ORIGINAL ARTICLE

Microbacterium lacusdiani sp. nov., a phosphate-solubilizing novel actinobacterium isolated from mucilaginous sheath of *Microcystis*

Bing-Huo Zhang^{1,2}, Nimaichand Salam², Juan Cheng^{1,2}, Han-Quan Li¹, Jian-Yuan Yang¹, Dai-Ming Zha¹, Qi-Gen Guo¹ and Wen-Jun Li^{2,3}

A novel actinobacterium, designated strain JXJ CY 01^T, was isolated from a mucilaginous sheath of *Microcystis aeruginosa* FACHB-905 collected from Lake Dianchi, south-west China. Taxonomic position of the isolate was determined by polyphasic approaches. Strain JXJ CY 01^T shared 16S rRNA sequence similarities of 98.9 and 98.0% with *Microbacterium marinilacus* YM11-607^T and *Microbacterium paludicola* US15^T, and less than 98% with other members of the genus *Microbacterium*. The DNA–DNA relatedness values between strains JXJ CY 01^T and *M. marinilacus* JCM 16546^T and *M. paludicola* JCM 14308^T were 53.5 ± 1.4 and $53.8 \pm 2.1\%$, respectively. L-Ornithine was detected in the cell wall, and rhamnose, galactose, glucose, arabinose, fucose and mannose as signature sugars in the whole-cell hydrolysates. Other chemotaxonomic characteristics determined were MK-12 and MK-11 as predominant menaquinones, anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0} and iso-C_{17:0} as major cellular fatty acids (>10%), and diphosphatidylglycerol, phosphatidylglycerol, an unidentified glycolipid and two unidentified phospholipids as the polar lipids. The DNA G+C content was 70.4 mol%. On the basis of the above taxonomic data, strain JXJ CY 01^T is determined to represent a novel species of the genus *Microbacterium*, for which the name *Microbacterium lacusdiani* sp. nov. is proposed. The type strain is JXJ CY 01^T (= KCTC 29655^T = DSM 29188^T). The type strain JXJ CY 01^T can solubilize both insoluble inorganic (calcium phosphate) and organic phosphate (L- α -phosphatidylcholine) and is possibly one of the mechanism for enhancement of growth of *M. aeruginosa* FACHB-905.

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INTRODUCTION

Eutrophication caused by dumping of agricultural, domestic and industrial waste is a major reason behind harmful cyanobacterial blooms in more than 60% of the freshwater lakes in China.^{1,2} In Lake Dianchi, the largest lake in Yunnan Province, southwestern China and a vital source of water of Kunming City (the capital of Yunnan Province), *Microcystis* especially *Microcystis aeruginosa* was found to be the main algal blooms during warm seasons.³ Mucilaginous investment of *Microcystis* provides an excellent ecological niches for many other bacteria, including Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacteroidetes.^{4–6} Mutualism is one of the main reasons of this coexistence of *Microcystis* and bacteria, whereby the bacteria provide the raw materials to *Microcystis* and are replenished with nutrient and safer environments.^{7–12}

During a study of bacteria associated with mucilage released by *M. aeruginosa* FACHB-905 (MAF) collected from Lake Dianchi, a novel actinobacterium designated strain JXJ CY 01^T belonging to the genus *Microbacterium* was isolated. This manuscript described the

polyphasic characterization of the novel strain. The manuscript also discusses the phosphate-solubilizing property of the strain in light to its *in vitro* interaction with MAF.

MATERIALS AND METHODS

Isolation and maintenance of strain

MAF culture was incubated in HGZ media¹³ under illumination of 30–50 μmol photon per m² per second in a 12-h light/dark cycle till the color turned into green. About 0.2 ml of this green algal culture broth was spread on International *Streptomyces* Project (ISP) medium 2 agar plates,¹⁴ and incubated at 28 °C for 5 days. Bacterial colonies were selected and repeatedly streaked on the same medium to obtain pure cultures. Purified strain was maintained on ISP 2 medium at 28 °C and stored as glycerol suspensions (30%, v/v) at –80 °C.

Phenotypic characterization

Morphology was observed using a light microscope (BX43; Olympus, Tokyo, Japan) and a scanning electron microscope (QUANTA200; FEI, Hillsboro, OR, USA). Gram staining was done by using the standard Gram's staining procedure. Growth at various pH (4.0–11.0), temperatures (4–50 °C) and NaCl contents (0–10%, w/v) were tested according to the method described by

¹College of Life Science, Jiujiang University, Jiujiang, PR China; ²State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, Guangzhou, PR China and ³Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Ürümqi, PR China

Correspondence: Professor W-J Li, State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, No 135, Xingang West Road, Guangdong, Guangzhou 510275, PR China.
E-mail: liwenjun3@mail.sysu.edu.cn

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Xu *et al.*¹⁵ using ISP 2 as the basal growth medium. Catalase activity was assessed from the bubble production on addition of a drop of 3% (v/v) H₂O₂. Oxidase activity was determined according to Kovacs.¹⁶ Other phenotypic characteristics were determined according to Goodfellow¹⁷ and Williams *et al.*¹⁸ Enzyme activities were measured by using the commercial API ZYM system (bioMérieux, Marcy l'Etoile, France). Assimilation of various substrates was determined using Biolog GN III Micro Plate assays as described by the manufacturer.

Chemotaxonomic analysis

The peptide subunit of peptidoglycan and whole-cell sugars was determined according to the procedures described by Hasegawa *et al.*¹⁹ and Tang *et al.*²⁰ Polar lipids were extracted as described by Minnikin *et al.*²¹ and analyzed according to the method described by Collins and Jones.²² Menaquinones were extracted as described by Collins *et al.*²³ and analyzed using HPLC.²⁴ Cellular fatty acids were extracted, methylated and analyzed by using the microbial identification system (Sherlock Version 6.1; MIDI database: TSBA6).²⁵ Biomass for fatty acid analysis was obtained from cells grown on tryptone soy agar (TSA; Difco, Berkeley, CA, USA) at 28 °C for 4 days. The G+C content of genomic DNA of strain JXJ CY 01^T was measured using HPLC.²⁶

Phylogenetic analysis

The genomic DNA extraction, PCR amplification and sequencing of 16S rRNA gene were performed according to Xu *et al.*¹⁵ The almost-complete 16S rRNA gene sequence of the strain was aligned with sequences of the most closely related taxa using CLUSTAL X version 1.83.²⁷ Phylogenetic dendrograms were generated by the neighbor-joining,²⁸ maximum-likelihood²⁹ and maximum-parsimony³⁰ tree making algorithms using the software package MEGA version 5.0.³¹ The topologies of the phylogenetic trees were evaluated by using bootstrap analysis of Felsenstein³² with 1000 replicates. The genomic DNA–DNA relatedness between strain JXJ CY 01^T and closely related members of the genus *Microbacterium* (*M. marinilacus* JCM 16546^T and *M. paludicola* JCM 14308^T) were performed as described by Ezaki *et al.*³³

Phosphate solubilization

Phosphate solubilization was determined by measuring the amount of soluble phosphorus released from an insoluble phosphate-containing growth (PG) medium (glucose 10.0 g, (NH₄)₂SO₄ 0.5 g, MgSO₄·7H₂O 0.3 g, NaCl 0.3 g, KCl 0.3 g, FeSO₄·4H₂O 0.036 g, MnSO₄·4H₂O 0.03 g, Ca₃(PO₄)₂ 10.0 g or L-α-phosphatidylcholine 2.0 g, distilled water 1 liter, pH 7.0). Aliquots of JXJ CY 01^T culture suspension were inoculated into PG medium to a final cellular concentration of ~1 × 10⁶ CFU ml⁻¹ and incubated for 6 days (28 °C, 180 r.p.m.). Uninoculated PG medium served as control. The cultures were harvested by centrifugation at 4860 g for 20 min. Soluble phosphorus in culture supernatant was estimated using a colorimetric method as described below: Reaction mixture containing 5 ml supernatant, 0.1 ml 2,4-dinitrophenol solution (0.011 M) and 5 ml Mo-Sb reagent solution were adjusted to a final volume of 50 ml with distilled water, briefly mixed and kept incubated at 25 °C for 30 min. Absorbance of the reaction mixture was monitored at 700 nm against a standard of potassium phosphate. The Mo-Sb reagent solution contained (per liter) sulfuric acid 2.87 mol, ammonium molybdate 8.1 mmol, antimonyl potassium tartrate 1.5 mmol and ascorbic acid 85.2 mmol (added into the solution just before use). Concentration of the soluble phosphorus in the culture media was calculated based on the following equation: $X = P \times V_1/V_2 \times K$, where X represent soluble phosphorus in the culture media; P the soluble phosphorus calculated for a reaction mixture; V₁ the total volume of reaction mixture; V₂ the volume of culture supernatant added in the reaction mixture, and K the dilution ratio used for measuring the absorbance.

Effect on the growth of MAF by co-culturing with strain JXJ CY 01^T under *in vitro* condition

Effect of strain JXJ CY 01^T on the growth of MAF was determined by co-culturing them in three different media: HGZ broth¹³ and modified HGZ media (KH₂PO₄ replaced with Ca₃(PO₄)₂ and L-α-phosphatidylcholine respectively) as detailed below. Aliquots of pure cultures of MAF (2 × 10⁶ CFU ml⁻¹)

were inoculated onto the three media. Each of these media was then co-inoculated with three different inoculums of strain JXJ CY 01^T (0.2 × 10⁹, 1 × 10⁹ and 5 × 10⁹ CFU ml⁻¹). For the control, bacterial cell suspension was replaced with sterilized water. The co-cultures were incubated under illumination of 30–50 μmol photon per m² per second in a 12-h light/dark cycle. Aliquots of the cultures were removed on the 7th and 60th days of incubation. From the aliquots, quantities of MAF cells were counted using a light microscope (BX43, Olympus). The amount of soluble phosphorus in the media was also concurrently measured by the above described method.

Statistical analysis

All the values for phosphate solubilization and growth promotion assay were expressed as means of three experimental data. These data were subjected to one-way ANOVA at *P* < 0.05 and *P* < 0.01 using SPSS software version 17 (SPSS, Beijing, China).

RESULTS AND DISCUSSION

Phenotypic characteristics

Cells of strain JXJ CY 01^T were observed to be Gram-staining-positive and non-endospore forming. Cells are short rods with a size of 0.4–0.6 × 0.6–1.1 μm (Figure 1). Growth of strain JXJ CY 01^T was observed at 4–45 °C, pH 6.0–8.0 and 0–5% (w/v) NaCl, with optimal growth at 25–28 °C, pH 7.0–8.0 and 0–2% (w/v) NaCl. The isolate was found to be positive for catalase and oxidase tests. Other differential phenotypic characteristics of strain JXJ CY 01^T and the related type strains *M. marinilacus* JCM 16546^T and *M. paludicola* JCM 14308^T are listed in Table 1, and the detailed characteristics of the proposed novel strain are given in the species description.

Chemotaxonomic characteristics

Strain JXJ CY 01^T contained L-ornithine as the diagnostic amino acid along with alanine, aspartic acid, glutamic acid and glycine in the peptidoglycan, and mannose, rhamnose, glucose, galactose, arabinose and fucose as whole-cell sugars. The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol, an unidentified glycolipid and two unidentified phospholipids (see Supplementary Figure S1). The respiratory menaquinones consisted of MK-12 (70.1%), MK-11 (21.2%) and MK-12 (H₄) (8.7%). The fatty acid methyl ester profiles were anteiso-C_{15:0}

Figure 1 Scanning electron micrograph of strain JXJ CY 01^T after cultured in ISP 2 media for 3 days. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 Differential characteristics of strain JXJ CY 01^T and members of the genus *Microbacterium*

	1	2	3
G+C content (mol%)	70.4	71.6	66.5
NaCl tolerance (% w/v)	0–5	0–9	0–8
Temperature for growth (°C)	4–45	4–37	4–40
pH range for growth	6–8	6–9	6–9
Tween 80 hydrolysis	–	–	+
Starch hydrolysis	+	–	–
<i>Utilization of</i>			
D-Sorbitol	+	–	–
D-Ribose	+	–	–
D-Xylitol	+	–	+
D-Melibiose	–	+	–
L-Cysteine	–	+	+
L-Histidine	–	+	+
L-Valine	–	+	+
D-Arabitol	+	–	–
D-Arabinose	+	+	–
<i>Acid produced from (API 50CH)</i>			
D-Arabinose	–	–	+
L-Arabinose	–	+	–
D-Galactose	–	+	–
D-Glucose	–	+	+
Salicin	+	–	–
Sucrose	+	–	+
Trehalose	–	+	–
D-Turanose	+	–	+
<i>API 20 NE results</i>			
Adipic acid	–	+	+
Citric acid	–	+	–
Gelatin	+	–	–
Malate	–	–	+
Mannose	–	+	+

Abbreviations: +, positive; –, negative.

Strains: 1, JXJ CY 01^T; 2, *M. marinilacus* JCM 16546^T; 3, *M. paludicola* JCM 14308^T. All data are from this study.

(42.9%), iso-C_{16:0} (20.1%), anteiso-C_{17:0} (16.9%), iso-C_{15:0} (11.5%), iso-C_{17:0} (3.3%), iso-C_{14:0} iso (2.1%), anteiso-C_{15:1} A (1.7%) and C_{16:0} (0.9%) (Supplementary Table S1). The G+C content of the genomic DNA was determined to be 70.4 mol%.

Phylogenetic characteristics

Analysis of the almost-complete 16S rRNA gene sequence (1538 bp) revealed that strain JXJ CY 01^T is a member of the genus *Microbacterium*. It shared 16S rRNA gene sequence similarities of 98.8, 98.0, 97.3 and 97.1% with *M. marinilacus* YM11-607^T, *M. paludicola* US15^T, *M. sediminicola* YM10-847^T and *M. bareri* DSM 20145^T, and less than 97% with other members of the genus *Microbacterium*. The strain formed a relatively stable clade with strains *M. marinilacus* YM11-607^T and *M. paludicola* US15^T in the phylogenetic dendrograms based on 16S rRNA gene sequence (Figure 2 and Supplementary Figure S2). DNA–DNA hybridization experiments showed that strain JXJ CY 01^T displayed a DNA–DNA reassociation values of 53.5 ± 1.4 and $53.8 \pm 2.1\%$ with *M. marinilacus* JCM 16546^T and *M. paludicola* JCM 14308^T, respectively (Supplementary Table

S2), thereby indicating that the whole genomic DNA relatedness values are below the 70% delineating limit for species identification.³⁴

The genotypic and phenotypic features described above suggest that strain JXJ CY 01^T could be clearly distinguished from its closest phylogenetic relatives. Besides low DNA–DNA relatedness with the closely related phylogenetic strains, the strain is also distinguished from them by several phenotypic properties as listed in Table 1. Based on the above findings, strain JXJ CY 01^T is considered to represent a novel species of the genus *Microbacterium*, for which the name *Microbacterium lacusdiani* sp. nov. is proposed.

Phosphate solubilization

The cultures in PG media on incubation for 6 days were turbid, indicating that the strains grew well in the phosphate-solubilizing media. The content of soluble phosphorus in the media containing Ca₃(PO₄)₂ and L-α-phosphatidylcholine increased from 0.8 ± 0.1 and $1.2 \pm 0.0 \text{ mg l}^{-1}$ at day 0 to 45.7 ± 0.8 and $5.8 \pm 0.4 \text{ mg l}^{-1}$, respectively, after 6 days incubation. However in the control, there was no increase in the amount of soluble phosphorus in Ca₃(PO₄)₂-containing medium while slight increase of $1.6 \pm 0.1 \text{ mg l}^{-1}$ was observed in the one containing L-α-phosphatidylcholine ($P < 0.01$). The results indicated that strain JXJ CY 01^T could dissolve insoluble phosphorus effectively.

Effect on the growth of MAF by co-culturing with strain JXJ CY 01^T under *in vitro* condition

The effect of cell density of MAF by co-inoculating with different inoculums of strain JXJ CY 01^T on three different media is shown in Table 2. In the absence of any co-inoculums, a 10-fold increase in the cell densities of MAF was observed on day 60 as compared with day 7 when the phosphate source is KH₂PO₄ while a little lesser than 10-fold in medium containing insoluble phosphate (Table 2). This result indicated that soluble phosphorus is more favorable for the growth of MAF. Additionally, MAF growth was better off using both insoluble inorganic and organic phosphate when co-cultured with strain JXJ CY 01^T (Table 2). Inoculum size also gave varying effect on the growth promotion activities. Cell density of MAF was higher with an inoculum size of $0.2 \times 10^9 \text{ CFU ml}^{-1}$ of strains JXJ CY 01^T on day 7 in medium containing KH₂PO₄. Similar pattern was observed in media containing L-α-phosphatidylcholine. On the contrary, higher MAF was observed with a higher inoculum density of strain JXJ CY 01^T in medium containing Ca₃(PO₄)₂ (Table 2). The cell density of MAF in both HGZ and modified HGZ media on day 60 increased with the increase in bacterial inoculums density (Table 2).

The content of soluble phosphorus increased significantly in modified HGZ media after inoculation of strain JXJ CY 01^T (Figure 3; $P < 0.05$, $P < 0.01$). This may partly contribute to the enhanced growth rates of MAF in modified HGZ media. Despite the fact that HGZ media have adequate soluble phosphorus for growth of MAF, co-inoculation of JXJ CY 01^T increased the cell density of MAF significantly (Table 2). This result indicated apart from phosphate solubilization, additional factors may be involved for growth promotion of MAF by strain JXJ CY 01^T. Similar findings have been reported by other research groups working on cyanobacterial mat. Zhao et al.³⁵ found that *Actinobacteria* strain *Gordonia* sp. txj1302RI produce unknown substances that promote the growth of *Microcystis*. de-Bashan et al.³⁶ reported that *Azospirillum* spp. produce indole-3-acetic acid, thereby promoting the growth of *Chlorella vulgaris*. *Microbacterium azadirachtiae* AI-S262^T was reported as a plant-growth-promoting actinobacterium by mechanisms of indole-3-acetic acid production and phosphorus solubilization.³⁷

Figure 2 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strain JXJ CY 01^T and its closely relative species of the genus *Microbacterium*. The strain *Curtobacterium pusillum* DSM 20527^T was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at the nodes. Asterisks indicate branches that were conserved using the maximum-parsimony and maximum-likelihood methods. Bar, 0.005 substitutions per nucleotide position.

Table 2 Cell density (mean ± s.d.; *n* = 3) of MAF after co-culturing with strain JXJ CY 01^T

A	Day 7 ($\times 10^6$ CFU ml ⁻¹)			Day 60 ($\times 10^7$ CFU ml ⁻¹)		
	a*	b*	c*	a*	b*	c*
0.0	9.03 ± 0.33	6.85 ± 0.31	6.72 ± 0.42	9.87 ± 0.32	5.58 ± 0.22	6.38 ± 0.37
0.2	11.40 ± 0.48**	7.83 ± 0.11**	9.35 ± 0.44**	10.34 ± 0.60	10.47 ± 0.51**	9.91 ± 0.40**
1.0	10.63 ± 0.67*	8.33 ± 0.35**	8.25 ± 0.47*	11.64 ± 0.73*	14.04 ± 0.67**	11.07 ± 0.55**
5.0	9.35 ± 0.45	9.40 ± 0.22**	7.25 ± 0.32	13.74 ± 0.53**	16.16 ± 0.80**	15.65 ± 0.99**

Statistical comparisons with the controls were made using ANOVA (**P* < 0.05, ***P* < 0.01). A, initial cell density of JXJ CY 01^T ($\times 10^9$ CFU ml⁻¹).
*Phosphate source in HGZ medium: a, KH₂PO₄; b, Ca₃(PO₄)₂; c, L-α-phosphatidylcholine.

Figure 3 Soluble phosphorus concentrations in HGZ and modified HGZ media after co-culturing of MAF and JXJ CY 01^T for day 7 (a) and day 60 (b). a, b, c and d represents initial cell densities of JXJ CY 01^T corresponding to 0×10^9 , 0.2×10^9 , 1×10^9 and 5×10^9 CFU ml⁻¹, respectively. Statistical comparisons with the controls were made by ANOVA (**P* < 0.05, ***P* < 0.01).

Description of *Microbacterium lacusdiani* sp. nov.

Microbacterium lacusdiani (la.cus.di.a'ni. L. gen. n. lacus, of a lake; N.L. gen. n. diani, of Dian; N.L. gen. n. *lacusdiani*, of Dian lake).

Cells are aerobic, Gram-staining positive, non-spore-forming and short rods ($0.4\text{--}0.6 \times 0.6\text{--}1.1$ μm). Growth is observed at 4–45 °C, pH 6.0–8.0 and 0–5% (w/v) NaCl, with optimal growth at 25–28 °C, pH 7.0–8.0 and 0–2% (w/v) NaCl. Utilizes D-arabinose, D-arabitol,

D-(+)-cellobiose, D-fructose, D-galactose, D-glucose, D-glycerol, L-lactose, D-mannitose, D-ribose, sodium acetate, D-sorbitol, sucrose, D-trehalose, D-xylitol and D-xylose as sole carbon sources, and L-alanine, L-aspartic acid, L-glutamic acid, L-glutamine, glycine, L-leucine, L-isoleucine, L-methionine, L-lysine, L-tyrosine, L-tryptophan, L-proline, L-serine and hypoxanthine as sole nitrogen sources. Does not utilize dulcitol, D-mannose, D-melibiose, D-myo-inositol, D-raffinose, L-rhamnose, sodium propionate, L-cysteine, L-histidine, L-phenylalanine, L-valine or L-threonine as either sole carbon or nitrogen sources. Positive in catalase and oxidase tests but negative in milk coagulation, milk peptonization, nitrate reduction, methyl red, Voges-Prokauer and H₂S production tests. Hydrolyzes gelatin and starch, but not casein or cellulose. Acid is produced from salicin, starch, sucrose and D-turanose (API 50CH). The cell wall peptidoglycan contains L-ornithine along with whole-cell sugars of mannose, rhamnose, glucose, galactose, arabinose and fucose. Polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, an unidentified glycolipid and two unidentified phospholipids. The major menaquinones were MK-11 and MK-12. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0} and iso-C_{15:0}. The G+C content of the genomic DNA of the type strain is 70.4 mol%. It solubilizes insoluble form of both inorganic and organic phosphate.

The type strain, JXJ CY 01^T (= KCTC 29655^T = DSM 29188^T), was isolated from mucilaginous sheath of *M. aeruginosa* FACHB-905, collected from Lake Dianchi, Yunnan province, China. The 16S rRNA gene sequence of strain JXJ CY 01^T has been deposited in GenBank under the accession number KP986565.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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