Paracoccus sphaerophysae sp. nov., a siderophore-producing, endophytic bacterium isolated from root nodules of *Sphaerophysa salsula*

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An aerobic, brown-pigmented, non-spore-forming, endophytic bacterium, designated strain Zy-3^T, was isolated from root nodules of *Sphaerophysa salsula*, a native leguminous herb belonging to the family Leguminosae growing in north-western China. Cells of strain Zy-3^T were non-motile, Gram-negative rods. Strain Zy-3^T produced siderophores and showed antifungal activity. Phylogenetic analysis of the 16S rRNA gene sequence indicated that the closest relative of this organism was *Paracoccus halophilus* HN-182^T (96.6 % sequence similarity). On the basis of genotype, fatty acid patterns and physiological characteristics, a novel species *Paracoccus sphaerophysae* sp. nov. is proposed, with Zy-3^T (=ACCC 05413^T =HAMBI 3106^T) as the type strain.

As an essential element for micro-organisms (Loper & Buyer, 1991), iron also plays an important part in some host–bacteria interactions (Mila *et al.*, 1996). Under iron-limited conditions, micro-organisms produce siderophores (low-molecular-mass biomolecules, 0.5–1.5 kDa) in order to scavenge ferric ions, which bind with high affinity to specific outer-membrane receptors. The major groups of siderophores for the chelation of Fe³⁺ include hydroxamates, thiohydroxamates and catecholates (Richardson *et al.*, 1999). Because of their role in biological control of plant diseases, the production of siderophores by plant-associated bacteria has received major attention (Loper & Buyer, 1991).

The genus *Paracoccus* was first described by Davis *et al.* (1969), and the description was later emended by Katayama *et al.* (1995). It consists of Gram-negative, oxidase- and catalase-positive bacteria that show substantial metabolic versatility. Phylogenetically, the genus belongs to the α -3 subgroup of the *Proteobacteria*. In this study, an endophytic, brown-pigmented, siderophore-producing bacterial strain, Zy-3^T, was isolated from root nodules of *Sphaerophysa salsula*, a native leguminous herb belonging to the family Leguminosae growing in north-western China. Based on phenotypic and genotypic studies,

it is concluded that this endophytic isolate represents a novel species within the genus *Paracoccus*.

The GPS coordinates for the sampling site from which strain Zy-3^T was obtained were 38° 45' 07" N 100° 22' 10" E. Healthy, non-ruptured nodules were carefully rinsed with sterile distilled water to remove soil, placed in a fine-mesh plastic holder and surface-sterilized by immersion in 95 % ethanol for 30 s and then in 5 % sodium hypochlorite for 3 min and finally rinsed eight times with sterile distilled water. The surface-sterilized root nodules were crushed on a sterile plate and bacteria were isolated on nutrient agar (NA) containing (per litre distilled water) 5.0 g NaCl, 5.0 g peptone, 1.5 g yeast extract, 1.5 g beef extract and 20 g agar, pH 7.2. Isolates were incubated at 28 °C, and the purity of the colonies was checked by repeated streaking of single colonies on plates and by microscopic examination. Pure cultures were preserved at 4 °C for temporary storage or in 20 % glycerol at –80 °C for long-term storage. Surface-sterilized nodules were rolled on NA plates and aliquots of the sterile distilled water from the final rinse solution were plated onto NA plates as controls to detect possible contaminants.

The Chromeazurol S (CAS) assay (Schwyn & Neilands, 1987) was used to detect siderophores. Routine cultivation was conducted at 28 °C on NA. The Gram reaction was determined by staining cells grown on NA at 28 °C for 24 h, according to the method described by Gerhardt *et al.* (1994). Endospore formation was detected by malachite green staining. Cultured cells were adsorbed onto a sterile

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Zy-3^T is GU129567.

Two supplementary figures are available with the online version of this paper.

filter after reaching the exponential growth phase and then fixed with 3 % glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) for 1 h at room temperature (Robinson *et al.*, 1984). The samples were washed, dehydrated with a graded ethanol series, critical-point-dried and sputter-coated with a gold film; samples were then examined in a JEOL JSM-6360 digital scanning electron microscope (Fig. 1).

Strain Zy-3^T and reference strain *Paracoccus halophilus* CGMCC 1.6117^T (Liu *et al.*, 2008) were grown on NA, and subjected to physiological and biochemical tests, including indole production from tryptophan, phosphate solubilization activity and chitinase activity according to Dong & Cai (2001). To test for antifungal activity, spores of fungal cultures (*Fusarium oxysporium*, *Alternaria burnsii* and *Rhizoctonia solani*) were grown on potato dextrose agar (PDA) plates. An agar plug (5 mm diameter) taken from an actively growing fungal culture was placed to one side on the surface of a PDA plate. Test bacteria were streaked perpendicular to the agar plug on the opposite side, towards the edge of the plate. Plates inoculated with fungal agar plugs alone were used as controls. Plates were incubated at 28 ± 2 °C until fungal mycelium covered the agar surface completely in the control plate, and zones of inhibition were recorded. A test was considered positive when bacteria interfered with the normal spread of the fungus and presented an inhibition zone in three replications. Sensitivity to antibiotics was examined by spreading cells (0.5 McFarland) onto NA and placing discs (Difco) on the plates containing the following antibiotics: ampicillin (100 µg), chloramphenicol (50 µg), kanamycin (50 µg), penicillin G (10 U) and streptomycin (50 µg). The diameters of inhibition zones were measured after 48 h of incubation at 28 °C.

To determine the optimal growth temperature of the test strain, cultures were incubated between 4 and 70 °C on NA plates for 7 days. The influence of pH on growth was determined between pH 3.0 and 11.5 by using appropriate

Fig. 1. Scanning electron micrograph of exponential-phase cells, showing the cell morphology of strain Zy-3^T. Original magnification ×10 000. Bar, 1 µm.

biological buffers (Chung *et al.*, 1995), and growth was monitored by the OD₅₉₅ of the culture in nutrient broth (NB; as NA without agar) at 28 °C for 7 days on a shaker. NaCl tolerance was measured in NB at 1–9 % (w/v) NaCl, with shaking at 28 °C for 7 days. Motility was determined with an optical microscope using the hanging-drop technique (Skerman, 1967). Acid production from some carbohydrates was detected using the method of Hugh & Leifson (1953). Filter-sterilized carbohydrate solutions were added at 1 % (w/v), and acid production was recorded after 7 and 14 days of incubation. To test for growth on reduced sulfur and elemental sulfur, the test strain was cultivated for 14 days in Allen's medium (Allen, 1959), which was modified by the addition of elemental sulfur, sulfide, thiosulfate or sulfite (each at 1 %) as energy sources, and the pH was adjusted to 6.5. Growth was monitored by measuring turbidity at 600 nm after 10 days of incubation at 28 °C with shaking. Starch hydrolysis was determined as described by Cowan & Steel (1965). Denitrification was determined by growth and gas formation in a stab culture of NA containing 0.1 % (w/v) agar. Catalase activity was determined by the formation of bubbles in a 3 % H₂O₂ solution. Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Urease production was determined as described by Lányi (1987). Strain Zy-3^T and *P. halophilus* CGMCC 1.6117^T were cultivated for 3 days at 28 °C on trypticase soy agar (TSA) adjusted to pH 8.0. Harvesting, saponification, methylation and extraction of fatty acid methyl esters were performed according to Sasser (1990). Fatty acid methyl esters were identified by GC-MS, as described previously (Lipski & Altendorf, 1997). Quinones were determined as described by Collins (1985) and Wu *et al.* (1989). The brown coloration of the bacterium was due to the biosynthesis of carotenoids within the cells. Pigments were extracted from the cells with acetone, and reversed-phase HPLC of the extracted pigment was carried out by using procedures described previously (Fraser *et al.*, 1997) with minor modifications. Carotenoids present in the sample were identified by comparing retention times with an individual reference standard (Sigma).

The DNA base compositions of strain Zy-3^T and *P. halophilus* CGMCC 1.6117^T were determined by thermal denaturation (Marmur & Doty, 1962) using 0.1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The midpoint of thermal denaturation of DNA (*T*_m) was determined graphically after correction of absorbance values for thermal solvent expansion. The G + C content (mol%) was calculated from the following equation: G + C = 2.08 *T*_m – 106.4, based on thermal denaturation in 0.1 × SSC (Owen *et al.*, 1969). Total DNA was extracted according to the method of Terefework *et al.* (2001) and used as a template to amplify the 16S rRNA gene with primers fd1 and rD1 (Weisburg *et al.*, 1991). Procedures described by Van Berkum *et al.* (1996) were used for PCR amplification of the full-length 16S rRNA gene. 16S rRNA gene sequences were aligned using the programs in

CLUSTAL_X version 1.83 (Thompson *et al.*, 1994, 1997). Phylogenetic trees were reconstructed with sequence alignments of 1360 nt by the neighbour-joining method and the Jukes–Cantor model and bootstrapped with 1000 replications using TREECON version 1.3b (Van de Peer & De Wachter, 1994), by maximum-likelihood analysis using the PHYLIP package (Felsenstein, 1989) and by maximum-parsimony analysis using MEGA version 4.0 (Tamura *et al.*, 2007) with 1000 bootstrap replications.

Compared with other *Paracoccus* species, strain Zy-3^T was characterized by growth at 4 and 60 °C. The optimal growth temperature was 28 °C. Growth was observed in 1–6 % NaCl, the optimum being 3 %; when the NaCl concentration in the medium was increased to 7 %, growth was slow, and no growth occurred in the presence of more than 8 % NaCl. Strain Zy-3^T was able to grow at pH 5–11, with optimum growth at pH 7.5. Further physiological and biochemical properties of strain Zy-3^T are provided in the species description and a comparison of strain Zy-3^T with

P. halophilus CGMCC 1.6117^T is given in Table 1. Strain Zy-3^T was capable of aerobic growth on CAS agar plates, and haloes formed around colonies, revealing that it produced siderophores. The antifungal activity of strain Zy-3^T was checked and the strain exhibited broad-spectrum activity against test fungi, with inhibition zones 18.2–23.7 mm in diameter. Strain Zy-3^T was resistant to ampicillin, chloramphenicol and penicillin G and sensitive to kanamycin and streptomycin.

Strain Zy-3^T contains ubiquinone 10 as the sole quinone. The fatty acid profile (C_{18:1}ω7c, 74.98 %; C_{18:0}, 5.15 %; C_{10:0} 3-OH, 1.47 %; anteiso-C_{15:0}, 6.10 %; C_{14:0} 3-OH, 0.44 %; C_{17:0}, 1.11 %; C_{16:0}, 3.04 %) was characteristic of alphaproteobacteria. The major cellular fatty acid was C_{18:1}ω7c, and the major hydroxy fatty acid was C_{10:0} 3-OH. The fatty acid profiles of strain Zy-3^T and *P. halophilus* CGMCC 1.6117^T are compared in Table 2. Carotenoid production was confirmed by HPLC analysis. One of the major carotenoids produced by strain Zy-3^T was β-carotene, which was identified by the retention time of the peak being almost identical to that of standard β-carotene (19.899 vs 19.988 min; see Supplementary Fig. S1, available in IJSEM Online). The DNA G+C content of strain Zy-3^T was 67.1 mol%. 16S rRNA gene sequence analysis indicated that strain Zy-3^T was related to *P. halophilus* HN-182^T with the highest similarity, 96.6 %. Other closely related species included *Paracoccus solventivorans* (96.1 % similarity to the type strain), *P. versutus* (96.0 %) and *P. pantotrophus* (95.3 %). Phylogenetic trees were constructed with the neighbour-joining (Fig. 2), maximum-parsimony (Supplementary Fig. S2a) and maximum-likelihood (Supplementary Fig. S2b) methods. These methods generated slightly different tree topologies, but strain Zy-3^T and *P. halophilus* HN-182^T generated a coherent cluster in all three trees.

Table 1. Differential properties of strain Zy-3^T and *P. halophilus* CGMCC 1.6117^T

All data are from this study. Both strains are non-motile, do not grow at pH 4.5 or reduce nitrate to nitrogen, are negative for utilization of sucrose, ethanol, Tweens and starch and for utilization of sulfide and thiosulfate as energy sources and are resistant to ampicillin and penicillin G. Both strains are positive for urease activity and utilization of L-arabinose, D-galactose, D-mannose, D-fructose and D-xylose and are sensitive to kanamycin and streptomycin.

Characteristic	Zy-3 ^T	<i>P. halophilus</i> CGMCC 1.6117 ^T
Pigmentation	+	–
Utilization of:		
Maltose	+	–
Lactose	+	–
Inositol	+	–
Sodium hippurate	+	–
α-Naphthylacetic acid	+	–
Chitosan	–	+
Antifungal activity	+	–
Phosphate solubilization activity	–	+
Sensitivity to chloramphenicol*	–	+
Methyl red test	–	+
Voges–Proskauer test	–	+
Growth at/in:		
4 °C	+	–
60 °C	+	–
pH 10.0	+	–
8 % NaCl	–	+
DNA G+C content (mol%)	67.1	67.2

*Zones of inhibition were measured from the edge of the disc to the edge of the clear zone. –, Resistance (inhibition zone <2 mm); +, sensitivity (inhibition zone ≥2 mm).

Table 2. Fatty acid compositions of strain Zy-3^T and *P. halophilus* CGMCC 1.6117^T

Values are percentages of total fatty acids and were obtained in this study. ND, Not detected.

Fatty acid	Zy-3 ^T	<i>P. halophilus</i> CGMCC 1.6117 ^T
C _{10:0} 3-OH	1.47	1.65
C _{14:0}	ND	1.72
C _{14:0} 3-OH	0.44	0.92
iso-C _{15:0}	1.27	0.35
anteiso-C _{15:0}	6.10	0.42
iso-C _{15:1} I	ND	0.93
C _{16:0}	3.04	10.03
C _{17:0}	1.11	3.11
anteiso-C _{17:0}	3.52	0.55
C _{17:1} ω8c	0.84	1.17
C _{18:0}	5.15	9.10
C _{18:1} ω7c	74.98	63.28

Fig. 2. Phylogenetic tree constructed with the neighbour-joining method based on 16S rRNA gene sequence evolutionary distances among strain Zy-3^T and other members of the genus *Paracoccus*. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at branching points. The sequence of *Roseobacter denitrificans* Och 114^T was used as an outgroup. Bar, 1% nucleotide substitution. Maximum-parsimony and maximum-likelihood trees are available in Supplementary Fig. S2.

Combining the phenotypic and genotypic differences stated above, it is concluded that strain Zy-3^T represents a novel species of the genus *Paracoccus*, for which the name *Paracoccus sphaerophysae* sp. nov. is proposed.

Description of *Paracoccus sphaerophysae* sp. nov.

Paracoccus sphaerophysae (sphae.ro.phy'sae. N.L. gen. n. *sphaerophysae* of *Sphaerophysa*, a genus of leguminous plants, referring to the origin of the type strain).

Cells are Gram-negative, non-motile, aerobic, non-spore-forming rods and grow as single cells (0.4–0.8 × 1.1–2.7 µm) or as pairs to short chains. Colonies on NA are circular, smooth, convex, non-glossy and cream-white to brown. Growth occurs at 4–60 °C (optimum 28 °C), 1–6% NaCl (optimum 3%) and pH 5–11 (optimum pH 7.5). Capable of producing siderophores and showing antifungal activity. Positive for catalase, oxidase and urease activities. Negative for L-phenylalanine dehydrogenase, chitinase and phosphate solubilization activities. The methyl red and Voges-Proskauer tests are negative. Resistant to ampicillin, chloramphenicol and penicillin G and sensitive to kanamycin and streptomycin. No indole is produced from tryptophan. Agar, casein, starch, peptone broth, Tweens and gelatin are not hydrolysed. Utilizes L-cystine, L-cysteine, L-methionine, L-alanine, L-tryptophan, L-tyrosine, L-leucine, L-threonine, L-serine, L-histidine, L-arginine, L-glutamic acid, L-asparagine, L-aspartic acid, malonic acid, succinic acid, α-naphthylacetic acid, hypoxanthine, L-arabinose, D-galactose, maltose, D-mannose, D-fructose, lactose, inositol, D-xylose, D-ribose, raffinose, sodium hippurate, (+)-melibiose, L-rhamnose monohydrate, sodium pyruvate, D-sorbitol and glycerol. Does not utilize ethanol or sucrose. Nitrate is not reduced to nitrogen. Reduced sulfur does not support autotrophic growth. Glucose is not fermented. The sole ubiquinone is Q-10. The major cellular fatty acid (~75% of the total fatty acids) is C_{18:1}ω7c; minor fatty acids are C_{18:0}, C_{10:0} 3-OH, anteiso-C_{15:0}, anteiso-C_{17:0}, C_{14:0} 3-OH, C_{17:0}, C_{16:0}, iso-C_{15:0} and C_{17:1}ω8c. Capable of producing β-carotene. The DNA G+C content of the type strain is 67.1 mol% (T_m).

The type strain, Zy-3^T (=ACCC 05413^T =HAMBI 3106^T), was isolated from root nodules of *Sphaerophysa salsula*, a native legume growing in north-western China.

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References

- Allen, M. B. (1959). Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. *Arch Mikrobiol* **32**, 270–277.
- Chung, Y. C., Kobayashi, T., Kanai, H., Akiba, T. & Kudo, T. (1995). Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. *Appl Environ Microbiol* **61**, 1502–1506.
- Collins, M. D. (1985). Isoprenoid quinone analysis in classification and identification. In *Chemical Methods in Bacterial Systematics*, pp. 267–287. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- Davis, D. H., Doudoroff, M., Stanier, R. Y. & Mandel, M. (1969). Proposal to reject the genus *Hydrogenomonas*: taxonomic implications. *Int J Syst Bacteriol* **19**, 375–390.
- Dong, X.-Z. & Cai, M. -Y. (2001). *Determinative Manual for Routine Bacteriology*. Beijing: Scientific Press (English translation).
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Fraser, P. D., Miura, Y. & Misawa, N. (1997). *In vitro* characterisation of astaxanthin biosynthetic enzymes. *J Biol Chem* **272**, 6128–6135.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

- Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *J Bacteriol* **66**, 24–26.
- Katayama, Y., Hiraishi, A. & Kuraishi, H. (1995). *Paracoccus thiocyanatus* sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of *Thiobacillus versutus* to the genus *Paracoccus* as *Paracoccus versutus* comb. nov. with emendation of the genus. *Microbiology* **141**, 1469–1477.
- Lányi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Lipski, A. & Altendorf, K. (1997). Identification of heterotrophic bacteria isolated from ammonia-supplied experimental biofilters. *Syst Appl Microbiol* **20**, 448–457.
- Liu, Z.-P., Wang, B.-J., Liu, X.-Y., Dai, X., Liu, Y.-H. & Liu, S.-J. (2008). *Paracoccus halophilus* sp. nov., isolated from marine sediment of the South China Sea, China, and emended description of genus *Paracoccus* Davis 1969. *Int J Syst Evol Microbiol* **58**, 257–261.
- Loper, J. E. & Buyer, J. S. (1991). Siderophores in microbial interactions on plant surfaces. *Mol Plant Microbe Interact* **4**, 5–13.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Mila, I., Scalbert, A. & Expert, D. (1996). Iron withholding by plant polyphenols and resistance to pathogens and rots. *Phytochemistry* **42**, 1551–1555.
- Owen, R. J., Hill, L. R. & Lapage, S. P. (1969). Determination of DNA base compositions from melting profiles in dilute buffers. *Biopolymers* **7**, 503–516.
- Richardson, N., Davies, J. A. & Radüchel, B. (1999). Iron (III)-based contrast agents for magnetic resonance imaging. *Polyhedron* **18**, 2457–2482.
- Robinson, R. W., Akin, D. E., Nordstedt, R. A., Thomas, M. V. & Aldrich, H. C. (1984). Light and electron microscopic examinations of methane-producing biofilms from anaerobic fixed-bed reactors. *Appl Environ Microbiol* **48**, 127–136.
- Sasser, M. (1990). Identification of bacteria through fatty acid analysis. In *Methods in Phytobacteriology*, pp. 199–204. Edited by Z. Klement, K. Rudolph & D. C. Sands. Budapest: Akademiai Kiado.
- Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.
- Skerman, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Terefework, Z., Kaijalainen, S. & Lindström, K. (2001). AFLP fingerprinting as a tool to study the genetic diversity of *Rhizobium galegae* isolated from *Galega orientalis* and *Galega officinalis*. *J Biotechnol* **91**, 169–180.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Van Berkum, P., Beyene, D. & Eardly, B. D. (1996). Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). *Int J Syst Bacteriol* **46**, 240–244.
- Van de Peer, Y. & De Wachter, R. (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Wu, C., Lu, X., Qin, M., Wang, Y. & Ruan, J. (1989). Analysis of menaquinone compound in microbial cells by HPLC. *Microbiology* [English translation of *Microbiology (Beijing)*] **16**, 176–178.