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ORIGINAL PAPER

Nitratireductor soli sp. nov., isolated from phenol-contaminated soil

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Abstract Strain ZZ-1^T, a Gram-negative, rod-shaped bacterium, motile by flagella, was isolated from phenol-contaminated soil. Strain ZZ-1^T was found to grow at 15–37 °C (optimum 25–30 °C), at pH 6.0–10.0 (optimum pH 7.5) and with 0–8.0 % (w/v) NaCl (optimum 0.5 %). The isolate was found to be able to reduce nitrate to nitrite, but not to nitrogen. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain ZZ-1^T is a member of the genus *Nitratireductor*, and shows high sequence similarities to *Nitratireductor pacificus* MCCC 1A01024^T (98.5 %) and lower (<97 %) sequence similarities to all other *Nitratireductor* species. Chemotaxonomic analysis revealed that strain ZZ-1^T possesses Q-10 as the predominant ubiquinone and Summed feature 8($C_{18:1}$ $\omega 6c$ and/or $C_{18:1}$ $\omega 7c$; 66.6 %), $C_{19:0}$ $\omega 8c$ cyclo (23.3 %), $C_{18:0}$ (3.4 %), iso-$C_{17:0}$ (2.3 %) and $C_{17:0}$ (1.0 %) as the major fatty acids. The polar lipids of strain ZZ-1^T were determined to be diphosphatidylglycerol, phosphatidylcholine, phospholipids, aminolipids, a glycolipid and an aminophospholipid. The DNA G+C content was determined to be 64.1 mol%. Based on the draft genome sequence, the DNA–DNA hybridization

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estimate value between strain ZZ-1^T and *N. pacificus* MCCC 1A01024^T was 46.5 ± 3.0 % and ANI was 75.9 %. The combination of phylogenetic analysis, phenotypic characteristics, chemotaxonomic data and DNA–DNA hybridization supports the conclusion that strain ZZ-1^T represents a novel species of the genus *Nitratireductor*, for which the name *Nitratireductor soli* sp. nov. is proposed. The type strain is ZZ-1^T (=JCM 30640^T = MCCC 1K00508^T).

Keywords *Nitratireductor soli* sp. nov. · Taxonomy · Phenol-contaminated · ANI estimate

Abbreviation

MCCC	Marine Culture Collection of China
ANI	Average nucleotide identity
DDH	DNA–DNA hybridization
MSM	Mineral salts medium

Introduction

Labbé et al. (2004) first described the genus *Nitratireductor*, which forms a novel lineage in the family *Phyllobacteriaceae* within the α2 subgroup of the *Proteobacteria* (Labbé et al. 2004). Members of the genus *Nitratireductor* are Gram-negative, rod-shaped, strictly aerobic, light-yellow or whitish-brown pigmented bacteria. Chemotaxonomic studies have demonstrated that *Nitratireductor* strains contain $C_{18:1}$ *ω7c* as the major fatty acid, $C_{19:0}$ *ω8c* cyclo as the main cyclopropane fatty acid and Q-10 as the main respiratory quinone. Currently, the genus contains 7 species: *Nitratireductor aquibiodomus*, isolated from a denitrification system (Labbé et al. 2004), *Nitratireductor kimnyeongensis*, from seaweed (Kang et al. 2009), *Nitratireductor basaltis*, from black sand (Kim et al. 2009), *Nitratireductor aquimarinus* from a diatom culture (Jang et al. 2011), *Nitratireductor indicus*, from deep-sea water (Lai et al. 2011a), *Nitratireductor pacificus* from an enriched deep-sea sediment sample (Lai et al. 2011b) and *Nitratireductor lucknowense* from pesticide contaminated soil (Manickam et al. 2012) (http://www.bacterio.net/Nitratireductor.html).

During screening for phenol-degrading isolates in phenol-contaminated soil, strain ZZ-1^T was isolated and characterised. The data presented here support the conclusion that strain ZZ-1^T represents a novel species of the genus *Nitratireductor*, for which the name *Nitratireductor soli* sp. nov. is proposed. The type strain is ZZ-1^T (=JCM 30640^T = MCCC 1K00508^T).

Materials and methods

Bacterial strains, isolation and cultivation

During screening for phenol-degrading isolates, a light-yellow pigmented bacterium, designated ZZ-1^T, was isolated from phenol-contaminated soil in Zaozhuang city, Shandong province, P. R. China (N34°42′ E117°33′). *N. pacificus* MCCC 1A01024^T, was used as the reference strain for phenotypic characterisation. Unless indicated otherwise, the morphological, physiological and biochemical characteristics of strain ZZ-1^T and the reference strain were determined using routine cultivation on TSA or TSB at 30 °C.

Phenotypic characterisation

The Gram reaction was determined using the non-staining method, as described by Buck (1982). Cell morphology was determined by light phase-contrast microscopy (IX70; Olympus) and transmission electron microscopy (H7650; Hitachi). The gliding motility was determined using the hanging-drop method (Bernardet et al. 2002). Growth at various temperatures (4, 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C), salt concentrations (0.5–10 % NaCl with increments of 0.5 %, w/v) and pH values (pH 4.0–10.5 with increments of 0.5 pH units) was assessed in TSB and after incubation for up to 5 days. The pH was maintained using four different buffers: 100 mM citric acid-sodium citrate buffer (pH 4.0–6.0), 50 mM phosphate buffer (pH 5.5–8.0), 50 mM Tris–HCl buffer (pH 7.5–9.0) and 20 mM glycine-NaOH buffer (pH 8.5–10.5). Catalase and oxidase was tested according to McCarthy and Cross (1984). Growth under anaerobic conditions was determined in TSB supplemented with or without 0.1 % (w/v) nitrate using the GasPak Anaerobic System (BBL) according to the manufacturer's instructions. Hydrolysis of Tweens 20, 40 and 80 was tested according to Arden Jones et al. (1979). Other biochemical tests were carried out using the API 20 NE, API ZYM systems and 32 GN Microplates, according to the manufacturers' instructions. *N. pacificus* MCCC 1A01024^T was tested at the same time as strain ZZ-1^T.

Sensitivity to antibiotics was tested on TSA plates using discs containing the following antibiotics: ampicillin (10 μg), streptomycin (10 μg), kanamycin (30 μg), gentamicin (10 μg), penicillin (100 μg), vancomycin (30 U), tetracycline (30 μg), piperacillin (100 μg), erythromycin (15 μg), chloramphenicol (30 μg), rifampicin (5 μg), cefoperazone (75 μg), novobiocin (30 μg), cephadrin (30 μg), clindamycin (2 μg), roxithromycin (15 μg), lincomycin (2 μg), carbenicillin (100 μg), norfloxacin (10 μg), amoxicillin (10 μg), and polymyxin B (30 μg).

Determination of 16S rRNA gene sequence and phylogenetic analysis

Genomic DNA was extracted according to standard procedures (Sambrook and Russell 2001). Amplification of the 16S rRNA gene was performed with the primer pair 27F (5′-GAGTTTGATCMTGGCTCAG-3′, positions 8-27 in *Escherichia coli* 16S rRNA) and 1492R (5′-ACGGYTACCTTGTTACGACTT-3′, positions 1492–1507 in *E. coli* 16S rRNA), originally described by Lane (1991). Pairwise sequence similarity was calculated using a global alignment algorithm, implemented in the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012). The 16S rRNA gene sequence alignment was performed using the CLUSTAL_X program (Thompson et al. 1997). Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei 1987) and maximum-likelihood (Felsenstein 1981) with Kimura's two parameter calculation model (Kimura 1980) in MEGA version 6.0 (Tamura et al. 2013). A bootstrap analysis of 1000 resamplings was used to evaluate the tree topology (Felsenstein 1985).

Genome sequencing, G+C content, DDH and ANI estimate

The draft genome sequence strain ZZ-1^T was determined by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China), using Solexa paired-end (500 bp library) sequencing technology (Illumina, SanDiego, CA, USA). The G+C content of the chromosomal DNA was determined by analysis of the draft genome sequence.

DNA–DNA hybridization (DDH) estimate value was analysed using the genome-to-genome distance calculator (GGDC2.0) with the alignment method of BLAST+ (Auch et al. 2010a, b; Meier-Kolthoff et al. 2013). The average nucleotide identity (ANI) was calculated using the algorithm of Goris et al. (2007) by the web service of EZGenome.

Determination of fatty acid, polar lipid analysis and isoprenoid ubiquinone

For chemotaxonomic determination, cells of strain ZZ-1^T and the reference strain were grown on TSB and harvested at the mid-exponential phase by centrifugation, washed with distilled water and freeze-dried. The fatty acid profiles were determined according to the method of manufacturer's instructions of the Sherlock Microbial Identification System (MIDI Corporation; Sasser 1990). The fatty acid methyl esters were obtained from cells by saponification, methylation and extraction, and separated in a gas chromatograph (Agilent 6890N). Peaks were automatically integrated and fatty acid names and percentages were determined using the MIDI Sherlock MIS system (Library: TSBA6; Version, 6.0B). The polar lipid analysis of strain ZZ-1^T was performed at the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described by Tindall (1990a, b). Analysis of the respiratory quinone was carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Quinones were extracted according to Collins et al. (1977) and separated by HPLC (Tamaoka et al. 1983).

Results and discussion

Phenotypic characteristics

Strain ZZ-1^T was found to be a Gram-negative, aerobic, rod-shaped bacterium that is motile by flagella. Cells were observed to be approximately 0.5–0.8 μm in width and 1.2–1.6 μm in length (see Supplementary Materials Fig. S1). Growth was observed at 0–8 % NaCl, at pH 6.0–10.0 and 15–37 °C, Strain ZZ-1^T was found to be sensitive to ampicillin, gentamicin, penicillin, tetracycline, piperacillin, erythromycin, chloramphenicol, rifampicin, novobiocin, clindamycin, roxithromycin, lincomycin, carbenicillin, amoxicillin and polymyxin B; and to be resistant to streptomycin, kanamycin, vancomycin,

Table 1 Physiological characteristics of strain ZZ-1^T and related type strains of *Nitratireductor* species

Characteristic	1	2	3	4	5	6	7	8
Isolation source	Soil	Sea sediment	Sea water	Denitrification system	Seaweed	Black sand	Pesticide contaminated soil	Culture of the diatom
NaCl for growth (%, w/v)	0–7	0–8	0–7	0–5	0–7	0–8	0–5	1–7
Temperature for growth (°C)	10–41	5–37	10–37	ND	10–45	15–45	20–42	20–40
Hydrolysis of								
Tween 20	−	+	NA	NA	NA	NA	NA	NA
Tween 40	w	+	NA	NA	NA	NA	NA	NA
Tween 80	w	−	+	NA	NA	NA	NA	NA
API 20NE								
Indole production	+	−	−	−	−	−	−	−
D-glucose fermentation	+	−	−	−	−	−	−	−
D-mannose	−	+	+	+	+	−	−	+
D-maltose	+	−	+	w	−	−	+	−
Malic acid	+	−	+	−	−	−	−	−
API ZYM								
Valine arylamidase	+	−	+	+	+	+	+	+
β-glucuronidase	+	−	−	−	−	−	−	−
API 32GN								
D-ribose	+	+	NA	+	+	+	+	NA
Suberic acid	+	−	NA	+	NA	NA	−	NA
Lactic acid	−	+	+	−	+	NA	+	NA
L-arabinose	+	−	−	+	−	+	+	NA
L-histidine	+	−	+	NA	NA	−	NA	NA
Sucrose	−	+	+	−	w	+	+	NA
Malate	−	w	−	w	NA	−	NA	NA
3-Hydroxybenzoic acid	−	+	NA	−	NA	NA	NA	NA
Acetate	−	+	NA	−	+	+	−	NA
L-proline	−	+	+	−	−	+	+	NA
L-alanine	−	+	+	+	+	−	NA	NA

Strains 1, ZZ-1^T (data from this study); 2, *N. pacificus* MCCC 1A01024^T(data from this study); 3, *N. indicus* C115^T (data from Lai et al. 2011a, b); 4, *N. aquibiodomus* NL21^T (data from Labbé et al. and Lai et al. 2011a, b); 5, *N. kimnyeongensis* KY 101^T (data from Kang et al. 2009); 6, *N. basaltis* J3^T (data from Kim et al. 2009); 7, *N. lucknowense* IITR-21^T (data from Manickam et al. 2012); 8, *N. aquimarinus* CL-SC21^T (data from Jang et al. 2011). Characteristics are scored as + positive, − negative, *w* weak, *NA* no data available

cefoperazone, cephadrin and norfloxacin. Other characteristics are given in Table 1.

16S rRNA gene sequence analysis

A nearly full-length 16S rRNA gene sequence of strain ZZ-1^T was determined (1448 nt, GenBank accession number KP639570). In the neighbour-joining phylogenetic tree (Fig. 1), strain ZZ-1^T grouped among *Nitratireductor* species and formed a subclade with *N. pacificus* MCCC 1A01024^T. According to sequence similarity calculations, the strain is closely related to *N. pacificus* MCCC 1A01024^T (98.5 %) and showed <97 % similaries with other currently described *Nitratireductor* species. Phylogenetic trees inferred by the maximum-likelihood method are shown in Fig. S2 (see

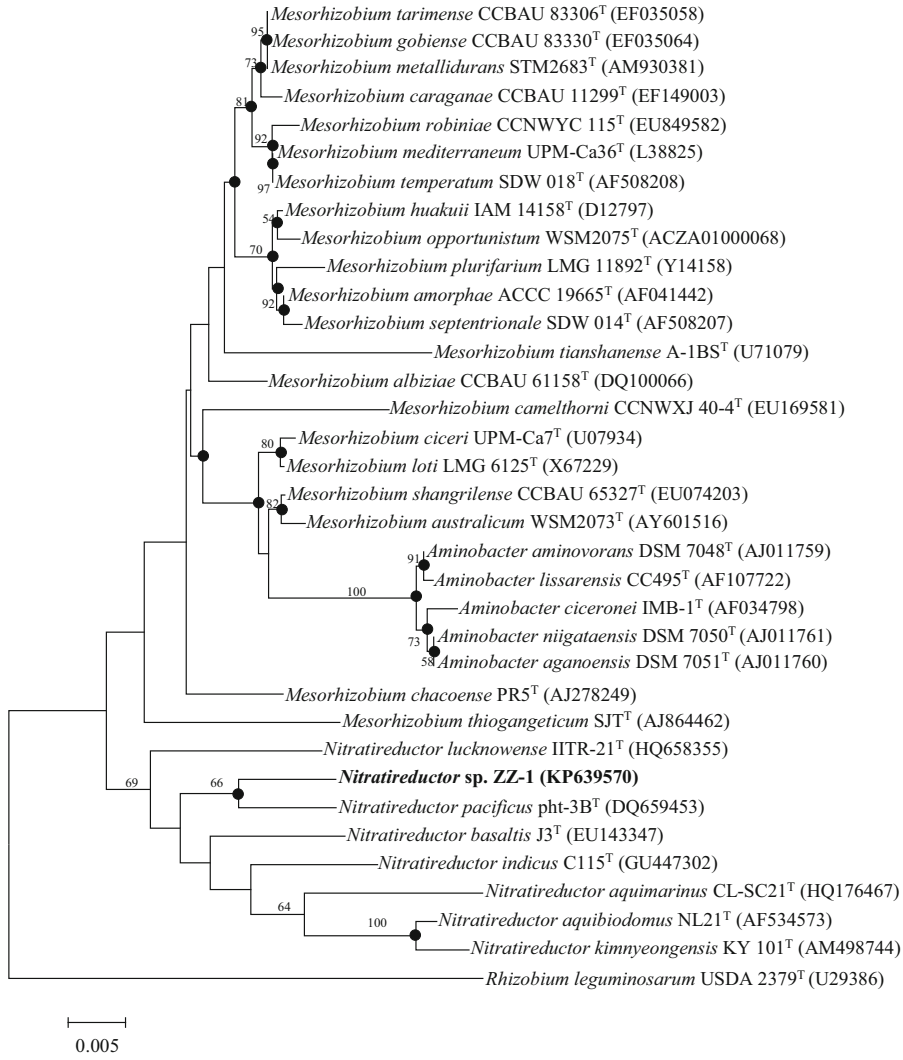

Fig. 1 Phylogenetic tree constructed by the neighbour-joining method based on 16S rRNA gene sequences of strain ZZ-1^T and close relatives. *Rhizobium leguminosarum* USDA 2379^T (U29386) was used as outgroup. *Dots* indicated that the corresponding nodes were also recovered in the trees generated with maximum-parsimony algorithms. Bootstrap values, generated from 1000 re-samplings, at or above 50 % are indicated at the branch points. *Bar*, 0.005 nucleotide substitutions per nucleotide position

Supplementary Materials Fig. S2). This branching pattern demonstrated that strain ZZ-1^T represents a novel species within the genus *Nitratireductor*.

Genome sequencing, DNA G+C content, DDH and ANI estimate chemotaxonomic characteristics

The draft genome of strains ZZ-1^T consists of 5,125,897 bp in length. The accession number for strain ZZ-1^T is LFVY00000000. The draft genome sequence of *N. pacificus* MCCC 1A01024^T (NZ_AMRM00000000.1) was obtained from NCBI. The chromosomal DNA G+C content of strain ZZ-1^T was determined to be 64.1 mol%, which is close to the value determined for *N. pacificus* MCCC 1A01024^T (63 mol%). The DDH estimated value between strain ZZ-1^T and *N. pacificus* MCCC 1A01024^T was 46.5 ± 3.0 %, which is below the standard cut-off value (70 %) (Wayne et al. 1987). The ANI value between strain ZZ-1^T and *N. pacificus* MCCC

1A01024^T was 75.9 %, which is below standard ANI criteria for species identity (95–96 %) (Richter and Rossello-Mora 2009). These data confirm that strain ZZ-1^T represents a novel species of the genus *Nitratireductor*.

Chemotaxonomic characteristics

Chemotaxonomic analysis revealed that strain ZZ-1^T possesses Q-10 as the predominant ubiquinone. This is consistent with the members of the genus *Nitratireductor* (Labbé et al. 2004). The fatty acid profiles of strain ZZ-1^T and *N. pacificus* MCCC 1A01024^T are shown in Table 2. The major fatty acids of strain ZZ-1^T are (> 1 %) Summed feature 8($C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$; 66.6 %), $C_{19:0}$ $\omega8c$ cyclo (23.3 %), $C_{18:0}$ (3.4 %), iso-$C_{17:0}$ (2.3 %) and $C_{17:0}$ (1.0 %). The profile for strain ZZ-1^T was similar to that of *N. pacificus* MCCC 1A01024^T, but some qualitative and quantitative differences in proportions could be observed. Compared to *N. pacificus* MCCC 1A01024^T, strain ZZ-1^T was found to possess a high level of $C_{19:0}$ $\omega8c$ cyclo, whilst iso-$C_{17:0}$ 3-OH was not detected in strain ZZ-1^T. The polar lipids were found to comprise of diphosphatidylglycerol, phosphatidylcholine, phospholipids, aminolipids, a glycolipid and an aminophospholipid (Supplementary Materials Fig. S3).

Table 2 Cellular fatty compositions of strain ZZ-1^T and *N. pacificus* MCCC 1A01024^T

Fatty acid	1	2
$C_{16:0}$	0.8	0.5
$C_{17:0}$	1.0	0.6
$C_{18:0}$	3.4	3.4
Iso-$C_{15:0}$ 3-OH	0.8	0.6
Iso-$C_{17:0}$ 3-OH	–	1.9
Iso-$C_{17:0}$	2.3	2.2
$C_{19:0}$ $\omega8c$ cyclo	23.3	14.9
$C_{18:0}$ 3-OH	0.5	Tr
$C_{20:1}$ $\omega7c$	1.0	0.7
$C_{17:1}$ $\omega8c$	0.5	Tr
Summed feature 3*	0.5	0.4
Summed feature 8*	63.6	71.4

Strains 1, ZZ-1^T; 2, *N. pacificus* MCCC 1A01024^T. All data were obtained in this study. Values represent percentages of total fatty acids; – not detected. tr, trace amounts of less than 0.3 %. Summed features are groups of two or three fatty acids that cannot be separated by GLC using theMIDI system. Summed feature 3* contains $C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7$; Summed feature 8*($C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$)

Taxonomic conclusion

The results of the phylogenetic analysis, phenotypic analysis, and chemotaxonomic studies presented above support the conclusion that strain ZZ-1^T belongs to the genus *Nitratireductor*. However, phylogenetic distinctiveness, some phenotypic differences (Table 1) and its low DDH and ANI values when compared with the type strain of the closely related species *N. pacificus* confirmed that strain ZZ-1^T represents a species distinct from the recognised *Nitratireductor* species. Therefore, strain ZZ-1^T represents a novel species of the genus *Nitratireductor*, for which the name *Nitratireductor soli* sp. nov. is proposed.

Description of *Nitratireductor soli* sp. nov

Nitratireductor soli (so′li. L. neut. gen. n. *soli* of soil, the source of the type strain)

Cells are Gram-negative, aerobic, rod-shaped and motile by flagella. Cells are approximately 0.5–0.8 μm in width and 1.2–1.6 μm in length. After 2 days of incubation on TSA, colonies are 1.5 mm in diameter, smooth, circular, convex and pale yellow. Growth occurs at 15–37 °C (optimum 25–30 °C), at pH 6.0–10.0 (optimum pH 7.5) and in 0–8 % (w/v) NaCl (optimum 0.5 %). Positive for oxidase, catalase, nitrate reduction, indole production, hydrolysis of Tweens 20 and 80 (weak), D-glucose fermentation, aesculin hydrolysis and *N*-acetyl-glucosamine utilisation, but negative for arginine dihydrolase, gelatin hydrolysis or urease. With the API ZYM system, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14) (weakly), cystine aminopeptidase, acid phosphatase, leucine aminopeptidase, valine aminopeptidase, α-chymotrypsin, naphthol-AS-BI-phosphoamidase α-glucosidase, β-glucosidase, β-glucuronidase (weakly), *N*-acetyl-β-glucosaminidase; negative for α-galactosidase, β-galactosidase, α-mannosidase and α-fucosidase. With the API 20 NE system, utilises *N*-acetyl-glucosamine, D-maltose and malic acid but not D-glucose, L-arabinose, D-mannose, D-mannitol, potassium gluconate,

capric acid, phenylacetic acid or adipic acid. With the API 32 GN Microplates, utilises D-ribose, suberic acid, potassium 5-ketogluconate, L-serine, L-arabinose, citrate and L-histidine as sole carbon sources but does not utilise, L-rhamnose, inositol, sucrose, malate, itaconic acid, 3-hydroxybenzoic acid, glycogen, D-mannitol, D-glucose, D-mannose, D-maltose, propionate, adipic acid, phenyl acetate, acetate, citrate, gluconate, malonate, lactic acid, L-alanine, caprate, salicoside, D-melibiose, L-fucose, D-sorbitol, propionic acid, potassium 2-ketogluconate, 4-hydroxybenzoic acid, valeric acid, succinic acid or L-proline as sole carbon sources. The quinone system contains large amounts of Q-10 and the major fatty acids are Summed feature 8* ($C_{18:1}$ $\omega 6c$ and/or $C_{18:1}$ $\omega 7c$), $C_{19:0}$ $\omega 8c$ cyclo, $C_{18:0}$ and iso-$C_{17:0}$. The polar lipids are diphosphatidylglycerol, phosphatidylcholine, phospholipids, aminolipids, a glycolipid and an aminophospholipid. The DNA G+C content of the type strain is 64.1 mol%.

The type strain is *Nitratireductor soli* ZZ-1^T (=JCM 30640^T = MCCC $1K00508^T$), which was isolated from phenol-contaminated soil from Zaozhuang city, Shandong province, P. R. China. The Genbank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZZ-1^T is KP639570. The accession number for the draft genome of strain ZZ-1^T is LFVY00000000.

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