

Description of *Sinobacter flavus* gen. nov., sp. nov., and proposal of *Sinobacteraceae* fam. nov.

Yu Zhou,¹ Yu-Qin Zhang,^{2,3} Xiao-Yang Zhi,² Xu Wang,¹ Jing Dong,¹ Yun Chen,² Ren Lai^{1,4} and Wen-Jun Li²

Correspondence

Wen-Jun Li
wjli@ynu.edu.cn

Ren Lai
rlai72@njau.edu.cn

¹Key Laboratory of Microbiological Engineering of Agricultural Environment, Ministry of Agriculture, Life Sciences College of Nanjing Agricultural University, Nanjing, Jiangsu 210095, PR China

²The Key Laboratory for Microbial Resources of the Ministry of Education, PR China, and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, 650091, PR China

³Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, PR China

⁴Biotoxin department of Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, PR China

A novel Gram-negative, non-motile, rod-shaped bacterium, designated CW-KD 4^T, was isolated from a polluted soil sample collected from Jiangsu Province, China, by using a classic enrichment method. Based on 16S rRNA gene sequence analysis, the novel strain represented a deep-rooting lineage within the class *Gammaproteobacteria* that was clustered with the genera *Hydrocarboniphaga* and *Nevskia* and some other unidentified bacteria. Polyphasic taxonomic studies revealed that strain CW-KD 4^T showed rather distant relationships to its phylogenetically closest neighbours, including the two recognized genera *Hydrocarboniphaga* and *Nevskia*. Strain CW-KD 4^T showed only 89.9% and 89.7% 16S rRNA gene sequence similarities to the type species of the genera *Hydrocarboniphaga* and *Nevskia*, respectively. The predominant isoprenoid quinone of strain CW-KD 4^T was Q-8 with minor components including Q-9, MK-7 and MK-6. The major fatty acids were C_{16:0}, C_{18:1ω7c} and summed feature 3. The G + C content of the DNA was 65.1 mol%. On the basis of its distinctive phenotypic and genotypic characteristics, strain CW-KD 4^T represents a new genus and a novel species in the class *Gammaproteobacteria*, for which the name *Sinobacter flavus* gen. nov., sp. nov. is proposed. The type strain is CW-KD 4^T (=DSM 18980^T=KCTC 12881^T=CCTCC AB 206145^T). In addition, a new family, *Sinobacteraceae* fam. nov., is proposed to house the genus *Sinobacter* gen. nov.

Modern agricultural practices have relied on the heavy, and sometimes unnecessary, application of chemicals. This has led to high levels of poisonous chemical and chemical intermediate residues in the environment. After pesticides come into contact with water, soils, plants or other environments, they can be cleaned up gradually by processes such as excursion, volatilization, photolysis, absorption, eluviation and biotic or abiotic decomposition (Petit *et al.*, 1995). Micro-organisms can play active roles in the bioremediation of pollutants and representatives of many different genera such as *Pseudomonas*, *Rhodococcus*,

Proteus, *Bacillus*, *Candida* and *Achromobacter* are well known as 'pollutant-eating microbes' that are able to utilize poisonous chemicals as sole sources of carbon and energy for growth (Zwillich, 2000; Ellis *et al.*, 2006).

The novel bacterial strain described in this study was isolated from a farmland soil near a chemical factory. The soil had been contaminated by different kinds of herbicides and insecticides over a long period (data not shown). The novel isolate represented a deep-rooting lineage belonging to the order *Xanthomonadales* which, at present, constitutes a single family, the *Xanthomonadaceae*. The novel isolate showed closest 16S rRNA gene sequence similarity with the established genera *Nevskia* and *Hydrocarboniphaga* (Stürmeyer *et al.*, 1998; Palleroni *et al.*, 2004). Based on the distinct genotypic and phenotypic properties when compared with members of the most closely related genera, the novel strain is proposed as representing a novel genus and

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain CW-KD 4^T is EF154515.

species for which the name *Sinobacter flavus* gen. nov., sp. nov. is proposed.

Strain CW-KD 4^T was isolated from a farmland soil sample from Jiangsu Province, China, using a classic enrichment culture technique (Rosenberg, 1992). Modified ISP 4 medium [consisting of 200 µg ml⁻¹ atrazine, 0.1% K₂HPO₄, 0.1% MgSO₄·H₂O, 0.1% NaCl, 0.2% (NH₄)₂SO₄, 0.2% CaCO₃, 0.0001% FeSO₄·H₂O and 0.0001% MnCl₂·7H₂O] in which one of the carbohydrates was substituted by atrazine as the sole source of carbon and energy, was selected as the isolation medium. Incubation was performed at 30 °C for 7 days and the strain was preserved in a 20% (v/v) glycerol solution in distilled water at -80 °C. Biomass for molecular systematic and chemotaxonomic studies was obtained by incubation at 30 °C for 4 days in shake flasks (about 180 r.p.m.) with yeast tryptone (YT) broth for strain CW-KD 4^T and *Hydrocarboniphaga effusa* AP103^T and synthetic medium for *Nevskia ramosa* Soe 1^T, as described by Stürmeyer *et al.* (1998).

The cellular morphology of the novel isolate was observed by scanning and transmission electron microscopy (SEM, model S-3000N, Hitachi; TEM, model H-7650, Hitachi) (Fig. 1) after incubation for 3 days at 30 °C on YT agar. Samples were prepared for SEM and TEM as described by Nedashkovskaya *et al.* (2005) and Busti *et al.* (2006). The method of Yamaguchi & Yokoe (2000) was followed to determine acid production from carbohydrates. The utilization of sole carbon sources was investigated as described by Zhou *et al.* (2007a). Growth was tested at 5, 10, 25, 30, 35, 37 and 40 °C on YT broth. NaCl and pH tolerance were tested using Luria-Bertani (LB) broth adjusted to various pH values (3.0, 5.0, 6.0, 7.0, 7.8, 8.0 and 10.0) and NaCl concentrations (1–5%) (Zhou *et al.*, 2007b). Antibiotic susceptibility was examined by placing antibiotic discs on YT agar plates as described by Nedashkovskaya *et al.* (2005). Gram-staining and other

physiological characteristics were tested according to the methods of Gerhardt *et al.* (1994). The morphological, physiological and biochemical properties of the novel strain are given in detail in the species description.

Isoprenoid quinones were extracted from lyophilized cells and the samples were purified and analysed by HPLC using the procedures described by Hu *et al.* (2001). The predominant isoprenoid quinone for strain CW-KD 4^T was Q-8 (>83%). Minor components were Q-9, MK-7 and MK-6. *H. effusa* AP103^T and *N. ramosa* Soe 1^T possessed only Q-8 as the isoprenoid quinone. Fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system as described by Sasser (1990). The major fatty acids for strain CW-KD 4^T were C_{16:0}, C_{18:1}ω7c and summed feature 3 (comprising i-C_{15:0} 2-OH and/or C_{16:1}ω7c). The major fatty acid profiles of strains CW-KD 4^T, *H. effusa* AP103^T and *N. ramosa* Soe 1^T are presented in Table 1.

Genomic DNA was prepared for the determination of the base composition following the procedure of Marmur (1961). The DNA G+C content of the strain CW-KD 4^T was 65.1 mol% as determined by the thermal denaturation method (Mandel & Marmur, 1968).

PCR amplification of the 16S rRNA gene was performed as described by Li *et al.* (2007). The 16S rRNA gene sequence was manually aligned with reference sequences retrieved from the GenBank database following BLAST searching (some unidentified and unpublished sequences with high gene sequence similarity values to strain CW-KD 4^T were also included). Phylogenetic analysis was performed using the PHYLIP (Felsenstein, 1993) and MEGA version 3.1 (Kumar *et al.*, 2004) software packages. Multiple alignments were performed with the CLUSTAL_X program (Thompson *et al.*, 1997) and gaps were edited with the BioEdit program (Hall, 1999). Distances (distance options

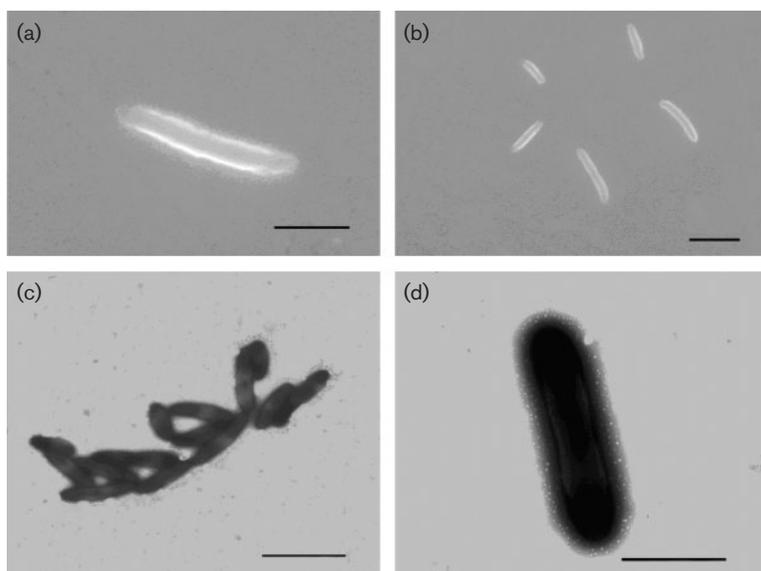


Fig. 1. SEM (a, b) and TEM (c, d) images of cells of strain CW-KD 4^T grown on LB agar for 3 days at 30 °C. Bars, 1000 nm (a, d); 2500 nm (b); 2000 nm (c).

Table 1. Phenotypic characteristics that differentiate strain CW-KD 4^T from related type strains

Taxa: 1, strain CW-KD 4^T; 2, *Hydrocarboniphaga effusa* AP103^T; 3, *Nevskia ramosa* Soe 1^T. +, Positive; –, negative; (+), weakly positive. Microcolonies of strain CW-KD 4^T are circular, convex and smooth. Colonies of *H. effusa* AP103^T are irregular shaped and smooth when small, but become circular later with a wrinkled and folded dome. Colonies of *N. ramosa* Soe 1^T have a rosette-like formation. All three genera have Q-8 as their predominant isoprenoid quinone. Summed feature 3 consists of i-C_{15:0} 2-OH and/or C_{16:1}ω7c. Data were obtained from this study, Palleroni *et al.* (2004) and Stürmeyer *et al.* (1998).

Characteristic	1	2	3
Length of rods (μm)	0.3–0.4 × 2.4–2.6	0.75–0.85 × 1.5–2.0	0.7–1.1 × 1.5–2.3
Cell morphology	Long rod	Rod	Bent Rod
Motility	–	+	+
Nitrate utilization	–	+	(+)
Ammonium salts utilization	–	+	+
Lipase (Tween 80)	–	+	+
Nutritional spectrum of organic or inorganic substrates	Narrow	Wide	Wide
Gelatinase	–	+	–
Catalase	–	(+)	(+)
Oxidase	+	–	–
Benzoate	–	–	+
Ethanol	–	+	+
DL-lactate	–	+	+
Acid production from:			
Glucose	(+)	–	+
Sucrose	(+)	(+)	+
Major cellular fatty acids	C _{18:1} ω7c, C _{16:0} and summed feature 3	C _{18:1} ω7c, i-C _{16:0} and summed feature 3	C _{18:1} ω7c, C _{16:0} and i-C _{16:0}
DNA G + C content (mol%)	65.1	60–61	67.8

according to the Kimura two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis (1000 resamplings) was used to evaluate the topology of the neighbour-joining tree (Felsenstein, 1985).

The almost complete 16S rRNA gene sequence (1487 nt) was determined for strain CW-KD 4^T. A neighbour-joining phylogenetic tree constructed based on 16S rRNA gene sequences showed that strain CW-KD 4^T belongs to the class *Gammaproteobacteria* and clustered together with two established genera, *Nevskia* and *Hydrocarboniphaga*. The novel strain and these two genera, along with some uncultured or unidentified bacteria, formed a deep-rooting lineage within the class *Gammaproteobacteria*. The deep-rooting lineage was given the name 'the HNS (*Hydrocarboniphaga/Nevskia/Sinobacter*) clade'. Obviously, the separated clade should not be included in the single family *Xanthomonadaceae* within the order *Xanthomonadales* (Fig. 2).

The HNS clade in the phylogenetic tree showed that strain CW-KD 4^T belongs to a phylogenetic cluster mainly consisting of uncultured and unidentified bacterial strains from contaminated soil, fresh water or other polluted environments (Fig. 2). An interesting characteristic observed was that many members in the cluster showed

degradation abilities for their corresponding pollutants (data were obtained from the GenBank database). The closest recognized strains were *H. effusa* AP103^T and *N. ramosa* Soe 1^T, but these strains showed only 89.9% and 89.7% 16S rRNA gene sequence similarity values to CW-KD 4^T, respectively.

The phenotypic characteristics that differentiate strain CW-KD 4^T from its closest phylogenetic neighbours *N. ramosa* Soe 1^T and *H. effusa* AP103^T are shown in Table 1. The results of this polyphasic taxonomy study showed that the novel strain could be easily distinguished from its closest relatives both on the basis of phenotypic features and on phylogenetic position. For strain CW-KD 4^T, only several carbohydrates were utilized as sole carbon or sole carbon/nitrogen sources from more than 50 carbohydrates tested; few enzyme activities were detected. The type strains of the type species of the two related genera (*Hydrocarboniphaga* and *Nevskia*) had a wide nutritional spectrum of organic or inorganic substrates. The novel isolate could not be attributed to either of these genera due to their almost complete phenotypic and phylogenetic distinctiveness. No bacterial fatty acid profile matching that of strain CW-KD 4^T was found in the TSBA40 4.10 library, further supporting the placement of the novel isolate in a new genus. It is concluded that strain CW-KD 4^T represents a new genus and novel species in the order

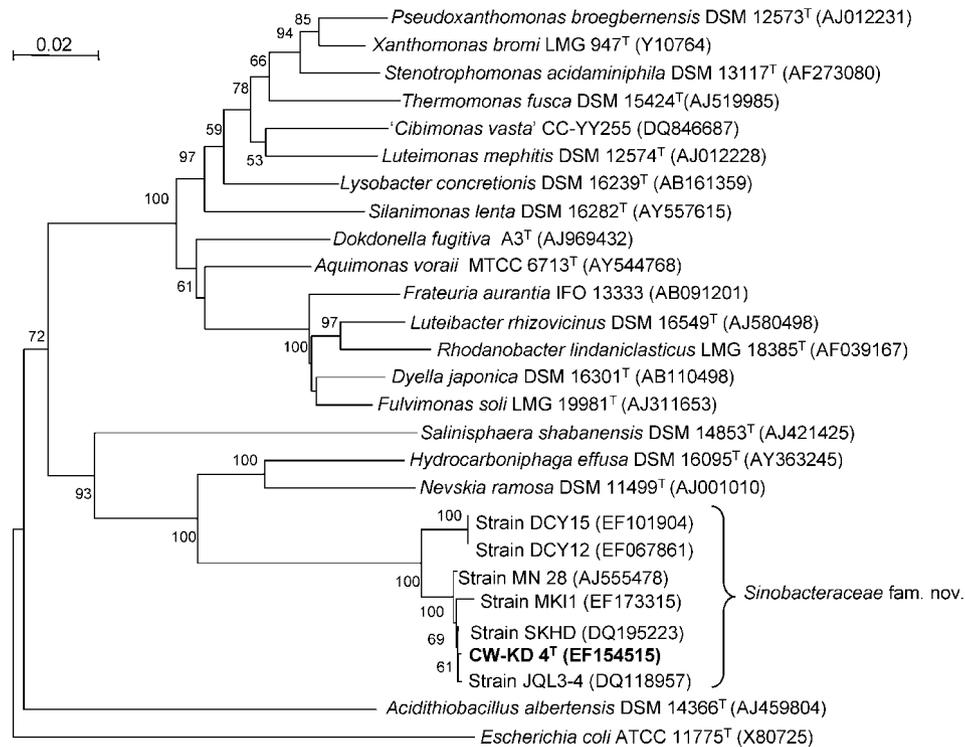


Fig. 2. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain CW-KD 4^T and other related taxa within the order *Xanthomonadales*. *Acidithiobacillus albertensis* in the class *Gammaproteobacteria* and *Escherichia coli* were selected as the outgroup. Numbers at nodes indicate bootstrap values (%) greater than 50%. Bar, 0.02 substitutions per nucleotide position. The maximum-likelihood tree showed essentially the same topology (data not shown).

Xanthomonadales, for which the name *Sinobacter flavus* gen. nov., sp. nov. is proposed.

At present, the order *Xanthomonadales* contains only one family, the *Xanthomonadaceae*. The phylogenetic analysis performed in this study showed that strain CW-KD 4^T of the HNS clade represented a deep-rooting lineage in the order *Xanthomonadales* which clustered with the genera *Hydrocarboniphaga* and *Nevskia* and some uncultured or unidentified bacterial strains. Furthermore, the deep-rooting lineage was separated from the family *Xanthomonadaceae* within the class *Gammaproteobacteria*. The *Sinobacter* clade was also shown to be a well separated lineage with more than 10% 16S rRNA gene sequence dissimilarity to the recognized genera *Hydrocarboniphaga* and *Nevskia*. Thus, based on the distinct phylogenetic position and 16S rRNA gene sequence signature nucleotides of the *Sinobacter* lineage in the class *Gammaproteobacteria*, a new family, *Sinobacteraceae* fam. nov., is proposed to house the newly proposed genus *Sinobacter*.

Description of *Sinobacter* gen. nov.

Sinobacter [Si.no.bac'ter. M.L. *Sinae* of China; N.L. masc. n. *bacter* (from Gr. n. *baktron*) rod; N.L. masc. n. *Sinobacter* rod-shaped microbe isolated from China].

Cells are Gram-negative, long rods, non-endospore-forming and non-motile. Aerobic and chemo-organotrophic. Oxidase positive and catalase negative. The major fatty acids are C_{16:0}, C_{18:1ω7c} and summed feature 3. The predominant isoprenoid quinone is Q-8. The DNA G+C content is about 65 mol%. The type and only species is *Sinobacter flavus* sp. nov.

Description of *Sinobacter flavus* sp. nov.

Sinobacter flavus (fla'vus. L. masc. adj. *flavus* yellow, the colour of colonies or pigment that the bacterium produces).

The following properties are also observed in addition to those given in the genus description. Cells are 0.4–0.5 μm in width and 2.4–2.8 μm in length (Fig. 1). Colonies are circular, convex and pale yellow-coloured on LB or YT agar and are only about 0.5–0.8 mm in diameter after 4 days cultivation at 30 °C. Colonies are ropy and not easy picked up with inoculation loops. Does not require Na⁺ or other special nutrition for growth. Growth occurs at 10–35 °C (optimum, 25–30 °C) and at pH 5–8 (optimum, 6.0–7.0). Growth is detected at 0–2% NaCl and better growth is seen without NaCl. Starch is hydrolysed, but casein, cellulose, chitin, DNA, gelatin and Tweens 80 are not hydrolysed.

Acid is produced from cellobiose, L-rhamnose, D-ribose and sucrose, but not from D-arabinose, adonitol, dulcitol, D-fructose, L-fucose, D-galactose, D-glucose, inositol, D-lactose, maltose, D-mannitol, D-melibiose, D-melezitose, L-raffinose, D-sorbitol, L-sorbose, trehalose or D-xylose. N-acetylglucosamine, cellobiose, D-glucose, glucosamine, D-ribose, starch and sucrose are utilized. Acetate, adonitol, adenosine, aesculin, D-arabinose, dextrin, dulcitol, D-erythrose, D-fructose, D-galactose, gelatin, inulin, inositol, D-lactose, D-mannose, maltose, malonate, D-mannitol, D-melezitose, melibiose, mucic acid, pyruvate, raffinose, salicin, D-sorbitol, D-sorbitose, sucrose, trehalose, Tween 80, turanose, tartrate, xylitol and D-xylose are not utilized. Oxidase and weak urease activities are present. Arginine decarboxylase, arginine dihydrolase, catalase, DNase, β -galactosidase, lysine decarboxylase, lipase (Tween 80 hydrolysis activity), methyl α -D-glucosidase, ornithine decarboxylase and phenylalanine decarboxylase activities are absent. Nitrate and nitrite are not reduced. H₂S (TSI reaction) is not produced. Methyl red and Voges-Proskauer tests are negative. KCN (0.0075%) is not tolerated. Susceptible to ampicillin, carbenicillin, chloramphenicol, doxycycline, erythromycin, kanamycin, lincomycin, neomycin, polymixin B, rifampicin, streptomycin and tetracycline. Resistant to gentamicin, penicillin G and vancomycin. The predominant isoprenoid quinone is Q-8; Q-9, MK-7 and MK-6 are present as minor components. The fatty acid profile contains C_{18:1 ω 7c} (28.92%), C_{16:0} (19.83%), summed feature 3 (12.04%, comprising i-C_{15:0} 2-OH and/or C_{16:1 ω 7c}), i-C_{16:0} (6.75%), C_{16:1 ω 5c} (5.8%), C_{14:0} (4.97%), C_{12:0} (4.08%), c-C_{19:0 ω 5c} (1.48%) and C_{12:0} 2-OH (1%). The DNA G + C content is 65.1 mol%.

The type strain, CW-KD 4^T (=DSM 18980^T=KCTC 12881^T=CCTCC AB 206145^T), was isolated from the surface layer of a polluted farmland soil from Nanjing, Jiangsu Province, China.

Description of *Sinobacteraceae* fam. nov.

Sinobacteraceae (Si.no.bac'ter.a.ce.ae. N.L. masc. n. *Sinobacter* type genus of the family; suff. -aceae ending to denote a family; N.L. fem. pl. n. *Sinobacteraceae* the *Sinobacter* family).

Cells are Gram-negative, non-motile and non-endospore-forming. Obligate aerobes with Q-8 as the predominant isoprenoid quinone and C_{16:0}, C_{18:1 ω 7c} and summed feature 3 as the major fatty acids. Oxidase positive and catalase negative. The pattern of 16S rRNA gene signatures of members of the family consists of nucleotides at positions 143 (C), 220 (A), 289–311 (A–U), 317–336 (C–G), 369–392 (G–A), 514–537 (U–A), 560 (U), 580–761 (U–A), 778–804 (U–U), 1129–1143 (C–A), 1163–1173 (A–U) and 1268 (A). The family is a member of the class *Gammaproteobacteria*, order *Xanthomonadales*. The type genus is *Sinobacter*.

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