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Pacificimonas aurantium sp. nov., Isolated from the Seawater of the Pacific Ocean

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Abstract A Gram-negative bacterium, denoted JLT2012^T, was isolated from the surface water of the Pacific Ocean. This aerobic bacterium was rod shaped and devoid of flagella, displayed gliding motility, and grew in characteristic orange colonies. The bacterium contained ubiquinone Q-10 as the major respiratory quinone, and spermidine and spermine as the major polyamine compounds. The dominant fatty acids were C18:1ω7c and/or C18:1ω6c (34.7 %), C16:0 (21.3 %), and C18:0 (15.9 %), whereas the polar lipids consisted mainly of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, four sphingoglycolipids, and several unknown glycolipids. The G + C content DNA was found to be 65.5 mol%. Comparative 16S rRNA gene sequence analysis revealed that strain JLT2012^T formed a distinct lineage within the genus *Pacificimonas* (formerly known as *Pacificamonas*) and shared the highest sequence similarity with the type strain of *Pacificimonas flava* JLT2015^T (96.0 %). Data combined from different studies on the phenotypic, phylogenetic, and genomic characteristics indicated that strain JLT2012^T is a representative of a novel species within *Pacificimonas* for which the name *Pacificimonas aurantium* sp. nov. (type strain JLT2012^T=LMG 27361^T=CGMCC 1.12399^T) is proposed.

Introduction

The genus *Pacificimonas*, belonging to the family *Sphingomonadaceae* [11], was first described by Liu et al. [13] and currently consists of a single species, *Pacificimonas flava*. Members of *Sphingomonadaceae* share several phenotypic traits, including smaller cell size, the presence of yellow colonies, predominant quinone profiles (Q-10), and spermidine as the major polyamine patterns [15, 25]. Species of the family *Sphingomonadaceae* display an oligotrophic strategy with low growth rates in the nutrient-limited marine environment [12]. Oligotrophic bacteria are the major contributors of microbial biomass in oligotrophic marine, and these organisms play vital roles in global cycling of carbon, nitrogen, and other biogeochemical processes [21]. With the development of advanced techniques, novel microbial species from the oligotrophic oceans have been discovered. These microbial species have been reported to contribute to novel metabolic pathways such as *alphaproteobacterium* "*Candidatus Pelagibacter ubique*" (SAR11 clade) and *gammaproteobacterium* HTCC2207 (SAR92 clade) isolates [22, 23]. The genome of *Pacificimonas flava* has been fully sequenced, and the analysis revealed that it contains abundant TonB-dependent transporter genes. The bacteria can adapt efficiently with the marine environment because of the presence of these transporter genes, which allow the bacteria to take up scarce resources from their surroundings [26]. In the present study, strain JLT2012^T was isolated from the surface water of the Pacific Ocean. Based upon the phylogenetic,

The GenBank accession number for the 16S rRNA gene sequence of strain JLT2012^T is JX878395.

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chemotaxonomic, and other phenotypic studies, JLT2012^T was considered to represent a novel species within the genus *Pacificimonas*.

Materials and Methods

Isolation and Cultivation of Strains

Surface seawater samples (at a depth of 50 m) were collected from the Pacific Ocean (102°35′W, 3°41′S). JLT2012^T was screened using the direct plating method. The surface seawater samples (200 µl) were taken and spread on a marine agar 2216 (MA; BD) plate, and the plates were incubated at 30 °C for 3 days. The strain was subsequently isolated and purified as single colonies on MA after incubation at 30 °C for 2 weeks, and stored in 15 % (v/v) glycerol suspensions in liquid nitrogen or at −80 °C. The culture was routinely grown in the marine broth 2216 (MB; BD).

Phenotypic and Chemical Characterization

Colony morphology and pigmentation were observed after cultivation of JLT2012^T on MA at 25 °C for 2 days. The cells were grown on MA for 2 days at 30 °C, following which, the cell size and morphology were observed under the transmission electron microscope (JEM-1230; JEOL USA) (Supplementary Fig. S1). A Gram-staining reaction was performed as described by Gerhardt et al. [5]. The gliding motility was determined by a semi-solid agar puncture method [3]. Hydrolysis of casein, starch, and gelatin was determined according to the method described by Smibert & Krieg [20]. Oxidase, catalase activity, and other biochemical and physiological properties were performed with the API 20E, API 20 NE, and API ZYM strips (bioMérieux) according to the manufacturer's protocol. Bacterial growth on sole carbon sources and nitrogen sources was tested by using Biolog GN2 microplates described by Rüger and Krambeck [18]. The presence of polyhydroxyalkanoates (PHAs) granules in the bacterial cells were studied with the Nile blue A staining method [17]. The antimicrobial agent susceptibility tests were performed by using the disk diffusion plate method described by Liu et al. [13].

Bacterial growth at various NaCl concentrations was investigated on the MB medium, with final NaCl concentrations of 0, 0.5, and 1.0–13.0 %, at intervals of 1.0 % (w/v) (at pH 7.8, 25 °C). Specifically, all the other components of the MB kept constant, and only the NaCl content was adjusted accordingly. The bacterial growth at different temperatures (4, 10, 20, 25, 30, 35, 40 and 50 °C, at pH 7.8, 2.0 % NaCl) was also measured in the MB medium. Bacterial growth at different pH values (pH 4.0–10.0, in increments of 1.0 pH unit) was observed after adjusting the final pH of the MB medium with HCl or NaOH (at 2.0 % NaCl, 25 °C). The following biological buffers were used to adjust the pH: Na_2HPO_4/NaH_2PO_4 for pH 4.0–7.0 and $Na_2CO_3/NaHCO_3$ for pH 8.0–10.0.

Chemotaxonomy

Respiratory quinones were extracted from 100 mg of freeze dried cell material using the two-stage method described by Tindall [27, 28] and analyzed by HPLC. For the polyamines analysis, cells were harvested on PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2), extracted, and analyzed as described as previously [1]. The genomic DNA was extracted according to the method of Marmur [14], and the genomic DNA G + C content of strain JLT2012^T was estimated using the HPLC method [16]. Cellular fatty acids were extracted from the cells grown on the MA medium for 2 days at 30 °C according to the protocol of MIDI, and analysis was carried out as described by Komagata and Suzuki [10] with cells grown on MA medium for 2 days at 30 °C. The MIDI version was Sherlock version 6.0, and the library was TSBA6 6.00. Polar lipids were measured by two-dimensional TLC, using the Merck silica gel 60F254 plates (10 by 20 cm), with chloroform–methanol–water (65:25:4, vol/vol) in the first dimension and chloroform–methanol–acetic acid–water (80:12:15:4, vol/vol) in the second dimension. Lipid spots were revealed by spraying the plates with 10 % molybdophosphoric acid in ethanol for phospholipids, and 5 % α-naphthol in concentrated sulfuric acid–ethanol (1:1, vol/vol) for glycolipids, followed by heating at 150 °C for 3–5 min [2, 7].

16S rRNA Gene Analysis and PCR Amplification

The 16S rRNA gene of strain JLT2012^T was amplified using universal bacterial primers [4]. Phylogenetic analysis based on 16S rRNA gene sequences was performed as described by Kim et al. [9]. To determine the approximate phylogenetic affiliation, the 16S rRNA gene sequence of strain JLT2012^T (1450 bp, GenBank accession number: JX878395) was compared with those available from the GenBank database by using the EzTaxon-e [8]. Phylogenetic analysis (with neighbor-joining, maximum-likelihood, and maximum-parsimony algorithms) was performed using BioEdit [6], and phylogenetic trees were constructed by using the neighbor-joining, maximum-parsimony, and maximum-likelihood methods within the software MEGA version 5 [24]. Distances and clustering obtained via the neighbor joining [19].

Results and Discussion

Phenotypic and Chemotaxonomic Characteristics

Growth of strain JLT2012^T occurred at 10–45 °C (optimum 30 °C), at pH 5–10 (optimum, pH 5–8) and in 0.5–4 % NaCl (w/v) (optimum 2 %). After incubation at 25 °C for 2 days on the MA, the colonies appeared as orange pigment and were circular with a diameter of 1–2 mm, as well as convex and shaped with intact margins. The cells were rod shaped, devoid of flagella with gliding motility, and catalase and oxidase positive. The results of the physiological characterization are summarized in Table 1 with the following species description: Strain JLT2012^T and the type strain of *P. flava* JLT2015^T were Gram negative, orange colored, and rod shaped; growth occurred optimally at 2 % NaCl; they were positive for activity of the alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsine, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, *N*-acetyl-*β*-glucosaminidase, and Voges–Proskauer reaction. Furthermore, both the strains were negative for the hydrolysis of starch and gelatin; nitrate reduction; H_2S and indole production;

Table 1 Characteristics that distinguish strain JLT2012^T from *P. flava* JLT2015^T

Characteristic	JLT2012^T	*P. flava* JLT2015^{Ta}
Growth conditions		
Temperature (°C)		
Range	10–40	20–30
Optimum	30	25
pH		
Range	5–10	5–12
Optimum	5–8	5–11
NaCl concentration (%)		
Range	0.5–4	0.5–7
Biochemical properties		
Poly-β-hydroxyalkanoate	−	+
API 20NE tests		
Oxidase	+	−
Utilization gelatin and gluconate	+	−
Enzymic activity (API ZYM)		
Lipase (C14)	−	+
Acid production from (API 20E)		
Sucrose	−	+
Gelatin	+	−
Assimilation of		
Potassium gluconate	+	−
Susceptibility to		
Ampicillin, carbenicillin, chloromycetin, gentamicin, kanamycin	+	−
Oxidation of (Biolog GN2)		
Acetic acid, *i*-erythritol, glycogen, lactulose, methyl pyruvate,	−	+
D-Galactose, D-raffinose, D-trehalose, turanose, α-keto glutaric acid, L-alanine, L-alanyl-glycine, L-glutamic acid, L-aspartic acid, hydroxy-L-proline	+	−

Both strains were Gram negative, orange colored, and rod shaped; growth occured optimally at 2 % NaCl; they were positive for activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsine, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, *N*-acetyl-*β*-glucosaminidase, and Voges–Proskauer reaction. Furthermore, all strains are negative for hydrolysis of starch, gelatin; nitrate reduction; H2S and indole production; assimilation of D-mannose, capric acid, malic acid, and trisodium citrate; acid production from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, and amygdalin; and activity of lipase (C14), cystine arylamidase, trypsine, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, and *β*-fucosidase

\+ positive, − negative, *ND* no data available

[a] Data of *P. flava* JLT2015^T were obtained from our previous work [13]

assimilation of D-mannose, capric acid, malic acid, and trisodium citrate; acid production from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, and amygdalin; and activity of lipase (C14), cystine arylamidase, trypsine, α- and β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and β–fucosidase. Strain JLT2012^T was susceptible to ampicillin, carbenicillin, chloromycetin, gentamicin, and kanamycin and found to be positive for the utilization of gelatin and gluconate, as well as acid production from gelatin and assimilation of potassium gluconate. Only the strain JLT2015^T could produce acid from sucrose and utilize acetic acid, *i*-erythritol, glycogen, lactulose and methyl pyruvate, and tested positive for the lipase activity (Table 1). However, only JLT2012^T strain could utilize D-galactose, D-raffinose, D-trehalose, turanose, α-keto glutaric acid, L-alanine, L-alanyl-glycine, L-glutamic acid, L-aspartic acid, and hydroxy-L-proline (Table 1).

The major polar lipids of strain JLT2012^T were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, sphingoglycolipids, and several other unknown glycolipids (Supplementary Fig. S2). The G + C content of strain JLT2012^T DNA was determined to be 65.5 mol%. Similar to the reference type strain of the genus *Pacificimonas*, the predominant respiratory quinone in strain JLT2012^T was found to be Q-10. The dominant cellular fatty acids of JLT2012^T were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), $C_{16:0}$, and $C_{18:0}$ (Table 2). The fatty acids $C_{17:0}$, anteiso-$C_{15:0}$ and $C_{17:1}$ $\omega6c$ were detected only in cells of strains JLT2012^T, whereas iso-$C_{13:0}$ 3-OH, $C_{16:1}$ $\omega5c$, $C_{16:0}$ *N* alcohol, anteiso-$C_{17:0}$, and $C_{19:0}$ cyclo $\omega8c$ were found only in cells of the *Pacificimonas* reference type strain (Table 2).

Table 2 Fatty acid compositions (%) of genus strain JLT2012^T and *P. flava* JLT2015^T

Fatty acids	JL2012^T	*P. flava* JL2015^{Ta}
$C_{12:0}$	1.1	3.9
$C_{14:0}$	2.9	3.2
$C_{16:0}$	21.3	17.3
$C_{17:0}$	1.6	–
$C_{18:0}$	15.9	8.4
iso-$C_{13:0}$ 3-OH	–	0.6
$C_{14:0}$ 2-OH	4.1	9.7
$C_{15:0}$ 2-OH	1.1	–
$C_{15:0}$ 3-OH	2.4	1.5
$C_{15:0}$ anteiso	–	2.0
$C_{16:1}$ $\omega5c$	–	2.1
$C_{17:1}$ $\omega6c$	7.1	–
$C_{18:1}$ $\omega9c$	2.5	1.2
$C_{16:0}$ *N* alcohol	–	1.0
Anteiso-$C_{17:0}$	–	0.7
$C_{19:0}$ cyclo $\omega8c$	–	4.2
Summed feature 3	5.4	14.1
Summed feature 8	34.7	30.2

Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 3, $C_{16:1}$ $\omega7c$ and/or $C_{16:1}$ $\omega6c$; Summed feature 8, $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$

Values are percentages of total fatty acids; values <0.5 % are not shown. – Not detected

[a] Data of *P. flava* JLT2015^T was obtained from our previous work [13]

Phylogenetic Analysis

Phylogenetic distances and clustering obtained via the neighbor-joining method [18] are shown in Fig. 1. The phylogenetic tree indicated that the strain JLT2012^T falls within the *Sphingomonadaceae* family formed a coherent cluster with *P. flava* JLT2015^T [13]. Similar results were obtained using the maximum-parsimony and maximum-likelihood methods (Fig. 1). A comparative 16S rRNA gene sequencing analysis revealed that JLT2012^T bacterium shared 96.0 % sequence similarity with *P. flava*.

Taxonomic Conclusions

Strain JLT2012^T exhibited 96.0 % 16S rRNA gene sequence similarity with *P. flava* JLT2015^T [13]. The phylogenetic association of strain JLT2012^T with the reference type strains of members of the genus *Pacificimonas* is consistent as indicated by the neighbor-joining, maximum-parsimony, and maximum-likelihood trees (Fig. 1). Strain JLT2012^T is distinguishable from the reference type strains of the genus *Pacificimonas* based on the differences in their polyphasic taxonomic characterizations including substrates used as sole sources of carbon and energy (Table 1), the temperature and NaCl concentration required for the optimum growth, acid production from sucrose and gelatin, enzymatic activity of lipase, susceptibility to antibiotics, DNA G + C content and fatty acid composition.

Based on the data from this polyphasic study, strain JLT2012^T represents a novel species and should be classified in the existing genus *Pacificimonas*. We, therefore, propose a name *Pacificimonas aurantium* sp. nov. for this newly identified bacterium.

Description of *Pacificimonas aurantium* sp. nov.

Pacificimonas aurantium sp. nov. (au'.ran.ti'.um. L. fem. adj. *aurantium*. orange colored, colony color of the type strain).

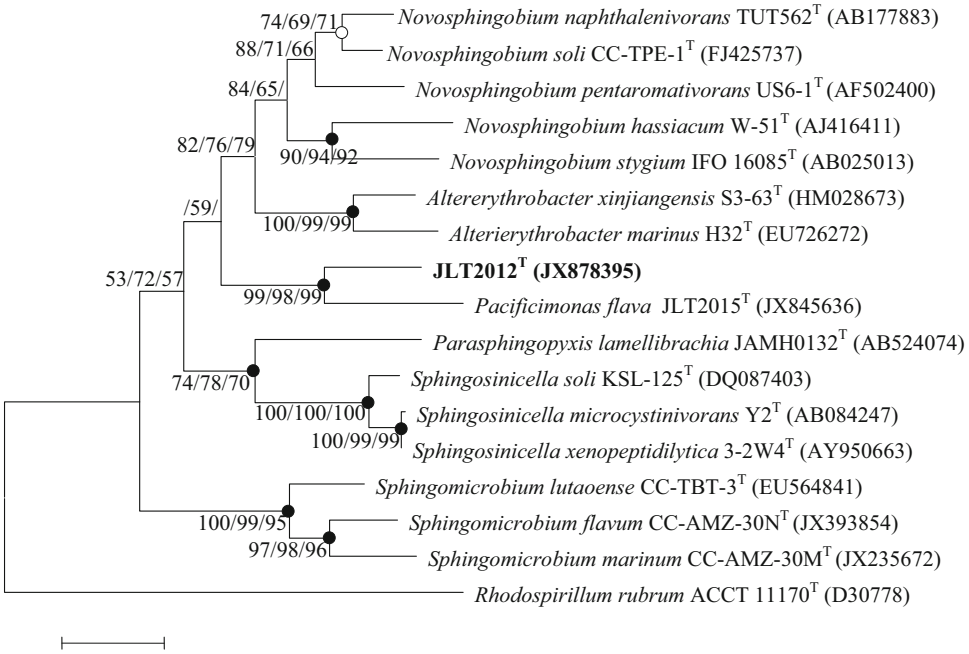

Fig. 1 Neighbor-joining phylogenetic tree showing the relationships between strain JLT2012^T and representatives of the family *Sphingomonadaceae* based on 16S rRNA gene sequences. Bootstrap percentages (above 50 %) from the neighbor-joining/maximum-parsimony/maximum-likelihood approach are shown (1000 replications). *Rhodospirillum rubrum* ATCC 11170^T was used as an outgroup to define the root of the tree. Bar, 0.02 estimated sequence divergence. Filled and open circles indicate the corresponding nodes recovered reproducibly by all treeing methods or by two treeing methods, respectively

Cells of strain JLT2012^T were Gram-negative, aerobic, and motile rods approximately 0.6–0.8 × 1.8–4.5 mm in size (Supplementary Fig. S1). Colonies were orange, circular, convex, and opaque on MA medium. Growth occurred between 10 and 45 °C (optimum, 30 °C), between salinities of 0–4 % (w/v) NaCl (optimum, 2 % NaCl), and between pH 5 and 10 (optimum, pH 5–8). Strain JLT2012^T was positive for catalase and oxidase. PHAs granules are not produced. Gelatin is hydrolyzed, but DNA, starch, casein, and urea are not. Indole or H_2S production is not detected. Nitrate and nitrite are not reduced. In API ZYM enzyme reactions, alkaline phosphatase (Supplementary Table S1), esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsine, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and *N*-acetyl-*β*-glucosaminidase are present. According to API 20E and 20NE tests (Supplementary Tables S2 and S3), positive reactions are seen for Voges–Proskauer reaction, gelatinase, and assimilation of potassium gluconate. According to Biolog GN2 tests (Supplementary Table S4), the following substrates are oxidized: dextrin, Tween 40, Tween 80, D-fructose, D-galactose, α-D-glucose, D-raffinose, D-trehalose, turanose, *β*-hydroxy butyric acid, α-keto glutaric acid, L-alanine, L-alanyl-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic, hydroxy-L-proline, L-proline, L-pyroglutamic acid, L-arabinose, D-cellobiose, D-mannitol, mono-methyl-succinate, D-gluconic, *γ*-hydroxy butyric acid, D,L-lactic acid, succinic acid, D,L-carnitine, *γ*-Amino butyric acid, 2-aminoethanol, glycerol, 2,3-butanediol, and glucose-6-phosphate. It is susceptible to ampicillin (10 μg), carbenicillin (100 μg), chloramphenicol (5 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin (10 μg), rifampicin (5 μg), and novobiocin (5 μg). Major fatty acids are summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) (34.7 %), $C_{16:0}$ (21.3 %), C18:0 (15.9 %), and $C_{17:1}\omega6c$ (7.1 %) (together representing 79.0 % of the total) (Table 2). The predominant polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, and four sphingoglycolipid (Supplementary Fig. S2). Spermidine and spermine are predominant in the polyamine pattern. The respiratory lipoquinone is ubiquinone Q-10. The DNA G + C content of the type strain is 65.5 mol%.

The type strain is JLT2012^T (=LMG 27361^T=CGMCC 1.12399^T), isolated from seawater of the Southeastern Pacific Ocean.

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