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EXPERIMENTAL ARTICLES

Advenella kashmirensis subsp. *methylica* PK1, a Facultative Methylotroph from Carex Rhizosphere

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Abstract—The strain (PK1) of facultative methylobacteria growing on methanol as a carbon and energy source was isolated from carex rhizosphere (Pamukkale National Park, Turkey). The cells were nonmotile gram-negative rods propagating by binary fission. The organism was a strict aerobe, oxidase- and catalase-positive. Optimal growth occurred at 29°C, pH 8.0–8.5, and 0.5% NaCl; no growth occurred at 2% NaCl. The organism used the ribulose bisphosphate pathway of C_1 assimilation. Predominant fatty acids were 11-octodecenoic (18:1ω7) and *cis*-hexadecenoic (16:1ω7c). Phosphatidylethanolamine and diphosphatidylglycerol were the dominant phospholipids. Q8 was the main ubiquinone. The DNA G+C content was 55.4 mol % mp. Sequencing of the 16S rRNA gene revealed that strain PK1 belonged to the genus *Advenella* with 98.8 and 99.2% similarity to the type strains *A. incenata* CCUG 45225^T and *A. kashmirensis* WT001^T, respectively. DNA–DNA homology of strain PK1 and *A. kashmirensis* WT001^T was 70%. While the MALDI analysis confirmed their close clusterization, RAPD analysis revealed the differences between strain PK1 and other *Advenella* strains. Based on its geno- and phenotypic properties, the isolate PK1 was classified as *A. kashmirensis* subsp. *methylica* PK1 (VKM-B 2850 = DSM 27514), the first known methylotroph of the genus *Advenella*.

Keywords: *Advenella*, facultative methylotroph, ribulose bisphosphate pathway

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The genus *Advenella* of the family *Alcaligenaceae* [1] was proposed by Coenye et al. [2] for description of gram-negative rod-shaped or coccoid bacteria growing at 3% NaCl in the medium and isolated from different clinical samples (veterinary and human). Later, the species *A. kashmirensis* WT001^T [3] and *A. mimigardefordensis* DPN7^T [5] isolated from orchard soil and compost, respectively, were described and reclassified from the genus *Tetrathiobacter* [3] to the genus *Advenella* [4]. *A. kashmirensis* WT001^T is a facultative chemolithotroph utilizing tetrathionate and thiosulfate as energy sources and growing at 6% NaCl. *A. mimigardefordensis* DPN7[7] can also grow on sulfur compounds, in contrast to *A. faeciporci* M-07^T isolated from the activated sludge of a bioreactor from piggery wastewater treatment (Japan) [6] and capable of growth at up to 4% NaCl. However, representatives of the genus *Advenella* capable of methylotrophic growth have not been described previously.

The unique natural object of Turkey, Pamukkale (meaning "cotton castle" in Turkish), is listed as a UNESCO World Heritage Site (1988). This complex includes a system of terrace water reservoirs formed by carbonate minerals (travertine) and 17 thermal springs with the water temperature of 35–100°C.

The goal of the present work was to provide taxonomic, physiological, and biochemical characterization of the first methylotrophic representative of the genus *Advenella* isolated from the rhizosphere of *Carex* sp. from Pamukkale.

MATERIALS AND METHODS

Research subject and cultivation conditions. The *Carex* sp. root taken under sterile conditions from water (37°C) on the territory of Pamukkale was placed into an Erlenmeyer flask (750 mL) with 200 mL of the medium K and 0.5% of methanol [7]. After three transfers for 3 days on a shaker (180 rpm) at 29°C, the suspension of the enrichment culture was plated to obtain single colonies on the agarized (Difco, United States, 2%) medium K with methanol. Separate colonies were transferred to agar slants, passed into a liquid medium, and again plated on the agar medium. The colony was then transferred to an agar slant. The purity of the isolated culture was confirmed by light and electron microscopy, as well as by homogeneity of the colonies grown on agar media with methanol or glucose/peptone.

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The collection type strains *A. kashmirensis* WT001^T and *A. incenata* CCUG 45225^T, as well as *A. incenata* 4GA2008, were used as reference strains for DNA–DNA hybridization, RAPD, and MALDI analysis.

Determination of the cultural, physiological, and biochemical properties of the isolate. For description of the colonies and studies of cell morphology and motility, the strain PK1 was grown on the K solid medium. The ability of the isolate to reduce nitrates was analyzed by the API 20NE tests and by incubation or 1, 2, or 3 days in the liquid medium K, where KNO_3 (1 g/L) was substituted for ammonium nitrogen. Indole production from tryptophan was assayed colorimetrically for the culture grown in the K medium with methanol and $(NH_4)_2SO_4$ replaced by KNO_3 (1 g/L), supplemented with 0.1% (w/v) L-tryptophan, using the Salkowski reagent [8]. The calibration curve was plotted using standard indole-acetic acid solutions. Starch hydrolysis was assessed by the reaction with Lugol's iodine solution after growing the culture on the K agar medium supplemented with soluble starch (0.2%, w/v). The presence of oxidase was detected using 1% (w/v) solution of tetramethyl-*p*-phenylenediamine dihydrochloride. Catalase activity was detected by pouring 3% hydrogen peroxide solution onto bacterial colonies on a solid medium.

The growth at different temperatures and pH values was analyzed as described previously [9].

Utilization of different organic compounds as a carbon and energy source was studied by replacing methanol in the mineral medium with 0.3% (w/v) of the tested substrates, followed by inoculation with the suspension of the culture washed from the K agar medium and 14-day incubation on a shaker at the optimal temperature. Volatile substrates were added at a concentration of 0.5% by vol. Dichloromethane utilization was determined as described previously [8].

To characterize the spectrum of the substrates and to reveal some biochemical properties of the investigated strain, the API 20E and 20NE tests (Biomerieux, France) were used according to the manufacturer's instructions.

Halotolerance of the isolate was determined in the liquid medium K, with the content of NaCl varying from 0 to 3%.

In the test for alternative nitrogen sources, $(NH_4)_2SO_4$ was replaced by other nitrogen compounds in equimolar amounts. The requirements for vitamins and antibiotic resistance were studied as described previously [10].

Electron microscopy was carried out by the known methods [11]. Enzymatic analysis was performed by the methods described previously [12].

MALDI assay. The MALDI spectrum of the bacteria was obtained using a MALDI-TOF Autoflex speed mass spectrometer (Bruker Daltonik GmbH, Germany) according to the procedure described [13].

Chemotaxonomic analysis. Phospholipid composition of the cells was assayed by thin-layer chromatography [11].

For fatty acid analysis, the bacteria were grown under optimal conditions, and the biomass (30 mg) was sampled in the exponential phase. Fatty acid composition was determined in an AT-5850/5973 GS-MS system (Agillent Technologies, United States) [11].

DNA extraction and assay. DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, United States) according to the manufacturer's instructions. The DNA G+C content was determined by the method of thermal denaturation in a Beckman DU-8B spectrophotometer (United States) at a heating rate of 0.5°C/min. The DNA of *Escherichia coli* K-12 was used as the standard. The level of DNA–DNA homology between the isolate and the reference cultures was determined by the method of DNA–DNA reassociation [14].

PCR amplification was performed using a MJ Mini DNA thermocycler (BioRad, United States). The 16S rRNA gene was amplified by PCR using the universal prokaryotic primers for the 16S rDNA: 27f: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r: 5'-AAGGAAGGTGATCCAGCTCGT-3' [15].

The fragment (550 bp) of the *mxa*F gene encoding the large subunit of the classical pyrroloquinoline quinone- (PQQ) dependent enzyme methanol dehydrogenase of gram-negative bacteria was amplified using the primers 1003f and 1561r, according to the protocol described previously [16].

Random amplified polymorphic DNA (RAPD) assay was carried out using the primer OPQ6 (GAGCGCCTTG) under the following conditions: 1 cycle— 95°C, 3 min; 30 cycles— 95°C, 1 min; 36°C, 1 min; 72°C, 90 s; the last cycle— 72°C, 5 min. The reaction mixture (15 μL) contained: double distilled water, 10 μL; ×10, PCR buffer, 2.2 μL; genomic DNA, 1 μL (10–100 ng); the mixture of dNTP, 0.2 mM; the primer, 4 μmol; and *Taq* DNA polymerase, 1 U.

Reaction products were separated by electrophoresis on 1% agarose gel. DNA fragments were isolated and purified from low-melting agarose using the ZymoClean Gel DNA Recovery Kit D4002 (Zymo Research, United States) according to the manufacturer's instruction. PCR fragments were sequenced with BigDye® Terminator v. 1.1 kits and an ABI PRISM® capillary analyzer (Applied Biosystems, United States).

Phylogenetic analysis. Preliminary screening for the similarity of the 16S rRNA gene sequences of the studied strains was performed as described [17].

The rooted phylogenetic tree was constructed by the neighbor-joining method (NEIGHBOR) using the TREECON software package [18]. Evolutionary distance was calculated as a number of substitutions per 100 nucleotides. The branching order was deter-

mined by bootstrap analysis of 100 alternative trees using the respective function of TREECON.

RESULTS AND DISCUSSION

Morphology. The strain PK1 was represented by nonmotile short rods (0.3–0.35 × 0.4–0.45 μm) with the gram-negative type of the cell wall. Capsules and spores were not formed. Reproduction occurred by binary fission. Small beige colonies were formed on a solid mineral medium with methanol, while yellowish, transparent, circular colonies with a flat profile, entire edges, smooth glistening surface, and homogenous structure developed on a rich peptone-containing medium.

Cultural, physiological, and biochemical properties. The strain grew in the K liquid medium with methanol, without cell aggregation and pigment formation. It was strictly aerobic; the growth was stimulated by folic acid. Growth occurred on methanol, formate, sodium acetate, glucose, arabinose, rhamnose, xylose, ribose, malate, fumarate, succinate, lactate, α-ketoglutarate, citrate, serine, alanine, glutamate, glycine, glucuronic acid, as well as on nutrient agar and wort agar. Weak growth was observed on methylamine, urea, isocitrate, and raffinose. No growth occurred on dimethylamine and trimethylamine, dichloromethane, dimethyl sulfoxide, mannitol, sorbitol, melezitose, thiosulfate, and tetrathionate. Soluble starch but not gelatin was hydrolyzed. The organism was oxidase- and catalase-positive. Glucose, rhamnose, and melibiose were fermented. The β-galactosidase, urease, and ornithine decarboxylase activities were not detected. Ammonium salts, nitrates, and some amino acids were used as nitrogen sources. Indole derivatives (~3.8 μg/mL of the culture liquid of the culture with OD_{600} = 1.0) were formed in the medium with nitrate as a nitrogen source, 0.5% methanol and 0.1% tryptophan. Growth occurred at 16–37°C and pH 6.5–9.0. Growth temperature optimum was 29°C; pH optimum was 8.0–8.5. The optimal NaCl concentration in the medium was 0.5%. Growth was inhibited by 2% NaCl. Phosphatidylethanolamine and diphosphatidyl glycerol were predominant in the phospholipid composition of the cells. The dominant fatty acids were 11-octadecanoic (18:1ω7, 31.3%) and *cis*-9-hexadecanoic (16:1ω7c, 29.2%), as well as hexadecanoic (16:0, 17.3%) (Table 1). The major quinone of the strain PK1 was ubiquinone Q_8. The strain was sensitive to gentamycin, resistant to oxacillin, kanamycin, novobiocin, erythromycin, neomycin, nalidixic acid, penicillin, chloramphenicol, tetracycline, and streptomycin.

Enzymological analysis (Table 2) revealed the activity of methanol dehydrogenase (PMS). In spite of this fact, it was impossible to amplify the fragment of the *mxaF* gene of the large subunit of methanol dehydrogenase in the strain PK1. The following activities were detected: formaldehyde dehydrogenases (PMS- and NAD^+-dependent, stimulated by reduced glu-

Table 1. Fatty acid composition of the cells of the strains under study (% of total content) grown on a peptone-containing medium

Fatty acids	Strain PK1	*A. kashmirensis* WT001^T
12:0	–	3.12
14:0	0.50	0.18
16:1ω7c and/or 15:0 iso 2-OH	29.20	28.02
16:1ω7t	0.6	–
16:00	17.30	21.69
14:0 3-OH and/or 16:1 iso I	5.20	10.13
17cyc	6.90	3.53
17:0	–	0.27
18:1ω7	31.30	28.31
18:00	1.60	1.86
16:0 3-OH	1.00	0.80
19cyc	6.40	–
19:0 cyc ω8c	–	1.32
19:0 10 methyl	–	0.18
20:1ω7c	–	0.34
Unknown	–	0.24

Table 2. Enzyme activities in the cell extract of strain PK1 grown on methanol

Enzyme	Cofactor	Activity, nmol/min^{-1} mg^{-1} protein
Methanol dehydrogenase	PMS	112
Formaldehyde dehydrogenase	PMS	42
	NAD^+	
	NAD^+, GSH*	3
		25
Formate dehydrogenase	PMS**	33
	NAD^+	10
Hydroxypyruvate reductase	NADPH	53
	NADH	0
Serine glyoxylate aminotransferase	NADPH	30
	NADH	0
Ribulose 1,5-bisphosphate carboxylase		56
Hexulosephosphate synthase	–	0
Glucose-6-phosphate dehydrogenase	NAD^+	6
	NADP	0
6-Phosphogluconate dehydrogenase	NAD^+	20
	NADP	0
α-Ketoglutarate dehydrogenase		30
Isocitrate dehydrogenase	NAD^+	6
	NADP	46
Glutamate dehydrogenase	NADH	0
	NADPH	16
Glutamate synthetase	ATP, Mn^{2+}	3
Glutamate synthase	NADH	0
	NADPH	0

* GSH, reduced glutathione.

** PMS, phenasine methosulfate.

tathione), formate dehydrogenases (PMS and NAD^+), and ribulose 1,5-bisphosphate carboxylase, the key enzyme of the ribulose bisphosphate pathway (RuBP). The NADPH forms of the serine pathway enzymes—hydroxypyruvate reductase and serine glyoxylate aminotransferase—were detected, but the NADH forms were not. Activities of hexulosephosphate synthase, the key enzyme of the ribulose monophosphate (RMP) pathway, glucose-6-phosphate dehydrogenase (NAD^+) and 6-phosphogluconate dehydrogenase (NAD^+) were absent. Glutamate dehydrogenase (NADPH) activity was detected. The activities of the glutamate cycle enzymes (glutamate synthase and glutamate synthetase) were absent or minor. Therefore, the strain PK1 oxidized methanol to CO_2 and autotrophically assimilated it in the carboxylation reaction of the RBP pathway of C_1 assimilation. Nitrogen assimilation occurred via reductive amination of α-ketoglutarate.

RAPD and MALDI analyses. RAPD analysis showed rather heterogeneous profiles of representatives of the genus *Advenella*: the profiles of *A. kashmirensis* WT001^T and *A. incenata* 4GA2008 were obviously similar, while the profile of the strain PK1 differed from all other profiles in molecular mass and by having a greater number of bands. Nevertheless, the strain PK1 clustered together with *A. kashmirensis* WT001^T on the dendrogram of the results of MALDI analysis (Fig. 1).

Genotypic characterization. The 16S rRNA gene sequencing demonstrated that the strain under study was related to the *Betaproteobacteria* and showed 96.6–99.2% similarity to the known species of the genus *Advenella* (Fig. 2). The level of DNA–DNA homology for the strain PK1 and the type strain *A. kashmirensis* WT001^T (70%) indicated that these strains belonged to the same species. The DNA G+C content was 55.4 mol %. In spite of the detected methanol dehydrogenase activity, it was impossible to

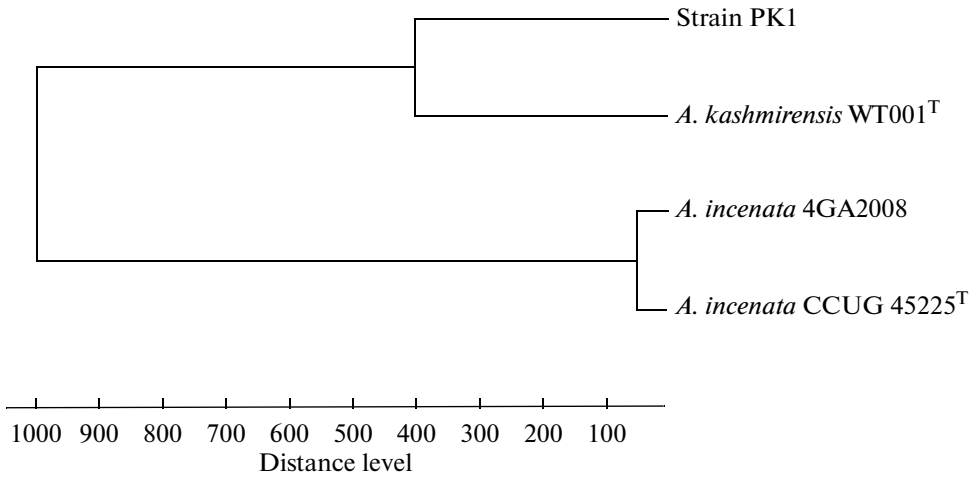

Fig. 1. The dendrogram of *A. kashmirensis* subsp. *methylica* PK1 plotted on the basis of MALDI analysis.

amplify the fragment of the *mxaF* marker gene of the large subunit of the classical PQQ-dependent methanol dehydrogenase in the strain PK1, which has been extensively used in the past decade for detection of methylotrophy. This fact was accounted for in a number of works showing the ability to utilize methanol spreading beyond the populations with the marker gene *mxaF* [19].

The strain PK1 is the first methylotrophic member of the genus *Advenella*. It may be assumed that this strain has become capable of methylotrophy as a result of horizontal gene transfer.

In turn, methylotrophy made it possible for strain PK1 to switch over to phytosymbiosis, because methanol is a natural plant metabolite and indole derivatives synthesized by the methylotroph (auxins) are

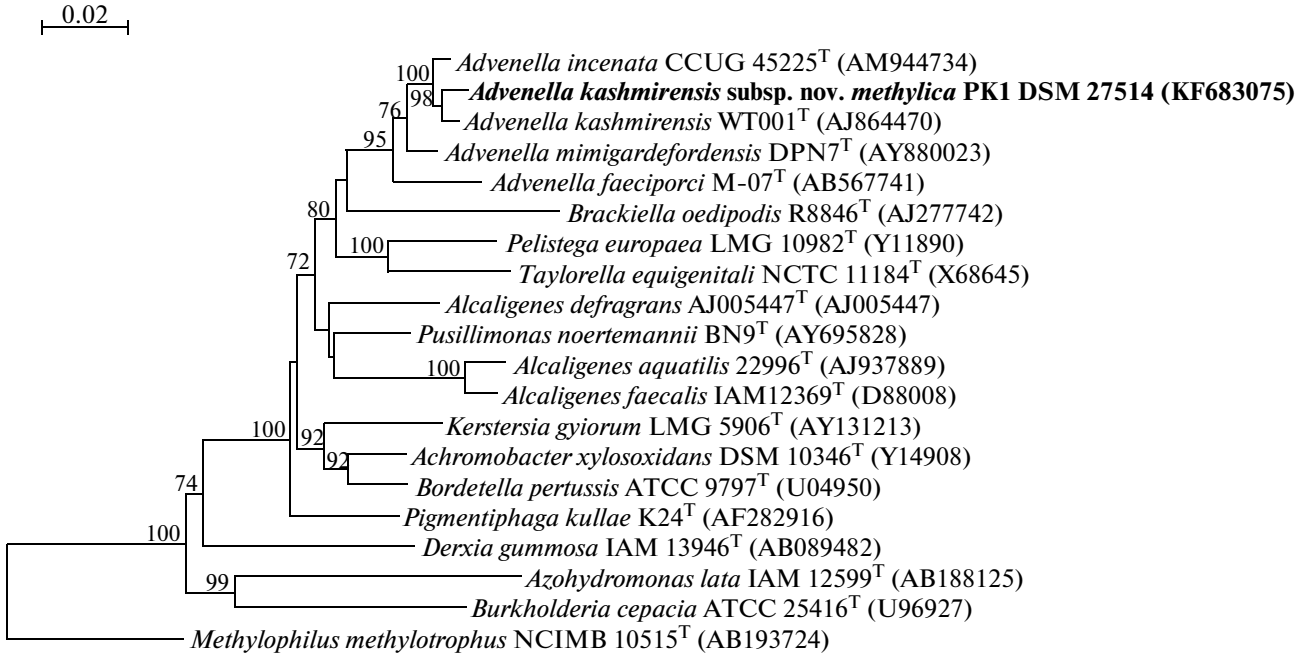

Fig. 2. Phylogenetic position of the strain *A. kashmirensis* subsp. *methylica* PK1 based on the comparison of the 16S rRNA sequences. The scale corresponds to 2 nucleotide replacements per every 100 nucleotides (evolutionary distance). The root was determined by including the sequence of *Methylophilus methylotrophus* as an outgroup. The numerals show statistical significance of the branching order determined by bootstrap analysis of 100 alternative trees.

Table 3. Comparative characteristics of *Advenella kashmirensis* subspecies

Characters	*A. kashmirensis* subsp. *methylica*	*A. kashmirensis* subsp. *kashmirensis*
Nitrate reduction	–	+
Denitrification	±	–
Urease API 20 NE	–	+
Growth substrates:		
Methanol	+	–
Formate	+	+
α-Ketoglutarate	+	–
Lactate	+	–
Thiosulfate	–	+
Tetrathionate	–	+
Starch hydrolysis	+	–
Raffinose	±	–
Succinate	+	+
Glucose	+	+
Fructose	+	+
Arabinose	+	+
Xylose	+	+
Ribose	+	+
Rhamnose	+	+
Glucuronic acid	+	+
Serine	+	+
Glycerol	–	–
Sucrose	–	–
Melezitose	–	–
Sorbitol	–	–
Dulcitol	–	–
Growth at 6% NaCl	–	+
Fatty acids:		
19cyc	+	–
12:0	–	+

phytohormones. Although sedge roots were submerged into saline water with 3–4% NaCl, strain PK1 inhabits within of plant tissues seems, however, to have lost its halotolerance.

In contrast to the type strain *A. kashmirensis* WT001^T, the methylotrophic strain PK1 grows on methanol, α-ketoglutarate, and lactate; it hydrolyzes starch, does not grow chemolithoautotrophically with thiosulfate and tetrathionate, has no urease activity, and does not reduce nitrates to nitrites. *A. kashmirensis* WT001^T grows in the presence of up to 6% NaCl in the growth medium; the strain PK1 endures only the concentrations up to 2% NaCl. The differences were revealed also in the fatty acid profiles. Cyclopropane nonadecanoic acid has been found in strain PK1 (Table 1) but is absent in the profile of *A. kashmirensis* WT001^T.

On the basis of geno- and phenotypic characteristics it was proposed to subdivide the *A. kashmirensis* species into two subspecies, with strain PK1 as a representative of a new methylotrophic subspecies, *A. kashmirensis* subsp. methylia. Capacity for methylotrophy is the key feature differentiating these subspecies. The differentiating characteristics of the subspecies are presented in Table 3 pupublications [3, 4]. Description of the subspecies *Advenella kashmirensis* subsp. *kashmirensis* coincides with the description presented in original publications [3, 4].

The strain PK1 was isolated from the rhizosphere of sedge growing on the territory of Turkey (Pamukkale) and deposited in the All-Russian and German Collections of Microorganisms and Cell Cultures (VKM B-2850 = DSM 27514).

The 16S rRNA gene sequence of the strain PK1 was deposited in the GenBank (access, no. KF683075).

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