Reclassification of Two Strains of *Arthrobacter oxydans* and Proposal of *Arthrobacter nicotinovorans* sp. nov.

**YUKIKO KODAMA,** * HIROSHI YAMAMOTO, NORIHIDE AMANO, AND TERUO AMACHI

*Institute for Fundamental Research, Suntory Ltd., Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan*

*Arthrobacter* strains are classified into seven groups according to peptidoglycan type (7). *Arthrobacter oxydans* and *Arthrobacter polychromogenes* strains have a lysine-serine-threonine-alanine type of peptidoglycan. Despite the importance of peptidoglycan composition in *Arthrobacter* systematics, *A. oxydans* DSM 419 and DSM 420 were classified mainly on the basis of their ability to oxidize nicotine (2, 5).

The results of subsequent comparative taxonomic studies indicated that *A. oxydans* DSM 419 and DSM 420 differ from the type strain of *A. oxydans*, strain DSM 20119. By using a new analytic method, the presence of particular dipeptides can be detected in cell wall peptidoglycan hydrolysates by comparing the retention times of peaks with the retention times of synthesized dipeptides.

In this study, we found that *A. oxydans* DSM 419 should be reclassified as *Arthrobacter ureafaciens* and that *A. oxydans* DSM 420 forms the nucleus of a new species, *Arthrobacter nicotinovorans*.

**MATERIALS AND METHODS**

**Bacterial strains.** Culture collection abbreviations are as follows: ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, Riken, Saitama, Japan; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; SAM, Culture Collection of the Institute for Fundamental Research, Suntory Limited, Osaka, Japan.

We used the following test strains: *Arthrobacter auripes* DSM 0298T (= IAM 12340T), *Arthrobacter citreus* DSM 0302T (= IAM 12341T), *Arthrobacter histidinolovorans* DSM 0296T (= JCM 2520T), *Arthrobacter ilicis* DSM 1581T (= ATCC 14264T), *A. oxydans* DSM 1562 (= DSM 419), *A. oxydans* DSM 0173 (= JCM 3873 = DSM 419), *A. oxydans* DSM 1563 (= DSM 420), *A. oxydans* DSM 0174 (= JCM 3874 = DSM 420), *A. oxydans* DSM 0156T (= JCM 2521T = DSM 20119T), *A. oxydans* DSM 1560T (= DSM 20119T), *A. polychromogenes* DSM 0161T (= JCM 2523T), and *A. ureafaciens* DSM 0299T (= IAM 1688T) (T = type strain).

**Cell morphology.** Cell shape and pleomorphism were determined by using cells grown in shake tubes containing Trypticase soy broth (catalog no. 11768; BBL Microbiology Systems, Cockeysville, Md.) at 30°C. The Gram reaction was determined by using cells grown on Trypticase soy broth supplemented with 1.8% agar at 30°C for 24 h. Motility was examined by using the hanging drop method and cells grown on L broth (1% Polypeptone [Daigo Eiyou], 0.5% yeast extract [Difco], 0.5% NaCl, 0.1% glucose; pH 7.0) supplemented with 1.8% agar at 30°C for 24 h. Flagellar distribution was observed by using negatively stained (1% wt/vol) phosphotungstic acid in distilled water, pH 7.0 preparations that were viewed with a transmission electron microscope (model JEM 1200 EX; JEOI Ltd., Tokyo, Japan).

**Physiological and biochemical characteristics.** Nitrate reduction and starch hydrolysis were tested in nutrient broth supplemented with 0.1% KNO₃ and 0.1% soluble starch, respectively. Tolerance to sodium chloride was tested in Trypticase soy broth supplemented with 10% NaCl; utilization of organic compounds as sole carbon sources was tested by using the method of Seiler et al. (11); assimilation of organic acids was tested by using the method of Yamada and Komagata (19); and vitamin requirements were tested by using the methods of Owens and Keddie (9). Urea formation from creatinine and urea formation from uric acid were examined by using the method of Krebs and Eggleston (8), and production of nicotine blue was tested by using the method described by Sguros (12). All of these tests were done at 30°C for 1 week.

**Preparation of cell wall peptidoglycan.** The test strains were grown in L broth. Bacterial cells were harvested by centrifugation in the logarithmic phase of growth, washed with 0.1 M phosphate buffer (pH 7.2), and then disrupted in a sonic oscillator (Branson model 250 Sonifier) for 10 to 30 min at 5°C. Unbroken cells were removed by centrifugation at 3,000 to 5,000 × g for 10 to 15 min. The supernatant solution was supplemented with 4% sodium dodecyl sulfate and heated at 100°C for 40 min. After cooling, it was centrifuged at 10,000 × g for 30 min, and the precipitate was washed with warm water by centrifugation at 90°C for 20 min. The precipitate was collected and washed three times with water by centrifugation, and then it was freeze-dried.

**Quantitative determination of amino acid composition.** The amino acid composition of cell walls was determined by
using the method of Komagata and Suzuki (7). Approximately 1 mg of cell walls was hydrolyzed for 16 h at 100°C in 1 ml of 6 N HCl in a tube that was tightly closed with a Teflon-lined screw cap. The hydrolysate was filtered and washed with water. The filtrate was dried and dissolved in 0.02 N HCl. The amino acid composition was determined by using a model 835-50 amino acid analyzer (Hitachi Ltd., Tokyo, Japan). The following abbreviations are used below for the amino acids: Ala, alanine; Lys, lysine; Ser, serine; Thr, threonine; Glu, glutamic acid.

Determination of peptidoglycan structure. A partial acid hydrolysate of the cell wall preparations was prepared by hydrolyzing approximately 1 mg of cell walls in 1 ml of 4 N HCl for 30 to 60 min at 100°C. The presence of a particular dipeptide in partial acid hydrolysates of cell walls was determined by comparing the peaks with the peaks of synthesized dipeptides, using the amino acid analyzer.

Synthesis of dipeptides. Two dipeptides, Ala-E-Lys and Thr-E-Lys, were synthesized with Z-Thr(Z-Ala) and Z-Lys-OMe • HCl by using the method of Bodanszky et al. (1).

Determination of cellular fatty acid composition. Cellular fatty acid composition was determined by using the method described by Suzuki and Komagata (15). Cells were cultivated in shake flasks containing F medium (1% Bacto Peptone [Difco], 0.5% yeast extract [Difco], 0.5% Casamino Acids [Difco], 0.5% malt extract [Nacalai tesque], 0.05% KH₂PO₄; pH 7.2) at 30°C for 24 h. Cellular fatty acids were extracted by methanolysis with benzene-methanol-sulfuric acid (10:20:1, vol/vol), and the methyl esters were analyzed by using a model 663-30 gas-liquid chromatograph (Hitachi Ltd.) equipped with a 10% diethylene glycol succinate column (length, 5 m) and a flame ionization detector at 180°C.

Determination of isoprenoid quinones. Isoprenoid quinones were extracted from freeze-dried cells with chloroform-methanol (2:1, vol/vol) and purified by thin-layer chromatography (TLC).
raphy. The purified menaquinones were determined by using reverse-phase high-performance liquid chromatography (17). The abbreviations used below for menaquinones are in the form MK-\(n(H_m)\), with \(n\) indicating the number of isoprene units in the side chain and \(m\) indicating the number of hydrogen atoms saturating the isoprenoid chain.

**Isolation of DNA.** DNA was isolated by using the method of Tamaoka et al. (16) and cells that were cultivated for 24 h at 30°C in shake flasks containing L broth supplemented with 0.5% glycine.

**Determination of DNA base composition.** DNA base composition was determined by reverse-phase high-performance liquid chromatography after enzymatic hydrolysis of the DNA (18).

**DNA-DNA hybridization.** DNA-DNA hybridization was performed at 45°C in microdilution wells (3). DNA was labeled with photobiotin (Photoprobe Biotin; Vector Laboratories).

### RESULTS

**Morphological characteristics.** *A. oxydans* SAM 1562 and SAM 1563 were pleomorphic; most cells in 2-day-old cultures were coccoid. The coccoid cells became irregularly rod shaped after 6 h when they were transferred to fresh medium; many cells were arranged at angles to each other to give V-shaped forms. As growth proceeded, the rod-shaped cells became shorter and were eventually replaced by coccoid cells. The rods were motile by means of a few lateral flagella. Both organisms were gram positive in young cultures but subsequently became gram variable. The organisms were asporogenous, not acid fast, and obligately aerobic.

**Physiological and biochemical characteristics.** The results of the physiological and biochemical tests are shown in Table 1. *A. oxydans* SAM 1562 differed from the type strain of *A. oxydans*, strain SAM 0156, in lacking nitrate reduction, starch hydrolysis, growth in the presence 10% NaCl, and motility. *A. oxydans* SAM 1563 could be distinguished from the type strain of *A. oxydans*, strain SAM 0156, by its lack of nitrate reduction and motility.

**Quantitative determination of amino acid compositions of cell wall peptidoglycans.** The Lys-Ala-Thr-Glu molar ratio of the cell wall peptidoglycans of *A. oxydans* SAM 1562 and SAM 1563 was 1:4:1:1, which placed these organisms in either the wall type Lys-Thr-Ala, group or the wall type Lys-Ala-Thr-Ala group (4). However, in *A. oxydans* type strain SAM 0156, which was in the wall type Lys-Ser-Thr-Ala group, the Lys-Ala-Thr-Ala group was 1:3:1:1:1.

**Determination of peptidoglycan structure.** The chromatograms of the partially hydrolyzed peptidoglycans obtained by using the automatic amino acid analyzer are shown in Fig. 1 and 2. An Ala-e-Lys peak was found in the partial hydrolysate of the Lys-Ala-Thr-Ala peptidoglycan type. In contrast, a Thr-e-Lys peak was found in the partial hydrolysate of the Lys-Thr-Ala, peptidoglycan type. The partially hydrolyzed peptidoglycans of *A. oxydans* SAM 1562 and SAM 1563 (Fig. 2) showed that these organisms belong to the peptidoglycan type Lys-Ala-Thr-Ala group.

**Fatty acid, menaquinone, and DNA base compositions.** The cellular fatty acids, the predominant menaquinones, and the DNA base compositions of *A. oxydans* SAM 1562 and SAM 1563 and related organisms are shown in Table 2. The major cellular fatty acid components were 12-methyltetradecanoic acid (anteiso C15) and 14-methylhexadecanoic acid (anteiso C17); the contents of normal acids varied among the test
strains. N-hexadecanoic acid (normal C16) was found in the species with the Lys-Thr-Ala2 peptidoglycan type and the Lys-Ser-Thr-Ala peptidoglycan type, where it accounted for approximately 10% of the total fatty acids detected. However, in the species with the Lys-Ala-Thr-Ala peptidoglycan type this acid accounted for only 1 or 2% of the total fatty acids. The cellular fatty acid compositions of *A. oxydans* SAM 1562 and SAM 1563 were similar to those of species with a Lys-Ala-Thr-Ala type of peptidoglycan. All of the strains had MK-9(H2) as the principal isoprenoid quinone. The G+C contents of the DNAs of the test strains ranged from 61.5 to 64.9 mol%.

**DNA-DNA hybridization.** The levels of DNA-DNA relatedness found among *A. oxydans* SAM 1562, SAM 1563, SAM 0173, and SAM 0174 and related organisms are shown in Table 3. *A. oxydans* SAM 1562 and SAM 0173 shared high degrees of DNA complementarity (97 to 100%) with *A. ureafaciens* SAM 0299T, while *A. oxydans* SAM 1563 and SAM 0174 shared moderate degrees of DNA complementarity (47 to 55%) with *A. histidinolovorans* SAM 0296T, but less than 40% complementarity with the other test strains.

**DISCUSSION**

The genus *Arthrobacter* is currently defined mainly on the basis of cell morphology and chemotaxonomic characteristics (6), notably peptidoglycan structure (4, 7). The two strains which we received as *A. oxydans* are aerobic, gram positive, motile, and pleomorphic, contain major amounts of dihydrogenated menaquinones with nine isoprene units, have major amounts of iso and anteiso fatty acids (with 12-methyltetradecanoic acid [anteiso C15] and 14-methylhexadecanoic acid [anteiso C17] predominating), and have DNA base compositions which range from 63.3 to 62.4 mol% G+C. It is clear from these data that *A. oxydans* DSM 419 and DSM 420 belong to the genus *Arthrobacter* (6).

When we used a new determinative method (see above), we found that both strains of *A. oxydans* had the Ala-E-Lys peak on chromatograms prepared from their partially hydrolyzed peptidoglycans. Therefore, both strains belong to the group with the Lys-Ala-Thr-Ala peptidoglycan type. *A. aurescens, A. ilicis, A. histidinolovorans,* and *A. ureafaciens* also have this peptidoglycan type (4, 7, 10).

*Arthrobacter* species have DNAs that are heterogeneous compared with the DNA of the type species of the genus, *Arthrobacter globiformis* (14). They are rather diverse even when they have the same peptidoglycan type (13). It is clear from DNA hybridization data that *A. oxydans* DSM 419 should be classified as *A. ureafaciens*. In contrast, *A. oxydans* DSM 420 forms the nucleus of a new genomic species although it does exhibit a moderately high similarity value with *A. histidinolovorans* SAM 0296T. It can also be distinguished from other *Arthrobacter* species by a number of physiological properties (Table 1).

Consequently, we propose that *A. oxydans* DSM 420 should be described as a new species, *A. nicotinovorans*.


Slight growth occurs in the presence of 10% NaCl. Starch
TABLE 2. Chemotaxonomic characteristics of *A. oxydans* SAM 1562 and SAM 1563 and related organisms

<table>
<thead>
<tr>
<th>Peptidoglycan type</th>
<th>Strain</th>
<th>Fatty acid composition (%)</th>
<th>G+C content of DNA (mol%)</th>
<th>Isoprenoid quinone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anteiso acids</td>
<td>Iso acids</td>
<td>Normal acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C12</td>
<td>C17</td>
<td>C14</td>
</tr>
<tr>
<td>Lys-Ala-Thr-Ala</td>
<td><em>A. aurescens</em> SAM 0298&lt;sup&gt;T&lt;/sup&gt;</td>
<td>66</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>A. histidinolovorans</em> SAM 0296&lt;sup&gt;T&lt;/sup&gt;</td>
<td>64</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>A. ilicis</em> SAM 1581&lt;sup&gt;T&lt;/sup&gt;</td>
<td>70</td>
<td>20</td>
<td>tr</td>
</tr>
<tr>
<td></td>
<td><em>A. oxydans</em> SAM 1562</td>
<td>68</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>A. oxydans</em> SAM 1563</td>
<td>68</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>A. ureafaciens</em> SAM 0299&lt;sup&gt;xy&lt;/sup&gt;</td>
<td>58</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>Lys-Thr-Ala&lt;sub&gt;2&lt;/sub&gt;</td>
<td><em>A. citreus</em> SAM 0302&lt;sup&gt;xy&lt;/sup&gt;</td>
<td>57</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Lys-Ser-Thr-Ala</td>
<td><em>A. oxydans</em> SAM 0156&lt;sup&gt;T&lt;/sup&gt;</td>
<td>45</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>A. polychromogenes</em> SAM 0161&lt;sup&gt;T&lt;/sup&gt;</td>
<td>44</td>
<td>25</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cellular fatty acid data from reference 15.
<sup>b</sup> tr, trace (less than 1%).

### ACKNOWLEDGMENTS

We are grateful to M. Goodfellow, Department of Microbiology, The Medical School, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, England, for his interest and very helpful discussions. We also thank N. Kawaguchi, Institute for Fundamental Research, Suntory Ltd., for synthesizing the standard dipetides and T. Katayama, Institute of Biomedical Research, Suntory Ltd., for technical assistance in the analysis of dipetides.

### REFERENCES


