

## Phylogenetic analysis of some *Aerococcus*-like organisms from urinary tract infections: description of *Aerococcus urinae* sp. nov.

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**Partial 16S ribosomal ribonucleic acid sequences of five *Aerococcus*-like organisms originally isolated from patients with urinary tract infections were determined using reverse transcriptase in order to clarify their taxonomic position. Analysis of the sequence data revealed that the clinical isolates represent a hitherto unknown line of descent within the genus *Aerococcus*. A new species, *Aerococcus urinae*, is proposed for these isolates. The type strain is NCFB 2893.**

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### Introduction

The genus *Aerococcus* was proposed by Williams *et al.* (1953) to accommodate some Gram-positive, microaerophilic, catalase-negative bacteria which differed from streptococci primarily by their characteristic tetrad cellular arrangements. Currently, the genus comprises a single species, *Aerococcus viridans* (Evans, 1986). Aerococci are widely distributed and occur naturally in air, dust and vegetation; they also form part of the flora of meat-curing brines (Deibel & Niven, 1960). Although aerococci are generally considered saprophytic, some are pathogenic for lobsters (Hitchner & Snieszko, 1947) and there have been various reports of *Aerococcus*-like organisms from human infections (e.g. subacute bacterial endocarditis: Colman, 1967; Janosek *et al.*, 1980; Parker & Ball, 1976; urinary tract infections: Colman, 1967; bacteraemia: Taylor & Trueblood, 1985; and meningitis: Nathavitharana *et al.*, 1983). The identification of aerococci is complex, due to the paucity of reliable discriminatory phenotypic traits. Their most characteristic feature is their tendency to divide in two planes at right angles to form tetrads. Aerocci also exhibit many biochemical and physiological similarities with pediococci, enterococci, lactococci and leuconostocs, and are often confused with streptococci (Facklam *et al.*, 1989). Problems with the identification of aerococci are further

compounded by evidence of some genetic heterogeneity within the species (Bosley *et al.*, 1990; Wiik *et al.*, 1986).

Christensen *et al.* (1989, 1991) recently reported the isolation and characterization of some *Aerococcus*-like organisms (ALOs) from urine samples of patients with urinary tract infections. These clinical ALOs differed from typical *Aerococcus viridans* strains in several phenotypic tests and their precise taxonomic status remained uncertain. 16S rRNA sequencing studies have done much to clarify the inter- and intra-generic relationships of aerococci, pediococci, streptococci and other lactic acid bacteria (Bentley *et al.*, 1991; Collins *et al.*, 1989, 1990, 1991; Martinez Murcia & Collins, 1990; Williams *et al.*, 1991; Yang & Woese, 1989). Therefore, in the present study we have investigated the partial 16S rRNA sequences of five ALOs from urinary tract infections (Christensen *et al.*, 1989, 1991) in an attempt to determine their true phylogenetic position.

### Methods

**Cultures and cultivation.** Five strains, designated E2, E3, B3, I3 and ES4, originally isolated from urine specimens, were received from Dr J. J. Christensen (Bispebjerg Hospital, Copenhagen, Denmark). The isolates were grown on blood agar plates and, for nucleic acid analysis, in glucose/yeast extract/phosphate broth (Garvie, 1978) at 37 °C. Cultures were harvested by centrifugation in late exponential phase and washed in deionized water.

**Analysis of 16S rRNA.** Cells (about 2 g) were mechanically broken using a Braun homogenizer, and RNA purified according to the procedure of Embley *et al.* (1988). Nucleotide sequences were determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) directly from rRNA using AMV reverse transcriptase (Lane *et al.*, 1985). Nucleotide fragments produced in the

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Abbreviation: ALO, *Aerococcus*-like organism.

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chain-elongation reactions were separated on 55 cm wedge-shaped 6% (w/v) acrylamide/7 M urea gels at 55 °C using an LKB MacroPhor 2010 sequencing unit operated at 50 W per gel.

The sequences generated were aligned, and homology values determined by the Beckman Microgenie Program (Queen & Korn, 1984). Nucleotide substitution rates ( $K_{\text{nuc}}$  values) were calculated and an unrooted phylogenetic tree produced using the algorithm of Fitch & Margoliash (1967).

**Biochemical tests.** Biochemical tests were performed on the five clinical strains and the type strain of *Aerococcus viridans* (NCDO 1225) by using the API 20 STREP and 50 CH systems according to the manufacturer's instructions. Blood agar was used as basal medium. Test preparations were incubated at 37 °C and read after 4, 24 and 48 h.

## Results and Discussion

The partial 16S rRNA nucleotide sequence from ALO strain E2 was determined using reverse transcriptase. The sequence is shown in Fig. 1; it consisted of a continuous stretch of 1481 nucleotides representing about 96% of the total molecule. The first and last nucleotides in the sequence correspond to positions 8 and 1482 of the *Escherichia coli* numbering system (Brosius *et al.*, 1978). The primary structures of two short fragments (approx. positions 50 to 450, which includes variable regions V1 and V2; and positions 950 to 1210, which includes variable region V6; see Neefs *et al.*, 1990, for nomenclature) of the 16S rRNA of four other ALOs (strains E3, B3, I3 and ES4) were also determined and found to be identical to that of strain E2, thereby confirming the genetic relatedness of the five clinical isolates.

In order to determine the taxonomic position of the clinical ALOs, the sequence of strain E2 was aligned and compared with those of *Aerococcus viridans* and 27 reference strains representing 14 low-mol% G+C genera. Percentage sequence similarities, shown in Table 1, were calculated from a continuous region of 1340 nucleotides (ranging from positions 107 to 1433 of the *E.*

*coli* sequence). Approximately 100 nucleotides at the 5' end of the rRNA molecule were not included in the calculation to eliminate possible alignment errors due to the extremely variable V1 region (Neefs *et al.* 1990) and to ensure that only homologous positions were compared. The unknown isolate exhibited the highest similarity (94.5%) with *A. viridans*. High sequence similarities (approx. 91 to 92%) were also shown with carnobacteria, enterococci and vagococci, but significantly lower values (approx. 88 to 90%) were shared with lactobacilli, pediococci and streptococci. Some representative sequence homologies are shown in Table 1. A phylogenetic tree constructed from a matrix of derived  $K_{\text{nuc}}$  values using the Fitch and Margolish algorithm is shown in Fig. 2. In this scheme, the unknown clinical bacterium showed a close affinity with *A. viridans*, and clearly represents a second line within the genus *Aerococcus*. The five ALOs possessed identical biochemical profiles using the API 20 STREP and 50 CH systems. The type strain of *A. viridans* differed from these strains in several tests. Differential tests are shown in Table 2.

Phylogenetic studies have shown that the genus *Aerococcus* represents a distinct line of descent within the lactic acid bacteria (Collins *et al.*, 1990, 1991). Currently the genus consists of a single species, *A. viridans* (Evans, 1986). There is, however, indication of some heterogeneity within aerococci. In an investigation of aerococci from clinical sources Bosley *et al.* (1990) reported that two groups were discernible within *A. viridans* on the basis of DNA-DNA pairing. The two groups, however, possessed very high overall chromosomal DNA sequence relatedness (60 to 78% homology under optimal hybridization conditions) and were phenotypically indistinguishable. 16S rRNA sequencing studies confirmed the close affinity between these two groups, with only two base differences detected for a comparison of 1486 nucleotides between *A. viridans* and a representative strain of the second DNA homology group (Collins *et al.*,

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8      AGAGUUUGAUCCUGGCUCAGGACGAACGCGUGGCGGCGGCCUAAUACAUGCAAGUCGAGCGAACCGACGAAGUCGUCACUUCUGACGUUAGCGGCGGA
110    CGGGUGAGUAAACACGUAAAGGAACCUACCGAUAAAGCGGGGACAAACAUCCGGAACCGGUGCUAAUACCGCAUAGGAAAGGUCACCNCAUGGUGACCUUUG
202    GAAAGACGGCUUUGUGUCACUUAUCGAUGGCCUUGCNGUGCAUUAAGCUCGUUGGUGGGUAAACGGCCUACCAAGGCAUAUGCAUAGCCGACCGGAGA
301    GGGUAAUCGGCCACAUUGGACUGAGACACGGCCCAAACUCCUACGGGAGGCAGCAGUAGGGAAUCUUCGCAUUGGGCGCAAGCCUGACGGAGCAACGC
401    CGCGUGAGUGAAGAAGGUUUUCGGAUCGUAAAGCUCUGUUGUAAGAGAAGAAACAAUUGGAGAGUAACUGCUCUACGGUACGUUACCUUACAGAAAG
501    CCACGGCUAAACUACGUGCCAGCAGCCGCGUAAUACGUAGGUGGCAAGCGUUGUCCGGAUUUUAUUGGGCGUAAAGGGGCGCAGGCGGUUUCUUAAGUCU
601    GAUGUGAAAGCCACGGCUUUAACCGUGGAAGUGCAUUGGAAACUGGGNAACUUGAGUACAGAGAAGAGAAAGUGGAAACUCCAUGUGUAGCGGUGGAAUGCG
701    UAGAUAUAUGGAAGAACACCAUGGCGGAAGGCGACUUCUGGUCUGUCACUGACGCGUGAGGCGCGAAAGCGUGGGUAGCAAACAGGAUUAAGAUACCCUGG
801    UAGUCCACGCGUAAACGAUGAGUGCUANGUGUUGGAGGGUUAUCCACCCUUCAGUGCCGGAGUUAACGCAUUAAGCACUCCGCCUGGGGAGUACGGCCGC
900    AAGGCUAGAAACUAAAGGAUUGACGGGGACCGCACAAAGCGGUGGAGCAUGUGGUUUAUUCGAAGCAACGCGAAGAACCUUACCAAGUCUUGACAUCG
1001   UUGACCACUCUAGAGAUAGAGCUUUCUUCGGGACNAAGUGACAGGUGGUGCAUGGUUGUCGUCAGCUCGUGUGAGAUUUGGGUUAAGUCCCGCAA
1103   CGAGCGCAACCCUUAUUGUUAUGUUGCCAGCAUUNAGUUGGCGACUUAAGCGAGACUGCCGGUGACAAACCGGAGGAAGGCGGGGAGUACGUAUUAUC
1204   AUGCCCCUUAUGACUUGGGCUACACAGUGCUACAAUGGAUGGUUACAAAGAGUCAGCGACCUUGUGAAAGCAAGCGAAUCUUAUAAAGCCAUUCUCAGUU
1303   CGGAUUGUAGUCUGCAACUCGACUACAUGAAGCGGAAUCGCUAGUAAUCGCGGAGCAGCAGCCCGGUGAAUACGUUCCCGGUCUUGUACACACCGC
1403   CCGUCACACCACGAGAGUUUGUAACCCUGAAGUGCGGUGAGGUAAACCUUUGGAGCCAGCCGCGAAGGUNNACAGAUUU

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Fig. 1. Primary structure of 16S rRNA from *Aerococcus urinae* (NCFB 2893) determined using reverse transcriptase. N, undetermined nucleotide. The number of the nucleotide at the beginning of each line in the sequence corresponds to the *E. coli* numbering system (Brosius *et al.*, 1978).

Table 1. Homology (lower left-hand triangle) and  $K_{\text{nuc}}$  values (upper right-hand triangle) for a 1340-nucleotide region of 16S rRNAs of *Aerococcus urinae* and other taxa

|  | 1    | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     | 13     | 14     |
|--|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 1. <i>Aerococcus urinae</i><br>NCFB 2893 <sup>T</sup> (= ALO E2) | ·    | 0.0571 | 0.1225 | 0.1284 | 0.0812 | 0.0891 | 0.1260 | 0.1513 | 0.1428 | 0.1120 | 0.1085 | 0.1143 | 0.1260 | 0.0925 |
| 2. <i>Aerococcus viridans</i><br>NCDO 1225 <sup>T</sup>          | 94.5 | ·      | 0.1355 | 0.1190 | 0.0790 | 0.0812 | 0.1476 | 0.1260 | 0.1367 | 0.1050 | 0.1143 | 0.1004 | 0.1225 | 0.0768 |
| 3. <i>Bacillus subtilis</i><br>rrNB                              | 88.7 | 87.6   | ·      | 0.1143 | 0.1062 | 0.1004 | 0.1274 | 0.1537 | 0.1636 | 0.0868 | 0.1225 | 0.0891 | 0.1476 | 0.1131 |
| 4. <i>Brochothrix thermosphacta</i><br>NCDO 1676 <sup>T</sup>    | 88.2 | 89.0   | 89.4   | ·      | 0.1050 | 0.0925 | 0.1525 | 0.1464 | 0.1649 | 0.0724 | 0.1213 | 0.1096 | 0.1331 | 0.0936 |
| 5. <i>Carnobacterium divergens</i><br>NCDO 2763 <sup>T</sup>     | 92.3 | 92.5   | 90.1   | 90.2   | ·      | 0.0560 | 0.1331 | 0.1343 | 0.1476 | 0.0846 | 0.0835 | 0.1096 | 0.1154 | 0.0658 |
| 6. <i>Enterococcus cecorum</i><br>NCDQ 2674 <sup>T</sup>         | 91.6 | 92.3   | 90.6   | 91.3   | 94.6   | ·      | 0.1154 | 0.1004 | 0.1331 | 0.0824 | 0.0835 | 0.1050 | 0.1027 | 0.0593 |
| 7. <i>Lactobacillus acidophilus</i><br>NCDO 1748 <sup>T</sup>    | 88.4 | 86.6   | 88.3   | 86.2   | 87.8   | 89.3   | ·      | 0.1661 | 0.1296 | 0.1428 | 0.1131 | 0.1574 | 0.1416 | 0.1272 |
| 8. <i>Lactococcus garvieae</i><br>NCDO 2155 <sup>T</sup>         | 86.3 | 88.4   | 86.1   | 86.7   | 87.7   | 90.6   | 85.1   | ·      | 0.1599 | 0.1440 | 0.1201 | 0.1699 | 0.0902 | 0.1331 |
| 9. <i>Leuconostoc mesenteroides</i><br>NCDO 523 <sup>T</sup>     | 87.0 | 87.5   | 85.3   | 85.2   | 86.6   | 87.8   | 88.1   | 85.6   | ·      | 0.1537 | 0.1476 | 0.1686 | 0.1488 | 0.1428 |
| 10. <i>Listeria innocua</i><br>NCTC 11288 <sup>T</sup>           | 89.6 | 90.2   | 91.8   | 93.1   | 92.0   | 92.2   | 87.0   | 86.9   | 86.1   | ·      | 0.1108 | 0.1027 | 0.1320 | 0.0947 |
| 11. <i>Pediococcus acidilactici</i><br>NCFB 2767 <sup>T</sup>    | 89.9 | 89.4   | 88.7   | 88.8   | 92.1   | 92.1   | 89.5   | 88.9   | 86.6   | 89.7   | ·      | 0.1272 | 0.1225 | 0.0925 |
| 12. <i>Staphylococcus xylosus</i><br>NCTC 11043 <sup>T</sup>     | 89.4 | 90.6   | 91.6   | 89.8   | 89.8   | 90.2   | 85.8   | 84.8   | 84.9   | 90.4   | 88.3   | ·      | 0.1550 | 0.1120 |
| 13. <i>Streptococcus pyogenes</i><br>NCDO 2381 <sup>T</sup>      | 88.4 | 88.7   | 86.6   | 87.8   | 89.3   | 90.4   | 87.1   | 91.5   | 86.5   | 87.9   | 88.7   | 86.0   | ·      | 0.1260 |
| 14. <i>Vagococcus fluvialis</i><br>NCDO 2497 <sup>T</sup>        | 91.3 | 92.7   | 89.5   | 91.2   | 93.7   | 94.3   | 88.3   | 87.8   | 87.0   | 91.1   | 91.3   | 89.6   | 89.0   | ·      |

Table 2. Characteristics useful in differentiating *A. urinae* and *A. viridans*

Data were obtained using the API 20STREP and 50CH systems; results are for five strains of *A. urinae* and the type strain of *A. viridans*.

| Test                    | <i>A. urinae</i> | <i>A. viridans</i> |
|-------------------------|------------------|--------------------|
| Production of:          |                  |                    |
| $\beta$ -Galactosidase  | —                | +                  |
| $\beta$ -Glucuronidase  | +                | —                  |
| Pyrrolidonylarylamidase | —                | +                  |
| Leucine aminopeptidase  | +                | —                  |
| Acid production from:   |                  |                    |
| Lactose                 | —                | +                  |
| Glycerol                | —                | +                  |
| Sorbitol                | +                | —                  |

1990). The results of the present study clearly demonstrate that the human ALOs isolated by Christensen *et al.* (1989, 1991) from urinary tract infections are distinct from the *A. viridans* and genotype 2 of Bosley *et al.* (1990). The 16S rRNA of ALO strain E2 showed 74 base differences with that of the type strain of *A. viridans* (based on a comparison of the same 1486 nucleotide stretch). The ALOs from urine are also phenotypically quite distinct from *A. viridans* (see Table 2). Therefore, on the basis of the present findings it is formally

proposed that the ALOs of Christensen and associates be classified as a new species of the genus *Aerococcus*, as *Aerococcus urinae* (u.ri'nae: L. fem. n. *urinae*, pertaining to urine).

#### Description of *Aerococcus urinae* sp. nov.

Cells are Gram-positive, coccoid, occurring mostly in clusters but also in pairs and tetrads. Non-pigmented and non-motile. Microaerophilic. Catalase-negative. Growth does not occur at 10 and 45 °C. Growth occurs in 6.5% (w/v) NaCl, and on 5% (v/v) horse blood agar, producing  $\alpha$ -haemolytic reaction. Acid is produced from D-arabitol (slow reaction), D-glucose, D-fructose, mannitol, ribose (slow reaction), sorbitol, sucrose and xylitol. Acid is not produced from amygdalin, arabinose, galactose, glycerol, glycogen, inositol, lactose, maltose, D-raffinose and trehalose. Hippurate is hydrolysed. Aesculin is not hydrolysed.  $\beta$ -Glucuronidase and leucine aminopeptidase positive.  $\alpha$ -Galactosidase,  $\beta$ -galactosidase, pyrrolidonylarylamidase, alkaline phosphatase and arginine dihydrolase negative. H<sub>2</sub>S is not produced. Nitrate is not reduced to nitrite. The G+C content of DNA is 44.4 mol%, as determined by melting temperature. Isolated from human sources (urinary tract specimens). The type strain is NCFB 2893 (NCTC 12142).

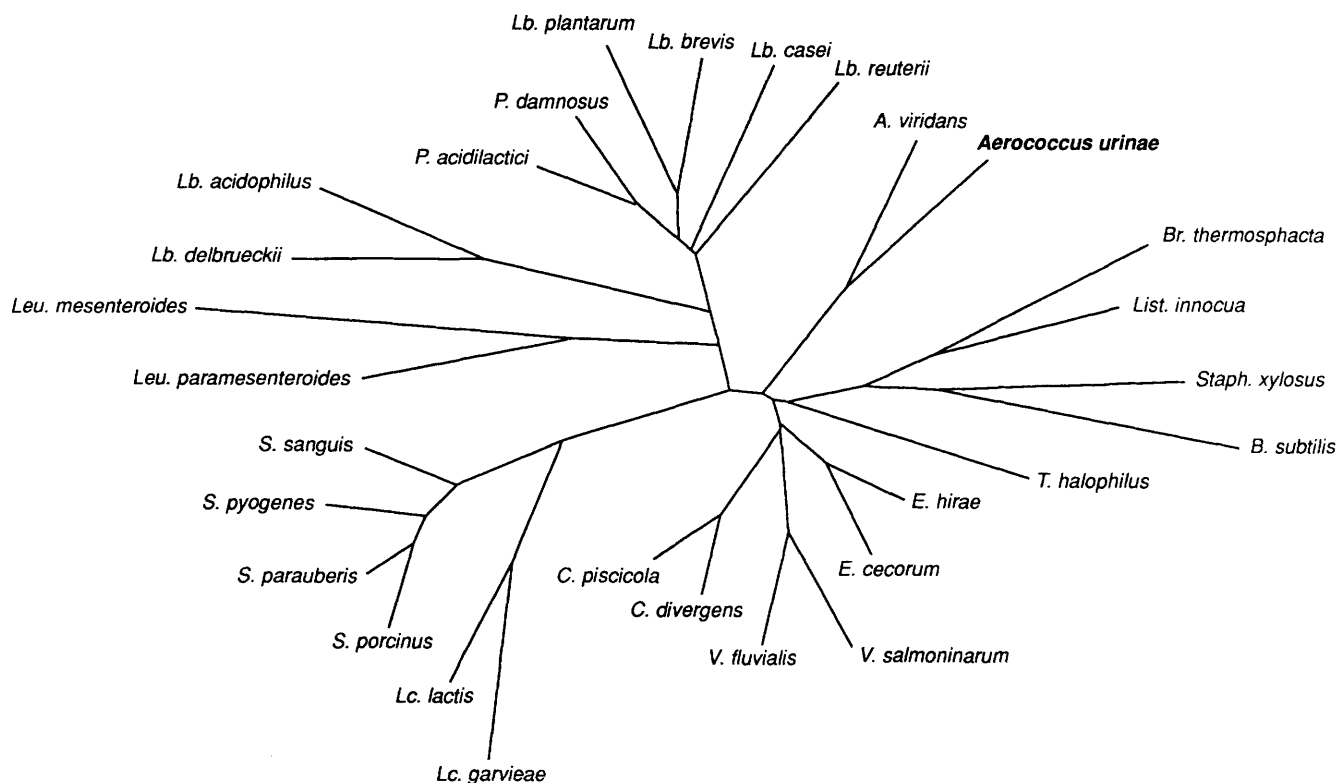


Fig. 2. Unrooted phylogenetic tree showing the position of *Aerococcus urinae* within a radiation of lactic acid bacteria. Genus abbreviations: A., *Aerococcus*; B., *Bacillus*; Br., *Brochothrix*; C., *Carnobacterium*; E., *Enterococcus*; Lb., *Lactobacillus*; Lc., *Lactococcus*; Leu., *Leuconostoc*; List., *Listeria*; P., *Pediococcus*; S., *Streptococcus*; Staph., *Staphylococcus*; T., *Tetragenococcus*; V., *Vagococcus*. Reference species corresponded to *C. piscicola* NCDO 2762<sup>T</sup>, *E. hirae* NCDO 1258<sup>T</sup>, *Lb. brevis* NCDO 1749<sup>T</sup>, *Lb. casei* NCDO 161<sup>T</sup>, *Lb. delbrueckii* NCDO 213<sup>T</sup>, *Lb. plantarum* NCDO 1752<sup>T</sup>, *Lb. reuteri* NCDO 2589<sup>T</sup>, *Lc. lactis* NCDO 2118<sup>T</sup>, *Leu. paramesenteroides* NCDO 803<sup>T</sup>, *P. damnosus* NCDO 1832<sup>T</sup>, *S. sanguis* NCTC 7863<sup>T</sup>, *S. parvauberis* NCDO 651, *S. porcinus* NCDO 600, *T. halophilus* NCDO 1635<sup>T</sup>, and *V. salmoninarum* NCFB 2777<sup>T</sup>. Strain details of other species examined are given in Table 1.

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