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EXPERIMENTAL ARTICLES

A Novel Plant-Associated Thermotolerant Alkaliphilic Methylotroph of the Genus *Paracoccus*

N. V. Doronina and Yu. A. Trotsenko

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

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Abstract—Strain GB isolated from the maize rhizosphere is a gram-negative, aerobic, non-spore-forming, nonpigmented, nonmotile, chemolithotrophic, facultatively methylotrophic bacterium. Cells are cocci or short rods. The strain does not require vitamins. Optimum growth in a medium with methanol occurs at 38–42°C at pH 8.0–9.2. The doubling time is 12 h. In addition to methanol, the bacterium can grow on methylamine, dimethylformamide, acetone, thiosulfate + $NaHCO_3$, and in an atmosphere of H_2 + CO_2 + O_2. Methanol and methylamine are oxidized by the respective dehydrogenases to CO_2 via formaldehyde and formate, respectively. The CO_2 produced is assimilated via the ribulose bisphosphate pathway. Fatty acids are dominated by cyclopropanoic (58–61%), palmitic (24–26%), and octadecanoic (8–9%) acids. The main phospholipids are phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylcholine. The major ubiquinone is Q_{10}. The bacterial genome contains genes controlling the synthesis and secretion of cytokinins. The culture liquid exhibits cytokinin activity. The G+C content of DNA is 62.5 mol %, as determined from the DNA thermal denaturation temperature (T_m). Strain GB shows a moderate degree of DNA–DNA homology (<40%) with the type representatives of the genus *Paracoccus.* Based on the data obtained, the bacterium was classified as a new species of this genus, named *P. kondratievae.*

Key words: *Paracoccus kondratievae* sp. nov., facultative methylotroph, autotroph, ribulose bisphosphate pathway, cytokinins

Methylotrophic bacteria, utilizing methanol as the sole source of carbon and energy, are permanently associated with plants, which produce and excrete this C_1-compound [1]. Pink-pigmentedbacteria of the genus *Methylobacterium* have been revealed in the phyllosphere of many plants [2, 3]. The presence of cytokinins, zeatin and zeatin riboside, in pink methylobacteria [4] suggests that they are phytosymbionts, whose association with plants is mediated by phytohormones and probably by other growth factors. In view of this, of interest is the detection of nonpigmented methylotrophic bacteria in the phyllosphere and rhizosphere of some plants [5].

The present work was aimed at identifying and studying a nonpigmented strain of methylotrophic bacteria isolated from the maize rhizosphere.

MATERIALS AND METHODS

Strain GB was isolated from the rhizoplane of a maize plant picked in the Stavropol region. The medium used for the isolation and cultivation of this strain contained (g/l) KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.125; NaCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.002; yeast extract, 0.1; and methanol, 1 vol % (pH 8.0–9.0). Reference strains of the genus *Paracoccus* used in this study were obtained from K. Suzuki, Japan (*P. denitrificans* ATCC 17441^T, *P. alcaliphilus* JCM 7364^T, *P. aminophilus* JCM 7686^T, and *P. aminovorans* JCM 7685^T), from Y. Katayama, Japan (*P. versutus* IAM 12814^T and *P. thiocyanatus* IAM 12816^T), from F. Rainey, United States (*P. pantotrophus* ATCC 35512 and *P. solventivorans* DSM 6637^T), and from E. Stackebrandt, Germany (*P. marcusii* DSM 11574^T and *P. alkenifer* DSM 11593^T). One of the reference strains, *P. methylutens* VKM B-2164^T, was isolated earlier in our laboratory.

Reference strains were maintained on PYG agar medium containing (%) peptone, 0.5; yeast extract, 0.5; glucose, 0.5; and agar, 2 (pH 7.5). *P. alcaliphilus* and, in some cases, strain GB were grown on medium D (pH 9.0) [6]; *P. solventivorans* was grown on a medium with acetone [7].

The investigation of morphological, physiological, and biochemical characteristics and the determination of quinones, fatty acids, phospholipids, and enzyme activities were performed as described earlier [8].

DNA was isolated by the method of Marmur [9]. The G+C content of DNA was determined by the thermal denaturation method on a DU-8B spectrophotom-

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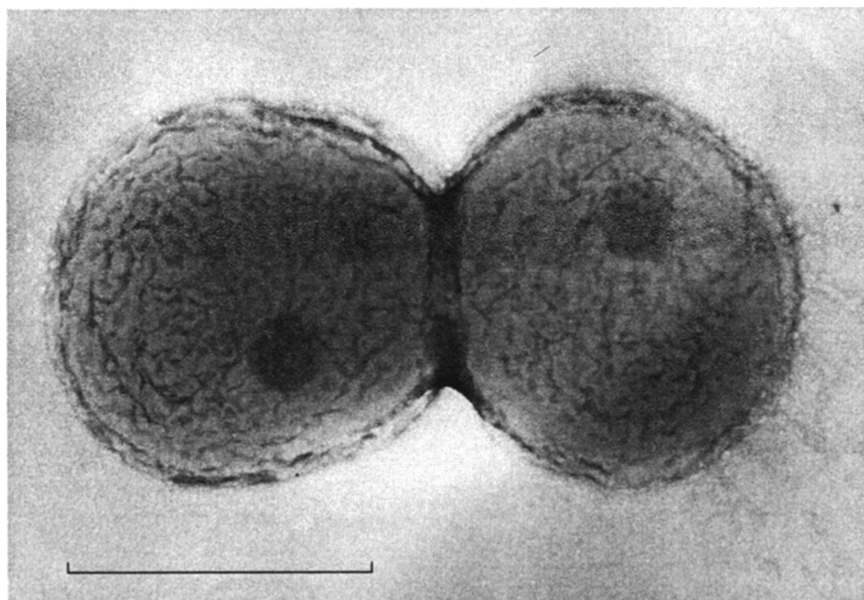

Morphology of strain GB cells (negative standing). Bar represents 0.5 μm.

eter (Beckman, United States), at a heating rate of 0.5 deg/min. For a more precise determination of the DNA thermal denaturation temperature (T_m), relevant measurements were carried out at a heating rate of 0.1 grad/min within a temperature range of 2°C around the rough estimate of T_m. T_m values were determined from the maximum of the first derivative of the thermal denaturation curves. The G+C content of DNA was calculated by the formula G+C = $T_m \times 2.08 - 106.4$ (mol %) [10].

The degree of DNA–DNA homology was evaluated from the DNA reassociation rates [11] under conditions described earlier [12].

The PCR-based analysis of genes controlling the synthesis and secretion of cytokinins was carried out using the following primers: 5'-GTTGATCGTGTGCAATGCTGT-3' (primer 1) [13] and 5'-ATTGAGAAGCGAAATCGACCC-3' (primer 2) [14]. DNA was isolated and PCR amplifications were conducted as described earlier [5], using the DNA of *Agrobacterium tumefaciens* as the positive control. PCR amplification products were analyzed by electrophoresis in 2% agarose gel.

The concentration of cytokinins in the culture liquid was estimated by testing the accumulation of amaranthin in the *Amaranthus caudatus* L. seedlings [15].

RESULTS

Morphological, cultural, physiological, and biochemical properties. The cells of strain GB are gram-negative cocci or short rods 0.5–0.6 by 1.3 μm in size. They occur singly, in pairs (see figure), or, sometimes, in short chains. Cells are nonmotile and do not form flagella, pigments, spores, or prosthecae. Three-day colonies grown on agar medium with 0.5 vol % methanol and 0.05% yeast extract at 37°C are white, opaque, circular, with even edge and smooth shiny surface, 0.7–1.0 mm in diameter. Two-day colonies grown on nutrient agar distinguihaveshed a larger size (2 mm in diameter) and butyrous consistency.

The isolate was found to be able to obtain energy through both respiration and fermentation. It could grow anaerobically on methanol in the presence of nitrates. Growth occurred at temperatures between 20 and 50°C, with an optimum at 37–42°C. Optimum pH for growth was found to be 8.0–9.0; no growth occurred at pH values lower than 7.0. Growth was completely inhibited by 3% NaCl. Yeast autolysate and vitamin mixture stimulated growth on methanol, although we failed to elucidate the requirements of strain GB for individual vitamins. Utilizable carbon and energy sources are L-arabinose, D-galactose, D-glucose, D-mannose, D-ribose, D-fructose, adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, ethanol, acetone, acetate, malate, α-ketoglutarate, succinate, fumarate, alanine, aspartate, betaine, glutamate, *N,N*-dimethylglycine, sarcosine, serine, methanol, and methylamine. Poor growth was also observed on maltose, arginine, formaldehyde, and formamide. The strain could grow autotrophically in an atmosphere of $H_2 + CO_2 + O_2$ and in Baalsrud medium [16] with thiosulfate and $NaHCO_3$ as the sources of energy and carbon, respectively.

Strain GB showed no growth on thiocyanate and in a methane–air (1 : 1) atmosphere and did not utilize D-xylose, D-lactose, L-rhamnose, raffinose, sucrose, D-trehalose, valine, glycine, tryptophan, di- and trimethylamine, dimethylformamide, dimethylsulfoxide, acetamide, pyruvate, propionate, tartrate, or citrate. Gelatin and starch were not hydrolyzed. The lipase,

indole, methyl red, and Voges–Proskauer tests were negative. The isolate alkalinized litmus milk and produced ammonia (but not H_2S) from peptone. Tests for catalase, oxidase, and urease were positive. Utilizable nitrogen sources were ammonium salts, nitrates, methylamine, urea, aspartate, glutamate, and alanine. The strain was sensitive to gentamicin, kanamycin, streptomycin, and erythromycin taken in the amount of 10 μg/disk and resistant to ampicillin and lincomycin taken in amounts of 2–10 μg/disk.

The major ubiquinone was Q_{10}. Phospholipids were represented by phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and cardiolipin. Fatty acids were dominated by cyclopropanoic ($\Delta C_{19:0}$), palmitic ($C_{16:0}$), octadecanoic ($C_{18:0}$), and *cis*-vaccenic (*cis*-$C_{18:1}$) acids (Table 1).

Table 1. Fatty acid profile of strain GB

Fatty acid	% of total content
$C_{16:1}$	0.1
$C_{16:0}$	25.5
Me-$C_{17:0}$	0.5
$C_{17:1}$	0.3
$C_{17:0}$	0.1
$C_{18:1}$	5.0
$C_{18:0}$	8.8
Me-$C_{19:0}$	0.1
Δ-$C_{19:0}$	59.4

Note: Cells were grown on soy–tryptone agar (pH 8.0) at 38°C for 48 h.

Metabolic characterization. The activities of enzymes of the primary and intermediary metabolism of methanol in cell extracts of strain GB are shown in Table 2. Cells grown in a medium with methanol or methylamine contained inducible PMS-dependent dehydrogenases for the primary oxidation of these substrates. The resultant formaldehyde could be oxidized by NAD^+- and glutathione-dependent formaldehyde dehydrogenase to formate, and then to CO_2 with the involvement of NAD^+- and PMS-dependent dehydrogenases. The presence of phosphoribulokinase and ribulose bisphosphate carboxylase suggests that the CO_2 formed from C_1-substrates is assimilated via the ribulose bisphosphate (RuBP) pathway. The key enzymes of the ribulose monophosphate (RuMP) pathway (hexulose-3-phosphate synthase) and the serine pathway (serine–glyoxylate aminotransferase and malate lyase) were absent. The presence of α-ketoglutarate dehydrogenase implies that the tricarboxylic acid cycle is closed and that the glyoxylate cycle does not function. Cells grown in a medium with ammonium sulfate as the nitrogen source contained glutamate dehydrogenase and the glutamate cycle enzymes glutamate synthase and glutamine synthetase.

Genotaxonomic characteristics. The G+C content of the DNA of strain GB was 62.5 mol %. The degree of DNA–DNA homology with the type representatives of the genus *Paracoccus*, estimated from the DNA–DNA reassociation rates, was 37–42% with *P. denitrificans* and *P. methylutens* and did not exceed 30% with *P. thiocyanatus, P. aminophilus, P. aminovorans, P. solventivorans, P. alcaliphilus, P. alkenifer, P. marcusii*, and *P. pantotrophus*.

The ability of strain GB to synthesize cytokinins was studied with allowance for the relevant data available in the literature concerning the well-studied *tmr* and *tzs* cytokinin genes of *Agrobacterium tumefaciens* [13] and the *ptz* gene of *Pseudomonas syringae* pv. *savantanoi* [14] and their products Ipt, Tzs, and Ptz. The 5'-terminal nucleotide sequences of these genes are highly homologous, as well as the *N*-terminal amino acid sequences of their protein products. The *C*-terminal regions of the cytokinins Ipt, Tzs, and Ptz and the 3'-terminal regions of their genes are strongly variable. However, the 25–38 and 92–104 amino acid sequences are conservative, and the 31–38 and 96–104 sequences completely coincide. The oligonucleotide primer 1 used in this work encodes the 31–38 amino acid sequence of the product of the *tzs* gene; the nucleotide sequence of primer 2 is complementary to the region of this gene coding for the amino acids 98–104 of Tzs. In these regions, the *ptz, tzs*, and *tmr* genes, as well as their products Ptz, Tzs, and Ipt, are completely homologous (100%). Therefore, genes controlling the synthesis and secretion of cytokinins can be analyzed simultaneously.

The PCR-based analysis of the genome of strain GB showed that it contains genes responsible for the synthesis and secretion of cytokinins. The biotest for the formation of amaranthin in *Amaranthus caudatus* L. seedlings showed that the culture liquid of strain GB possessed cytokinin activity.

DISCUSSION

Representatives of the genus *Paracoccus* are gram-negative cocci or short rods capable of aerobic growth on a wide range of one- and polycarbon compounds [6, 8, 17–24]. Some species of this genus can grow anaerobically with nitrate as the electron acceptor. Facultatively chemolithotrophic species of the genus *Paracoccus* can utilize reduced sulfur compounds and molecular hydrogen as energy sources. Members of this genus are mesophilic, nonhalophilic, and usually neutrophilic. The major ubiquinone is Q_{10}. The G+C content of DNA is 64–70 mol % [20, 22].

Taken together, the morphological, physiological, and biochemical properties of strain GB allow it to be assigned to the genus *Paracoccus*, within which 13 species are presently known, namely, *P. denitrificans* [17], *P. alcaliphilus* [6], *P. aminophilus, P. aminovorans* [18], *P. kocurii* [19], *P. solventivorans* [7], *P. methylutens* [8], *P. thiocyanatus, P. versutus* [20], *P. alkenifer* [21], *P. marcusii* [22], *P. pantotrophus* [23],

Table 2. Activities of some enzymes in extracts of strain GB cells (nmol/(min mg protein))

Enzyme	Cofactor	Growth substrate		
		methanol	methylamine	glucose
Alcohol oxidase		0	0	0
Methanol dehydrogenase	PMS	40	0	7
Methylamine dehydrogenase	PMS	0	83	0
Formaldehyde dehydrogenase	PMS	0	0	0
	NAD^+	0	0	0
	NAD^+, GSH	60	59	16
Formate dehydrogenase	PMS	60	77	0
	NAD^+	55	49	0
Hydroxypyruvate reductase	NADH	20	33	20
	NADPH	16	13	13
Serine–glyoxylate aminotransferase	NADH	0	0	0
	NADPH	0	0	0
Phosphoribulokinase		184	175	0
Ribulose 1,5-bisphosphate carboxylase		1310	1450	0
Glucose-6-phosphate dehydrogenase	NAD^+	20	22	95
	$NADP^+$	40	38	95
6-Phosphogluconate dehydrogenase	NAD^+	0	0	0
	$NADP^+$	13	15	90
Fructose-1,6-bisphosphate aldolase		20	30	21
Pyruvate dehydrogenase	NAD^+	22	21	24
α-Ketoglutarate dehydrogenase	NAD^+	33	38	31
Isocitrate dehydrogenase	NAD^+	0	0	0
	$NADP^+$	40	40	48
Isocitrate lyase		0	0	0
Citrate synthase		15	15	17
Glutamate dehydrogenase	NADH	0	0	0
	NADPH	49	55	51
Glutamate synthase	NADH	12	10	11
	NADPH	14	15	7
Glutamine synthetase	ADP, Mn^{2+}	62	73	47

and *P. carotinifaciens* [24]. The species *P. thiocyanatus, P. marcusii*, and *P. carotinifaciens* are pigmented; *P. versutus* and *P. carotinifaciens* are motile.

Cells of strain GB have no flagella and do not produce pigments. This strain differs from the known members of the genus *Paracoccus* by its high thermotolerance (growth at temperatures of up to 50°C), prevalence of palmitic (16 : 0) and cyclopropanoic ($\Delta_{19:0}$) acids in the fatty acid profile, and a relatively low G+C content of DNA (62.5 mol %). At the same time, strain GB is similar to the alkaliphilic species *P. alcaliphilus* in having a high of pH growth optimum (8.0–9.0) and to *P. denitrificans* in its abilities to use nitrate as the electron acceptor, to grow autotrophically in the H_2 + CO_2 + O_2 atmosphere, and to utilize thiosulfate as the energy source, as well as in the presence of urease and the absence growth factor of requirements. On the other hand, the degree of DNA–DNA homology of strain GB with these and other species of the genus *Paracoccus* does not exceed 42%. All this allows strain GB to be considered a new species of this genus.

We propose to name this new species *P. kondratievae* in honor of the late Russian microbiologist E.N. Kondratieva, who substantially contributed to the investigation of autotrophic and methylotrophic bacteria. *P. kondratievae* sp. nov. is a typical facultative methylotroph capable of autotrophic utilization of methanol and methylamine, common products of plant metabolism. The presence of genes controlling the synthesis and secretion of cytokinins in this bacterium and the cyto-

kinin activity of its culture liquid suggest that *P. kondratievae* is a plant-associated symbiont. A taxonomic description of *P. kondratievae* sp. nov. is given below.

Paracoccus kondratievae sp. nov. (the species name refers to the Russian microbiologist E.N. Kondratieva). Cells are cocci 0.6 μm in diameter or short rods 0.6 by 1.3 μ in size, occurring singly, in pairs, or in short chains. Gram-negative, nonmotile, nonpigmented, asporogenous. Multiply by binary fission. Alkaliphilic and thermotolerant: growth occurs at 20–50°C and pH 7.5–10.5, optimally at 38–42°C and pH 8.0–9.0. No growth occurs in the presence of 3% NaCl. Aerobe, facultative chemolithotroph and methylotroph. Assimilates C_1-compounds via the ribulose bisphosphate pathway. Utilizable carbon and energy sources are L-arabinose, D-galactose, D-glucose, D-ribose, D-fructose, adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, ethanol, acetone, acetate, malate, α-ketoglutarate, succinate, fumarate, L-alanine, L-aspartate, L-glutamate, sarcosine, *N*,*N*-dimethylglycine, betaine, serine, methanol, methylamine, formaldehyde, $CO_2 + H_2 + O_2$, and thiosulfate + $NaHCO_3$. Methane, di- and trimethylamine, mono- and dichloromethane, dimethylsulfoxide, formate D-xylose, D-lactose, L-rhamnose, raffinose, sucrose, D-trehalose, propionate, citrate, pyruvate, and tartrate do not support growth. Yeast extract (0.01%) stimulates growth. Utilizable nitrogen sources are ammonium salts, nitrates, urea, methylamine, and amino acids. Does not hydrolyze cellulose, gelatin, or starch. The methyl red, Voges–Proskauer, and lipase tests are negative. Indole and hydrogen sulfide are not produced. Alkalinizes litmus milk. Urease-, oxidase-, and catalase-positive. Capable of anaerobic growth in the presence of nitrate. The major phospholipids are phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and phosphatidylcholine. Fatty acids are dominated by cyclopropanoic ($\Delta C_{19:0}$), palmitic ($C_{16:0}$), octadecanoic ($C_{18:0}$), and *cis*-vaccenic (*cis*-$C_{18:1}$) acids. The main ubiquinone is Q_{10}. The G+C content of DNA is 62.5 mol % (T_m). DNA–DNA reassociation values with the type cultures *Paracoccus denitrificans* ATCC 17441^T and *P. methylutens* VKM B-2164^T is about 40%. The type strain *P. kondratievae* GB was isolated from the maize rhizosphere and deposited in the All-Russia Collection of Microorganisms as VKM B-2222.

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