Phylogenetic Analysis of Some *Aerococcus*-Like Organisms from Clinical Sources: Description of *Helcococcus kunzii* gen. nov., sp. nov.

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16S rRNA gene sequencing studies were performed on some unusual gram-positive catalase-negative cocci of unknown taxonomic position isolated from human clinical sources. Comparative analysis of the sequence data demonstrated that the clinical isolates represent a hitherto-unknown line of descent within the low-G+C-content gram-positive bacteria. On the basis of the phylogenetic findings and the phenotypic distinctiveness of the organisms, it is proposed that they be classified in a new genus, *Helcococcus*, as *Helcococcus kunzii* sp. nov. The type strain of *H. kunzii* is NCFB 2900.

A collection of nine strains of a gram-positive coccoid organism isolated from human sources appeared to represent a previously undescribed bacterial group. The strains, catalase-negative facultative anaerobes showing some phenotypic resemblance to *Aerococcus viridans*, were all isolated from wound cultures. Seven of the strains were recovered from lower-extremity wounds (six from feet and one from a lower-leg ulcer); the remaining isolates were found in cultures of breast masses. Most cultures were mixed, containing organisms such as staphylococci in addition to the aerococcus-like strains. However, these isolates differed from typical aerococci by virtue of their variable growth in 6.5% NaCl and their lipophilic nature, as evidenced by the stimulation of their growth by horse serum or Tween 80. Their slow growth and small colony size may have precluded their recognition in mixed cultures containing other more vigorously growing bacterial strains.

16S rRNA is universally recognized as a powerful molecular chronometer. Different degrees of sequence conservation (ranging from very conserved to highly variable) enable the molecule to measure small genealogical distances as well as span great ones (25). Over the past few years, 16S rRNA sequencing studies have greatly improved the taxonomy of the genera *Aerococcus*, *Enterococcus*, *Lactococcus*, and *Streptococcus* and other catalase-negative lactic acid bacteria (2–4, 6, 21, 24). 16S rRNA sequence comparisons have not only proved invaluable in clarifying the inter- and intrgeneric relationships among these taxa, but because of the cumulative nature of the sequence data, they have also provided a firm basis for the recognition of several new species and genera (1, 3, 5, 6, 21–23). Therefore, in the present study we have further characterized these unusual cocci from human clinical sources, using phenotypic tests and partial 16S rRNA sequencing. On the basis of phylogenetic analysis and the phenotypic distinctiveness of these organisms, we propose that they be classified in a new genus, *Helcococcus*.

MATERIALS AND METHODS

DNA studies. Chromosomal DNA was isolated as described by Pitcher et al. (18). DNA base composition was estimated by thermal denaturation in standard saline citrate as described by Garvie (13), with DNA from *Escherichia coli* NCDO 1984 (K-12) (51.5 mol% G+C) as the standard. Chromosomal DNA hybridization studies were performed as described by Farrow et al. (10).

16S rRNA gene sequence determination and analysis. Approximately 2.5 µg of DNA was subjected to polymerase chain reaction amplification in a total volume of 100 µl containing 2 U of *Taq* polymerase (Amersham International, Aylesbury, United Kingdom). The reaction involved 36 cycles of denaturation at 92°C for 2 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min. DNA was extracted with chloroform and purified with a Geneclean II kit (Bio 101, Inc.) according to the manufacturer's instructions. Qualitative analysis of the DNA fragments was performed by agarose gel electrophoresis. The amplified product was sequenced by using α-³⁵S-dATP and a Sequenase version 2.0 sequencing kit (U.S. Biochemicals) as described previously (15). Reaction products were separated on 55-cm-long wedge-shaped (0.2 to 0.6-mm-thick) 6% acrylamide–7 M urea gels at 55°C using an LKB MacroPhor 2010 sequencing unit operated at 50 W per gel.

Sequences were aligned and similarity values were determined by using the Wisconsin Molecular Biology package (7). Nucleotide substitution rates (K_{nuc} values) were calculated, and an unrooted phylogenetic tree was produced by using the algorithm of Fitch and Margoliash (12) contained in a program written by Felsenstein (11).

Biochemical tests. Isolates were routinely grown on brucella agar containing horse blood (Becton Dickinson Microbiology Systems, Cockeysville, Md.), which yielded inocula for biochemical tests. The strains were examined with the API 20 Strep system (Analytab Products, Plainview, N.Y.) and by conventional methods. The following media, obtained from BBL, Cockeysville, Md., were supplemented with horse serum (Bio-Whittaker, Walkersville, Md.) and used to examine the isolates: Andrade's broths containing glucose, maltose, sucrose, mannitol, and xylose; nitrate broth; urea agar; nutrient gelatin; motility test medium; and

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GAGAGUUUGAUCCUGGCUCAGGACGAACGCGUGCGCGGUGCUUAAACACAUGCAAGUUGAACGAGAAUUUUUUCAGUUGAUUUUCUGGAAUGAAACCGAAA
 ANGGAAGUAGCGAAGCGGUGAGUAAACACGUGAGAAACCGCCUUUCACAAAGGAUAGCCUCGGGAAACCGGGAUUAUACCUUAUGAUACAUAUUAU
 CGCAUGAUGUAAUAGUAAUAGUAAUUAAGAAUUUCGGUGAAAGUUGGUCGCGUCUGAUUAGCUAGUUGGUAAGGUACUGGCCUUAACCAAGGCAACGAU
 CAGUAGCCGGAUUGAGAGGUUGAACGGCCACACUGGAACUGAGACACGGUCCAGACUCCUACGGGAGGCAGAGUGGGGAAUUUUGCACAUGGGGGA
 CCCUGAUGCAGCGACGCCGCGUGAACGAUGAAGGUCUUCGGAUUGUAAAGUUCUGUCCUUAAGCGAAGAUAAUGACAGUAGCUAAGAAGCAAGCCCGUGCU
 AAUACGUGCCAGCAGCCGCGGUAUACGUAUGGGGCAAGCGUUGUCCGGAUUAUUGGGCGUAAAGGUANGUAGGCGGUAAUUAAGUCUGAAUUUA
 AGGCGUGGCUCAACACCGGUUCGGUUUAGAAAACUGGUUAACUUGAGUAGUAGAGGGGAAAGUGGAAUUCUAGUGUAGCGGUGAAUUGCGUAGAUUA
 UGGAGGAACACCAGUGGCGAAGGCGACUUUCUAGAUCUAAACUGACGCGUGAGAUACGAAGCGUGGGUAGCAACAGGAUUAAGAUACCCUGGUAGUCCAC
 GCAGUAAACGAUGAGUGCUAGUUGUUGGGAGUCAAAUCUCAGUGACGCGACUAACGCAUUAAGCACUCCGCCUGGGGAGUACGUACGCAAGUAUGAAACU
 CAAAGGAUUGACGGGACCGCACAGCAGCGGAGAUUGGUUUAUUCGAAGCAACGCGAAGAACCUUACCAAGGCUUGAAGGGANAUCUAGAGAUUA
 GGAUAGUCUUUUCGGAGACUUGUAUACAGGUGGUGCAUGGUUGUCGUCAGCUCGUGUCGUGAGAUUGGGUUAAGUCCCGUAACGAGCGCAACCCCUAU
 CUUAGUUUACUAGCGAGUAAAGUCGAGGACUCUAGAGAGACUGCCGGUAGUAAACCGGAGGAAGGUGGGGAGUACGUCAAAUCAUACGCCCUAUUAGUC
 UUGGGCUACACACGUGCUACAAUGGUCUAGCAAAAGCGUAGCUACUCCUGGAGAGCAAGCGAAUCGCAUAAACAGAUUCUAGUUCGGAUUGCAGGCUGC
 AACUCGCCUGCAUGAAGUCGGAGUUGCUAGUAAUCGUGGAUCAGAACGCCAGGAGAAUGCGUUCGCCGGUCUUGUACACACCGCCCGUCACCAUGGG
 AGUUGGCAUACCGGAAGUCGUCGAGCUAACGCUAAGGAGGACGCGCGAAGGUUGGUAGAU

FIG. 1. Nucleotide sequence of derived 16S rRNA of *H. kunzii* (NCFB 2900).

brain heart infusion (BHI) broth containing 6.5% NaCl. The method of Wong (26) was employed to assay the ability of the isolates to synthesize porphyrins. The strains were tested for pyrazinamidase production by using a slight modification of the method of Sulea et al. (20). All isolates were tested for susceptibility to vancomycin by using standard disk diffusion methods and blood-supplemented Mueller-Hinton agar (16). The effects of different atmospheres on growth were examined by streaking 1 drop of a saline suspension of the isolates onto replicate blood agar plates and then incubating them for 48 h in either 5% CO₂, an ambient atmosphere, or an anaerobic chamber. The plates were examined for overall colony growth and size of individual colonies.

Biochemical tests performed on three of the strains at the Centers for Disease Control were carried out by the methods of Facklam and Collins (9). Included were tests for growth in the presence of elevated NaCl concentrations; growth at 10 and 45°C; acid production from trehalose, sorbitol, lactose, mannitol, sucrose, inulin, raffinose, glycerol, arabinose, salicin, melibiose, maltose, and sorbose; hydrolysis of arginine, starch, esculin, urea, and hippurate; motility; production of acetoin; and susceptibility to vancomycin.

Nucleotide sequence accession number. The determined 16S rRNA gene sequence has been deposited in GenBank under accession number X69837.

RESULTS AND DISCUSSION

Cells of the nine clinical isolates were coccoid in shape and formed pairs, short chains, or irregular groups. The strains were gram-positive, catalase-negative, facultative anaerobes that were susceptible to vancomycin and unable to synthesize porphyrin. Upon original examination, the isolates grew poorly (if at all) on BHI agar and broth. All the strains grew well, forming pinpoint colonies after 24 h, on brucella agar supplemented with 5% horse blood. Horse serum (1%) or Tween 80 (0.1%) greatly stimulated their growth on BHI agar or in BHI broth. There was no discernible enhancement of growth in either increased CO₂ or an anaerobic atmosphere; cultures incubated on blood agar for 48 h in different atmospheres showed equally good growth, forming tiny greyish colonies. Only two of the specimens yielding these isolates were cultured both aerobically and anaerobically, with only one specimen yielding the isolate in both incubation atmospheres. The anaerobic culture of the second specimen contained a mixture of anaerobic bacteria that may have overgrown the isolate in question. Seven of the nine strains produced the enzyme pyrazinamidase.

When tested with serum-supplemented Andrade's broths,

the isolates produced acid from glucose and maltose, but they gave negative reactions in all other serum-supplemented media. The three strains examined at the Centers for Disease Control showed negative reactions in all tests performed, including the test for growth at 10 and 45°C, with the exception of the test for hydrolysis of esculin. The strains tested at the Centers for Disease Control grew in media supplemented with 2.0 and 4.0% NaCl, but growth in the presence of 6.5% NaCl was variable. When all strains were tested with serum-supplemented BHI broth containing 6.5% NaCl, two of the nine isolates failed to grow after 7 days of incubation, and the remaining isolates showed a lag period before visible growth was noted.

All the isolates gave an API Rapid Strep profile of 4100413, corresponding to "doubtful" *A. viridans*. This profile reflected positive reactions for hydrolysis of esculin and pyrrolidonyl-2-naphthylamide and acidification of lactose, trehalose, starch, and glycogen. Although the phenotypic traits of the isolates most closely resembled those of aerococci (pyrrolidonyl-2-naphthylamide positive and leucine aminopeptidase negative), their lipophilic nature, variable growth in 6.5% NaCl, and lack of a preference for aerobic incubation atmospheres distinguished them from typical aerococci. These isolates can be differentiated from streptococci and most other nonaerococcal genera of catalase-negative gram-positive cocci by their inability to produce leucine aminopeptidase. Other leucine aminopeptidase-negative organisms include leuconostocs, distinguished by their resistance to vancomycin, and some gemellae, which fail to hydrolyze esculin, in contrast to the aerococcus-like strains described here. Thermal denaturation experiments on chromosomal DNA from two of the isolates (no. 7 and 22) gave a G+C content of 29.5 to 30 mol%. This DNA base composition value is significantly lower than those reported for aerococci (ca. 35 to 40 mol% [19]), gemellae (33 mol% [8]), and most streptococci (ca. 34 to 46 mol% [14]).

The partial 16S rRNA gene sequences of three of the unknown strains were investigated to determine the strains' relationship to other low-G+C-content gram-positive bacteria. The 16S rRNA gene sequence of strain 22, determined by direct polymerase chain reaction DNA sequencing, is shown in Fig. 1. The sequence consisted of a continuous stretch of 1,464 nucleotides, representing approximately 96% of the total 16S rRNA gene. Similarity values were determined for a 1,330-nucleotide region (ranging from positions 107 to 1410 of the *E. coli* numbering system) of this new sequence and homologous sequences of over 40 reference strains from the genera *Aerococcus*, *Gemella*, and *Streptococcus* and other major phylogenetic lines within the

TABLE 1. Sequence similarities for a 1,340-nucleotide region of 16S rRNAs of *H. kunzii* and some other representative low-G+C-content gram-positive organisms

Organism and strain	% Sequence similarity																
	<i>H. kunzii</i>	<i>A. urinae</i>	<i>A. viridans</i>	<i>C. piscicola</i>	<i>E. faecalis</i>	<i>E. rhusiopathiae</i>	<i>G. haemolysans</i>	<i>G. sanguis</i>	<i>L. acidophilus</i>	<i>L. delbrueckii</i>	<i>L. fermentum</i>	<i>L. oris</i>	<i>L. garvieae</i>	<i>L. mesenteroides</i>	<i>L. paramesenteroides</i>	<i>P. damnosus</i>	<i>P. pentosaceus</i>
<i>Helcococcus kunzii</i> NCFB 2900																	
<i>Aerococcus urinae</i> NCFB 2893	79.5																
<i>Aerococcus viridans</i> NCDO 1225	80.8	94.8															
<i>Carnobacterium piscicola</i> NCDO 2762	79.7	91.4	92.0														
<i>Enterococcus faecalis</i> NCDO 581	79.4	90.2	91.6	93.4													
<i>Erysipelothrix rhusiopathiae</i> NCTC 8163	77.3	82.5	83.3	82.3	81.7												
<i>Gemella haemolysans</i> NCTC 10243	79.1	87.3	88.0	87.2	86.2	82.3											
<i>Globicatella sanguis</i> NCFB 2835	80.0	90.9	91.1	91.4	91.0	83.7	87.4										
<i>Lactobacillus acidophilus</i> NCDO 1748	79.4	87.9	87.2	87.8	88.1	81.0	84.6	88.1									
<i>Lactobacillus delbrueckii</i> NCDO 213	79.3	86.7	87.4	87.3	87.5	81.7	84.3	86.9	94.2								
<i>Lactobacillus fermentum</i> NCDO 1750	78.7	90.2	90.2	89.9	88.7	81.5	84.7	88.7	89.8	89.2							
<i>Lactobacillus oris</i> NCDO 2160	78.8	88.4	88.6	89.0	88.6	82.3	84.6	89.1	90.2	89.2	93.8						
<i>Lactococcus garvieae</i> NCDO 2155	79.5	86.1	87.7	87.6	86.8	81.9	85.1	88.4	85.0	85.8	86.6	86.5					
<i>Leuconostoc mesenteroides</i> NCDO 523	78.1	86.7	87.6	85.7	86.4	81.6	83.1	86.3	86.6	86.0	87.0	87.3	84.6				
<i>Leuconostoc paramesenteroides</i> NCDO 803	79.8	88.4	89.5	89.5	88.7	81.9	84.7	89.0	87.6	87.0	89.7	90.5	86.8	91.0			
<i>Pediococcus damnosus</i> NCDO 832	79.7	89.6	90.2	92.0	90.5	82.7	86.3	89.9	89.7	89.3	92.6	92.3	87.8	87.0	89.9		
<i>Pediococcus pentosaceus</i> NCDO 990	79.1	89.6	89.7	91.9	90.0	82.7	86.0	89.9	89.3	88.7	92.4	92.6	88.1	86.7	89.5	96.6	
<i>Streptococcus agalactiae</i> NCDO 1348	79.9	87.7	88.1	89.3	89.4	82.9	85.9	88.3	85.7	85.5	86.1	86.5	90.3	85.5	87.2	88.2	88.8

lactic acid bacteria. Approximately 100 nucleotides proximal to the 5' end of the rRNA gene were omitted from the similarity calculations in order to remove alignment problems arising from considerable variations between species in the length of the V1 variable region (see the work of Neefs et al. [17] for nomenclature). A matrix of representative sequence similarities is shown in Table 1. Isolate 22 exhibited very low levels of sequence similarity with aerococci, gemellae, streptococci, and related species (ca. 77 to 82%). In view of this remote relationship to the lactic acid and related bacteria, the comparative analysis was extended to include other low-G+C-content gram-positive sequences available in the EMBL data base. Isolate 22 displayed only distant relationships (<80% similarity) to all the organisms examined (including mycoplasmas), thereby confirming its genealogical distinctiveness. A phylogenetic tree depicting the position of the unknown bacterium within a radiation of low-G+C-content gram-positive bacteria is shown in Fig. 2. Fragments (ca. positions 7 to 530, which includes the diagnostic variable regions V1 and V2) of the 16S rRNA genes of two further isolates (no. 7 and 14) were also investigated and found to be identical to that of strain 22, consistent with the high degree of biochemical uniformity shown among the clinical isolates. Furthermore, chromosomal DNA-pairing studies revealed 75% homology between isolates 7 and 22, consistent with the isolates' belonging to a single species.

We have phenotypically characterized nine catalase-negative, facultatively anaerobic cocci isolated from human wound infections. On the basis of their overall biochemical profiles, the strains resemble aerococci but can be distinguished from them by their lipophilic nature. Comparative 16S rRNA sequence analyses clearly demonstrate that the clinical isolates from human sources are phylogenetically only remotely related to aerococci, streptococci, and other

catalase-negative, facultatively anaerobic, low-G+C-content gram-positive taxa. In view of the presented phylogenetic evidence and the phenotypic distinctiveness of these unknown clinical isolates, it is proposed that they be classified in a new genus, *Helcococcus*, as *Helcococcus kunzii* sp. nov.

Description of *Helcococcus* gen. nov. *Helcococcus* (Hel.co.coc'cus. Gr. n. *helkos*, wound; Gr. mas. n. *coccus*, berry, sphere; N.L. mas. n. *Helcococcus*, a sphere found in wounds). Cells consist of gram-positive cocci and are non-motile. Catalase negative. Facultatively anaerobic. Acid but not gas is produced from glucose and some other sugars. Lactate and acetate are the major end products of glucose metabolism. Growth may or may not occur in 6.5% NaCl. No growth on bile-esculin agar. Lipophilic (growth greatly stimulated by serum or Tween 80). Pyrrolidonylarylamidase positive. Arginine dehydrolase and leucine arylamidase negative. Susceptible to vancomycin. The G+C content of DNA is 29.5 to 30 mol%. The type species is *Helcococcus kunzii*.

Description of *Helcococcus kunzii* sp. nov. *Helcococcus kunzii* (kunzi.i. M.L. gen. n. *kunzii*, named after Lawrence J. Kunz, an American bacteriologist). Cells are nonpigmented and nonmotile. Nonhemolytic. Growth may or may not occur in 6.5% NaCl. No growth on bile-esculin agar. Lipophilic. Catalase negative. Facultatively anaerobic. Acid is produced from lactose and trehalose; some strains produce acid from glycogen. Acid is not produced from arabinose, inulin, raffinose, ribose, or sorbitol. Esculin is hydrolyzed in the API test system as well as the conventional test system; starch is hydrolyzed in the API test system but not in the conventional Centers for Disease Control test system. Gelatin, hippurate, and urea are not hydrolyzed. Arginine is not deaminated. Pyrrolidonylarylamidase positive. Some strains produce pyrazinamidase. Alkaline phosphatase, α -galactosi-

FIG. 2. Unrooted tree, based on a comparison of ca. 1,330 nucleotides, showing the phylogenetic interrelationships of *H. kunzii* and some other low-G+C-content gram-positive bacteria. Abbreviations: *A.*, *Aerococcus*; *At.*, *Atopobium*; *Cb.*, *Carnobacterium*; *Ec.*, *Enterococcus*; *Ery.*, *Erysipelothrix*; *G.*, *Gemella*; *Gl.*, *Globicatella*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Leu.*, *Leuconostoc*; *M.*, *Mycoplasma*; *P.*, *Pediococcus*; *S.*, *Streptococcus*; *V.*, *Vagococcus*.

dase, β -galactosidase, β -glucuronidase, and leucine arylamidase negative. Voges-Proskauer negative. Nitrate is not reduced. Vancomycin sensitive. The G+C content of DNA of the type strain is 30 mol%, as determined by melting temperature. Isolated from human sources (foot wounds, leg ulcers, purulent breast mass). The type strain is NCFB 2900 (= no. 22).

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