Sphingobium qiguonii sp. nov., a carbaryl-degrading bacterium isolated from a wastewater treatment system

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A Gram-staining-negative, catalase-positive, carbaryl-degrading, non-spore-forming, non-motile, rod-shaped bacterium, designated strain X23^T, was isolated from a wastewater treatment system. Phylogenetic analysis based on 16S rRNA gene sequence indicated that the strain belongs to the genus *Sphingobium*. The highest 16S rRNA gene sequence similarity observed for the isolate was 96.6% with the type strain of *Sphingobium amiense*. Chemotaxonomic data [major ubiquinone: Q-10; major polar lipids: diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, sphingoglycolipid, phosphatidylethanolamine and unknown aminolipids and phospholipids; major fatty acids: summed feature 7 (C_{18:1}ω7c, C_{18:1}ω9t and/or C_{18:1}ω12t), C_{16:1}ω5c, C_{14:0} 2-OH and C_{16:0} 2-OH] as well as the inability to reduce nitrate and the presence of spermidine as the major polyamine supported the affiliation of the strain to the genus *Sphingobium*. Based on the phylogenetic analysis, whole-cell fatty acid composition and biochemical characteristics, the strain could be separated from all recognized species of the genus *Sphingobium*. Strain X23^T should be classified as a novel species of the genus *Sphingobium*, for which the name *Sphingobium qiguonii* sp. nov. is proposed, with strain X23^T (=CCTCC AB 208221^T =DSM 21541^T) as the type strain.

Carbamate pesticides, such as carbaryl (1-naphthyl *N*-methylcarbamate), have been used extensively for the control of pests in agriculture. These pesticides can inhibit the activity of acetyl cholinesterase in an irreversible manner and thereby cause insect death (Fahmy *et al.*, 1970). As the acetyl cholinesterases are also present in all vertebrates, the potential for damage by this class of insecticides to non-target organisms could be very high (Kuhr & Dorough, 1976). Soil micro-organisms play a significant role in the bioremediation of pesticide-contaminated soil, and a variety of carbaryl-degrading soil bacteria have been isolated and characterized (Chapalamadugu & Chaudhry, 1991; Hayatsu & Nagata, 1993; Doddamani & Ninnekar, 2001).

The genus *Sphingobium* was first proposed by Takeuchi *et al.* (2001) to accommodate a part of the genus *Sphingomonas* (Yabuuchi *et al.*, 1990; Takeuchi *et al.*, 1993, 2001), due to the latter containing species that were genetically relatively diverse based on 16S rRNA gene sequences. Members of the genus *Sphingobium* represent environmental isolates that play an important role in the bioremediation and biodegradation of pollutants. At the time of writing, this genus comprised 17 recognized species (www.bacterio.cict.fr/s/sphingobium.html). In this paper, the taxonomic characterization of a carbaryl-degrading bacterium, designated strain X23^T, is presented. Based on the results of a polyphasic taxonomic study, this strain was proposed to represent a novel species of the genus *Sphingobium*.

A conventional enrichment method was employed to isolate the carbaryl-degrading strain (Ou & Sharma, 1989). A carbamate-pesticide-contaminated sludge sample (1.0 g, pH 6.0) collected from the wastewater treatment system of a pesticide manufacturing company in Xuzhou, Jiangsu province, PR China, was inoculated into mineral salts

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain X23^T is EU095328.

A transmission electron micrograph of a cell of strain X23^T, polar lipid profiles, a neighbour-joining tree based on 16S rRNA gene sequences, and cellular fatty acid profiles are available with the online version of this paper.

medium [MM; 4.8 g K₂HPO₄, 1.2 g KH₂PO₄, 1.0 g NH₄NO₃, 0.2 g MgSO₄ · 7H₂O, 0.4 g Ca(NO₃)₂ · 4H₂O, 0.5 g NaCl and 0.001 g Fe₂(SO₄)₃ per litre] supplemented with 30 mg carbaryl l⁻¹ as the sole carbon source and incubated at 28 °C on a rotary shaker at 150 r.p.m. for approximately 1 week. Every 7 days, 5 ml enrichment culture broth was subcultured into 100 ml fresh MM containing 30 mg carbaryl l⁻¹. After five rounds of transferring, the enrichment culture was serially diluted and spread on modified LB/10 agar (MLB; 1.0 g tryptone l⁻¹, 0.5 g yeast extract l⁻¹, 1.0 g NaCl l⁻¹, pH 7.0) supplemented with 100 mg carbaryl l⁻¹. Strain X23^T was maintained on MLB slants at 4 °C and stored as 20 % glycerol suspensions at -80 °C.

Cell morphology and motility were observed by light microscopy (BH-2; Olympus) and transmission electron microscopy (H-7650; Hitachi). Growth at different pH (5–10, in increments of 0.5 pH units) and temperatures (4, 10, 15, 20, 25, 28, 37, 42, 45, 50 and 55 °C) was determined on MLB medium. Catalase activity was determined by assessing bubble production in 3 % (v/v) H₂O₂, and oxidase activity was determined using a 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine (Kovacs, 1956). The effect of oxygen on the growth of strain X23^T was carried out according to the method described by Slack (1968). Nitrate reductase, H₂S production, indole production, urease and gelatinase tests were performed by using the API 20E kit (bioMérieux). Assimilation and oxidation tests were performed using the API 20NE kit (bioMérieux) and Biolog GN2 system, respectively, following the manufacturers' recommendations. Some physiological and biochemical tests were also done according to Holt *et al.* (1994) to confirm the identification. The carbaryl-degrading activity of the strain was analysed by HPLC as described by Yan *et al.* (2007).

Biomass for quantitative analysis of fatty acids, polar lipids, polyamines and isoprenoid quinones of strain X23^T was prepared by scraping cells from MLB plates that had been incubated for 5 days to stationary state at 28 °C. Polar lipids were extracted as described by Minnikin *et al.* (1984) and identified by two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980). Phosphatidylcholine and lipids containing free amino groups were identified by spraying with Dragendorff reagent (Sigma) and ninhydrin reagent, respectively. Sugar-containing lipids were detected by using alpha-naphthol reagent (Sigma). The total polar lipid profile was identified by using molybdophosphoric acid reagent. Polyamines were extracted as described by Takeuchi *et al.* (2001) and analysed by HPLC as described by Hamana *et al.* (1994). Isoprenoid quinones were extracted by using the method of Collins *et al.* (1977) and analysed by HPLC as described by Kroppenstedt (1982). Analysis of whole-cell fatty acids followed the methods described by Sasser (1990) using the Microbial Identification System (MIDI). Chromosomal DNA was extracted and purified as described previously (Cui *et al.*, 2001). The G + C content of DNA was determined by melting-point analysis (Marmur & Doty, 1962) using *Escherichia coli* DH5α DNA as a reference.

The nearly complete 16S rRNA gene sequence was obtained by PCR amplification using a set of universal primers, 5'-AGAGTTTGATCCTGGCTCAG-3' (*E. coli* bases 8–27) and 5'-TACCTTGTTACGACTT-3' (*E. coli* bases 1507–1492), originally presented by Lane (1991). The PCR operating conditions were similar to those described by Suzuki & Yamasato (1994). The PCR product was ligated into the vector pMD18-T (TaKaRa Biotechnology) and then transformed into cells of *E. coli* DH5α. An automatic sequencer (Applied Biosystems, model 3730) was used to determine the 16S rRNA gene sequence. Multiple sequence alignment of 16S rRNA gene sequences was performed by using CLUSTAL X software (Thompson *et al.*, 1997). Phylogenetic analysis was performed by using two tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. A maximum-likelihood tree topology was evaluated by means of a bootstrap analysis based on 100 resamplings.

Cells of strain X23^T were aerobic, Gram-staining-negative, asporogenous, non-flagellated rods (Supplementary Fig. S1, available in IJSEM Online). The strain required about 3 days to form visible colonies on MLB agar at 28 °C and the colonies were circular, slightly convex with entire margins, opaque, greyish-white and 0.5–0.8 cm in diameter after 5 days of incubation at 28 °C. The strain could grow at pH 5.0–8.0; optimum growth was at pH 6.0–7.0. Growth was not observed at temperatures below 4 or above 42 °C. The optimum growth temperature was around 28 °C. Strain X23^T degraded carbaryl. Other phenotypic characteristics of strain X23^T are given in Table 1 and in the species description.

Strain X23^T possessed the following chemotaxonomic characteristics that supported its assignment to the genus *Sphingobium*. Cellular fatty acid analysis revealed the presence of summed feature 7, consisting of one or more of C_{18:1}ω7c, C_{18:1}ω9t and C_{18:1}ω12t (63.64 %) as the major non-hydroxylated fatty acids and C_{14:0} 2-OH (6.97 %) as the major hydroxylated fatty acid. Other fatty acids detected were C_{16:1}ω5c (7.45 %), C_{16:0} 2-OH (5.42 %), C_{16:0} (3.62 %), C_{17:0} (3.48 %), summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 2.86 %) and C_{18:0} (1.22 %). The summed amount of unknown fatty acids was 5.34 %. The detailed fatty acid profiles of strain X23^T and other species of the genus *Sphingobium* are shown in Supplementary Table S1. The respiratory quinone of strain X23^T was ubiquinone Q-10 and the polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, sphingoglycolipid and unknown aminolipids and phospholipids (Supplementary Fig. S2a). These polar lipids are also commonly found in other species of the genus *Sphingobium*, e.g. *S. amiense* YT^T (Supplementary Fig. S2b), *S. fuliginis* TKP^T, *S. xenophagum* BN6^T, *S. cloacae* S-3^T, *S. olei* IMMIB HF-1^T and *S. ummariense* RL-3^T. Strain X23^T contained 3.09 μmol spermidine (g wet cells)⁻¹ as the major polyamine. The genomic DNA G + C content of strain X23^T was 62.7 ± 1 mol%, which fell within the range

Table 1. Differential biochemical characteristics of strain X23^T and the type strains of other species of the genus *Sphingobium*

Strains: 1, *Sphingobium qiguonii* sp. nov. X23^T; 2, *S. amiense* YT^T; 3, *S. herbicidovorans* MBIC 3166^T; 4, *S. fuliginis* TKP^T (Prakash & Lal, 2006); 5, *S. yanoikuyae* GIFU 9882^T (Ushiba *et al.*, 2003); 6, *S. cloacae* S-3^T; 7, *S. xenophagum* BN6^T; 8, *S. olei* IMMIB HF-1^T (Young *et al.*, 2007); 9, *S. ummariense* RL-3^T (Singh & Lal, 2009); 10, *S. aromaticiconvertens* RW16^T (Wittich *et al.*, 2007); 11, *S. rhizovicinum* CC-FH12-1^T; 12, *S. chlorophenolicum* ATCC 33790^T (Ushiba *et al.*, 2003); 13, *S. chungbukense* DJ77^T (Kim *et al.*, 2000); 14, *S. japonicum* UT26^T (Pal *et al.*, 2005); 15, *S. francense* SP + ^T (Pal *et al.*, 2005); 16, *S. indicum* B90A^T (Pal *et al.*, 2005). +, Positive reaction; –, negative reaction; ND, no data available. Data for strains 1, 2, 3, 6, 7 and 11 from this study; data for other strains from references given. GW, Greyish-white; Y, yellow; CW, cream-white; LY, light yellow; CY, cream-yellow.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Colony pigmentation	GW	CY	Y	Y	LY	CW	Y	Y	Y	LY	Y	ND	Y	Y	Y	Y
Motility	–	+	+	–	+	–	+	ND	–	ND	+	–	ND	–	–	–
Oxidase	+	+	–	+	–	+	+	+	–	+	+	+	+	+	+	+
Nitrate reduction	–	–	–	+	–	–	–	–	–	–	+	–	–	ND	ND	ND
β-Galactosidase	–	–	+	–	+	–	+	–	+	+	+	+	–	ND	ND	ND
Acid from glucose	–	+	+	+	+	–	–	+	–	ND	–	+	–	+	+	+
Growth on:																
L-Arabinose	+	–	+	+	+	–	+	+	+	–	+	–	+	+	+	+
D-Mannose	–	–	–	ND	–	–	–	–	–	–	+	–	+	–	–	–
Citrate	+	–	–	ND	+	–	–	–	–	–	+	–	+	–	–	ND
Hydrolysis of:																
Urea	+	–	–	–	–	+	–	–	–	ND	–	–	–	ND	ND	+
Aesculin	–	–	+	–	+	–	+	+	+	–	+	+	–	+	+	–

of 62–67 mol% observed for other members of the genus *Sphingobium*.

Phylogenetic analysis (Fig. 1) showed that strain X23^T formed a monophyletic clade within the genus *Sphingobium*; this relationship was also maintained in the tree based on the neighbour-joining algorithm (Supplementary Fig. S3). The 16S rRNA sequence similarity value between strain X23^T and *Sphingobium amiense* was 96.6%. Strain X23^T was also closely related to the type strains of other species of the genus *Sphingobium*: *S. herbicidovorans* (96.4%), *S. yanoikuyae* (96.2%), *S. xenophagum* (96.0%), *S. fuliginis* (96.0%), *S. cloacae* (95.9%), *S. olei* (95.5%), *S. lactosutens* (95.5%) and *S. ummariense* (95.4%). Determination of DNA–DNA relatedness between strain X23^T and other type strains representing species of the genus *Sphingobium* was not carried out as the 16S rRNA gene sequence similarities did not exceed 97%, the threshold value for the delineation of bacterial species (Stackebrandt & Goebel, 1994). In addition, the nucleotide signatures specific to the 16S rRNA of species of the genus *Sphingobium*, i.e. a U–A pair at position 52:359, an A–G pair at position 987:1218, a U–G pair at position 990:1215 and the presence of U at position 593 (Takeuchi *et al.*, 2001), were all found in the sequence of strain X23^T. This set of nucleotide signatures might be useful for the placement of strain X23^T in the genus *Sphingobium*.

The phylogenetic tree based on 16S rRNA gene sequences, the G + C content of the genomic DNA, the presence of 2-hydroxymyristic acid in the fatty acid profile, the detection of ubiquinone Q-10 and the presence of sphingoglycolipid clearly suggested that strain X23^T belonged to the genus

Sphingobium. In addition, Takeuchi *et al.* (2001) have shown that the inability to reduce nitrate and the presence of spermidine as the major polyamine are good diagnostic markers for the genus *Sphingobium*. Comparison of phenotypic properties between strain X23^T and closely related type strains (Table 1 and Supplementary Table S1) revealed significant differences, supporting the conclusion that strain X23^T was distinct from all known species of the genus *Sphingobium*. Hydrolysis of urea and acid production from glucose in strain X23^T were positive and negative respectively, in contrast to *S. amiense* YT^T, *S. herbicidovorans* MBIC 3166^T, *S. fuliginis* TKP^T and *S. yanoikuyae* GIFU 9882^T. Unlike these type strains, strain X23^T did not possess C_{17:1ω6c}, and the quantity of summed feature 4 was much lower. On the basis of phylogenetic analysis, phenotypic differences and chemotaxonomic data, strain X23^T is suggested to represent a novel species of the genus *Sphingobium*, for which the name *Sphingobium qiguonii* sp. nov. is proposed.

Description of *Sphingobium qiguonii* sp. nov.

Sphingobium qiguonii (qi.guo'ni.i. N.L. masc. gen. n. *qiguonii* of Qi-Guo, to honour Qi-Guo Zhao, a respected Chinese pedologist, for his enormous contributions to pedology and soil biology in China).

Cells are aerobic, Gram-staining-negative, catalase- and oxidase-positive, non-motile, non-sporulating rods with rounded ends, approximately 0.6–0.8 µm wide and 1.0–1.2 µm long without flagellum. Colonies on MLB agar are circular (0.5–0.8 cm in diameter after 5 days), entire, low

Fig. 1. Phylogenetic tree based on complete 16S rRNA gene sequences showing the relationship between strain X23^T and related species of the genus *Sphingobium*. The tree was reconstructed by the maximum-likelihood method and rooted by using *Zymomonas mobilis* as the outgroup. Numbers at nodes represent bootstrap values (based on 100 resamplings). Accession numbers are given in parentheses. Bar, 10 substitutions per nucleotide position.

convex, opaque and greyish-white. Optimum temperature and pH for growth are 28 °C and 6.0–7.0, respectively. Positive for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, gelatinase and urease (API 20E and 20NE). Negative for nitrate reduction, β -galactosidase, β -glucosidase, tryptophan deaminase, Voges–Proskauer reaction, H₂S and indole production. Acid is not produced from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. Utilizes glucose, maltose, arabinose, gluconate and citrate, but not mannose, *N*-acetylglucosamine, mannitol, capric acid, adipic acid, malate and phenylacetic acid. The following carbon sources are oxidized (Biolog GN2 system): L-arabinose, D-arabinose, D-fructose, dextrin, Tweens 40 and 80, α -D-glucose, maltose, succinic acid monomethyl ester, acetic acid, citric acid, DL-lactic acid, L-alaninamide, DL-alanine, L-alanyl glycine, L-glutamic acid, L-aspartic acid, pyruvic acid methyl ester, D-gluconic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, propionic acid, D-saccharic acid, sebacic acid, glycol L-aspartic acid, glycol L-glutamic acid, inosine, uridine, phenylethylamine, putrescine, α -D-glucose 6-phosphate and D-glucose 1-phosphate; the following carbon sources are not oxidized: adonitol, L-fructose, D-galactose, *myo*-inositol, D-psicose, L-rhamnose, D-sorbitol, xylitol, formic acid, succinic acid, bromosuccinic acid, L-asparagine, L-proline, L-ornithine, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, cellobiose, i-erythritol, gentiobiose, α -lactose, lactulose, melibiose, methyl β -D-glucoside, raffinose, trehalose, turanose, *cis*-aconitic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, *p*-hydroxyphenylacetic acid, itaconic acid, malonic acid, quinic acid, succinamic acid, glucuronamide, L-histidine, L-hydroxyproline, L-leucine, L-phenylalanine, L-pyroglyutamic acid, D-serine, L-serine, L-threonine, DL-

carnitine, γ -aminobutyric acid, urocanic acid, thymidine, 2-aminoethanol, 2,3-butanediol and DL- α -glycerol phosphate. Ubiquinone 10 (Q-10) is the predominant respiratory quinone. Major cellular fatty acids are C_{18:1} ω 7c (within summed feature 7), C_{16:1} ω 5c, C_{14:0} 2-OH, and C_{16:0} 2-OH and minor fatty acids are C_{16:0}, C_{17:0}, C_{18:0} and summed feature 4 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH). The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, sphingoglycolipid and unknown aminolipids and phospholipids. Spermidine is the major polyamine. The genomic DNA G+C content of the type strain is 62.7 mol%.

The type strain, X23^T (=CCTCC AB 208221^T =DSM 21541^T), was isolated from a wastewater treatment system in a pesticide manufacturing company in Xuzhou, Jiangsu province, PR China.

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