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Acinetobacter antiviralis sp. nov., from Tobacco Plant Roots

Lee, Jung-Sook[1]*, Keun Chul Lee[1], Kwang Kyu Kim[1], In Cheon Hwang[2], Cheol Jang[2], Nam Gyu Kim[2], Woon Hyung Yeo[3], Beom Seok Kim[4], Yong Man Yu[5], and Jong Seog Ahn[1]

[1]*Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea*
[2]*Central Research Institute, Kyung Nong Co. Ltd., Gyeongju 780-110, Korea*
[3]*KT&G Central Research Institute, Daejeon 305-308, Korea*
[4]*College of Life and Environmental Sciences, Korea University, Seoul 136-713, Korea*
[5]*Department of Applied Biology, Chungnam National University, Daejeon 305-764, Korea*

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Acinetobacter **strain KNF2022^T was isolated from tobacco plant roots during the screening of antiviral substances having inhibitory effects on Tobacco mosaic virus (TMV) and examined by phenotypic, chemotaxonomic, and genetic characterization. It was a nonmotile, Gram-negative bacterium. This strain contained Q-9 as the main respiratory quinone. The major cellular fatty acids of the isolate were 16:0, 18:1 w9c, and 16:1 w7c/15 iso 2OH. The DNA base composition was 44 mol%. Phylogenetic analysis based on the 16S rRNA sequence revealed that the isolate formed an evolutionary lineage distinct from other *Acinetobacter* species. Based on the evaluation of morphologic, physiologic, and chemotaxonomic characteristics, DNA-DNA hybridization values, and 16S rRNA sequence comparison, we propose the new species *Acinetobacter antiviralis* sp. nov., the type strain of which is KNF2022^T (=KCTC 0699BP^T).**

Keywords: *Acinetobacter antiviralis* sp. nov., antiviral activity, polyphasic taxonomy

The genus *Acinetobacter* was first described by Brisou and Prévot [2]. The genus *Acinetobacter* comprises nonmotile, aerobic, Gram-negative bacteria. *Acinetobacter* spp. represented a well-defined genus by 16S rRNA sequence analysis [8]. However, species delineation has been more problematic, and 15 genomic species were left unclassified. It indicates that the genus would extend beyond the described groups. At the time of writing, the genus *Acinetobacter* consists of 17 species: *A. baumannii, A. baylyi, A. bouvetii, A. calcoaceticus, A. gerneri, A. grimontii, A. haemolyticus, A. johnsonii, A. junii, A. lwoffii, A. parvus, A. radioresistens, A. schindleri, A. tandoii, A. tjernbergiae, A. towneri,* and *A. ursingii* [1, 3, 14–16].

The authors isolated a novel bacterium from tobacco plant roots. In this study, we describe the morphologic, biochemical, and phylogenetic distinctiveness of the isolate, and propose that it should be assigned to a new species, *Acinetobacter antiviralis* sp. nov.

*Corresponding author
Phone: +82-42-860-4618; Fax: +82-42-860-4677;
E-mail: jslee@kribb.re.kr

MATERIALS AND METHODS

Microorganisms and Culture Conditions

We screened the bacteria having antiviral activity against plant virus. We selected strain KNF2022^T on Mueller-Hinton agar (Difco) from tobacco plant roots, Daejoen, Korea. The isolate was not only effective against TMV, but also potato virus Y (PVY) and cucumber mosaic virus (CMV) [10]. The isolate and *Acinetobacter* reference strains were cultured on tryptic soy agar for 24 h at 30°C for further analyses.

Inhibitory Activity of Culture Filtrate

Xanthi-nc was used for local lesion assay of TMV infection in the greenhouse. For the virus inoculum, tobacco leaves (0.1 g) of NC82, systemically infected with TMV, were grounded in 20 ml of phosphate buffer (0.02 M, pH 7.3). The sap was filtered through two layers of cheesecloth. The extract was then centrifuged at 3,000 rpm for 15 min to remove debris. The supernatant was used as TMV inoculum. Antiviral activity in local lesion host was tested by using the half-leaf method [4, 7]. The culture filtrate was applied with cotton swabs on the upper surface of half leaf, while distilled water was applied on the remaining half as control. The virus was inoculated 24 h after application of the culture filtrate by using ordinary Carborundum (600 mesh) methods [4, 7]. The symptom on the inoculated plants was observed at 3 to 4 days after the inoculation, and the effects of the treatment were measured by comparing the number of local lesions on plants between the treated and untreated (control) ones.

Phenotypic Characterization

Cultural characteristics were observed on tryptic soy agar (TSA, Difco). The cell morphology was examined under a light microscope (1,000× magnification; Nikon) on TSA. Motility was determined with an optical microscope using the hanging drop technique [20]. Anaerobic growth was recorded in an anaerobic chamber on TSA. Growth at different temperatures was observed in a nutrient broth at 10, 15, 20, 25, 30, 37, and 42°C by the methods of Yumoto *et al*. [27]. Oxidase activity was determined using Bactident-Oxidase strips (Merck), and catalase activity was tested using 3% H_2O_2. API (bioMerieux, Marcy l'Etoile, France) 20E, 20NE, and 50CHB were used for the physiologic and biochemical characterization. All API tests were performed in accordance with the manufacturer's directions. Duplicate antibiotic-susceptibility tests were performed by using Sensi-Discs (6 mm; BBL) containing the following: ampicillin (10 mg), chloramphenicol (30 mg), erythromycin (15 mg), kanamycin (30 mg), neomycin (30 mg), penicillin G (10 IU), rifampicin (5 mg), streptomycin (10 mg), tetracycline (30 mg), vancomycin (30 mg), and ticarcillin (clavulanic acid) (85 mg); zone diameter interpretive standards of the NCCLS (2003) were used for interpretation [13].

Chemotaxonomic Characterization

For a total cellular fatty acid analysis, the cells were cultured on TSA for 24 h at 30°C, and then the fatty acids were extracted following the Microbial Identification System instructions, as previously described [11, 26]. The isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), and purified by thin-layer chromatography (TLC) on Merck Kieselgel 60 F_{254} plates (20×20 cm, 0.5 mm thickness) using petroleum ether:diethyl ether (9:1, v/v) as the solvent. The identities of the quinones were determined by HPLC analysis, as described by Shin *et al*. [19].

DNA Base Composition

DNA was extracted and purified by a modification of the method of Marmur [12]. The G+C content in the DNA was determined using

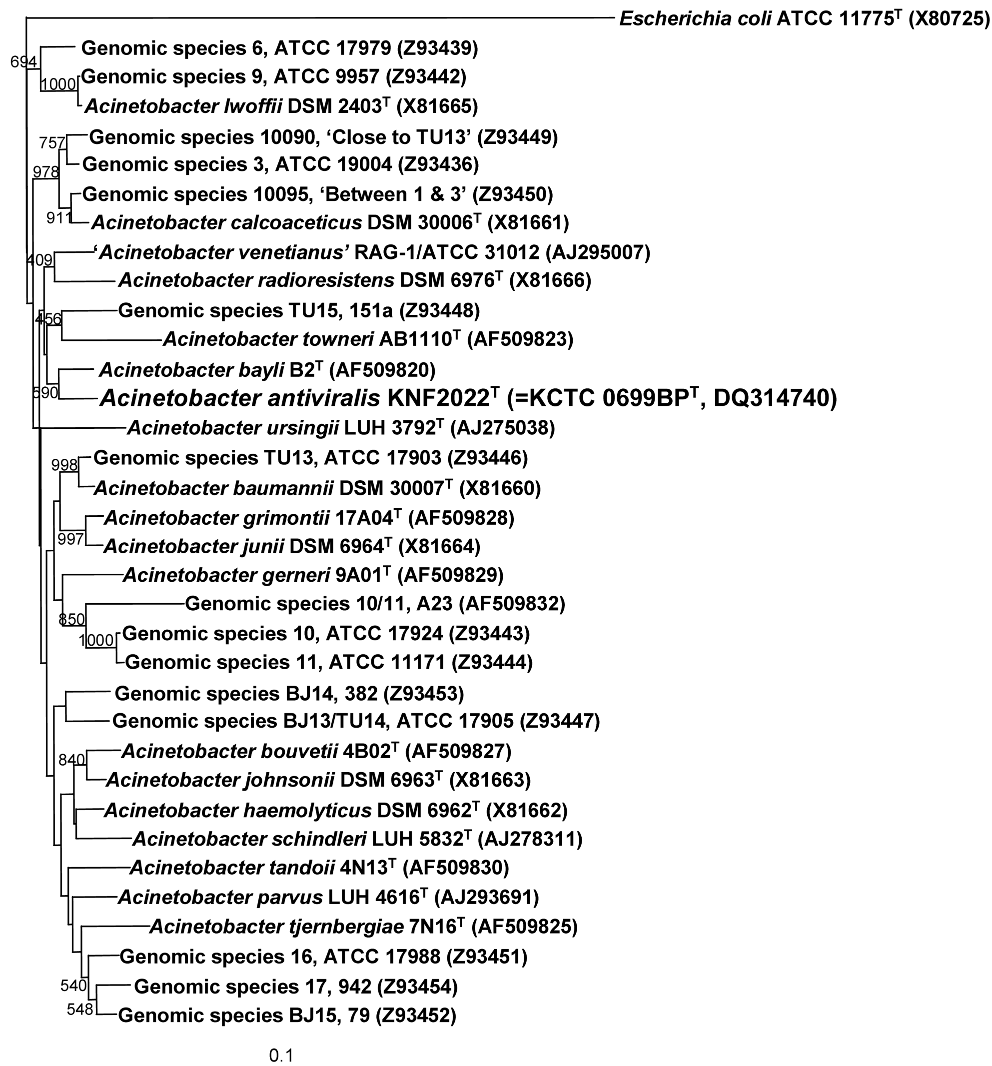

Fig. 1. Phylogenetic tree showing the position of isolate KNF2022^T with other species from genus *Acinetobacter* and related taxa based on 16S rRNA gene sequences.

The numbers indicate bootstrap values >400. Scale bar, 0.01 accumulated changes per nucleotide.

Table 1. Differential characteristics of the KNF2022^T isolate and *Acinetobacter* species.

Characteristics	KNF 2022^T	*A. bayli* KCTC 12413^T	*A. towneri* KCTC 12419^T	*A. ursingiii* KCTC 12410^T	Genomic species TU15 KCTC 12687	*A. radioresistens* KCTC 12411^T	*A. johnsonii* KCTC 12405^T	*A. calcoaceticus* KCTC 2357^T	*A. lwoffii* KCTC 12407^T	*A. baumannii* KCTC 2508^T	*A. gerneri* KCTC 12415^T	*A. tandoii* KCTC 12417^T
Arginine dihydrolase	–	–	–	+	–	–	–	+	–	+	+	–
Citrate utilization	+	+	–	+	–	–	–	–	–	+	–	+
Acetoin production	+	–	+	+	+	+	+	+	+	+	+	+
Assimilation of:												
Gluconate	–	+	w	w	–	w	w	–	–	w	w	–
Adipic acid	–	+	w	+	w	+	w	+	+	+	+	–
Citrate	+	+	w	+	w	w	w	+	+	+	+	+
Arabinose	w	–	w	w	–	w	w	–	w	+	w	w
Glucose	w	+	w	w	–	w	w	–	w	w	w	w
Phenylacetate	–	w	w	+	+	+	w	+	+	+	+	+
Fermentation from:												
Rhamnose	+	w	–	–	–	–	–	w	–	w	w	–
Glucose	+	+	–	–	–	–	–	–	–	+	–	–
Melibiose	+	+	–	–	w	–	–	+	–	+	+	–
Arabinose	+	+	–	+	+	–	–	+	–	–	–	–
Galactose	+	+	–	–	–	–	–	+	–	+	–	–
Mannose	w	+	–	–	–	–	–	+	–	+	–	–
D-Lyxose	+	–	–	–	–	–	–	w	–	w	–	–

Abbreviations: +, positive reaction; w, weakly positive; –, negative reaction.

Table 2. Susceptibility of the KNF2022^T isolate and *Acinetobacter* species to antibiotics.

Susceptibility to:	KNF2022^T	*A. bayli* KCTC 12413^T	*A. towneri* KCTC 12419^T	*A. radioresistens* KCTC 12411^T	*A. calcoaceticus* KCTC 2357^T	*A. baumannii* KCTC 2508^T	*A. gerneri* KCTC 12415^T
Chloramphenicol	+	+	+	+	+	+	v
Vancomycin	–	–	+	v	+	–	v
Ampicillin	+	+	+	+	+	–	+
Penicillin G	–	+	+	v	+	–	+

Abbreviations: +, positive; –, negative; v, variable.

the reversed-phase high-performance liquid chromatography (HPLC) method described by Tamaoka and Komagata [24].

16S rRNA Sequence Analysis

Two universal primers (9F and 1492R) described by Stakebrandt and Liesack [23] were used for the PCR amplification of the 16S rRNA gene, and the amplified PCR product was purified using a QIAquick PCR purification kit (Qiagen). The purified 16S rRNA gene was then sequenced using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems), with an automatic DNA sequencer (Model 377; Applied Biosystems).

Phylogenetic Analysis

Nearly complete 16S rRNA sequences (1,410 bp) were determined for isolate KNF2022^T, and aligned with the 16S rRNA and rDNA sequences of representatives from the genus *Acinetobacter* and related taxa using the PHYDIT program (available at http://plaza.snu.ac.kr/~jchun/phydit/). A phylogenetic tree was constructed using the neighbor-joining method [18] based on distance matrix data. The evolutionary distances were calculated using the Jukes and Cantor model [9]. The PHYLIP software package [6] was used for all the analyses. The topology of the phylogenetic tree was evaluated using a bootstrap analysis [5] of the neighbor-joining method based on 1,000 replications.

DNA-DNA Hybridization

The DNA-DNA hybridization was determined by the membrane filter technique using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Molecular Biochemicals, Germany). The signal intensities were determined using the TINA 2.0 program. The signal produced by self-hybridization was taken as 100%, and percentage homology values were calculated from duplicate samples.

Results and Discussion

During the screening of microorganisms for antiviral materials inhibiting tobacco mosaic virus (TMV) infection, the culture filtrate of isolate KNF2022^T was found to significantly suppress the virus infection on tobacco plant, *Nicotiana tabacum* cv. Xanthi nc., a local lesion host of TMV. The culture filtrate of isolate KNF2022^T was treated onto the upper surface of tobacco leaves, *N. tabaccum* cv. Xanthi nc., at the same time of or 24 h before TMV inoculation, and local lesion formation on the leaves was inhibited completely.

Isolate KNF2022^T was an aerobic, nonmotile, Gram-negative organism. The colonies were circular, convex, smooth, and slightly opaque with entire edges on TSA. Cells were rods of about 0.4–0.5 µm in width by 0.7–1.0 µm in length at 24 h incubation on TSA, and coccoid cells occurred in old culture. The isolate did not grow in an anaerobic chamber at 37°C. Growth occurred at 10–42°C, and the optimal growth temperature was 25–30°C. Morphologic, physiologic, and chemotaxonomic characteristics and the susceptibility to antibiotics of the isolate and the other *Acinetobacter* species are shown in Tables 1 and 2, respectively.

The whole-cell fatty acid profiles for the isolate and reference strain, *A. bayli* KCTC 2413^T, are shown in Table 3. The major fatty acids in the isolate were 16:0, 18:1 w9c, and summed feature 3. The major quinone of isolate KNF2022^T was Q-9. The DNA base composition of isolate KNF2022^T was 44 mol%.

The primary structure of the 16S rRNA sequence of the strain was compared with those of closely related reference strains. A phylogenetic tree, based on the K_{nuc} value (Fig. 1), indicated that isolate KNF2022^T belonged to the genus *Acinetobacter*. The GenBank accession numbers for the 16S rRNA sequences used for the phylogenetic analysis are shown in Fig. 1.

The isolate KNF2022^T was phylogenetically closely related to *A. bayli* B2^T (98.1% sequence similarity). The levels of sequence similarity between the isolate and other *Acinetobacter* species ranged from 94.4% to 97.9%.

The DNA-DNA hybridization values between isolate KNF2022^T and reference strains, *A. bayli* KCTC 12413^T, *A. calcoaceticus* KCTC 2357^T, and *A. baumannii* KCTC 2508^T were 37%, 29%, and 33%, respectively. The phylogenetic definition of a species generally includes "strains with approximately 70% or greater DNA-DNA relatedness" [17, 21, 22, 25]. Therefore, the phylogenetic and DNA-DNA hybridization results demonstrated that the isolate was phylogenetically closer to the genus *Acinetobacter* and not related to any previously described *Acinetobacter* taxa at the species level.

Based on the above results, a new species *Acinetobacter antiviralis* sp. nov. with type strain KNF2022^T (=KCTC 0699BP^T) is proposed.

The new *Acinetobacter* species is described below.

Table 3. Cellular fatty acid composition of the KNF2022^T isolate and *Acinetobacter* species.

Fatty acid	KNF 2022^T	*A. bayli* KCTC 12413^T	*A. towneri* KCTC 12419^T	*A. ursingiii* KCTC 12410^T	Genomic species TU15 KCTC 12687	*A. radioresistens* KCTC 12411^T	*A. johnsonii* KCTC 12405^T	*A. calcoaceticus* KCTC 2357^T	*A. lwoffii* KCTC 12407^T	*A. baumannii* KCTC 2508^T	*A. gerneri* KCTC 12415^T	*A. tandoii* KCTC 12417^T
12:0	10.4	5.4	9.6	7.4	4.5	7.3	9.5	3.7	6.3	7.6	4.3	6.8
12:0 2OH	1.2	1.7	1.1	1.8	tr	2.7	1.2	1.3	tr	3.0	4.7	1.4
12:0 3OH	4.2	4.3	6.9	3.7	2.8	5.9	7.8	3.0	4.4	4.7	6	5.1
14:0	tr[a]	tr	1	1.2	tr	1.6	tr	tr	tr	1.3	tr	tr
Summed feature 2[b]	2.9	1.9	tr	4.2	3	2.6	tr	2.1	tr	4.8	1.3	tr
16:1 N alcohol	1	1.5	tr	ND	ND	ND	ND	1.5	ND	ND	ND	1.2
16:1 ω9c	ND[c]	1.1	tr	tr	ND	tr	ND	tr	ND	1.2	tr	ND
Summed feature 3[b]	22.4	18.7	14.1	14.6	30.9	18.1	34	17.4	38.5	5.2	15.3	20.8
16:0	24.9	23.3	17.1	18.3	20.9	19.5	18.7	19.5	18.5	18.1	19.6	22.7
18:1 ω9c	21.7	35.2	44.3	44.3	28.1	37.2	19	30.6	24.1	44.6	41.9	30.4
18:1 ω7c	4.6	tr	tr	tr	2.7	1.1	4.5	2.3	3.9	tr	1	3
18:0	2.6	2.9	1.6	tr	3.1	tr	1.4	2.8	1.4	1.6	2.9	3.1

[a]tr, Trace (<1%).
[b]Summed features represent groups of two or three fatty acids that could not be separated by gas-liquid chromatography with the MIDI system. Summed feature 2 contained one or more of the following fatty acids: 12:0 ALDE? and/or 16:1 iso I/14:0 3OH. Summed feature 3 contained one or more of the following fatty acids: 16:1 ω7c and/or 15:0 iso 2OH.
[c]ND, not detected.

Description of *Acinetobacter antiviralis* sp. nov.

Acinetobacter antiviralis (an.ti.vi.ra' lis. Gr. prep. *anti* against, N. L. adj. *viralis* pertaining to virus, N. L. masc/ fem. adj. *antiviralis* antiviral) is an aerobic, nonmotile, Gram-negative organism. In young cultures, cells are rods of about 0.4−0.5 μm in width by 0.7−1.0 μm in length. In old cultures, rods become shorter or spherical elements. It forms circular, convex with entire margins, moist, shiny and light yellow-colored colonies. The strain does not grow in an anaerobic chamber at 37°C. The temperature range for growth is 10−42°C with optimal growth at 30°C. The pH range for growth is 6.0−8.0 with optimal growth at 7.0. No growth occurs with a NaCl concentration of more than 5%, whereas optimal growth occurs without NaCl concentration. The strain gives positive results for urease, catalase, citrate utilization, acetoin production, assimilation of citrate, and fermentation from glycerol, L-arabinose, ribose, D-xylose, galactoside, glucose, melibiose, rhamnose, and D-lyxose. However, it gives negative results for arginine dihydrolase, oxidase, nitrate reduction, gelatin hydrolysis, β-galactosidase, assimilation of gluconate, adipic acid, and phenyl-acetate, and fermentation from L-xylose, adonitol, β-methyl-D-xyloside, fructose, sorbose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, arbutin, salicin, inuline, melezitose, raffinose, xylitol, gentibiose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate, and 5-ketogluconate. It gave positive reactions for the susceptibility to ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin, rifampicin, streptomycin, tetracycline, and ticarcillin (clavulanic acid). However, it gave negative reactions for the susceptibility to vancomycin and penicillin.

The G+C content in the DNA is 44 mol%, the major isoprenoid quinone is Q-9, and the major cellular fatty acids are 16:0, 18:1 w9c, and summed feature 3.

Source: isolated from tobacco plant roots, Daejeon, Korea. The type strain is KNF2022^T (=KCTC 0699BP^T).

The GenBank accession number for the 16S rRNA sequence of *Acinetobacter antiviralis* KNF2022^T is DQ314740.

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