Phylogenetic Analysis of *Alloiococcus otitis* gen. nov., sp. nov., an Organism from Human Middle Ear Fluid

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The partial 16S rRNA sequence of an unknown bacterium that was originally isolated from middle ear fluids of children with persistent otitis media was determined by reverse transcription. A comparison of this sequence with sequences from other gram-positive species having low guanine-plus-cytosine contents revealed that this bacterium represents a new line of descent, for which the name *Alloiococcus otitis* gen. nov., sp. nov., is proposed. The type strain is strain NCFB 2890.

Faden and Dryja (7) recently reported the isolation of an unknown organism from tympanocentesis fluid collected from middle ears of young children who were suffering from chronic otitis media. The cells of this bacterium were large gram-positive cocci (often present as diplococci or tetrads) which phenotypically most closely resembled aerococci and streptococci (7). However, the organism differed from aerococci and streptococci in being catalase positive. Despite possessing catalase, the bacterium could not be identified by using the Staph-Ident system (Analytab Products), and its taxonomic position remained unresolved (7).

16S rRNA sequence analysis has assumed a central role in ascertaining the phylogenetic relationships of bacteria (17).

MATERIALS AND METHODS

Cultures and biochemical tests. Strains 3621, 4491, 7213, and 7760^T (T = type strain) were received from D. H. Batts and R. R. Hinshaw, Upjohn Laboratories, Kalamazoo, Mich. Cultures were grown on blood agar plates and Todd-Hewitt broth (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 5% horse serum at 37°C. Biochemical tests were performed by using the API 20 STREP system (API-Biomerieux, Basingstoke, United Kingdom) according to the instructions of the manufacturer. Test preparations were incubated at 37°C, and tests were read after 4 and 24 h and 7 days.

GAGUUGAUCCUGGUCAGGACGAAACGUGCGGCAUGCCUAAUACAUGCAAGUCGAAACGCCAAAUCUCCUGCUUGCAGGAAGAGCGGAGUGGCGAAC
GGGUGAGUAAACACGUGAGGAACUUGCCCAUAGAGGGGGAUAAACCCCGAAACGGGUGCUAAUACCGCAUAAUUCUCCAGUGCAUGCUGGGAAAAUA
AAAGGUGGCUUACCGCUUUAUGGAUANNUGCGCGCAUUAAGCUAGUUGGUAGGGUANAGGCCUACCAAGGCCAUGAUGCGUAGCCGACUUGAGAGGG
UGAUCGGCCACACUGGAACUGAGACACGGUCCAGACUCCUACGGGAGGCAGCAGUAGGGAAUUCUCCACAAUGGGUGCAAGCCUGAUGGAGCAAUGCCGC
GUGAGUGAAGAAGGACUUCGGUUCGUAAGCUCUGUUGUUGGGGAAGAACACGGGAUAGGAGUCACUGCCUAUCCUUGACGGUACCCAAACAGAAAGUCA
CGGCUAAUCUAGUGCCAGCAGCCGCGGUAUACGUAGGUGACAAGCGUUGUCGGAUUUAUUGGGCGUAAAGCGAGCGCAGGCCGUGCCGUAAGUCUGAU
GUGAAAGCCACCGCUCACCGUGGAACGGCAUUGGAAACUGGCGGACUUGAAUGUAGCAGAGGAAAGUGGAAUUCUAGUGUAGCGGUGGAAUGCGUAG
AUAAUUGGAGGAACACCCAGUGGGGAAAGCGACUUCUGGGCUAUGAUUGACGUGAGGCUCAAAGCGUGGGGAGCGAACAGGAUUAAGAUACCCUGGUAG
UCCACGCCGUAACGAUGAGUGCUAAGUGUUGGAGGGUUCGCGCCUUCAGUGCUGGAGUUAACGCAUUAAGCACUCCGCCUGGGGAUACGACCGCAAG
GUUGAAACUCAAAGAAUUGACGGGGACCGCACAAAGCGGUGGAGCAUGUGGUUUAUUCGAAGCAACCGCAAGAAACCUUACAGCUCUUGACAUUCU
GCUAGCUUCAGAGAUGAAGCGUUCUUCGGGGACAGAAUGACAGGUGGUGCAUGGUUGUCGUCAGCUCGUGUGGAGAUUGGGUUAAGUCCCGUAA
CGAGCGCAACCCUUAUUGUAGUUGCCAGCAUUGAGUUGGGCACUCUAGCAAGACUGCCGGUGAUAAACCGGAGGAAGGCGGGGAUGACGUCAAUUAUC
AUGCCCCUUAUGAGCUGGGCUACACAGUGCUACAAGGAGUGGUACAACGAGUGGCGAGACCGCGAGGUUAGCUAAUCUCCAAAGCCAUCUCAGUUC
GGAUUGCAGGCUGCAACUCGCGUGCAUGAAGUCGGAUUCGUAUUCGCGGAUAGCCGCGGUGAAUUCGUUCCCGGUGCUUGUACACACCGCC
CGUCACACCACGAGAGUCUGAAACACCCGAAGCCGCGGGCAACCAUUGGAGCCAGCCGUCGAA

FIG. 1. Primary structure of 16S rRNA from *Alloiococcus otitis*. The first nucleotide and the last nucleotide in the sequence correspond to positions 9 and 1469, respectively, of the *E. coli* numbering system (2).

In recent years this molecule has done much to clarify the inter- and intrageneric relationships of low-guanine-plus-cytosine (G+C)-content gram-positive cocci (viz., the genera *Aerococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*) (1, 3–5, 10, 12–16). Because of the possible role of the unknown coccus of Faden and Dryja in middle ear infections (7), we determined the partial 16S rRNA sequence of this organism in order to ascertain its precise phylogenetic position.

DNA base composition. DNA was prepared as described by Garvie (9). DNA base composition was determined by subjecting the preparations to thermal denaturation in standard saline citrate as described by Garvie (9), using DNA from *Escherichia coli* NCDO 1984 as the standard.

Analysis of 16S rRNA. Cells were mechanically broken by using a Braun homogenizer, and rRNA was purified by using the procedure of Embley et al. (6). Nucleotide sequences were determined by the Sanger dideoxynucleotide chain termination method directly from rRNA by using avian myeloblastosis virus reverse transcriptase (6). The sequences generated were aligned, and homology values were determined by using the Beckman Microgenie program (11). Nucleotide substitution rates (K_{nuc} values) were calculated, and an unrooted phylogenetic tree was produced by using the algorithm of Fitch and Margoliash (8).

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TABLE 1. Homology values for a 1,340-nucleotide region of 16S rRNAs of *Alloiococcus otitis* and other taxa

rRNA from:	% Homology with rRNA from:											
	<i>Alloiococcus otitis</i> NCFB 2890 ^T	<i>Aerococcus viridans</i> NCDO 1225 ^T	<i>Bacillus subtilis</i> rrNB	<i>Brochothrix</i> <i>thermosphacta</i> NCDO 1676 ^T	<i>Carnobacterium</i> <i>divergens</i> NCDO 2763 ^T	<i>Carnobacterium</i> <i>piscicola</i> NCDO 2762 ^T	<i>Enterococcus</i> <i>cecorum</i> NCDO 2674 ^T	<i>Enterococcus hirae</i> NCDO 1258 ^T	<i>Lactobacillus</i> <i>acidophilus</i> NCDO 1748 ^T	<i>Lactobacillus brevis</i> NCDO 1749 ^T	<i>Lactobacillus casei</i> NCDO 161 ^T	<i>Lactobacillus</i> <i>delbrueckii</i> NCDO 213 ^T
<i>Aerococcus viridans</i> NCDO 1225 ^T	88.7											
<i>Bacillus subtilis</i> rrNB	87.8	87.6										
<i>Brochothrix</i> <i>thermosphacta</i> NCDO 1676 ^T	85.7	89.0	89.4									
<i>Carnobacterium</i> <i>divergens</i> NCDO 2763 ^T	89.9	92.5	90.1	90.2								
<i>Carnobacterium</i> <i>piscicola</i> NCDO 2762 ^T	89.5	91.9	90.5	90.7	97.5							
<i>Enterococcus cecorum</i> NCDO 2674 ^T	89.9	92.3	90.6	91.3	94.6	94.1						
<i>Enterococcus hirae</i> NCDO 1258 ^T	89.1	91.6	90.3	91.7	94.8	94.6	97.0					
<i>Lactobacillus</i> <i>acidophilus</i> NCDO 1748 ^T	87.2	86.6	88.3	86.2	87.8	87.9	89.3	89.1				
<i>Lactobacillus brevis</i> NCDO 1749 ^T	89.0	90.7	88.8	88.1	91.7	92.9	91.6	92.9	89.8			
<i>Lactobacillus casei</i> NCDO 161 ^T	88.9	90.0	88.5	88.5	91.9	92.0	90.7	91.1	89.7	93.5		
<i>Lactobacillus delbrueckii</i> NCDO 213 ^T	86.5	87.7	87.2	85.7	87.2	87.2	88.9	89.3	94.3	90.0	89.7	
<i>Lactobacillus plantarum</i> NCDO 1752 ^T	87.5	90.1	89.1	87.3	91.1	92.1	90.8	91.6	88.9	95.4	93.5	89.0
<i>Lactobacillus reuteri</i> NCDO 2589 ^T	88.6	89.9	87.7	87.3	90.5	90.4	90.6	91.0	91.5	93.1	93.6	91.2
<i>Lactococcus garvieae</i> NCDO 2155 ^T	85.0	88.4	86.1	86.7	87.7	88.1	90.6	89.4	85.1	88.1	87.3	87.1
<i>Lactococcus lactis</i> NCDO 2118 ^T	86.5	88.7	86.9	87.6	88.7	88.8	90.4	90.3	87.1	89.4	88.5	87.9
<i>Leuconostoc</i> <i>mesenteroides</i> NCDO 523 ^T	85.4	87.5	85.3	85.2	86.6	85.7	87.8	87.2	88.1	87.0	86.9	87.2
<i>Leuconostoc paramesen-</i> <i>teroides</i> NCDO 803 ^T	85.9	89.3	86.8	87.9	90.1	89.9	90.7	90.7	87.8	90.4	90.4	87.5
<i>Listeria innocua</i> NCTC 11288 ^T	87.8	90.2	91.8	93.1	92.0	91.9	92.2	92.0	87.0	88.3	88.4	86.9
<i>Pediococcus acidilactici</i> NCFB 2767 ^T	89.9	89.4	88.7	88.8	92.1	92.0	92.1	92.4	89.5	94.6	93.8	86.9
<i>Pediococcus damnosus</i> NCDO 1832 ^T	89.0	89.9	89.6	88.1	91.8	92.0	91.3	91.6	89.9	94.6	94.9	89.5
<i>Staphylococcus xylosus</i> NCTC 11043 ^T	86.7	90.6	91.6	89.8	89.8	89.6	90.2	90.5	85.8	87.2	87.5	86.1
<i>Streptococcus sanguis</i> NCTC 7863 ^T	86.9	89.4	89.7	88.2	89.6	89.9	91.1	91.2	86.5	88.8	88.9	86.7
<i>Streptococcus</i> <i>parauberis</i> NCDO 651	86.7	88.9	87.0	87.8	89.3	90.0	90.6	90.9	86.5	88.6	88.4	86.2
<i>Streptococcus porcinus</i> NCDO 600	86.2	88.6	86.9	87.8	89.0	89.7	90.4	90.4	87.0	87.8	88.3	86.6
<i>Streptococcus pyogenes</i> NCDO 2381 ^T	86.6	88.7	86.6	87.8	89.3	89.6	90.4	90.4	87.1	87.8	88.6	86.5
<i>Tetragenococcus</i> <i>halophilus</i> NCDO 1635 ^T	89.2	90.8	91.2	90.0	92.4	93.1	93.5	93.4	88.4	89.9	90.1	88.4
<i>Vagococcus fluvialis</i> NCDO 2497 ^T	88.9	92.7	89.5	91.2	93.7	93.2	94.3	94.3	88.3	91.3	90.0	88.3
<i>Vagococcus</i> <i>salmoninarum</i> NCFB 2777 ^T	88.5	91.7	90.0	90.1	94.4	93.9	94.1	94.6	87.0	90.2	90.5	86.9

TABLE 1—Continued

% Homology with rRNA from:															
<i>Lactobacillus plantarum</i> NCDO 1752 ^T	<i>Lactobacillus reuteri</i> NCDO 2589 ^T	<i>Lactococcus garvieae</i> NCDO 2155 ^T	<i>Lactococcus lactis</i> NCDO 2118 ^T	<i>Leuconostoc mesenteroides</i> NCDO 523 ^T	<i>Leuconostoc paramesenteroides</i> NCDO 803 ^T	<i>Listeria innocua</i> NCTC 11288 ^T	<i>Pediococcus acidilactici</i> NCFB 2767 ^T	<i>Pediococcus damnosus</i> NCDO 1832 ^T	<i>Staphylococcus xylosus</i> NCTC 11043 ^T	<i>Streptococcus sanguis</i> NCTC 7863 ^T	<i>Streptococcus parauberis</i> NCDO 651	<i>Streptococcus porcinus</i> NCDO 600	<i>Streptococcus pyogenes</i> NCDO 2381 ^T	<i>Tetragenococcus halophilus</i> NCDO 1635 ^T	<i>Vagococcus fluvialis</i> NCDO 2497 ^T
92.7															
87.5	88.0														
88.5	87.9	95.0													
82.2	88.5	85.6	85.7												
90.4	90.8	87.0	88.3	91.0											
88.6	87.9	86.9	87.8	86.1	88.1										
94.3	94.2	88.9	89.0	86.6	90.2	89.7									
94.6	93.7	88.0	89.6	87.1	89.6	89.0	96.6								
87.1	85.9	84.8	85.0	84.9	86.9	90.4	88.3	88.1							
88.4	89.4	90.8	92.0	87.8	89.2	88.4	89.4	89.3	86.9						
87.6	88.2	91.6	92.5	86.8	88.4	87.4	88.0	88.4	85.7	96.7					
87.5	88.0	91.8	92.5	86.4	87.8	87.5	87.5	88.1	85.9	95.5	97.7				
87.8	88.9	91.5	92.7	86.5	87.6	87.9	88.7	88.7	86.0	96.0	97.1	96.6			
89.0	88.5	87.2	88.2	87.6	89.5	90.3	91.0	89.8	89.5	90.5	91.2	90.2	89.0		
91.0	89.1	87.8	89.4	87.0	88.3	91.1	91.3	91.5	89.6	89.9	88.6	88.4	89.0	91.7	
90.6	88.4	88.6	89.3	86.0	88.9	90.8	90.6	91.1	89.9	89.8	89.0	88.4	89.3	91.8	96.3

FIG. 2. Unrooted phylogenetic tree showing the position of *Alloiococcus otitis* within a group of gram-positive bacteria. The tree is based on a comparison of 1,340 bases. *Lb.*, *Lactobacillus*; *P.*, *Pediococcus*; *Leu.*, *Leuconostoc*; *S.*, *Streptococcus*; *A.*, *Aerococcus*; *Br.*, *Brochothrix*; *List.*, *Listeria*; *Staph.*, *Staphylococcus*; *B.*, *Bacillus*; *T.*, *Tetragenococcus*; *C.*, *Carnobacterium*; *E.*, *Enterococcus*; *V.*, *Vagococcus*; *Lc.*, *Lactococcus*.

Nucleotide sequence accession number. The partial 16S rRNA sequence of strain 7760^T has been deposited in the EMBL data base under accession number X59765.

RESULTS AND DISCUSSION

The four unknown strains formed pinpoint colonies which were off-white and alpha-hemolytic. Gram staining revealed that the cells were gram positive and were arranged in pairs, tetrads, and clumps. The strains were catalase positive and failed to produce acid from sugars. Pyrrolidonylarylamidase, β -galactosidase, and leucine arylamidase were produced. The estimated G+C content was 44 to 45 mol%. These characteristics were in good agreement with the characteristics given in the description of Faden and Dryja (7).

The partial 16S rRNA sequence of human strain 7760^T is shown in Fig. 1. The reverse transcriptase sequence consisted of a continuous stretch of 1,466 bases representing approximately 96% of the total primary sequence. The first nucleotide and the last nucleotide in the sequence correspond to positions 9 and 1469, respectively, of the *E. coli* 16S rRNA sequence (2). The sequences of short fragments (ca. position 50 to position 450, which included variable regions V1 and V2) of the 16S rRNAs of the other isolates (strains 3621, 4491 and 7213) were also determined and were found to be identical to the sequence of strain 7760^T, confirming the relatedness of the four human strains. In order to determine the phylogenetic position of the unknown bacterium, the sequence of strain 7760^T was aligned and compared with the sequences of representative species belonging to the low-G+C-content genera *Aerococcus*, *Bacillus*, *Brochothrix*,

Carnobacterium, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Staphylococcus*, *Streptococcus*, and *Vagococcus* (1, 3–5, 10, 12–15). Percentages of similarity were calculated for a stretch of 1,340 nucleotides (ranging from position 107 to position 1433 of the *E. coli* sequence) (Table 1). The human strain exhibited relatively low levels of sequence relatedness with all of the reference species examined. The highest levels of homology were observed with aerococci, enterococci, carnobacteria, and pediococci (ca. 89 to 90%). Significantly lower levels of homology were observed with lactococci, staphylococci, and streptococci (ca. 85 to 87%). An unrooted tree constructed from derived K_{nuc} values is shown in Fig. 2. The results of a distance matrix analysis confirmed the distinctiveness of the unknown bacterium. In this scheme the human strain formed a long, distinct line of descent, showing no specific relationship with any of the other low-G+C-content taxa examined (Fig. 2).

Faden and Dryja (7) believed that the unknown bacterium isolated from human patients with middle ear infections phenotypically more closely resembled *Aerococcus viridans* and streptococci than other gram-positive cocci (e.g., enterococci, micrococci, staphylococci). However, unlike aerococci and streptococci, the unknown bacterium was catalase positive. In our phylogenetic analysis we demonstrated that the unknown organism falls unambiguously within the low-G+C-content gram-positive bacteria. In addition, it is evident from the sequence data that the bacterium not only is genealogically distinct from aerococci and streptococci, but also exhibits no specific relationship with any of the other low-G+C-content genera examined. Be-

cause of the phenotypic and phylogenetic distinctiveness of the unknown organism, we formally proposed that this bacterium should be classified in a new genus, *Alloiococcus* (Al.loi.o.coc'cus. Gr. adj. *alloios*, different; N.L. n. *coccus*, coccus; *Alloiococcus*, different coccus, referring to the phylogenetic distinctiveness of the organism), as *Alloiococcus otitis* (o.ti'tis. M.L. n. *otitis*, inflammation of the ear). The descriptions below are based on the results of Faden and Dryja (7) and our results.

Description of *Alloiococcus* gen. nov. Cells are ovoid and occur mostly in pairs or tetrads. Cells are gram positive and nonsporeforming. Grows at 45°C, in the presence of 6.5% NaCl, and on bile esculin agar. Aerobic. Catalase positive and oxidase negative. Acid is not produced from glucose and other sugars. Pyrrolidonylarylamidase positive. The DNA base composition is 44 to 45 mol% G+C. The type species is *Alloiococcus otitis*.

Description of *Alloiococcus otitis* sp. nov. Cells are ovoid and often occur as diplococci and tetrads. Grows slowly on solid media; after 48 h at 37°C small alpha-hemolytic colonies are produced on blood agar. Growth occurs in the presence of 6.5% NaCl and on bile esculin agar. No growth occurs at 45°C or in an anaerobic environment. Acid is not produced from arabinose, dulcitol, glycerol, glucose, inulin, lactose, maltose, mannose, mannitol, raffinose, rhamnose, ribose, salicin, sucrose, sorbitol, trehalose, or xylose. Catalase positive and oxidase negative. Starch and esculin are not hydrolyzed; hippurate may or may not be hydrolyzed. Pyrrolidonylarylamidase, β -galactose, and leucine arylamidase are produced. Arginine dihydrolase is not produced. The DNA base composition is 44 to 45 mol% G+C. Isolated from middle ear fluids of children with persistent otitis media with effusion. The type strain is strain NCFB 2890 (= 7760).

ACKNOWLEDGMENTS

We are grateful to R. R. Hinshaw and D. H. Batts, The Upjohn Co., for the gift of cultures.

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