Pandoraea thiooxydans sp. nov., a facultatively chemolithotrophic, thiosulfate-oxidizing bacterium isolated from rhizosphere soils of sesame (*Sesamum indicum* L.)

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A facultatively chemolithoautotrophic, thiosulfate-oxidizing, Gram-negative, aerobic, motile, rod-shaped bacterial strain, designated ATSB16^T, was isolated from rhizosphere soils of sesame (*Sesamum indicum* L.). 16S rRNA gene sequence analysis demonstrated that this strain was closely related to *Pandoraea pnomenusa* LMG 18087^T (96.7 % similarity), *P. pulmonicola* LMG 18016^T (96.5 %), *P. apista* LMG 16407^T (96.2 %), *P. norimbergensis* LMG 18379^T (96.1 %) and *P. sputorum* LMG 18819^T (96.0 %). Strain ATSB16^T shared 96.0–96.4 % sequence similarity with four unnamed genomospecies of *Pandoraea*. The major cellular fatty acids of the strain ATSB16^T were C_{17:0} cyclo (33.0 %) and C_{16:0} (30.6 %). Q-8 was the predominant respiratory quinone. The major polar lipids were phosphatidylmethylethanolamine, diphosphatidylglycerol, phosphatidylethanolamine and two unidentified aminophospholipids. Hydroxyputrescine and putrescine were the predominant polyamines. The genomic DNA G + C content of the strain was 64.0 mol%. On the basis of the results obtained from this study, strain ATSB16^T represents a novel species of the genus *Pandoraea*, for which the name *Pandoraea thiooxydans* sp. nov. is proposed. The type strain is ATSB16^T (=KACC 12757^T =LMG 24779^T).

Currently, the genus *Pandoraea* consists of five species, namely *Pandoraea pnomenusa*, *P. pulmonicola*, *P. apista*, *P. norimbergensis* and *P. sputorum*, and four genomospecies (Coenye *et al.*, 2000; Daneshvar *et al.*, 2001). During a study of thiosulfate-oxidizing bacterial populations in sesame rhizosphere soils, one of the isolates, strain ATSB16^T, was subjected to phenotypic, genotypic and chemotaxonomic investigations in order to determine its taxonomic position.

Rhizosphere soil samples were collected from sesame (*Sesamum indicum* L.) cultivated in fields in Jung ha-dong (35° 10–17' N 128° 49–56' E), Republic of Korea.

Rhizosphere soil (soil still attached to the roots after gentle shaking) and fine roots (approximately 1 cm in length) from five plants were pooled into one sample, homogenized by thorough hand-mixing and used for enrichment isolation. Each rhizosphere soil sample (10 g) was added to 100 ml liquid mineral salts thiosulfate (MST) medium (Mukhopadhyaya *et al.*, 2000) and incubated in a rotary shaker at 30 °C, in the dark to avoid any growth of phototrophic bacteria, until the colour of the indicator in the medium, bromocresol purple, changed to yellow. To isolate pure cultures, 10-fold dilutions (10²–10⁶) were made with sterile deionized water and 0.2 ml aliquots were spread with sterile glass sticks on MST agar. Colonies that developed a yellow halo against a purple background, indicative of the production of sulfuric acid resulting from

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ATSB16^T is EF397578.

the oxidation of thiosulfate, were selected, streaked on solid MST medium and subcultured until one unique type of colony was observed. Strain ATSB16^T was one of the isolates that could be distinguished in terms of colony morphology and rate and extent of acid production in MST medium. The strain was maintained on MST agar and subcultured every week.

The 16S rRNA gene sequence of strain ATSB16^T was determined by PCR amplification and direct sequencing as described previously (Anandham *et al.* 2008a, b). A phylogenetic analysis was performed with multiple sequence alignments using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed with neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods using the program MEGA3 (Kumar *et al.*, 2004) and bootstrap values based on 1000 replications were calculated (Felsenstein, 1985).

Gram-staining was performed by using a Gram-staining kit (Difco) and qualitative tests for catalase and oxidase activities were performed using catalase and oxidase strips (Difco), according to the manufacturer's instructions. Cell morphology was observed under transmission and scanning electron microscopes. Growth under heterotrophic conditions was monitored for 10 days at 30 °C in mineral salts medium without thiosulfate with several organic compounds (sodium succinate, sodium acetate, sodium citrate, D-glucose, D-fructose, α -lactose, D-mannose, sucrose, xylose, glycerol, mannitol, L-glutamic acid, sorbitol, malic acid, L-glutamine, L-cysteine, yeast extract, methanol and starch; 0.2 %, w/v, for each carbohydrate and 0.1 %, w/v, for each organic acid, sugar alcohol and yeast extract). Chemo-organotrophic growth was tested on Luria-Bertani agar, nutrient agar, tryptic soy agar (TSA) and R2A agar (all from Difco). The ability to oxidize and grow on different reduced sulfur compounds was tested by using mineral salts medium supplemented with one of the following compounds: thiosulfate (20 mM), tetrathionate (10 mM), sulfite (3 mM in 50 mM EDTA to prevent autooxidation), thiocyanate (2 mM) and elemental sulfur (0.1 %, w/v). Mixotrophic growth (i.e., concurrent utilization of organic and inorganic substrates) was examined in mixotrophic medium as described by Anandham *et al.* (2007). The pH range for growth was determined in R2A broth from pH 4.0 to 10.0, in increments of 0.5 pH units. All of the basic physiological and biochemical tests were performed at 30 °C according to Gerhardt *et al.* (1994). Growth at various NaCl concentrations (1–7 %) and temperatures (4–50 °C) was investigated in R2A broth. Physiological and biochemical properties were further determined with the API ZYM, API 20NE and ID 32 GN systems (bioMérieux), according to the manufacturer's instructions.

For the analysis of the cellular fatty acids, cells grown on TSA plates at 30 °C for 48 h were used. Fatty acid methyl esters were extracted and prepared by using the standard

protocol of the Microbial Identification System (Microbial ID). The G + C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989) using a reversed-phased column (Supelcosil LC-18-S; Supelco). Polar lipids were extracted and analysed by two-dimensional TLC according to Minnikin *et al.* (1984). Polyamines and isoprenoid quinones were extracted and analysed in HPLC, as described by Busse *et al.* (1997) and Groth *et al.* (1996), respectively.

An almost-complete 16S rRNA gene sequence of strain ATSB16^T was obtained (1410 bp). Preliminary sequence comparisons with 16S rRNA gene sequences retrieved from the GenBank database indicated that strain ATSB16^T belonged to the family *Burkholderiaceae* of the *Betaproteobacteria*. 16S rRNA gene sequence analysis revealed that strain ATSB16^T is closely related to *P. pnumenusa* LMG 18087^T (96.7 % similarity), *P. pulmonicola* LMG 18016^T (96.5 %), *P. apista* LMG 16407^T (96.2 %), *P. norimbergensis* LMG 18379^T (96.1 %), *P. sputorum* LMG 18819^T (96.0 %), *Pandoraea* genomospecies 1 R-5199 and *Pandoraea* genomospecies 2 CDC G5084 (96.0 %), *Pandoraea* genomospecies 3 CDC G9805 (96.2 %) and *Pandoraea* genomospecies 4 CDC H652 (96.4 %). The overall topologies of the maximum-parsimony and maximum-likelihood trees were similar to that of the neighbour-joining tree. The phylogenetic analysis revealed that strain ATSB16^T formed a line of descent that was separate from the genus *Pandoraea*, which was supported by a high bootstrap value (Fig. 1).

After 2 days on R2A, colonies of strain ATSB16^T were white, circular and convex with clear margins. Cells were Gram-negative, aerobic, rod-shaped and motile by means of a single polar flagellum (Fig. 2). The strain grew chemolithoautotrophically on reduced sulfur compounds such as thiosulfate, sulfur and sulfite and chemo-organoheterotrophically on several single carbon sources. The strain did not require yeast extract or vitamins under either of these conditions. Both strain ATSB16^T and *P. norimbergensis* DSM 11628^T exhibited mixotrophic growth in mineral salts medium containing 20 mM thiosulfate plus 20 mM sodium acetate or glucose. *P. norimbergensis* DSM 11628^T could also grow chemolithoautotrophically with thiosulfate (Table 1). Mixotrophic growth may be metabolically advantageous, because low concentrations of sulfur compounds can limit growth and so the use of organic carbon for biomass synthesis, or even the co-oxidation of sulfur compounds with organic substrates, may ensure the better survival and growth of sulfur-oxidizing bacteria in the rhizosphere (Anandham *et al.*, 2007). In a previous study, we reported that strain ATSB16^T could use the tetrathionate-intermediate (S₄-I) pathway for thiosulfate oxidation and that extracellular accumulation of elemental sulfur was observed during thiosulfate oxidation (Anandham *et al.*, 2008b). Strain ATSB16^T was able to fix nitrogen in nitrogen-free Nfb medium but did not yield a positive amplicon with *nifH*-specific primers (Anandham *et al.*, 2008a).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain ATSB16^T. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.005 substitutions per nucleotide position.

In the present study, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α - and β -galactosidases, β -glucuronidase, α - and β -glucosidases, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase were not found in strain ATSB16^T or any of the recognized *Pandoraea* species. The absence of aesculin and gelatin hydrolysis and *N*-acetylglucosamine assimilation and the presence of leucine arylamidase activity and utilization of

phenylacetic acid were observed in strain ATSB16^T and all of the recognized *Pandoraea* species. These results are in good agreement with previous results (Coenye *et al.*, 2000). However, Coenye *et al.* (2000) reported the absence of assimilation of substrates such as L-arabinose and D-mannitol and the presence of alkaline phosphatase activity in *Pandoraea* species, for which the present study observed discrepancies. Such laboratory-to-laboratory discrepancies show the importance of obtaining and testing valid reference strains to verify their reactions with in-house procedures (Daneshvar *et al.*, 2001). Strain ATSB16^T can be differentiated from other *Pandoraea* species on the basis of its ability to assimilate D-mannose, maltose, L-rhamnose and L-fucose and inability to assimilate capric acid. The characteristics that differentiate strain ATSB16^T from other recognized *Pandoraea* species are shown in Table 1.

The fatty acid analysis revealed that strain ATSB16^T had a similar fatty acid profile to those of other *Pandoraea* species. However, it can be differentiated from other *Pandoraea* species on the basis of large amounts of C_{16:0} (30.6%) and C_{17:0} cyclo (33.0%) and a small amount of C_{18:1 ω 7c} (3.0%) (Table 2). The major quinone in strain ATSB16^T was ubiquinone 8 (Q-8) and the predominant polyamines were 2-hydroxyputrescine and putrescine. The DNA G+C content of strain ATSB16^T was 64.0 mol%, which is within the range reported for other *Pandoraea* species (61.2–64.3 mol%) (Coenye *et al.*, 2000). Strain ATSB16^T contained the polar lipids phosphatidylmethylethanolamine, diphosphatidylglycerol, phosphatidylethanolamine and unidentified aminophospholipids (Fig. 3).

The genotypic, phenotypic and chemotaxonomic data obtained from this study clearly demonstrate that strain ATSB16^T represents a novel *Pandoraea* species, for which we propose the name *Pandoraea thiooxydans* sp. nov.

Description of *Pandoraea thiooxydans* sp. nov.

Pandoraea thiooxydans (thi.o.ox'y.dans. Gr. n. *theion* sulfur; N.L. v. *oxydo* to make acid, oxidize; N.L. part. adj. *thiooxydans* oxidizing sulfur).

Fig. 2. Scanning (a) and transmission (b) electron microscope images of cells of strain ATSB16^T. Bars, 200 nm (a) and 1 μ m (b).

Table 1. Differential phenotypic and chemotaxonomic characteristics of strain ATSB16^T and type strains of the genus *Pandoraea*

Strains: 1, *Pandoraea thiooxydans* sp. nov. ATSB16^T; 2, *P. pnomenusa* LMG 18087^T; 3, *P. apista* LMG 16407^T; 4, *P. pulmonicola* LMG 18016^T; 5, *P. sputorum* LMG 18819^T; 6, *P. norimbergensis* DSM 11628^T. Data were obtained in this study unless indicated. All strains were positive for assimilation of malic acid, trisodium citrate, phenylacetic acid, sodium acetate, L-alanine, valeric acid, 3-hydroxybutyric acid and L-proline and activities of leucine arylamidase and naphthol-AS-BI-phosphohydrolase. All strains were negative for indole production, hydrolysis of aesculin and gelatin, assimilation of *N*-acetylglucosamine, D-ribose, inositol, sucrose, itaconic acid, potassium 5-ketogluconate, potassium 2-ketogluconate, salicin and melibiose and activities of lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α - and β -galactosidases, β -glucuronidase, α - and β -glucosidases, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6
Catalase	–	+	+	+	+	+
Growth with 4.5 % NaCl	+	–	–	–	–	–
Glucose fermentation	+	–	–	–	–	–
Chemolithoautotrophic oxidation of thiosulfate	+	–	–	–	–	+
Mixotrophic growth with thiosulfate*	+	–	–	–	–	+
Assimilation of:						
L-Arabinose	–	–	–	–	–	+
L-Fucose	+	–	–	–	–	–
D-Glucose	+	w	+	+	–	+
D-Mannose	+	–	–	–	–	–
D-Mannitol	+	–	–	–	+	–
Maltose	+	–	–	–	–	–
L-Rhamnose	+	–	–	–	–	–
D-Sorbitol	+	–	–	–	–	+
Potassium gluconate	w	+	+	+	+	+
Sodium malonate	w	–	–	–	–	–
Adipic acid	+	+	–	–	–	w
Capric acid	–	+	+	+	+	+
3-Hydroxybenzoic acid	–	w	+	+	+	+
4-Hydroxybenzoic acid	–	w	+	+	+	+
Lactic acid	+	+	+	+	–	w
Propionic acid	+	w	+	+	+	+
Suberic acid	w	+	–	–	–	–
Glycogen	w	–	–	–	–	–
L-Serine	+	+	+	+	+	w
Enzyme activity						
Esterase (C4)	w	+	+	+	+	+
Esterase lipase (C8)	–	–	–	–	–	w
Acid phosphatase	–	+	+	+	w	+
Alkaline phosphatase	–	–	w	+	–	+
Arginine dihydrolase	+	–	–	–	–	–
DNA G + C content (mol%)	64.0	64.3†	61.8†	61.8†	61.9†	63.2†

*Result with mineral salts medium amended with 20 mM thiosulfate plus 20 mM sodium acetate or glucose.

†Taken from Coenye *et al.* (2000).

Displays the following properties in addition to those given in Table 1. After 2 days on R2A, colonies are white, circular and convex with clear margins. Cells are Gram-negative, aerobic, rod-shaped and motile by means of a single polar flagellum, 0.5–0.7 μ m wide and 1.2–4.5 μ m long. Negative for catalase. Positive for oxidase and urease. Reduces nitrate and hydrolyses Tween 80. Grows at 4–42 °C (optimum 30 °C) and pH 4.0–10.0 (optimum pH 7.0). Produces acid in O/F glucose medium and grows with 5 % NaCl. Sensitive to ampicillin, kanamycin, chloramphenicol and trimethoprim and resistant to nalidixic acid. Grows

chemolithoautotrophically with thiosulfate, tetrathionate, sulfur and sulfite but not with thiocyanate. Elemental sulfur is deposited extracellularly during growth on media containing thiosulfate. Yeast extract or vitamins are not required for growth. Grows chemo-organoheterotrophically on tryptic soy agar, Luria–Bertani agar, nutrient agar and cetrimide agar. Assimilates D-mannose, maltose, L-rhamnose and L-fucose; does not assimilate capric acid. The predominant polar lipids are phosphatidylmethylethanolamine, diphosphatidylglycerol, phosphatidylethanolamine and unidentified aminophospholipids. The

Table 2. Cellular fatty acid compositions of strain ATSB16^T and type strains of the genus *Pandoraea*

Strains: 1, *Pandoraea thiooxydans* sp. nov. ATSB16^T; 2, *P. pnomenusa* LMG 18087^T; 3, *P. apista* LMG 16407^T; 4, *P. pulmonicola* LMG 18016^T; 5, *P. sputorum* LMG 18819^T; 6, *P. norimbergensis* DSM 11628^T. Data were obtained in this study. Cells were harvested after growth on TSA for 2 days at 30 °C. ECL, Equivalent chain-length; tr, trace (less than 0.5 %).

Fatty acids (%)	1	2	3	4	5	6
C _{10:0}	tr	—	tr	tr	tr	tr
C _{12:0}	3.5	2.1	2.2	3.1	3.0	2.4
C _{12:0} 2-OH	—	tr	tr	tr	tr	tr
C _{12:0} 3-OH	tr	—	—	—	—	—
C _{13:0}	tr	—	—	—	—	—
C _{13:1} AT 12–13	tr	tr	tr	tr	tr	tr
C _{14:0}	0.8	0.7	tr	tr	tr	tr
iso-C _{14:0} 3-OH	tr	—	—	—	—	—
C _{14:1} ω5c	—	—	tr	—	—	—
C _{15:0} 3-OH	tr	—	—	—	—	—
C _{15:1} ω6c	tr	—	—	—	tr	tr
C _{16:0}	30.6	24.7	24.8	20.3	18.9	17.8
C _{16:0} 2-OH	—	—	—	tr	tr	—
C _{16:0} 3-OH	1.7	4.7	5.9	5.8	6.4	4.7
C _{16:1} 2-OH	tr	—	—	tr	—	—
C _{16:1} ω5c	0.8	tr	—	—	tr	—
iso-C _{16:0}	2.1	—	—	—	—	—
C _{17:0}	0.6	tr	tr	tr	0.7	0.6
C _{17:0} cyclo	33.0	10.7	9.9	8.0	13.3	4.5
iso-C _{17:0} 3-OH	—	—	—	tr	—	—
C _{18:0}	tr	1.4	1.1	1.4	1.5	2.5
C _{18:1} 2-OH	tr	tr	0.5	0.5	—	tr
C _{18:1} ω5c	tr	tr	—	tr	tr	tr
C _{18:1} ω7c	3.0	23.7	24.1	25.3	28.1	35.5
C _{18:1} ω7c 11-methyl	—	tr	—	—	—	—
C _{19:0} ω8c cyclo	7.7	7.8	3.8	11.2	—	3.0
C _{19:0} 10-methyl	—	—	—	tr	—	—
C _{20:2} ω6,9c	tr	—	—	tr	—	—
ECL 13.957	tr	—	—	—	—	—
Summed features*						
1	tr	—	—	—	—	—
2	9.7	5.6	6.2	7.0	7.1	5.8
3	3.4	16.2	20.1	14.8	19.1	21.4
7	0.6	—	—	—	—	—

*Summed features represent groups of fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 consisted of iso-C_{15:1} I and/or C_{13:0} 3-OH. Summed feature 2 consisted of one or more of C_{12:0} aldehyde, an unknown fatty acid of ECL 10.928, iso-C_{16:1} I and C_{14:0} 3-OH. Summed feature 3 consisted of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature 7 consisted of one or more of an unknown fatty acid of ECL 18.846, C_{19:0}ω10c cyclo and C_{19:1}ω6c.

polyamine pattern contains 2-hydroxyputrescine and putrescine as the predominant compounds and the quinone system contains exclusively ubiquinone 8 (Q-8).

Fig. 3. Polar lipid pattern of strain ATSB16^T after separation by two-dimensional TLC. DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmethylethanolamine; APL1 and APL2, unidentified amino phospholipids.

The major cellular fatty acids are C_{16:0} and C_{17:0} cyclo. The DNA G + C content of the type strain is 64.0 mol%.

The type strain, ATSB16^T (=KACC 12757^T =LMG 24779^T), was isolated from rhizosphere soils of sesame in Jung ha-dong, Republic of Korea.

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