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Imperialibacter roseus gen. nov., sp. nov., a novel bacterium of the family *Flammeovirgaceae* isolated from Permian groundwater

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A novel bacterial strain, designated P4^T, was isolated from Permian groundwater and identified on the basis of its phylogenetic, genotypic, chemotaxonomic and phenotypic characteristics. Cells were aerobic, Gram-stain-negative rods. 16S rRNA gene sequence-based phylogenetic analysis revealed that P4^T is affiliated with the family *Flammeovirgaceae* in the phylum *Bacteroidetes*, but forms a distinct cluster within this family. The DNA G+C content of strain P4^T was 45.2 mol%. The predominant cellular fatty acids were $C_{16:1}\omega6c$/$C_{16:1}\omega7c$ and iso-$C_{15:0}$. MK-7 was the main respiratory quinone. The polar lipids were phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, unidentified phospholipids, an unidentified aminolipid, unidentified glycolipids and unidentified polar lipids. Based on our extensive polyphasic analysis, a novel species in a new genus, *Imperialibacter roseus* gen. nov., sp. nov., is proposed. The type strain of *Imperialibacter roseus* is P4^T (=CICC 10659^T=KCTC 32399^T).

Bacteria affiliated with the family *Flammeovirgaceae* of the phylum *Bacteroidetes* are widely distributed in various environments, particularly in aquatic systems. Detailed study has led to the identification of a number of new genera in the family *Flammeovirgaceae* in recent years (Khan *et al.*, 2007; Nedashkovskaya *et al.*, 2007; Seo *et al.*, 2009; Srisukchayakul *et al.*, 2007; Yoon *et al.*, 2007, 2008, 2011). A bacterial strain, designated P4^T, was isolated from Permian groundwater as part of a study to use this novel water source for cultivation of biofuel-producing microalgae (Wang *et al.*, 2012). Polyphasic taxonomic tests including phylogenetic, genotypic, chemotaxonomic and phenotypic assays were performed in order to identify the novel strain described here. The bacterial strain P4^T belongs to a novel genus and species in the family *Flammeovirgaceae*.

Permian groundwater used for isolation of bacteria was collected from the Pecos Cenozoic Trough in Imperial, TX, USA (31° 16′ 16.93″ N 102° 40′ 48.35″ W). Strain P4^T was successfully isolated on Difco marine agar 2216 plates (BD Bioscience) at 30 °C and cryo-preserved at −80 °C in marine broth 2216 supplemented with 30 % (v/v) glycerol. After 48 h of incubation on marine agar plates, the aerobic bacterium formed circular, flat, pink colonies. Gram staining was performed according to the method described by Gerhardt *et al.* (1994). Scanning electron microscopy was applied to observe the morphology of strain P4^T. The results indicate that strain P4^T is a Gram-stain-negative, rod-shaped bacterium (Fig. 1).

Genomic DNA was extracted using an Ultra-Clean microbial DNA isolation kit (MoBio Laboratories). PCR amplification and 16S rRNA gene sequencing were performed as described previously (Enticknap *et al.*, 2006). The almost full-length 16S rRNA gene was analysed using the EzTaxon server 2.1 (Chun *et al.*, 2007). The 16S rRNA gene sequence of strain P4^T was aligned with those of representative members of selected genera belonging to the families *Cyclobacteriaceae* and *Flammeovirgaceae* in the phylum *Bacteroidetes*. Phylogenetic analysis was conducted using the MEGA 4 software package (Tamura *et al.*, 2007). A phylogenetic tree was reconstructed using the neighbour-joining algorithm (with Jukes–Cantor correction). The robustness of the inferred tree topology was evaluated after 1000 bootstrap replicates of the neighbour-joining data.

The taxonomic analysis using EzTaxon showed that the 16S rRNA gene sequence of strain P4^T was no more than 88 % similar to that of any previously identified type strain. The closest cultured strains, *Algoriphagus halophilus* JC 2051^T (88.18 %) and *Fulvivirga kasyanovii* KMM 6220^T (88.16 %), were affiliated with different families, *Cyclobacteriaceae* and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P4^T is KC800928.

A supplementary table is available with the online version of this paper.

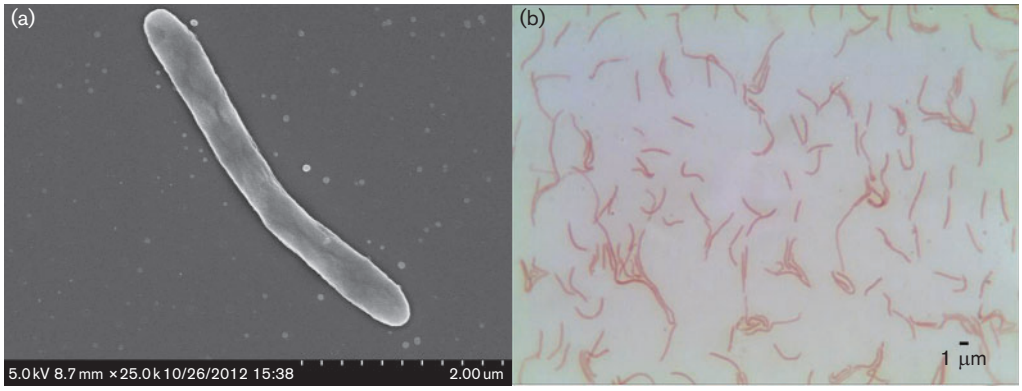

Fig. 1. Morphological characterization of strain P4^T observed by transmission electron microscopy (a) and fluorescence microscopy (b) after 2 days of incubation in ZoBell 2216E medium at 30 °C. Bars, 2 μm (a) and 1 μm (b).

Flammeovirgaceae, in the phylum *Bacteroidetes*. Phylogenetic analysis of strain P4^T and all type strains in the two families indicated that the novel bacterium was not affiliated with the family *Cyclobacteriaceae* (not shown), and formed a distinct lineage in the family *Flammeovirgaceae* (Fig. 2).

Temperature, pH and salinity ranges for growth of strain P4^T and two reference strains (*A. halophilus* JC 2051^T and *F. kasyanovii* CCTCC AB 206119^T) were tested according to previously described methods (Nedashkovskaya *et al.*, 2004b; Yi & Chun, 2004). Physiological and biochemical characterizations were conducted using API ZYM, API 50 CH, API 20NE, API 20E and API 50CHB strips (bioMérieux), while some media used for tests were prepared as described previously (Yi *et al.*, 2003). Sensitivity to antibiotics was tested by adding discs (Oxoid) containing different antibiotics on marine agar plates spread with fresh cultures of strain P4^T. Antibiotics and the amounts used in this

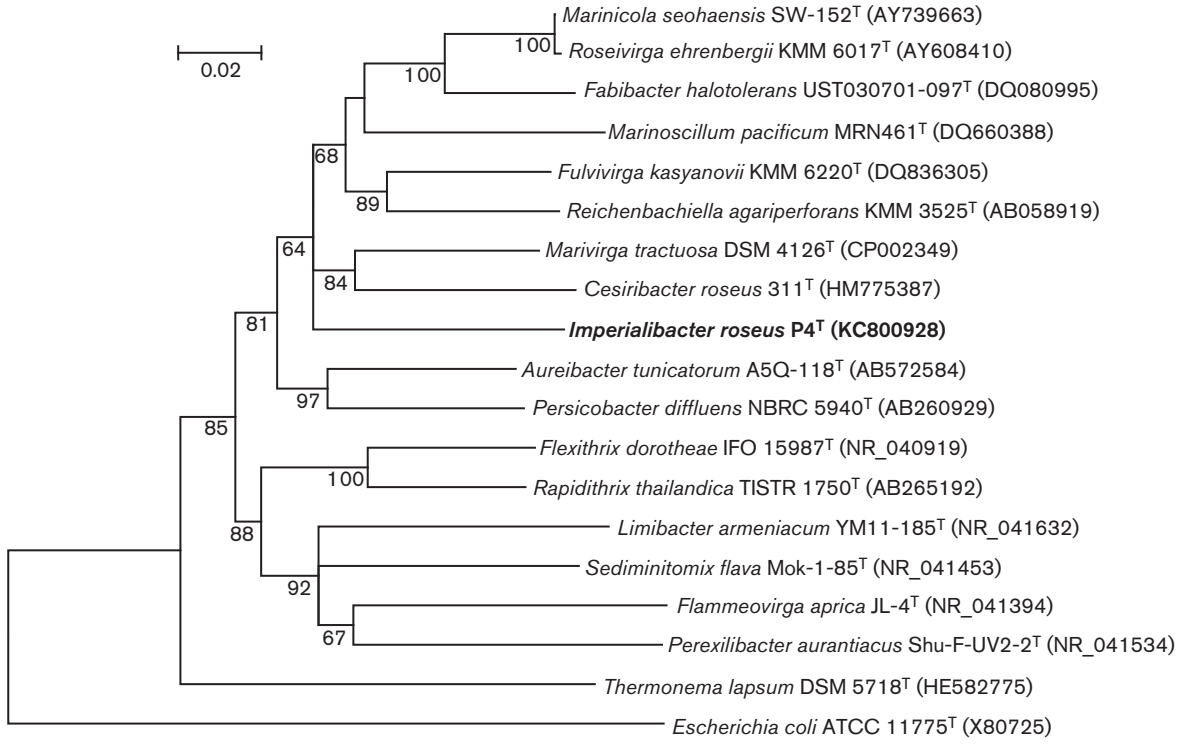

Fig. 2. Rooted neighbour-joining tree of partial 16S rRNA gene sequences of strain P4^T and representative members of selected genera belonging to the family *Flammeovirgaceae* in the phylum *Bacteroidetes*. The tree was reconstructed using MEGA software. *Escherichia coli* ATCC 11775^T was used as the outgroup. Bar, 0.02 substitutions per nucleotide position.

experiment were as follows: ampicillin (10 μg), penicillin G (10 IU), carbenicillin (100 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), polymyxin B (300 IU), streptomycin (10 μg) and tetracycline (30 μg). The effect of antibiotics on growth of cells was assessed after 48 h based on the methods described by Chou *et al.* (2008). The physiological and biochemical characteristics of strain P4^T are given in the species description and in Tables 1 and S1 (available in IJSEM Online). All parameters mentioned above were tested simultaneously on the two reference strains.

The DNA G+C content of strain P4^T was determined by using the thermal denaturation method (Mandel & Marmur, 1968). The results showed that the DNA G+C content of strain P4^T was 45.2 mol%. *A. halophilus* JC 2051^T had a lower DNA G+C content (37 mol%) and *F. kasyanovii* KMM 6220^T showed a higher DNA G+C content (59.9 mol%).

Table 1. Differential characteristics of strain P4^T and reference strains *A. halophilus* JC 2051^T and *F. kasyanovii* CCTCC AB 206119^T

Strains: 1, P4^T; 2, *A. halophilus* JC 2051^T; 3, *F. kasyanovii* CCTCC AB 206119^T. +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3
Pigmentation	Pink	Yellow	Red
pH for growth	5–9*	5–10	5–10
Temperature for growth (°C)	10–37	10–40	15–45†
Salinity for growth (%, w/v)	2	3–7	2
Hydrolysis of:			
Casein	−	−	+
Starch	−	−	+
Tween 80	−	+	+
Enzyme activities			
Trypsin	−	+	w
Chymotrypsin	+	+	−
α-Galactosidase	−	+	w
β-Galactosidase	−	+	w
β-Glucuronidase	−	+	−
N-Acetylglucosaminidase	+	+	−
α-Mannosidase	−	+	w
β-Fucosidase	−	−	w
Acid production from:			
D-Arabinose	−	−	+
Galactose	+	w	−
Glucose	+	+	−
Methyl α-D-mannopyranoside	+	+	−
Methyl α-D-glucoside	+	−	−
N-Acetylglucosamine	+	+	−
Arbutin	+	+	−
Lactose	+	w	−
Melibiose	+	w	−
Starch	−	−	+
Glycogen	−	−	+
5-Keto-D-gluconate	−	−	+

*Strain P4^T showed weak growth at pH 5 and 9.
†*F. kasyanovii* CCTCC AB 206119^T showed weak growth at 45 °C.

The Sherlock Microbial Identification System (MIDI) was applied for identifying and quantifying the cellular fatty acids of strain P4^T and the two reference strains. The predominant cellular fatty acids of strain P4^T were $C_{16:1}\omega6c$/$C_{16:1}\omega7c$ (39.88 %) and iso-$C_{15:0}$ (24.24 %), while the proportions of these two components in *A. halophilus* JC 2051^T were 22.42 and 17.54 %, respectively. *F. kasyanovii* CCTCC AB 206119^T contained two other major cellular fatty acids in addition to $C_{16:1}\omega6c$/$C_{16:1}\omega7c$ (36.12 %) and iso-$C_{15:0}$ (14.59 %): iso-$C_{17:0}$ 3-OH (13.97 %) and $C_{16:1}\omega5c$ (10.45 %). The quinone composition was characterized by HPLC as described previously (Nedashkovskaya *et al.*, 2004a). The main isoprenoid quinone of strain P4^T was MK-7 (approx. 100 %). Polar lipids were determined by using TLC. The polar lipid profile was composed of phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, an unidentified phospholipid, an unidentified aminolipid, unidentified glycolipids and unidentified polar lipids (Fig. 3). Since phosphatidylcholine is not commonly found in members of the phylum *Bacteroidetes*, the finding of phosphatidylcholine requires further confirmation by GC/MS analysis.

Although the main isoprenoid quinone of strain P4^T is consistent with that of *F. kasyanovii* KMM 6220^T (Nedashkovskaya *et al.*, 2007), the great differences shown in phylogenetic, genotypic, chemotaxonomic and phenotypic analyses distinguished the novel bacterium from the closest related strains, *A. halophilus* JC 2051^T and *F. kasyanovii* KMM 6220^T. Based on the polyphasic taxonomic analysis in this study, a new genus and novel species affiliated with the family *Flammeovirgaceae* are proposed, for which we propose the name *Imperialibacter roseus* gen. nov., sp. nov.

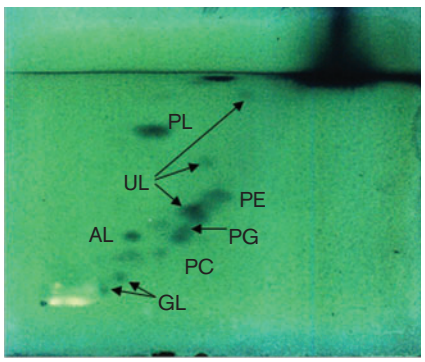

Fig. 3. Total polar lipid profile of strain P4^T after two-dimensional TLC. PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; GL, unidentified glycolipids; PL, unidentified phospholipid; UL, unidentified polar lipids.

Description of *Imperialibacter* gen. nov.

Imperialibacter (Im.pe.ri.a′li.bac′ter. N.L. n. *Imperial* Imperial, a town in Texas where the type strain of the type species was isolated; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Imperialibacter* a rod isolated from Imperial, TX, USA).

Cells are aerobic, Gram-stain-negative rods. The predominant cellular fatty acids are $C_{16:1}\omega6c/C_{16:1}\omega7c$ and iso-$C_{15:0}$. The main respiratory quinone is MK-7. The polar lipids include phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, an unidentified phospholipid, an unidentified aminolipid, unidentified glycolipids and unidentified polar lipids. 16S rRNA gene sequence-based phylogenetic analysis indicates that the genus *Imperialibacter* is affiliated with the family *Flammeovirgaceae* and is distinct from all known genera in the family. The type species is *Imperialibacter roseus*.

Description of *Imperialibacter roseus* sp. nov.

Imperialibacter roseus (ro′se.us. L. masc. adj. *roseus* rose-coloured).

The main characteristics are the same as given for the genus. In addition, cells are about 4 μm long and 0.4 μm wide. Colonies are circular, flat and pink on marine agar plates after 48 h of cultivation. The temperature for growth is 10–37 °C. Grows at pH 5–9 and at 2 % (w/v) NaCl. Capable of hydrolysing casein, starch and Tween 80. Enzyme activities possessed by the type strain belong to the chymotrypsin and *N*-acetylglucosaminidase families, but not trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase or β-fucosidase. Acids are produced from galactose, glucose, methyl α-D-mannopyranoside, methyl α-D-glucoside, *N*-acetylglucosamine, arbutin, lactose and melibiose, but not D-arabinose, starch, glycogen or 5-keto-D-gluconate. Assimilation of glucose, fructose, mannose, arbutin, aesculin, melibiose, L-fucose and 5-keto-D-gluconate is observed among 54 tested carbon sources included in the API 20NE and API 20E strips.

The type strain is P4^T (=CICC 10659^T=KCTC 32399^T), isolated from Permian groundwater from the Pecos Cenozoic Trough in Imperial, TX, USA. The DNA G+C content of the type strain is 45.2 mol%.

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