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ORIGINAL PAPER

Sinomonas halotolerans sp. nov., an actinobacterium isolated from a soil sample

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Abstract A novel actinobacterial strain, designated CFH S0499^T, was isolated from a soil sample collected from Catba island in Halong Bay, Vietnam. The cells were observed to be Gram-stain positive, aerobic, non-motile, curved rods. The strain was found to grow optimally at 28 °C and pH 7.0. Growth was found to occur at 0–7 % NaCl. Chemotaxonomically, the peptidoglycan type was determined to be of the A3α type, with glutamic acid, glycine, alanine and lysine as the major cell wall amino acids. The whole cell sugars were found to contain mannose, galactose, glucose, ribose and rhamnose. The polar lipids were identified as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, glycolipids and two unidentified phospholipids. The major fatty acids were identified as anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$ and the predominant respiratory quinone as MK-9 (H_2), with a minor amount of MK-10 (H_4) and MK-8 (H_2). The G+C content of the genomic DNA was determined to be 71.8 mol%. The 16S rRNA gene sequence analysis showed that strain CFH S0499^T should be assigned to the genus *Sinomonas* and is closely related to members of the species *Sinomonas atrocyanea* DSM 20127^T (98.3 %), *Sinomonas soli* CW 59^T (98.28 %), *Sinomonas flava* CW 108^T (98.26 %), *Sinomonas mesophila* MPLK 26^T (97.5 %) and *Sinomonas notoginsengisoli* SYP-B 575^T (95.8 %). DNA–DNA hybridizations showed low values (49.1–54.5 %)

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between strain CFH S0499^T and its four closest neighbours. Based on phenotypic, chemotaxonomic and phylogenetic analysis, strain CFH S0499^T is concluded to represent a novel species of the genus *Sinomonas*, for which the name *Sinomonas halotolerans* sp. nov. is proposed, with CFH S0499^T as the type strain (=CCTCC AB2014300^T = KCTC 39116^T).

Keywords *Sinomonas halotolerans* sp. nov. · Family *Micrococcaceae* Halong Bay

Introduction

The genus *Sinomonas*, a member of the family *Micrococcaceae* in the phylum *Actinobacteria*, was first proposed by Zhou et al. (2009) with the description of newly isolated strain *Sinomonas flava* CW 108^T and the reclassification of *Sinomonas atrocyanea* DSM 20127^T (previously as *Arthrobacter atrocyaneus*) (Zhou et al. 2009; Kuhn and Starr 1960). Shortly after the genus was described, another two species *Arthrobacter echigonensis* and *Arthrobacter albidus* were reclassified into the genus *Sinomonas* as *Sinomonas echigonensis* and *Sinomonas albida* (Ding et al. 2009; Zhou et al. 2012). At the time of, nine species have been described in the genus *Sinomonas*, including *S. flava* (Zhou et al. 2009), *S. atrocyanea* (Kuhn and Starr 1960; Zhou et al. 2009), *S. echigonensis* (Ding et al. 2009; Zhou et al. 2012), *S. albida* (Ding et al. 2009; Zhou et al. 2012), *S. soli* (Zhou et al. 2012), *S. notoginsengisoli* (Zhang et al. 2014), *S. mesophila* (Prabhu et al. 2014), and the newly described *S. susongensis* (Bao et al. 2015) and *S. humi* (Lee et al. 2015). All the species of the genus *Sinomonas* are aerobic and rod-shaped, and characterised chemotaxonomically by the presence of galactose, mannose and ribose as the major cell wall sugars; A3α as the peptidoglycan type; MK-9 (H_2) as the predominant menaquinone; diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol as the major phospholipids; and iso-$C_{15:0}$, anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ as the major fatty acids.

During the course of studying the diversity of actinobacteria, strain CFH S0499^T was isolated from a soil sample, collected from Catba island in Halong Bay, Vietnam. Based on its phenotypic, chemotaxonomic and phylogenetic analysis, strain CFH S0499^T can be classified as representing a novel species of the genus *Sinomonas*, for which the name *Sinomonas halotolerans* sp. nov. is proposed.

Materials and methods

Isolation of bacterial strain and culture conditions

Strain CFH S0499^T was isolated from a soil sample collected from Catba island (E 20°46′24″, N 107°07′14″) in Halong Bay, Vietnam, through the serial dilution plating method using Reasoner′s 2A agar medium (BD; Becton, Dickinson and Company). Nalidixic acid (25 mg L^{-1}) and nystatin (50 mg L^{-1}) were added during the isolation process. The purified colonies were maintained on modified T5 medium (tryptone 0.5 g, yeast extract 2.0 g, glucose 1.0 g, lotus root starch 1.0 g, water 1000 mL, agar 20 g, pH 7.0) (Yu et al. 2013) and preserved as glycerol suspensions (20 %, v/v) at −80°C. The reference type strain *S. atrocyanea* DSM 20127^T was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany); *S. flava* CW 108^T and *S. soli* CW 59^T were obtained from the Institute of Quality and Standard for Agro-products, Zhejiang Academy of Agricultural Sciences (China); *S. mesophila* MPLK 26^T and *S. notoginsengisoli* SYP-B 575^T were obtained from Yunnan Institute of Microbiology, Yunnan University, respectively. Reference strains were grown in parallel for comparative testing.

Phenotypic characteristics

Gram-reaction was determined using the standard Gram staining method according to Cerny (1978) after growing the strain for 72 h on modified T5 medium at 28 °C. Growth and colony morphological characteristics were observed after 1 week incubation at 28 °C on LB agar, PYES (Wieser et al. 2002), YDC (Kuhn and Starr 1960) and TYB (Zhou et al. 2009). Colony colours were compared with the ISCC–NBS colour chart (standard samples, No. 2106) (Kelly 1964). Cell motility was observed depending on turbidity of a tube of semi-solid medium (Leifson 1960). Cell morphological properties were examined by light microscopy (model BH2; Olympus) and scanning electron microscopy (ESEM-TMP). The SEM sample was prepared as described by Ming et al. (2014). Modified T5

medium was used to determine growth at various temperatures (4, 10, 15, 20, 28, 37, 45, 50 and 60 °C). Tolerance to NaCl was examined on modified T5 medium containing different NaCl concentrations (0–10.0 %, w/v, at intervals of 1.0 %) after being incubated at 28 °C for 7 days. The pH range for growth (pH 4.0–10.0 at intervals of 1.0 pH units) was tested in modified T5 broth with the pH adjusted according to Xu et al. (2005). For carbon source utilisation and chemical sensitivity tests, the Biolog GIII microplate™ system was used according to the manufacturer's instructions. Nitrate source utilisation was tested as described by Williams (1989). Catalase activity was detected by assessing bubble production in 3 % (v/v) H_2O_2. Oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine (Kovacs 1956). Methyl red and Voges–Proskauer reactions, hydrolysis of Tweens 20, 40, 60 and 80, cellulose, xylan, gelatin, casein, and starch, milk coagulation and peptonization, utilisation of urea, H_2S production and nitrate reduction were performed as described by Gonzalez et al. (1978). Antibiotic susceptibility was observed by the disc (Himedia) diffusion plate method (Groth et al. 2004). Other tests for metabolic properties and enzyme activities were determined using the API ZYM, API 20 NE and API 50CHB test strips (bioMérieux, France) following the manufacturer's instructions.

Chemotaxonomy

To check the chemotaxonomic characteristics, purified cell walls were prepared and hydrolysed as described by (Schleifer and Kandler 1972). Procedures for the identification of cell wall amino acids and whole cell sugars were as described by Tang et al. (2009). Respiratory quinones were extracted from lyophilized cells and the extracts were purified and analysed by HPLC using the method as described by Hu et al. (2001). Polar lipids were examined by two-dimensional thin layer chromatography using the procedures of Minnikin et al. (1979) and Collins and Jones (1980). The fatty acids were prepared and analysed by following the instructions of Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6) (Sasser 1990). Biomass for fatty acid analysis was harvested from tryptose soy agar (TSA; Difco) after incubation at 28 °C for 3 days.

The DNA G+C content was determined by using HPLC method according to Mesbah et al. (1989) with *Escherichia coli* DH5α as the reference.

Molecular analysis

The 16S rRNA gene was amplified from the chromosomal DNA of strain CFH S0499^T using the universal bacterial primers (Li et al. 2007) and the PCR product was sequenced by Sangon Biotech (Shanghai, China). The sequence obtained was compared with available 16S rRNA gene sequences of validly named species in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). Phylogenetic analysis was conducted using the software package MEGA version 6.0 (Tamura et al. 2013) after multiple alignment of the sequence data by CLUSTAL_X (Thompson et al. 1997). Phylogenetic trees were constructed with the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) methods using bootstrap values based on 1000 replicates (Felsenstein 1985). Evolutionary distance matrices were calculated using Kimura's two-parameter model (Kimura 1980).

DNA–DNA hybridizations between strain CFH S0499^T and its close relatives *S. atrocyanea* DSM 20127^T, *S. soli* CW 59^T, *S. flava* CW 108^T and *S. mesophila* MPLK 26^T were carried out using the fluorometric micro-well method (Ezaki et al. 1989; Christensen et al. 2000) with the optimal hybridization temperature (46.9 °C).

Results and discussion

Phenotypic characteristics

Strain CFH S0499^T was observed to be Gram-stain positive, aerobic and non-motile. Light microscopy and scanning electron microscopy showed that the cells form curved rods in 20 h-old cultures and then fragmented into cocci after 48 h (Supplementary Fig. S1). The colonies were observed to be circular, opaque, convex and cream-coloured on modified T5 medium after 3 days incubation at 28 °C. Good growth was observed on the LB agar, YDC, PYES and TYB medium. Colonies were observed to be creamy-white colour on LB and YDC media, and pale-yellow on PYES and TYB media. Strain CFH S0499^T was found

to grow at 10–37 °C, 0–7.0 % NaCl (w/v) and pH 5.0–9.0, with optimal growth at 28 °C and pH 7.0. Strain CFH S0499^T was found to be able to hydrolyse xylan, cellulose and esculin but not Tweens 20, 40, 60, 80, casein, starch or tryptophan. The strain was found to be negative for oxidase, catalase and urease activities, nitrate reduction and H_2S production, while positive results were found for milk coagulation and peptonization, gelatinase, esterase, esterase lipase and alkaline phosphatase activities. Strain CFH S0499^T shows sensitivity to amikacin (30 μg), cefurosimc sodium (30 μg), ciprofloxacin (5 μg), chloroamphenicol (30 μg), erythromycin (5 μg), gentamicin (10 μg), novobiocin (5 μg), penicillin (10 IU), piperacillin (100 μg), polymyxinB (300 IU), sulfamethoxazde (23.75/1.25 μg), tetracycline (30 μg) and vancomycin (30 μg), but resistance to norfloxacin (10 μg), oxacillin (1 μg), ethylhydrocupreine (5 μg).

The differential characteristics to distinguish strain CFH S0499^T from the five reference strains are shown in Table 1, and the detailed characteristics of the strain was summarised in the species description.

Chemotaxonomic characteristics

The total hydrolysate of the cell wall peptidoglycan (4 N HCl, 16 h at 100 °C) revealed the presence of the amino acids lysine (Lys), glycine (Gly), alanine (Ala) and glutamic acid (Glu) in a molar ratio of 2.1 Lys:1.9 Gly:2.1 Ala:1.5 Glu. These results suggest that the new isolate has peptidoglycan type A3α. The whole cell sugars were found to contain major amounts of mannose and galactose and minor amounts of glucose, ribose and rhamnose. MK-9 (H_2), MK-10 (H_4) and MK-8 (H_2) (approx. 86.6, 7.2 and 3.8 %, respectively) were detected as the respiratory quinone system. The major fatty acids (≥5.0 %) were identified as anteiso-$C_{15:0}$ (39.1 %), iso-$C_{15:0}$ (19.0 %), anteiso-$C_{17:0}$ (10.8 %), iso-$C_{16:0}$ (14.7 %) and summed feature 4 ($C_{17:1}$ iso I and/or anteiso B; 9.2 %). The fatty acid profile of strain CFH S0499^T was found to be similar to those of the reference type strains of the genus *Sinomonas* (Supplementary Table S1), which support assigning strain CFH S0499^T to the genus *Sinomonas*. The polar lipids were found to consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, five glycolipids and two unidentified phospholipids (Fig. 1). However, the spot GL3 of strain CFH S0499^T (arrow, Fig. 1) was found to be positive with alpha-naphthol staining but negative with both ninhydrin staining and Dittmer and Lester staining. This component was previously identified to be phosphatidylmethylethanolamine by Zhou et al. (Zhou et al. 2009, 2012) and Bao et al. (2015). We repeated this experiment several times using strain CFH S0499^T and the reference strains *S. atrocyanea* DSM 20127^T and *S. flava* CW 108^T and obtained the same results (Fig. 1). Therefore, we identified the spot as a glycolipid with the same chromatographic position as the phosphatidylmethylethanolamine reported previously in some species of the genus *Sinomonas*.

The genomic DNA G+C content of strain CFH S0499^T was determined to be 71.8 mol%, which is within the range (66.6–71.8 mol%) previously obtained for species of the genus *Sinomonas*.

Molecular analysis

The 16S rRNA gene sequence (1502 nt, GenBank accession number KP232916) of strain CFH S0499^T was determined and compared with the corresponding 16S rRNA gene sequences in the GenBank/EMBL/DDBJ database, which clearly demonstrated that strain CFH S0499^T belongs to the genus *Sinomonas*. On the basis of 16S rRNA gene sequence similarities, four *Sinomonas* type strains were found to be close phylogenetic neighbours including *S. atrocyanea* DSM 20127^T (98.3 %), *S. soli* CW 59^T (98.28 %), *S. flava* CW 108^T (98.26 %) and *S. mesophila* MPLK 26^T (97.5 %); other members of the genus *Sinomonas* showed low similarities (<97 %). The results from EzTaxon-e and the neighbour-joining phylogenetic tree indicated strain CFH S0499^T forms an independent cluster with its neighbour *S. mesophila* MPLK 26^T (Fig. 2). Phylogenetic trees constructed by the maximum-parsimony and maximum-likelihood algorithms also supported these results (Supplementary Figs S2 and S3). The determined DNA–DNA relatedness values between strain CFH S0499^T and *S. atrocyanea* DSM 20127^T, *S. soli* CW 59^T, *S. flava* CW 108^T and *S. mesophila* MPLK 26^T were 54.4 ± 1.9, 53.7 ± 2.5, 53.0 ± 1.9 and 49.1 ± 1.4 %, respectively, which are well below the 70 % cut-off point for recognition of genomic prokaryotic species (Wayne et al. 1987). Thus the strain can be considered to belong to a species separate from other species of the genus *Sinomonas*.

Table 1 Differential phenotypic characteristics of strain CFH S0499^T and its closest phylogenetic neighbours

Characteristic	1	2	3	4	5	6
pH value for growth	5.0–9.0	6.0–9.0	5.0–9.0	6.0–9.0	6.0–8.0	6.0–9.0
NaCl for growth (%)	0–7	0–7(3–6)	0–7(3–6)	0–6	0–6	0–6
Nitrate reduction	−	+	+	+	−	−
Catalase	−	+	+	+	+	+
Degradation of						
Tween 40	−	−	−	+	−	+
Tween 80	−	−	−	−	−	+
Xylan	+	−	−	−	−	−
Cellulose	+	−	−	−	−	−
Urea	−	−	−	−	−	−
Esculin	+	+	−	−	w	−
Gelatin	+	−	−	−	−	−
Carbon utilization						
D-adonitolfucose	−	+	+	−	+	+
D-fructose	w	w	−	−	−	+
D-mannose	−	w	−	−	−	−
D-maltose	−	w	−	−	+	+
D-melibiose	w	+	+	−	w	+
D-melezitose	−	w	−	−	−	−
D-raffinose	−	w	−	−	−	−
D-saccharose	w	w	+	−	−	−
D-turanose	w	−	−	−	−	−
Fucose	−	w	−	−	−	−
Galactose	w	w	−	+	−	+
Glucose	−	w	−	+	−	+
Glycerol	−	w	−	−	w	−
Glycogen	w	−	−	−	−	−
Nitrogen utilization						
L-Valine	+	+	+	−	+	+
L-Tyrosine	−	−	−	−	−	+
L-Methionine	−	+	+	+	−	−
L-Serine	+	+	+	+	−	−
L-Proline	+	+	+	+	−	+
L-Lysine	+	+	+	+	+	−
L-Isoleucine	+	+	+	−	+	+
L-Glutamine	−	−	+	+	−	w
Glycine	+	+	+	+	+	−
N-Acetyl-D-glucosamine	−	w	−	−	−	−
G+C mol %	71.8	70.3[a]	66.9[b]	71.1[a]	68.8[c]	66.6[d]

Data were obtained during this study under identical growth conditions. +, positive; −, negative; w, weakly positive

1, CFH S0499^T; 2, *S. atrocyanea* DSM 20127^T; 3, *S. soli* CW 59^T; 4, *S. flava* CW 108^T; 5, *S. mesophila* MPLK 26^T; 6, *S. notoginsengisoli* SYP-B 575^T

[a] Data from Zhou et al. (2009)

[b] Data from Zhou et al. (2012)

[c] Data from Prabhu et al. (2014)

[d] Data from Zhang et al. (2014)

In conclusion, chemotaxonomic characteristics (major fatty acids, polar lipids and predominant menaquinones) and phylogenetic trees support the conclusion that strain CFH S0499^T belongs to the genus *Sinomonas*. However some phenotypic characteristics, listed in Table 1, and the low DNA–DNA relatedness values showed the difference compared with other described *Sinomonas* species. Therefore, based on these physiological and phylogenetic data, we conclude that strain CFH S0499^T represents a

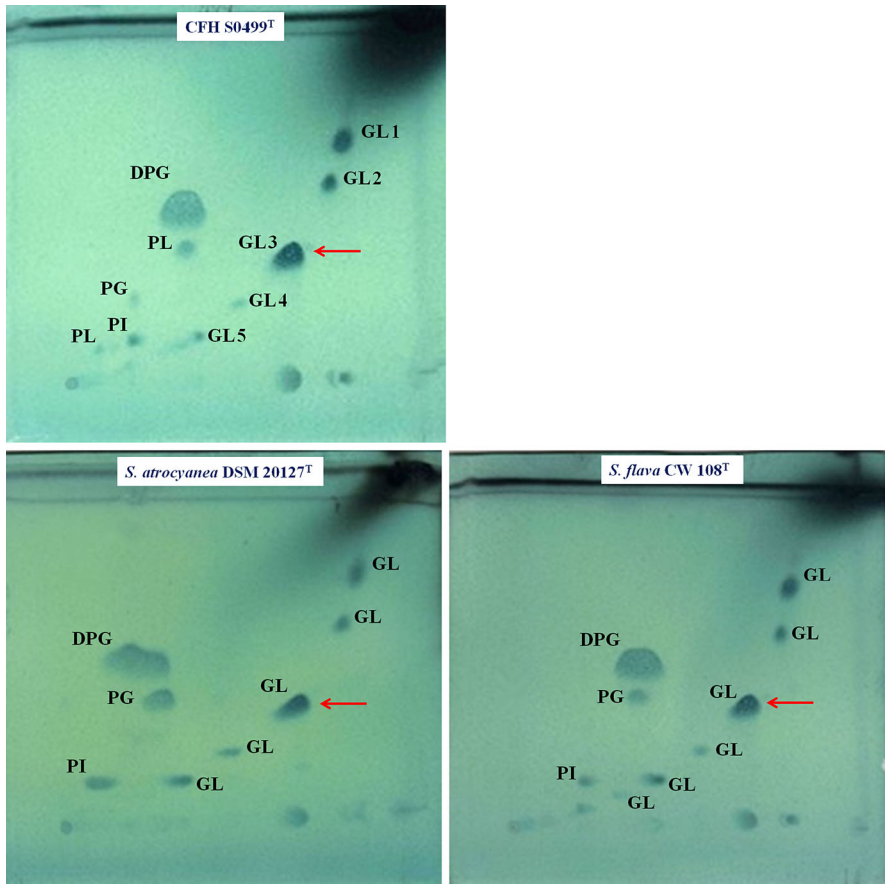

Fig. 1 Two-dimensional thin-layer chromatogram of polar lipids of strain CFH S0499^T and its close relatives of the genus *Sinomonas*. *Note*: DPG, diphosphatidylglycerol; PG, phatidylglycerol; PI, phosphatidylinositol; GL, glycolipid; PL, unknown phospholipid. The chromatographic conditions were as follow: Silica Gel 60 thin-layer plates (10 × 10 cm) were spotted with 10.0 µl of a whole-cell lipid extract. Chloroform–methanol-water (65:25:4, vol/vol/vol) was used in the first direction, and chloroform–acetic acid–methanol-water (80:18:12:5, vol/vol/vol/vol) was used in the second direction. The spray reagent was molybdatophosphoric acid

novel species of the genus *Sinomonas*, for which the name *Sinomonas halotolerans* sp. nov. is proposed.

Description of *Sinomonas halotolerans* sp. nov

Sinomonas halotolerans (ha.lo.to'le.rans. Gr. n. *hals halos* salt; L. pres. part. *tolerans* tolerating, enduring; N. L. part. adj. *halotolerans* salt-tolerating).

Gram-stain positive, aerobic, non-motile, curved rods at the beginning phase of growth, then fragments into coccoid shaped cells. Colonies are circular, opaque, convex and cream-coloured on modified T5 agar. Growth occurs at NaCl (0–7 %), pH 5.0–9.0 (optimum, pH 7.0) and 10–37 °C (optimum, 28 °C). Good growth on LB agar, YDC, PYES and TYB medium. Creamy-white coloured colonies are formed on LB and YDC media and pale-yellow on PYES and TYB media. Able to hydrolyse xylan, cellulose, esculin but not Tweens 20, 40, 60, 80, casein, starch or tryptophan. Negative for oxidase, catalase, urease activity, nitrate reduction and H_2S production; positive for milk coagulation and peptonization, and gelatinase activities. Methyl red and Voges–Proskauer reactions are negative. In API ZYM tests, acid phosphatase, alkaline phosphatase, esterase, esterase lipase, α-glucosidase, β-glucuronidase and leucine arylamidase activities are positive but *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, lipase, α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are negative. In API 20 NE tests, positive for utilisation of *N*-acetyl-glucosamine, L-arabinose (weakly), D-glucose, malic acid, D-maltose, D-mannitol, D-mannose and phenylacetic acid but negative for glucose fermentation and utilisation of capric acid, gluconate, potassium adipic acid and trisodium citrate. Able to use acetic acid, acetoacetic acid, γ-aminobutyric acid, D-cellobiose, dextrin, D-fructose, D-

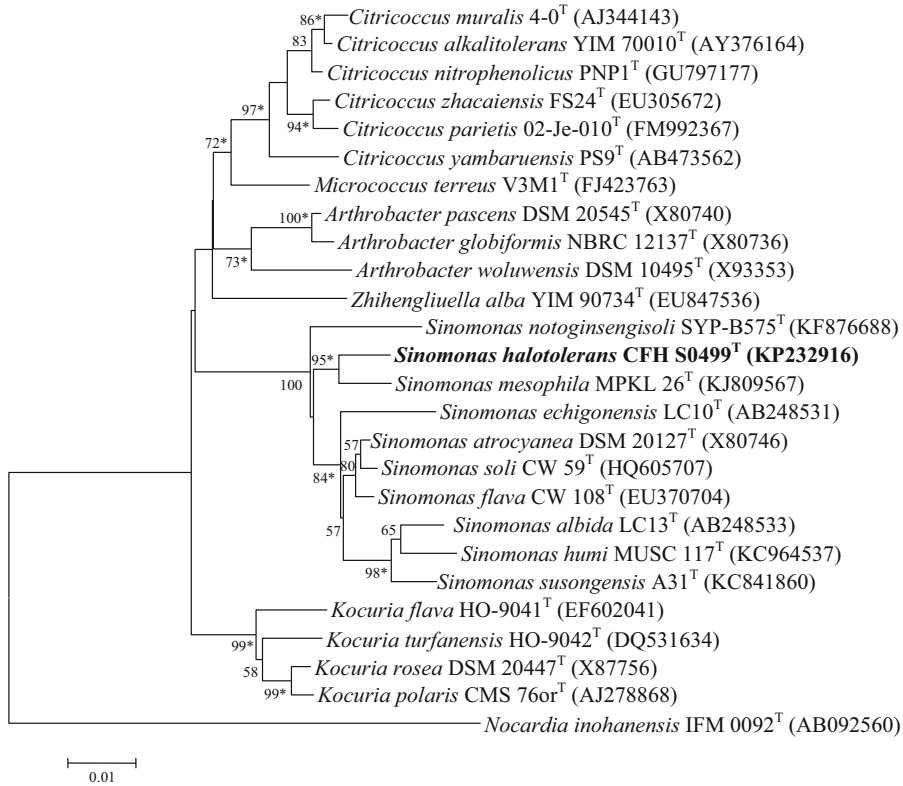

Fig. 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain CFH S0499^T, members of the genus of *Sinomonas* and the type species from each genus within the family *Micrococcaceae*. Bootstrap percentages (≥50 %) based on 1000 resamplings are listed at the nodes. Asterisks indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. *Bar* 0.01 substitutions per nucleotide position

galactose, gentiobiose, D-gluconic acid, α-D-glucose, D-glucuronic acid, glycerol, p-hydroxy-phenylacetic acid, β-hydroxy-D,L butyric acid, inosine, α-D-lactose, D-malic acid, D-maltose, D-mannose, D-melibiose, β-methyl-D-glucoside, propionic acid, quinic acid, D-raffinose, D-salicin, stachyose, sucrose, D-trehalose and D-turanose as the sole carbon source but unable to use D-arabitol, bromo-succinic acid, citric acid, formic acid, D-fructose-6-PO_4, D-fucose, L-fucose, D-galacturonic acid, L-galactonic acid lactone, gelatin, D-glucose-6-PO_4, α-hydroxy-butyric acid, α-keto-butyric acid, α-keto-glutaric acid, L-lactic acid, D-lactic acid methyl ester, L-malic acid, D-mannitol, 3-methyl glucose, methyl pyruvate, mucic acid, myo-inositol, pectin, L-rhamnose, D-saccharic acid or D-sorbitol. Able to use *N*-acetyl-β-D-mannosamine, L-alanine, L-arginine, D-aspartic acid, L-glutamic acid, L-histidine, L-isoleucine, L-lysine, L-proline, L-serine and L-valine as the sole nitrogen source but unable to use *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl neuraminic acid, L-aspartic acid, glucuronamide, L-glutamine, glycine, glycyl-L-proline, L-methionine, L-pyroglutamic acid, D-serine or L-tyrosine. The peptidoglycan type is A3α type, with Glu, Gly, Ala and Lys as the major cell wall amino acids. The whole cell sugars contain major amounts of mannose and galactose, with minor amounts of glucose, ribose and rhamnose. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, glycolipids and two unidentified phospholipids. The major fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The predominant respiratory quinone is MK- 9 (H_2), with a minor amount of MK-10 (H_4) and MK-8 (H_2). The G+C content of the genomic DNA of the type strain is 71.8 mol%.

The type strain CFH S0499^T (=CCTCC AB2014300^T = KCTC 39116^T) was isolated from a soil sample collected from Catba island (E 20°46′24″,

N 107°07′14″) in Halong Bay, Vietnam. The GenBank accession number for the 16S rRNA gene sequence of strain CFH S0499^T is KP232916.

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