d] Data for type strain only; no clinical isolates.

our ability to say that *Leuconostoc* spp. are primarily opportunistic pathogens of debilitated individuals. But considering their wide distribution in the environment and the relatively few infections they cause, it is assumed that these bacteria have very little virulence for healthy human beings.

THE *AEROCOCCUS* GENUS

Aerococcus (air coccus) is a gram-positive coccus that divides on two planes at right angles, resulting in tetrad and cluster arrangements on Gram stains; some pairs and singles may also be found. The bacteria are facultatively anaerobic and catalase negative, although some strains can produce a weakly positive catalase-like reaction. Cytochrome enzymes are absent.

Identification of Genus and Species

The aerococci were first described in 1953 (157). The organism was isolated from settle plates placed in a hospital environment. Although the bacteria had characteristics similar to those of the streptococci, especially the enterococci, they were clearly different from the streptococci in Gram stain characteristics. Because of the cellular morphology, the original authors thought that the cultures resembled pediococci more than streptococci. Similarities to *Gaffkya* and *Pediococcus* strains were also noted by other investigators (43), and it was suggested that the strains were so similar that they should all be combined into one *Pediococcus* species. Clausen (28) described yet another group of bacteria with cellular morphology that resembled the aerococci described by Williams et al. (157), but these bacteria were catalase positive. Clausen suggested that there were two species of aerococci, one catalase-negative *Aerococcus viridans* and one catalase-positive *A. catalasicus*. This idea was not officially accepted by any taxonomic committee. Whittenbury (155) found that the *Aerococcus* and *Pediococcus* spp. could be clearly distinguished by phenotypic characteristics. Whittenbury also found that *Pediococcus urinae-equi* was similar to *A. viridans* and believed that these bacteria should be combined into a single species, *A. viridans* (155). Other investigators have applied additional biochemical and serologic analyses, as well as DNA-DNA relatedness studies, to show that the aerococci and pediococci are separate genera (17, 53, 115, 143, 156) and that *Gaffkya homari*, *P. homari*, and *A. viridans* are sufficiently similar to be combined into one taxon, *A. viridans*.

Initially, specific identification of the aerococci was difficult because of the controversy over the proper classification. Early investigators used growth tolerance tests (growth at 45°C, growth in the presence of 40% bile, and growth at pH 9.6) and enzyme tests (hydrolysis of hippurate, esculin, and arginine) to identify the aerococci (43, 51, 52, 155). During the development of the miniaturized tests for identification of gram-positive cocci, particularly the streptococci and enterococci, it was noted that the aerococci were more accurately identified than other gram-positive cocci (37, 54, 59, 63, 162). The reason for this phenomenon was that these systems incorporated one or both of the PYR and LAP tests. A unique feature of these two tests is that until recently, when the *Helcococcus* and *Globicatella* genera were described, *A. viridans* was the only gram-positive coccus known to give positive PYR and negative LAP results. The tests described in Table 7 can be used to identify the *Aerococcus* genus (17, 58). More recently, a genetic probe that will identify *A. viridans* has been described (80). This probe reacts with the type strain of *P. equinae-equi*, which also lends evidence that these two bacteria are the same species. In addition, the 16S rRNA sequences of these two bacteria were 99.9% homologous (35).

A proposal to add a second species to the *Aerococcus* genus, *A. urinae*, has been made (2, 3). However, at this time we believe that there is only one valid species of *Aerococcus*, *A. viridans* (see section on *Aerococcus*-like organisms [ALOs]).

In addition to the phenotypic characteristics listed in Table 7, *A. viridans* does not hydrolyze arginine and is Voges-Proskauer negative. Hippurate is hydrolyzed by about 85% of the strains. *A. viridans* is also strongly alpha-hemolytic on blood agar plates. The colonies are large and comparable in size to those of the enterococci on conventional blood agar plates.

All cultures received at the CDC were consistent in the following physiologic characteristics: all were susceptible to vancomycin, did not produce gas in MRS broth, produced PYR but not LAP, and failed to grow at 10°C. Some strains grew very poorly, but most did not grow, at 45°C; none were motile, and all were strongly alpha-hemolytic on blood agar plates (Table 7). A wide variety of reactions were observed with acid formation in lactose, mannitol, melibiose, raffinose, sorbitol, sucrose, and trehalose broths. None of the strains produced acid in arabinose, glycerol, or sorbose broth. About two-thirds of the cultures hydrolyzed hippurate.

Clinical Significance, Disease, and the CDC Experience

Aerococci have been isolated from the air (92, 157), soil (98), and human infections (36, 37, 122). *A. viridans* has been isolated from blood cultures of patients with endocarditis (89, 125, 149), urine cultures of patients with urinary tract infec-

tions (36), synovial fluid of septic arthritis patients (148), blood cultures of patients with bacteremia (36, 37, 93, 121, 122), and cultures of CSF from patients with meningitis (116). *A. viridans* has been reported in 4 of 317 cases of endocarditis (122) and, more recently, in 4 of 71 cases of endocarditis (19).

In the past 10 years, we have identified 36 strains of *A. viridans* from human sources: 25 from blood, 3 from urine, 2 from spinal fluid, 2 from wound cultures, and 1 culture each from ear, cyst, synovial fluid, and an unknown source. The two strains from the CSF were from patients with meningitis, and the blood cultures, one each, were from patients with endocarditis and pneumonia.

A. viridans is, for the most part, susceptible to penicillins, macrolides and related drugs, and chloramphenicol. These bacteria, like many gram-positive bacteria, are resistant to the aminoglycosides (22). Resistance to erythromycin, tetracycline, minocycline, and chloramphenicol has been documented (22, 108).

ALO

The ALO described by Christensen and colleagues (25, 26) are bacteria that morphologically and phenotypically resemble aerococci but, unlike the aerococci, do not have PYR activity. Aguirre and Collins (2) showed that the rRNA sequences of the ALO and aerococci were 94.5% similar. These investigators proposed that the ALO were a second species of the *Aerococcus* genus, *A. urinae* (2). Results from DNA-DNA hybridization studies in our laboratories indicate relatedness of less than 10% between strains of *A. urinae* (ALO) and *A. viridans*. We believe that these data together with the differences in phenotypic characteristics suggest that the ALO are insufficiently similar to *A. viridans* to belong to the *Aerococcus* genus.

The ALO can be identified by the reactions listed in Table 7. ALO are LAP positive and PYR negative and grow at 45°C, the opposite of the reactions observed with the aerococci. Like the aerococci, ALO are susceptible to vancomycin, do not form gas in MRS broth, grow in broth with 6.5% NaCl, do not grow at 10°C, and are nonmotile.

The ALO were first associated with urinary tract infections (25, 26), and later reports indicated that the organism may be isolated from blood cultures of patients with endocarditis (24). Very little information on the antimicrobial susceptibility of the ALO is available. Limited data indicate that the ALO are susceptible to penicillin and erythromycin but resistant to trimethoprim and aminoglycosides (24).

We have identified five strains that closely resemble ALO from our culture collection. All five strains have been isolated from the urine of patients with urinary tract infections.

THE *HELCOCOCCUS* GENUS

The genus *Helcococcus* (*helkos*, wound) contains gram-positive cocci that are catalase negative and facultatively anaerobic and are arranged in pairs, tetrads, and clusters.

Identification of Genus and Species

A second group of bacteria similar to the aerococci has been designated helcococci (32). These gram-positive cocci are similar to the aerococci in that they are vancomycin susceptible, do not form gas from MRS broth, are PYR positive and LAP negative, usually grow in broth containing 6.5% NaCl (this may depend to some extent on the formulation of the base medium, but comparative studies of media and growth of helcococci have not been done), do not grow at 10 or 45°C, and are not motile (Table 7). The only difference between the helcococci and aerococci noted to date is growth of these two genera on blood agar plates. The helcococci grow more slowly, are usually nonhemolytic, and form small viridans streptococcus-like colonies, whereas the aerococci form large (enterococcuslike) colonies surrounded by a large zone of alpha hemolysis after overnight incubation. Only one species of *Helcococcus* is described, *Helcococcus kunzii* (32).

Clinical Significance and the CDC Experience

All isolates described to date have been isolated from cultures of wounds showing signs of infection. We have identified 4 *H. kunzii* cultures among 50 cultures that had been previously identified as *A. viridans*. There are no data on antimicrobial susceptibility of the helcococci.

THE *ALLOIOCOCCUS* GENUS

Alloiococcus (*alloios*, different) spp. are aerobic, gram-positive cocci arranged in pairs, tetrads, and clusters (1). Alloiococci do not grow under anaerobic atmospheres. Cultures usually give a weak catalase reaction in the catalase test, but experiments performed in the CDC *Streptococcus* Laboratory indicate that these bacteria do not contain the cytochrome enzymes.

Identification of Genus and Species

Only one species of *Alloiococcus* is currently known, *Alloiococcus otitidis* (*otitis*, inflammation of the ear). The initial isolations of these bacteria were from the ear fluids of children with otitis (61). The bacterium is difficult to grow, often taking 2 to 3 days for growth to develop on blood agar plates. Most strains grow better on heart infusion rabbit blood agar plates than on Trypticase soy sheep blood agar plates. Most strains do not grow in thioglycolate or Todd-Hewitt broth. Gram stains prepared from agar plates or from motility medium show the typical cellular arrangement. Cultures grow in heart infusion and brain heart infusion broths very slowly, often requiring 48 h of incubation for growth.

Alloiococci are susceptible to vancomycin, do not form gas in MRS broth, and are PYR and LAP positive. Alloiococci grow very slowly in 6.5% NaCl broth; some strains show visible growth in 48 h, while others require 7 days of incubation. These bacteria do not grow at 10 or 45°C, are not motile, and are nonhemolytic after a few days of growth, although cultures become alpha-hemolytic after extended incubation (Table 7).

Since only one species of the genus *Alloiococcus* is currently described, identification of the genus identifies the species. *Alloiococcus* cultures do not form acid in any of the carbohydrate broths that we tested. Variable reactions were observed with hydrolysis of hippurate, with 9 of 23 cultures being positive. Only one strain hydrolyzed esculin. Most of the isolates were capable of growing on bile-esculin medium but did not give positive reactions.

Clinical Significance and the CDC Experience

The first strains of alloiococci were isolated from middle ear fluids of children with otitis media (61). In our collection of alloiococci, 18 of the 23 strains were isolated from middle ear fluid cultures, 4 were from blood cultures, and 1 was from sputum. Only the middle ear fluid cultures have been confirmed as *A. otitidis*. The remaining blood and sputum isolates have phenotypic characteristics of the alloiococci, but DNA-DNA relatedness studies have not been performed. The pa-

tient with the sputum isolate was diagnosed with pneumonia, and the clinical diagnosis for two of the four blood culture isolates was sepsis. No diagnosis was provided with the remaining two blood culture isolates. There are no published data on the antimicrobial susceptibility of *Alloiococcus* sp.

THE *GEMELLA* GENUS

Gemella (*gemellus*, twin) spp. are facultatively anaerobic, gram-variable cocci, arranged in pairs, tetrads, clusters, and sometimes short chains. Some strains decolorize quite readily in the Gram stain and may appear gram negative (128). In addition, some strains may require strict anaerobic conditions for primary isolation and become aerotolerant after transfer to laboratory media.

Identification of Genus and Species

The origin and history of the members of this genus are important for understanding the varied nature of the Gram stain and cellular arrangement characteristics of the species included in the *Gemella* genus. The *Gemella* genus was officially proposed by Berger in 1961 (12). At that time, the new genus included only one species, *Gemella haemolysans*, which was previously known as *Neissera haemolysans*. The establishment of the genus was made because this species was quite different from the other members of the genus *Neisseria*. *G. haemolysans* is easily decolorized in the Gram stain and appears gram negative, although some cells do not decolorize and appear gram positive. Thus, it is not difficult to understand why the *G. haemolysans* strains appearing as gram-negative diplococci were originally thought to be *Neisseria* spp.

A second member of the *Gemella* genus was added in 1988 (95). This species, *G. morbillorum*, was transferred from the *Streptococcus* genus. It had originally been identified as an anaerobic *Peptostreptococcus* sp. (85). As an anaerobe, *Peptostreptococcus morbillorum* had a preference for anaerobic growth conditions and had a tendency to form chains. Using 16S rRNA sequencing (100, 154), and DNA hybridization (154), investigators have shown that the two species are distinct from each other and belong to the same genus. The history of the *Gemella* genus was recently outlined by Ruoff (133).

These bacteria grow very poorly on blood agar plates and will often take 48 h to grow. On Trypticase soy sheep blood agar, most strains are either alpha- or nonhemolytic. Wide-zone alpha hemolysis may be observed with some strains, especially those resembling *G. haemolysans*, on blood agar bases containing rabbit or horse blood. The colonial morphology on sheep blood agar plates resembles slowly growing viridans streptococci; colonies are small and grayish to colorless.

Identifying these bacteria is difficult because of their slow growth characteristics. All cultures are susceptible to vancomycin and do not produce gas in MRS broth; most strains are LAP and PYR positive, fail to grow in broth containing 6.5% NaCl, fail to give positive bile-esculin reactions, do not grow at 10 and 45°C, and are nonmotile (Table 7). The positive reactions for LAP and PYR are weak when tested by the disk test, and in some cases, when the PYR disk test was negative, PYR reactions were positive only when the broth PYR test was used or the disk test was repeated with a very heavy inoculum.

The tests listed in Table 8 are those suggested by Berger (12) and Berger and Pervanidis (14) to differentiate between the species. There is controversy over the value of any of these tests in the differentiation of the species. There is poor correlation between the dehydrated substrate (API 20 Strep or API-ZYM) versus conventional tube tests (11, 12). We have

TABLE 8. Identification of the *Gemella* species

Species	Test result[a]				Cellular arrangement
	MAN	SOR	ALK	NO_2 reduction	
G. haemolysans	−	−	+	+	Pairs, tetrads, clusters
G. morbillorum	(+)	(+)	(−)	−	Pairs, short chains

[a] Acid from: MAN, mannitol; SOR, sorbitol; ALK, alkaline phosphatase. NO_2, nitritic oxide reduction. −, negative; +, positive; (+), weakly positive.

encountered difficulty in several aspects of both the genus and species identification. First, there appears to be an overlap in the physiologic characteristics that describe nutritionally variant streptococci (NVS) and *Gemella* species. Both the NVS and *Gemella* species are positive only in the LAP and PYR tests listed for genus identification (Tables 2 and 7, respectively). The only difference between the NVS and *Gemella* spp. is the cellular arrangement and the requirement by the NVS for pyridoxal for growth. The requirement for pyridoxal or satellitism is medium dependent. Some strains of NVS will grow on fresh medium but not on medium that is older than 10 to 20 days. Eleven of 45 strains we identified as *Gemella* were arranged in pairs and short chains (streptococcuslike); 3 of the 11 strains required pyridoxal for growth after retrieval from storage (NVS-like). If strains that are arranged in chains are placed in the *Gemella* genus, then considerable confusion will result.

With regard to species identification, most strains of NVS and all strains of *Gemella* spp. do not hydrolyze arginine, esculin, hippurate, starch, or urea. Acid formation in carbohydrate broths, using conventional tests, is somewhat varied, but the NVS are more active in inulin, lactose, melibiose, raffinose, and trehalose broths than are the *Gemella* spp., which rarely form acid in any of these broths. Most strains of NVS and *Gemella* spp. form acid in maltose and sucrose broths. However, we have identified strains of both groups that fail to form acid in these broths or in any of the 12 other carbohydrate broths we test. Among the 34 strains that were arranged in pairs, tetrads, and clusters, 27 strains did not form acid in either mannitol or sorbitol broth, indicating that these strains were *G. haemolysans*; 5 strains formed acid in both mannitol and sorbitol broth, indicating that these strains were *G. morbillorum*; and 1 strain formed acid in mannitol but not sorbitol and one strain formed acid in sorbitol but not mannitol broth. The identities of these two strains are unknown. Among the 11 strains of *Gemella*-like streptococci (Table 2), 8 strains did not form acid in either mannitol or sorbitol broth (*G. haemolysans*-like) and 3 strains formed acid in mannitol only (unidentified). From these results, it can be summarized that neither the identification of the species nor the identification of the genus is well defined.

Clinical Significance and the CDC Experience

The *Gemella* species are part of the normal flora of humans. *Gemella haemolysans* bacteria have been isolated from nearly 30% of pharyngeal swabs (13). *G. morbillorum* can be found in the gastrointestinal tract of humans (85). *G. haemolysans* has been isolated from blood cultures of patients with endocarditis (9, 18, 65, 91, 113). It has also been isolated from CSF cultures of patients with meningitis (5, 111, 112). *G. morbillorum* has been isolated from blood cultures of patients with endocarditis (110, 119) as well as from cultures of synovial fluid from a patient with septic arthritis (151) and cultures of the CSF of a

patient with meningitis (42). It is likely that the same type of infections caused by the viridans streptococci are also caused by the *Gemella* species (151).

Information regarding the antimicrobial susceptibility of these bacteria is limited. Buu-Hoi and coworkers (21) reported that all strains of *G. haemolysans* were susceptible to penicillin, ampicillin, vancomycin, rifampin, and chloramphenicol. Low-level resistance to aminoglycosides was found, but higher levels of resistance to trimethoprim were detected. Some strains were resistant to erythromycin and tetracycline, whereas others were susceptible to these drugs. These results indicate that the antimicrobial susceptibilities resemble those of the viridans streptococci (22).

Among the 34 *Gemella* cultures identified in our laboratory, 26 were blood culture isolates and 1 isolate each came from the following sources: synovial fluid, peritoneal fluid, lung, wound, abscess, and ear. No source was provided with submissions of two cultures. Eight of the 26 patients with blood cultures had subacute bacterial endocarditis: six patients infected with *G. haemolysans* and two patients infected with *G. morbillorum* strains. Four patients had bacterial sepsis, *G. morbillorum* in two cases, *G. haemolysans* in one case, and an unidentified *Gemella* strain (mannitol positive only) in one case. Two patients with positive blood cultures had pneumonia, *G. haemolysans* being the organism in both cases. The remaining 12 patients with positive blood cultures had no diagnosis submitted with their culture.

THE *PEDIOCOCCUS* GENUS

Pediococcus (*pedium*, plane surface cocci) spp. are facultatively anaerobic, catalase-negative, gram-positive cocci arranged in pairs, tetrads, and clusters.

Taxonomy and Identification of Genus

The taxonomy of this genus and its members has had a varied and confusing past. A review of the *Pediococcus* genus in 1949 noted that bacteria resembling pediococci had been included in the *Sarcina*, *Micrococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus* genera (123). Whittenbury was the first to report that at least one of the species included in the *Pediococcus* genus was more closely related to the *Aerococcus* genus (155). In addition, it was also noted that the initial name of the species (*Pediococcus cerevisiae*, isolated from beer), suggested to represent the type strain for the genus, had not been validly published and the same name was used to describe two different species (68). Even as late as 1985, *Bergey's Manual* (71) listed eight species of *Pediococcus*. DNA-DNA relatedness (44), fatty acid composition (11), and 16S rRNA analysis (35) have been used to more precisely define the genus and species of pediococci. The initial species name, *P. cerevisiae*, was omitted from the genus, and strains phenotypically and genetically resembling this species were combined with the species *P. damnosus*. The species *P. urinae-equi* was shown to be related to *A. viridans* (35, 44). The species *P. halophilus* was shown to be sufficiently unrelated to the remaining members of the genus to merit establishment of a new genus for that species, *Tetragenococcus halophilus* (35). Currently, there are five species included in the *Pediococcus* genus: *P. acidilactici*, *P. damnosus*, *P. dextrinicus*, *P. parvulus*, and *P. pentosaceus.*

Like the leuconostocs, all *Pediococcus* species are intrinsically resistant to vancomycin. Pediococci do not form gas in MRS broth, they are PYR negative but LAP positive, and some strains grow in 6.5% NaCl broth while others do not. Most strains grow at 45°C but not at 10°C, and most clinical cultures give positive bile-esculin reactions. All strains are nonmotile and appear alpha- or nonhemolytic on blood agar plates (Table 7) (58).

TABLE 9. Identification of the *Pediococcus* species

Species	Test result[a]						
	ARA	MAL	SUC	TRE	ST	ARG	NaCl
P. acidilactici	+	−	−	v	−	+	v
P. damnosus	−	+	+	+	−	−	−
P. dextrinicus	−	+	+	−	+	−	−
P. parvulus	−	−	−	v	−	−	+
P. pentosaceus	v	+	−	+	−	+	+

[a] Acid formation in: ARA, arabinose; MAL, maltose; SUC, sucrose; TRE, trehalose; ARG, arginine. ST, hydrolysis of starch; NaCl, growth in broth containing 6.5% NaCl. +, positive; −, negative; v, variable.

Identification of Species

Table 9 lists the species of pediococci and some selected phenotypic characteristics that can be used to identify them. Of the 47 strains of pediococci identified at the CDC (Table 7), 25 had characteristics of *P. acidilactici*, 7 had characteristics of *P. pentosaceus*, and 15 could not be placed in a species according to the phenotypic characteristics listed in Table 9. Of the 15 unidentified species, 3 were arginine negative, indicating that they were not *P. acidilactici* or *P. pentosaceus*, but they did not have phenotypic characteristics of the remaining *Pediococcus* species. The remaining 12 clinical cultures resembled *P. acidilactici* and *P. pentosaceus*; however, neither of these species is supposed to form acid in sucrose broth. Except for the formation of acid in sucrose broth, four strains resembled *P. acidilactici* and eight strains resembled *P. pentosaceus*. If acid in sucrose broth is accepted as a variable characteristic of these two species, then only 3 of the 47 strains of pediococci that we have received could not be identified as either *P. acidilactici* or *P. pentosaceus.*

Clinical Significance and the CDC Experience

The pediococci were first discovered in food products such as beer, cheese, meats, and plants (114, 123, 126). Colman and Efstratiou in all likelihood were the first to note that pediococci were isolated from human infections (38). This was followed by several reports indicating that pediococci could be found in stools of normal humans (132, 134), in stools of neutropenic patients undergoing antimicrobial digestive tract decontamination (109), and in blood cultures of patients with bacteremia (107), septicemia (78), abdominal abscesses (129), and a hepatic abscess (142). Most of the reports identified only *P. acidilactici*, but at least one report included *P. pentosaceus* as the infecting agent (41). In our previous study, *P. acidilactici* was isolated five times more frequently than *P. pentosaceus* (58). Documenting the role of the pediococci in human infection is problematic (107, 129) and merits further investigation.

P. acidilactici and *P. pentosaceus* have similar antimicrobial susceptibilities. Most strains are moderately susceptible to the beta-lactam antimicrobial agents and susceptible to clindamycin, erythromycin, chloramphenicol, rifampin, imipenem, gentamicin, daptomycin, and ramoplanin but resistant to the quinolones, vancomycin, and teicoplanin (29, 109, 144, 146, 160).

Clinical sources and diagnosis were provided with 31 of the 47 *Pediococcus* strains submitted to the CDC laboratory. Seventeen strains were blood culture isolates: two were from re-

ported cases of endocarditis, four patients had sepsis, two patients were reported with peritonitis, and the remaining nine blood cultures were from bacteremic patients. The two endocarditis cases were associated with *P. acidilactici* and a sucrose-positive *P. pentosaceus* strain. Four strains were isolated from the urine of patients with urinary tract infections, and two strains each were isolated from catheter tips, wounds, and peritoneal fluids. One strain each was isolated from CSF, cyst, lung, and bone. Although this group of strains is not the same as that in our earlier report (58), nearly the same species distribution was noted; 25 strains of *P. acidilactici* and 7 strains of *P. pentosaceus* were identified among the human strains. If the sucrose-positive variants are included in each species, the ratio is 2:1 *P. acidilactici* to *P. pentosaceus*.

THE *TETRAGENOCOCCUS* GENUS

Tetragenococcus (four producing) spp. are facultatively anaerobic, catalase-negative, gram-positive cocci arranged in pairs, tetrads, and clumps.

Taxonomy and Identification of Genus and Species

The genus *Tetragenococcus* contains only one species, *Tetragenococcus halophilus*, previously identified as *Pediococcus halophilus* (35). It has been proposed that one of the *Enterococcus* species, *E. solitarius*, be transferred to this genus, but the confirmation is not available at this time. *E. solitarius* is a single isolate, and there are no confirmed strains of *E. solitarius* other than the type strain. It is not known to which, if any, of the established genera this bacterium belongs. The tetragenococci differ from the pediococci by vancomycin resistance. The pediococci are vancomycin resistant and the tetragenococci are vancomycin susceptible. Other characteristics are similar (Table 7).

As far as is known at this time, no strains of tetragenococci have been isolated from humans. The data included in Table 7 for the tetragenococci were generated from the type strain only.

SUGGESTED LABORATORY PROCEDURES FOR DIFFERENTIATION

Acid Formation in Carbohydrate Broths

The carbohydrates that may be tested are arabinose, lactose, maltose, mannitol, melibiose, raffinose, ribose, sorbitol, sorbose, sucrose, and trehalose (60).

Acid from carbohydrates is determined in heart infusion broth containing the specific carbohydrate (1%) and bromcresol purple indicator. The carbohydrate broths can be inoculated with a drop or a loopful of an overnight broth culture or with several colonies taken from a blood agar plate. The inoculum should be from a fresh culture.

The carbohydrate broth is then incubated at 35°C for up to 14 days. A positive reaction is recorded when the broth turns yellow (if bromcresol purple is the indicator).

Deamination of Arginine

The deamination of arginine is determined in Moeller's decarboxylase medium. The medium is commercially available (Carr-Scarborough, Stone Mountain, Ga.). The medium is inoculated with a fresh culture and immediately overlaid with sterile mineral oil (about 1 to 2 ml/5 ml of arginine medium). The medium is then incubated at 35°C for up to 7 days. A positive reaction is recorded when the broth turns deep purple, indicating an alkaline reaction; NH_3 is released. The development of a yellow color or no change in color of the broth indicates a negative reaction.

Esculin Hydrolysis Test

Hydrolysis of esculin is determined on nutrient agar slants containing 1% esculin with ferric citrate (60). The medium is inoculated with a fresh culture and incubated at 35°C for up to 7 days. A positive reaction is recorded when the medium turns black. No change in the color of the agar indicates a negative reaction.

Gas from MRS Broth

The production of gas from glucose is determined in *Lactobacillus* MRS broth (Difco Laboratories, Detroit, Mich.) (45, 60). The broth is inoculated with two or more colonies from a plate or with a drop of broth culture. The broth is then sealed with melted petroleum jelly, and the tube is incubated at 35°C for up to 7 days. Gas production is indicated when the wax plug is pushed upward. Small bubbles that may accumulate over the incubation period are not read as positive. Most *Leuconostoc* strains are positive at 24 h but some strains require longer incubation periods.

Test for Growth at 10 and 45°C

Growth at 10 and 45°C is determined in heart infusion broth base medium with 0.1% dextrose and bromcresol purple indicator (60). As in the NaCl tolerance test, dextrose and indicator are added to aid in the visual interpretation of the test. Color changes are not required for a positive test. An increase in turbidity indicates growth and a positive test. The broths are inoculated with one or two colonies or a drop of an overnight broth culture and incubated at the respective temperatures. For the 10°C incubator, a refrigerator is adjusted to hold a temperature of 10°C. For the 45°C incubator, a hot water bath is adjusted to hold a temperature of 45°C. The caps of the tubes are carefully tightened and sealed with Parafilm to prevent moisture and contaminants from penetrating the medium tube. The tests are held for a minimum of 7 days and for up to 14 days in the case of slowly growing strains.

Hemolysis

The hemolytic reaction is determined on agar media containing 5% animal blood. The most commonly used base medium is Trypticase soy agar containing 5% sheep blood (BBL). Trypticase soy base is used because it supports the growth of all bacteria discussed in this review, although the *Alloiococcus* strains grew better on heart infusion agar plates containing rabbit blood. Other base media may be substituted if control strains of all genera are tested for growth. The agar plates are inoculated with the cultures of unknown bacteria as they are received in the laboratory. The plates are streaked in a conventional manner to test for purity, and the agar is stabbed with the inoculating loop to test for hemolysis. The plates are placed in a carbon dioxide (5%) incubator for 24 h and examined for purity and hemolysis when sufficient growth is apparent. If growth is scant or not visible, the plate should be returned to the CO_2 incubator for another 24 h. Plates with sufficient growth are examined for destruction of erythrocytes (alpha hemolysis) around the stab in the agar. Experienced microbiologists can examine the plates macroscopically to determine the hemolytic reaction; however, if there is any question about the hemolytic reaction, a broad-field microscope is

used to examine the stabbed area of growth. If destruction of some but not all of the erythrocytes is apparent, the hemolytic reaction is recorded as alpha. If no destruction of cells is apparent, the hemolytic reaction is interpreted as nonhemolytic. For a complete description of the hemolytic reactions, see reference 60.

Hippurate Hydrolysis Test

Hydrolysis of hippurate is tested in broth containing 1% sodium hippurate (see reference 60, p. 1275).

The broth is inoculated with a drop of fresh broth culture or one to two colonies from an agar plate. The broth is incubated at 35°C until the end of the incubation period (7 days for regular cultures and up to 10 to 14 days for slowly growing cultures). The broth is centrifuged to pellet the bacteria, 0.8 ml of the clear supernatant is transferred by pipette into a tube (13 by 100 mm), and 0.2 ml of ferric chloride reagent is added to the supernatant, with mixing. A heavy precipitate that remains after 10 min indicates a positive test. If the tube clears and remains clear, a negative reaction is recorded. Weak positive reactions are observed with some strains; this is noted when precipitates form and remain but are not heavy.

LAP and PYR Tests

The LAP and PYR disk tests are discussed together because they are performed in the same manner. The disks are commercially available from several commercial suppliers. The PYR and LAP disks we used were obtained from Carr-Scarborough Microbiologicals, Inc., Decatur, Ga. The reactions for LAP and PYR tests for other disks or devices should not differ substantially (79). The strains to be tested are grown on blood agar plates until sufficient growth is obtained to heavily inoculate the disks. For most strains, overnight incubation is sufficient, but for the gemellae, alloiococci, and helcococci, the inoculum plates are incubated for 2 or sometimes 3 days. The disks are placed on the blood agar plate in an area of little or no growth. The disks are inoculated heavily; two or more loopfuls of culture were used for strains that grew satisfactorily after 24 h, but several loopfuls of inoculum are necessary for satisfactory results when the cultures require 2 or 3 days of incubation. The plates with the disks are incubated on the bench at room temperature for 10 min, the detection reagent is added, and the reactions are read after 3 min. The development of a red color is positive, no change in color or a yellow color is negative, and the development of a pink color indicates a weak positive reaction. The color usually develops immediately; the test should be discarded after 10 min if still negative (60).

Litmus Milk Reaction

The litmus milk reaction is tested in 5 ml of broth-type medium. We obtain litmus milk broth from Carr-Scarborough.

Tubes containing the litmus milk are inoculated with a fresh culture of the unknown strain. The inoculum is either 1 drop of a 24-h broth culture or one or two colonies taken from a blood agar plate. The tubes are incubated at 35°C for up to 7 days. Acid formation is read as positive when a red or white color appears. A negative litmus milk reaction is recorded when the tube contents remain a blue color.

Motility Test

Motility is determined in modified Difco motility medium (27). The medium is prepared by adding 16 g of motility test medium (Difco), 4 g of nutrient broth powder (Difco), and 1 g of NaCl to 1 liter of distilled water. The medium is inoculated with an inoculating needle, not a loop. The needle is inserted into the center of the medium in the tube for about 1 in. (2.54 cm). The inoculated tube is placed in a 30°C incubator. Do not incubate the motility test at 37°C. Some strains become nonmotile at 37°C but are motile at temperatures from 25 to 30°C. Strains that are motile grow outward to the edge of the tube and downward toward the bottom of the tube. The motility medium should be incubated until good growth is observed; in most cases, 24 to 48 h is sufficient.

NaCl Tolerance Test

Growth in broth containing 6.5% NaCl is determined in heart infusion broth base with an addition of 6% NaCl (56, 60). Heart infusion base contains 0.5% NaCl. To make the test easier to read, 0.5% glucose and bromcresol purple indicator are added (56, 60). When the growth is luxuriant, the glucose is fermented and the broth changes from purple to yellow in color. However, an obvious increase in turbidity without a change in color is also interpreted as a positive test. One or two colonies or a drop of an overnight broth culture is inoculated into the broth containing 6.5% NaCl. The inoculated broth is incubated at 35°C for up to 14 days depending on the growth characteristics of the strain being tested. Other base media (brain heart infusion and Trypticase soy) have been used for determining NaCl tolerance of the enterococci and viridans streptococci, but these bases have not been tested with the other genera discussed in this review and should not be used without testing reference strains to determine specificity and sensitivity.

Pigmentation Test

Cultures of the unknown strains are grown on Trypticase soy–5% sheep blood agar plates for 24 h in a CO_2 atmosphere at 35°C. A cotton swab is used to pick up about a 50-mm smear of bacteria. The swab and smear are examined for a yellow color (positive pigmentation). A cream, white, or gray color is a negative test. The test should be repeated after 48 h of incubation if there is any question about the results at 24 h.

Pyruvate Utilization Test

The formula for the pyruvate utilization test is given in reference 60, p. 1270.

The pyruvate broth is inoculated with 1 drop of an overnight broth culture of the unknown organism. The broth is incubated at 35°C for 7 days. A positive reaction is indicated by the development of a yellow color. If the broth remains green or greenish yellow, the test is interpreted as negative. A yellow color with only a hint of green is interpreted as positive.

Slime Formation on 5% Sucrose Agar

Production of slime from 5% sucrose is tested on heart infusion agar containing 5% sucrose (see reference 60, p. 1276). The agar plates are inoculated with a drop of fresh broth culture or one or two colonies taken from an agar plate. The agar plate is incubated for 48 h in a CO_2 incubator. A positive reaction is indicated by the formation of large mucoid runny material external to the colonies. In some cases large opaque gumdrop-like colonies are indicative of slime production. A negative reaction on 5% sucrose agar is recorded when no slime or gumdrop-like colonies are apparent by visual inspection.

TABLE 10. Phenotypic characterization of facultatively anaerobic, catalase-negative, gram-positive cocci

Genus	Phenotypic characteristic[a]									
	Vancomycin susceptibility	Gas[b]	PYR	LAP	NaCl[c]	Bile-esculin reaction	Growth at:		Motility	Hemolysis[d]
							10°C	45°C		
Chains										
Streptococcus	S	−	−[e]	+*	v	v	−	v	−	α, β, n
Enterococcus	S*	−	+	+*	+	+	+*	+	v	α, β, n
Lactococcus	S	−	+*	+*	v	+*	+*	v	−	α, n
Vagococcus	S	−	+	+	+	+	+	−	+	α, n
Leuconostoc	R	+	−	−	+	v	v	v	−	α, n
Globicatella	S	−	+	−	+*	v	−	v	−	α
Pairs and tetrads										
Aerococcus	S	−	+	−	+	v	−	−*	−	α
Helcococcus	S	−	+	−	+	−	−	−	−	n
Alloiococcus	S	−	+	+	+	−	−	−	−	n
Gemella	S	−	+	+*	−	−	−	−*	−	α, n
Pediococcus	R	−	−	+	v	+	−*	v	−	α
Tetragenococcus	S	−	−	+	+	+	−	+	−	α

[a] S, susceptible; R, resistant. +, positive reaction or growth; −, negative reaction or no growth; v, variable reactions (11 to 89% positive); *, occasional exceptions occur (≤10% of observations); α, alpha hemolysis; β, beta hemolysis; n, nonhemolytic.
[b] Gas, gas from glucose in MRS broth.
[c] NaCl, growth in broth containing 6.5% NaCl.
[d] Hemolysis on Trypticase soy agar containing 5% sheep blood.
[e] Group A streptococci and nutritionally variant streptococci are PYR positive; all other streptococci are negative.

Starch Hydrolysis Test

Hydrolysis of starch is determined by inoculating a heart infusion agar plate containing 2% soluble starch (see reference 60, p. 1276).

The starch agar plate is inoculated with a loopful of broth, or with one or two colonies picked up with a loop, by making a single streak across the center of the plate. The plate is incubated in a CO_2 incubator for 48 h and then flooded with Gram's iodine. A completely clear area surrounding the organism growth indicates hydrolysis of starch. A partially cleared area surrounding the growth indicates partial hydrolysis and is interpreted as a weak positive reaction. If the plate stains uniformly dark purple because of the reaction of the iodine and starch, a negative reaction is indicated.

Tellurite Tolerance Test

The formula for the tellurite tolerance test is described in reference 57.

Tolerance to tellurite is determined on agar medium containing 0.04% potassium tellurite. The agar may be contained in a tube or plate. The medium is inoculated with 1 drop of a fresh broth culture or one or two colonies of the unknown culture. The inoculated medium is incubated at 35°C for 7 days. Tolerance (a positive result) is indicated whenever black colonies form on the surface. Typical and variant strains of *E. faecalis* usually form black colonies (positive tolerance) after 48 h of incubation. Some strains of *E. faecium* may form gray colonies (a negative reaction), but most strains fail to grow on tellurite medium.

Vancomycin Susceptibility Identification Test

The vancomycin test is performed in a manner similar to the bacitracin susceptibility test (54, 58). Several colonies of the strain are transferred to one-half of a Trypticase soy agar plate containing 5% sheep blood (BBL). The vancomycin susceptibility testing disk (30 μg; BBL) is placed in the heavy part of the streak. The inoculated agar plate is incubated in a carbon dioxide-enhanced atmosphere at 35°C for 18 h. Some strains (alloiococci, gemellae, and helcococci) require 48 or 72 h to show sufficient growth to interpret the test. Any zone of growth inhibition is considered susceptible. This is not a susceptibility test; it is a test for identification. The test is interpreted as resistant only if there is growth right up to the edge of the disk.

Voges-Proskauer Test

The Colbentz modification of the Voges-Proskauer test is used to determine the production of acetylmethyl carbinol (63). The unknown strains are grown on blood agar plates overnight at 35°C. Some strains of *Alloiococcus*, *Gemella*, and *Helcococcus* spp. may need to be incubated longer for sufficient growth. With a loop, all growth on the plate is transferred to 2 ml of Voges-Proskauer broth. The broth is incubated at 35°C for 6 h and then tested. No harm to the test will result if the broth is allowed to incubate overnight. Ten drops of solution A (α-naphthol) and 10 drops of solution B (sodium hydroxide and creatine) are added to the tube. The tube is shaken vigorously, and a positive reaction is indicated when a red color develops within 30 min. The tube must be vigorously shaken several times during the 30-min period. A pink or rust color is interpreted as a weak positive reaction.

SUMMARY AND CONCLUSIONS

Several reasons account for the expansion in the number of genera of facultatively anaerobic, catalase-negative, gram-positive cocci that are now being identified from human clinical sources. Recent advances in taxonomy through the use of DNA-DNA, RNA-DNA hybridization, 16S rRNA gene sequence comparisons, and whole-cell protein analysis have led to the recognition that several genera of bacteria originally considered nonpathogenic for humans are responsible for infections in debilitated or immunocompromised persons. Previously, these bacteria had been considered variants of identified human pathogens, and only through the use of the molecular techniques have they been recognized as new hu-

man pathogens. As examples, the lactococci and vagococci were considered variants of *Enterococcus*, a genus known to cause human disease.

Another reason that several new genera have been added to the list of human pathogens is that some of these bacteria are difficult to grow in the laboratory because they either are slow growers or grow best on media not commonly used in the laboratory. Because of these characteristics, bacteria such as the alloicocci have escaped detection, even though they have probably always been associated with ear infections.

A third reason for the increase in the number of recognized human pathogenic genera has been the selective pressure caused by the treatment of patients with antimicrobial agents. The intrinsically vancomycin-resistant *Leuconostoc* spp. and *Pediococcus* spp. were not recognized as pathogens until there was an increase in the use of vancomycin to treat infections caused by penicillin-resistant bacteria. These ubiquitous bacteria found an ecological niche that was devoid of bacteria that would have normally inhibited their growth. This, and the absence of penicillin-based antimicrobial agents, allowed them to multiply and cause disease.

Taxonomy is dynamic, and additional changes are sure to follow. Other genera of bacteria previously considered to be nonpathogenic for humans will undoubtably be added as isolation and classification techniques improve. It can be deduced from some of the information provided here that there are areas of bacterial classification that will need to be clarified by application of genetic techniques. Although several new genera have been added to the list of human pathogens that now must be identified by the clinical microbiologist, most of these changes have helped in the identification of the most difficult to identify strains of gram-positive cocci.

The clinical microbiologist should have little difficulty in adopting the additional tests used to differentiate the facultatively anaerobic, catalase-negative, gram-positive cocci. The tests listed in Table 10, performed as described in the section Suggested Laboratory Procedures for Differentiation, will differentiate the most commonly encountered gram-positive cocci. Since most clinical laboratories are already performing the tests necessary to determine catalase production, Gram stain reaction and cellular arrangement, and hemolysis on blood agar medium, as well as bile-esculin and 6.5% NaCl tolerance tests, only the three disk tests (vancomycin, PYR, and LAP), the two temperature growth tests (growth at 10 and 45°C), and the motility tests are needed to complete the identification of the genera listed in Table 10.

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