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

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Arthrobacter oxydans DSM 419 and DSM 420 have chemical and microbiological properties that are consistent with assignment to the genus *Arthrobacter*. Both organisms have the lysine-alanine-threonine-alanine peptidoglycan type. DNA-DNA pairing studies indicated that *A. oxydans* DSM 419 should be reclassified as *Arthrobacter ureafaciens* and that *A. oxydans* DSM 420^T forms the nucleus of a distinct genomic species. We propose that *A. oxydans* DSM 420 should be reclassified as *Arthrobacter nicotinovorans* sp. nov. The type strain is strain DSM 420.

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 aureescens* SAM 0298^T (= IAM 12340^T), *Arthrobacter citreus* SAM 0302^T (= IAM 12341^T), *Arthrobacter histidinovorans* SAM 0296^T (= JCM 2520^T), *Arthrobacter ilicis* SAM 1581^T (= ATCC 14264^T), *A. oxydans* SAM 1562 (= DSM 419), *A. oxydans* SAM 0173 (= JCM 3873 = DSM 419), *A. oxydans* SAM 1563 (= DSM 420), *A. oxydans* SAM 0174 (= JCM 3874 = DSM 420), *A. oxydans* SAM 0156^T (= JCM 2521^T = DSM 20119^T), *A. oxydans* SAM 1560^T (= DSM 20119^T), *A. polychromogenes* SAM 0161^T (= JCM 2523^T), and *A. ureafaciens* SAM 0299^T (= IAM 1658^T) (T = type strain).

Cell morphology. Cell shape and pleomorphism were de-

termined 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 Eiyō], 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; JEOL 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 Sgueros (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 room temperature, suspended in 5% trichloroacetic acid, and heated 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

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FIG. 1. Chromatograms of synthesized dipeptides and partial hydrolysates of peptidoglycans, as determined by using an automatic amino acid analyzer. (a) Lysine and the synthesized dipeptides Thr- ϵ -Lys and Ala- ϵ -Lys. (b) Partial acid hydrolysate of the Lys-Ala-Thr-Ala peptidoglycan prepared from *A. aurescens* SAM 0298^T. (c) Partial acid hydrolysate of the Lys-Thr-Ala₂ peptidoglycan prepared from *A. citreus* SAM 0302^T.

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- ϵ -Lys and Thr- ϵ -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% diethyleneglycol 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 chromatog-

FIG. 2. Chromatograms of partial acid hydrolysates of peptidoglycans, as determined by using an automatic amino-acid analyzer. (a) Partial acid hydrolysate of peptidoglycan prepared from strain SAM 1562. (b) Partial acid hydrolysate of peptidoglycan prepared from strain SAM 1563. The arrows indicate the peaks of the Ala- ϵ -Lys dipeptide.

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-Ala₂ 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-Ser-Glu ratio 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-ε-Lys peak was found in the partial hydrolysate of the Lys-Ala-Thr-Ala peptidoglycan type. In contrast, a Thr-ε-Lys peak was found in the partial hydrolysate of the Lys-Thr-Ala₂ 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

TABLE 1. Phenotypic characteristics of *A. oxydans* SAM 1562 and SAM 1563 and related organisms

Peptidoglycan type	Strain	Nitrate reduced to nitrate	Hydrolysis of starch	Growth in the presence of 10% NaCl	Vitamin requirement: none or only biotin	Production of nicotine blue	Utilization of:					
							L-Arginine	L-Asparagine	L-Histidine	L-Leucine	L-Arabinose	D-Galactose
Lys-Ala-Thr-Ala	<i>A. aurescens</i> SAM 0298 ^T	+	+	+	+	+	+	+	+	+	+	+
	<i>A. histidinovorans</i> SAM 0296 ^T	+	+	+	+	+	+	+	+	+	+	+
	<i>A. ilicis</i> SAM 1581 ^T	+	+	+	+	+	+	+	+	+	+	+
	<i>A. oxydans</i> SAM 1562	+	+	+	+	+	+	+	+	+	+	+
	<i>A. oxydans</i> SAM 1563	+	+	+	+	+	+	+	+	+	+	+
	<i>A. ureafaciens</i> SAM 0299 ^T	+	+	+	+	+	+	+	+	+	+	+
Lys-Thr-Ala ₂	<i>A. citreus</i> SAM 0302 ^T	+	+	+	+	+	+	+	+	+	+	+
Lys-Ser-Thr-Ala	<i>A. oxydans</i> SAM 0156 ^T	+	+	+	+	+	+	+	+	+	+	+
	<i>A. polychromogenes</i> SAM 0161 ^T	+	+	+	+	+	+	+	+	+	+	+

^a +, positive reaction; -, negative reaction; w, weak reaction; ND, not determined.

TABLE 2. Chemotaxonomic characteristics of *A. oxydans* SAM 1562 and SAM 1563 and related organisms

Peptidoglycan type	Strain	Fatty acid composition (%)										G+C content of DNA (mol%)	Isoprenoid quinone
		Anteiso acids		Iso acids				Normal acids					
		C ₁₅	C ₁₇	C ₁₄	C ₁₅	C ₁₆	C ₁₇	C ₁₄	C ₁₅	C ₁₆	C ₁₈		
Lys-Ala-Thr-Ala	<i>A. aurescens</i> SAM 0298 ^{Ta}	66	26		2	4	1			1		61.9	MK-9(H ₂)
	<i>A. histidinolorans</i> SAM 0296 ^T	64	22	1	3	5	1	1		2	tr ^b	62.6	MK-9(H ₂)
	<i>A. ilicis</i> SAM 1581 ^T	70	20	tr	1	3		1		1		61.5	MK-9(H ₂)
	<i>A. oxydans</i> SAM 1562	68	20	2	1	5	tr	1		2	1	63.3	MK-9(H ₂)
	<i>A. oxydans</i> SAM 1563	68	24		2	3	1			2		62.4	MK-9(H ₂)
	<i>A. ureafaciens</i> SAM 0299 ^{Ta}	58	28	1	5	6	2			1	1	63.6	MK-9(H ₂)
Lys-Thr-Ala ₂	<i>A. citreus</i> SAM 0302 ^{Ta}	57	8	2	7	7	1	2	1	11		64.9	MK-9(H ₂)
Lys-Ser-Thr-Ala	<i>A. oxydans</i> SAM 0156 ^T	45	17	2	9	11	2	2	1	11		63.1	MK-9(H ₂)
	<i>A. polychromogenes</i> SAM 0161 ^T	44	25	1	3	6	1	1	1	13		62.9	MK-9(H ₂)

^a Cellular fatty acid data from reference 15.^b tr, trace (less than 1%).

is hydrolyzed, and nitrate is not reduced. Nicotine blue is produced from nicotine. Urea is formed from creatinine and uric acid.

Utilizes L-arabinose, D-galactose, D-glucose, *meso*-inositol, D-ribose, D-xylose, 4-aminobutyrate, L-arginine, L-asparagine, L-histidine, and *p*-hydroxybenzoate, but not L-leucine.

Citric, formic, malonic, uric, and propionic acids are assimilated, but glutaric, adipic, pimelic, and benzoic acids are not.

The cell wall peptidoglycan is of the Lys-Ala-Thr-Ala type, and the principal isoprenoid quinone is MK-9(H₂). The fatty acids are mainly straight-chain, anteiso- and iso-methyl-branched acids. The major fatty acids are 12-methyltetradecanoic (anteiso C₁₅) and 14-methyl-hexadecanoic (anteiso C₁₇) acids.

The G+C content of the DNA is 62.4 mol%.

The type strain is strain DSM 420.

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TABLE 3. DNA-DNA homology values for *A. oxydans* SAM 1562 and SAM 1563 and related organisms

Fixed DNA from:	% Homology with biotin-labeled DNA from:				
	Strain SAM 1562	Strain SAM 0173	Strain SAM 1563	Strain SAM 0174	<i>A. ureafaciens</i> SAM 0299 ^T
Strain SAM 1562	100	112	25	21	87
Strain SAM 0173	88	100	13	31	70
Strain SAM 1563	27	40	100	104	29
Strain SAM 0174	24	24	110	100	12
<i>A. aurescens</i> SAM 0298 ^T	41	35	38	39	23
<i>A. histidinovorans</i> SAM 0296 ^T	25	20	47	55	21
<i>A. ilicis</i> SAM 1581 ^T	36	33	31	34	17
<i>A. ureafaciens</i> SAM 0299 ^T	100	97	20	31	100
<i>A. citreus</i> SAM 0302 ^T	13	16	9	10	0
<i>A. oxydans</i> SAM 1560 ^T	9	13	14	20	7

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