Kushneria sinocarnis sp. nov., a moderately halophilic bacterium isolated from a Chinese traditional cured meat

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A Gram-negative, aerobic, moderately halophilic bacterium, designated strain Z35^T, was isolated from a Chinese traditional cured meat produced in Wuhan. The isolate grew with 1–20 % NaCl (optimum 10 %), at 4–42 °C (optimum 37 °C) and at pH 4.5–8.5 (optimum pH 7.0). Cells of strain Z35^T were not motile and were rod or oval shaped. The genomic DNA G + C content was 59.1 mol%. The isoprenoid quinones were Q-9 (88.96 %), Q-8 (9.46 %) and Q-10 (1.58 %). The major fatty acids were C_{19:0} cyclo ω 8c, C_{16:0} and C_{17:0} cyclo. Phylogenetic analysis based on 16S rRNA gene sequence analysis indicated that strain Z35^T was closely related to the type strains of *Kushneria* species with 93.7–95.3 % sequence similarities. Phylogenetic analysis based on 23S rRNA gene sequence similarity values also confirmed the phylogenetic position of the isolate. Therefore, based on the phenotypic, chemotaxonomic and phylogenetic evidence, strain Z35^T is affiliated to *Kushneria*, but is clearly differentiated from other species of this genus and represents a new member, for which the name *Kushneria sinocarnis* sp. nov. is proposed. The type strain is Z35^T (=CCTCC AB 209027^T =NRRL B-59197^T =DSM 23229^T).

The family *Halomonadaceae*, within the class *Gammaproteobacteria*, was proposed by Franzmann *et al.* (1988). Because of the diversity of the family *Halomonadaceae* and the lack of typical differential phenotypic traits, many Gram-negative halophilic micro-organisms that were previously classified as members of genera *Deleya*, *Arthrobacter*, *Flavobacterium*, *Volcaniella*, *Halovibrio*, *Paracoccus*, *Alcaligenes* and *Pseudomonas* are now assigned to the family *Halomonadaceae* (Ventosa *et al.*, 1998). Based on 16S and 23S rRNA gene sequence relationships and phenotypic features, this heterogeneous group was re-evaluated (Arahal *et al.*, 2002; Dobson *et al.*, 1993; Mata *et al.*, 2002). At the time of writing, there were nine genera in the family *Halomonadaceae*: *Chromohalobacter*, *Carnimonas*, *Cobetia*, *Halomonas*, *Halotalea*, *Modicisalibacter*, *Salinicola*, *Zymobacter* and *Kushneria*. *Halomonas* was the largest genus in this family and contained 56 species with validly published names. *Chromohalobacter* contained nine species. The six genera, *Carnimonas*, *Cobetia*, *Halotalea*, *Modicisalibacter*, *Salinicola* and *Zymobacter*, each contained only one species (Arahal & Ventosa, 2008).

Recently, Sánchez-Porro *et al.* (2009) described a new genus, *Kushneria*, with the type species *Kushneria aurantia*, and reclassified *Halomonas marisflavi* (Yoon *et al.*, 2001), *Halomonas indalinina* (Cabrera *et al.*, 2007) and *Halomonas avicenniae* (Soto-Ramirez *et al.*, 2007) as *Kushneria marisflavi*, *Kushneria indalinina* and *Kushneria avicenniae*, respectively. The distinct characteristics of the genus were being oxidase-negative and having small amounts of Q-8 and Q-10 in the respiratory quinone system relative to the most closely related genus, *Halomonas*.

The majority of members of the family *Halomonadaceae* have been isolated from marine and saline environments (Ventosa *et al.*, 1998) and rarely from cured-meat products. To our knowledge, there are only three species within the family *Halomonadaceae* isolated from this type of environment: *Carnimonas nigrificans* (Garriga *et al.*, 1998) from raw cured-meat products; *Halomonas halodenitrificans* (Dobson & Franzmann, 1996) from meat-curing brines; and *Halomonas alimentaria* (Yoon *et al.*, 2002) from a traditional Korean fermented seafood. This study describes a Gram-negative, moderately halophilic bacterium, strain Z35^T, isolated from a piece of Chinese traditional salty pork that was cured with salt in Wuhan, and concludes that strain Z35^T represents a novel member of the genus *Kushneria*.

For the isolation of strain Z35^T, the cured meat was cut into small pieces, added to sterile HGM liquid medium [containing (all l⁻¹): 19.5 g MgCl₂·6H₂O, 29.0 g

The GenBank/EMBL/DDBJ accession numbers for the 16S and 23S rRNA gene sequences of strain Z35^T are FJ667549 and FJ797433, respectively.

A supplementary figure showing a scanning electron micrograph of cells of strain Z35^T is available with the online version of this paper.

MgSO₄·7H₂O, 1.1 g CaCl₂·2H₂O, 6.0 g KCl, 0.5 g NaBr, 10.0 g (NH₄)₂SO₄, 174.0 g NaCl, 3.0 g yeast extract, with 10 mM Tris-HCl buffer to maintain the pH at 7.0; Fairley *et al.*, 2002] and incubated at 37 °C for 24 h with rotation at 200 r.p.m. After this enrichment cultivation, an aliquot of the solution was diluted with 10 % NaCl solution and streaked onto HGM agar plates and incubated at 37 °C for 2 weeks. Single colonies were subcultivated several times to obtain a pure culture.

For taxonomic characterization, strain Z35^T was grown in MH medium (Ventosa *et al.*, 1982) at 37 °C unless otherwise stated. Cell morphology was observed using a scanning electron microscope (JSM-6390/LV; JEOL) with 20 kV accelerating voltage and at magnification ×10 000. Before observation, bacterial colonies from the plates were bound to slides and fixed with 2.5 % (v/v) glutaraldehyde solution. After washing, cells were dehydrated with ethanol and coated with gold.

The optimal conditions for growth were determined in MH medium containing 0, 0.5, 1, 3, 5, 7.5, 10, 12.5, 15, 20, 25 and 30 % (w/v) total salts, as well as in MH medium in which only the NaCl concentration was changed. The specific ionic requirement was examined in MH medium without Mg²⁺, Ca²⁺ and/or K⁺. Growth was also tested with NaCl substituted by KCl. The pH range for growth was determined in filter-sterilized MH medium buffered with hydrogen acetate/sodium acetate (pH 4.0–4.5), 10 mM MES (pH 5.0–6.0), 10 mM PIPES (pH 6.5–7.0), 10 mM Tris/HCl (7.5–9.0) and adjusted with NaOH or HCl. The pH range for growth was tested at pH 4.0–10.0 in increments of pH 0.5. Growth was determined at A₆₀₀. Growth at 0, 4, 15, 20, 28, 37, 42 and 45 °C was also determined.

Phenotypic characterizations were performed following the recommended minimal standards for describing new taxa of the family *Halomonadaceae* (Arahal *et al.*, 2007). Gram-staining reaction was determined using the method described by Dussault (1955). Growth under anaerobic conditions was studied in semi-solid MH medium (0.6 %, w/v, agar) by inoculation at the bottom of the tube, sealing with 2 ml agar (2 %, w/v) and 2 ml paraffin. Acid production from carbohydrates was determined using bromocresol purple supplemented with 1 % of the carbohydrate and liquid MH medium. Nutritional tests were analysed according to Arahal *et al.* (2007). Substrates were added as filter-sterilized solutions to basal medium [(all l⁻¹): 75 g NaCl, 2 g KCl, 0.2 g MgSO₄·7H₂O, 1 g KNO₃, 1 g (NH₄)₂HPO₄, 0.5 g KH₂PO₄] to give a final concentration of 1 g l⁻¹, except for carbohydrates which were used at 2 g l⁻¹. When the substrate was an amino acid, it was tested as a carbon, nitrogen and energy source and therefore the basal medium was prepared without KNO₃ and (NH₄)₂HPO₄. Other morphological, physiological and biochemical characteristics were determined as described by Mata *et al.* (2002). Oxidase reaction and additional biochemical tests were performed using the API 20 NE system (bioMérieux), according to the manufacturer's

instructions, except that the culture was suspended in artificial seawater solution (w/v: 10 % NaCl, 0.594 % MgSO₄·7H₂O, 0.453 % MgCl₂·6H₂O, 0.064 % KCl, 0.013 % CaCl₂). All phenotypic characterizations were done using *K. avicenniae* MW2a^T as a reference (Soto-Ramirez *et al.*, 2007).

The nearly full-length 16S rRNA gene sequence was amplified by PCR with universal primers Uni-27F and Uni-1492R (Wilson *et al.*, 1990) and the PCR products were ligated into the pGEM-T vector (Promega). The full-length 23S rRNA gene sequence was obtained by PCR with two sets of primer pairs: 1310V (5'-GAGTCTGCAACTCGACT-3', forward, positions 1310–1326, *Escherichia coli* 16S rRNA gene sequence numbering; this work) and 985R (5'-CCGGTCCTCTCGTACT-3', reverse; Arahal *et al.*, 2002; *E. coli* positions 2654–2669); and 1023V (5'-GCGTAAYAGCTCACT-3', forward; *E. coli* positions 1091–1105) and 504R (5'-SWGTTTCGRVAWGGA-3', reverse; *E. coli* positions 33–49) (Arahal *et al.*, 2002). DNA sequencing was performed at the Beijing Genomics Institute (Beijing, PR China). The sequences were initially compared with the sequences in the GenBank database. Subsequently, the 16S and 23S rRNA gene sequences were aligned with those of closely related strains of the family *Halomonadaceae* using the CLUSTAL W algorithm with default parameters (Thompson *et al.*, 1994). Phylogenetic analysis was carried out using MEGA 4.0 (Tamura *et al.*, 2007). Distances and clustering were determined using the neighbour-joining and maximum-parsimony methods with bootstrap analyses based on 1000 replications.

The genomic DNA G+C determination and chemotaxonomic study of strain Z35^T, including whole-cell fatty acid and isoprenoid quinone analyses, were carried out by the China Center for Type Culture Collection (Wuhan, PR China). G+C content was determined by HPLC according to the method of Mesbah *et al.* (1989). Respiratory lipoquinone analysis was performed by HPLC as described by Xie & Yokota (2003). For whole-cell fatty acid analysis, strain Z35^T and the type strains of the genus *Kushneria* were grown on SW10 agar at 37 °C for 48 h supplemented with 10 % NaCl and analysed by GC (Hewlett Packard 6890) according to the Sherlock Microbial Identification System (MIDI) instructions. Fatty acids in strain Z35^T after growth on TSB agar were also analysed.

The cell size and morphology of strain Z35^T is shown in Supplementary Fig. S1 (available in IJSEM Online). Detailed results of the morphological, physiological and biochemical characteristics of strain Z35^T are given in the species description. Strain Z35^T shared some characteristics with the type strains of the other *Kushneria* species but also many differences: the main phenotypic differences are shown in Table 1.

The 16S rRNA gene sequence analysis indicated that strain Z35^T was closely related to *K. aurantia* A10^T (95.3 % 16S rRNA gene sequence similarity), *K. avicenniae* MW2a^T (94.1 %), *K. marisflavi* SW32^T (93.7 %) and *K. indalinina*

Table 1. Differential characteristics of strain Z35^T and the type strains of the genus *Kushneria*

Strains: 1, *Kushneria sinocarnis* sp. nov. Z35^T (data from this study); 2, *K. avicenniae* MW2a^T (Soto-Ramirez *et al.*, 2007); 3, *K. marisflavi* SW32^T (Yoon *et al.*, 2001; Mata *et al.*, 2002); 4, *K. indalinina* CG2.1^T (Cabrera *et al.*, 2007); 5, *K. aurantia* A10^T (Sánchez-Porro *et al.*, 2009). +, Positive; +/–, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Cell morphology	Rods or oval shapes	Rods or oval shapes	Rods	Rods or oval shapes	Rods
Pigmentation	Yellow	Orange	Yellow	Orange	Orange
NaCl for growth (% w/v)					
Range	1.0–20.0	0–25.0	0.5–27.0	3.0–25.0	5.0–17.5
Optimum	10.0	5.0	0.5–12.0	7.5–10.0	10.0
pH range for growth	4.5–8.5	5.0–9.0	5.0–10.0	5.0–9.0	5.5–8.5
Temperature range for growth (°C)	4.0–42.0	12.0–40.0	4.0–37.0	15–40.0	20.0–40.0
Reduction of nitrate	+	–	–	+	–
Hydrolysis of:					
Aesculin	+	+	+	–	+
Gelatin	–	+	+	+	+
Acid production from:					
L-Arabinose	+	+	+	+	–
Maltose	+	+ / –	+	–	+
D-Mannose	+	+*	+	–	+
D-Mannitol	+	+	+	–	–
Trehalose	+	+*	+	–	–
D-Xylose	+	+	+	–	+
Growth on:					
Cellobiose	–	+	+	+	+
Lactose	–	–*	+	+	–
D-Xylose	+	+*	+	–	+
Acetate	–	–*	–	+	+
Formate	–	–*	–	+	–
Fumarate	–	+*	+	+	–
Gluconate	+	+*	+	+	–
Adonitol	–	–*	+	+	+
D-Mannitol	–	+*	+	+	+
Sorbitol	+	–*	+	–	+
DL-Isoleucine	+	–*	–	+	–
L-Lysine	–	–*	–	+	–
L-Valine	–	–*	–	+	–

*Data determined in this study.

CG2.1^T (93.7 %). The phylogenetic tree constructed using the neighbour-joining algorithm revealed that strain Z35^T belongs to the cluster of strains of the genus *Kushneria* with high bootstrap support (Fig. 1a). The maximum-parsimony algorithm gave a similar result (data not shown). The 16S rRNA gene sequence of strain Z35^T contained the 15 signature nucleotides defined for members of the family *Halomonadaceae* (Ben Ali Gam *et al.*, 2007).

A complete 23S rRNA gene sequence (2919 bp) of strain Z35^T was obtained and compared with those of type strains of the family *Halomonadaceae*. The 23S rRNA gene sequence similarities of strain Z35^T with its closest neighbours were as follows: *K. aurantia* A10^T, 93.4%; *K. avicenniae* MW2a^T, 92.5%; *K. marisflavi* DSM 15357^T, 92.1%; and *K. indalinina* CG2.1^T, 92.1%. These results were very similar to those with the 16S rRNA gene, which

confirmed the phylogenetic affinity of strain Z35^T to the genus *Kushneria* (Fig. 1b). The maximum-parsimony algorithm gave a similar result (data not shown).

The G+C content of the strain Z35^T was 59.1 mol%, which was very similar to those of the other species of the genus *Kushneria*. The isoprenoid quinones of strain Z35^T consisted of Q-9 (88.96%), Q-8 (9.46%) and a small amount of Q-10 (1.58%). The major cellular fatty acids of strain Z35^T were C_{19:0} cyclo ω8c, C_{16:0} and C_{17:0} cyclo (Table 2). Comparing the amounts found in strain Z35^T with those found in the type strains of the genus *Kushneria* (Table 2) showed that strain Z35^T contained lower levels of C_{18:1}ω7c and C_{12:0} 3-OH and higher levels of C_{12:0}, C_{17:0} cyclo and C_{19:0} cyclo ω8c. In addition, strain Z35^T contained small amounts of C_{18:1}ω9c and iso-C_{13:0} 3-OH that were not found in the other type strains. TSB

Fig. 1. Neighbour-joining trees based on (a) 16S and (b) 23S rRNA gene sequences showing the phylogenetic positions of strain Z35^T and species of the genera *Chromohalobacter*, *Camimonas*, *Cobetia*, *Halomonas*, *Zymobacter* and *Kushneria*. Percentages at nodes are bootstrap values based on 1000 replications. Bars, 0.01 substitutions per nucleotide position.

medium was also used for the fatty acid analysis of strain Z35^T and very similar results were obtained (data not shown).

On the basis of phylogeny, whole-cell fatty acid and quinone compositions and phenotypic traits, we conclude that strain Z35^T represents a novel species of the genus *Kushneria*, for which we propose the name *Kushneria sinocarnis* sp. nov.

Table 2. Whole-cell fatty acids of strain Z35^T and the type strains of the genus *Kushneria*

Strains: 1, *Kushneria sinocarnis* sp. nov. Z35^T; 2, *K. avicenniae* MW2a^T; 3, *K. marisflavi* DSM 15357^T; 4, *K. indalinina* CG2.1^T; 5, *K. aurantia* A10^T. Data for strains 2–5 were taken from Sánchez-Porro *et al.* (2009). All strains were cultured on SW10 medium at pH 7.0 and 37 °C for 24 h.

Fatty acid	1	2	3	4	5
C _{10:0}	0.2	1.0	2.4	1.6	1.1
C _{12:0}	3.3	0.8	—	0.8	0.8
C _{12:0} 2-OH	1.0	3.2	3.2	3.6	0.4
C _{12:0} 3-OH	4.5	11.3	11.0	12.1	8.8
iso-C _{13:0} 3-OH	0.7	—	—	—	—
C _{14:0}	1.7	0.4	0.5	0.7	1.9
Summed feature 3*	2.3	2.6	3.5	4.2	3.5
C _{16:0}	27.3	37.4	33.4	40.7	40.4
C _{17:0} cyclo	11.2	3.2	2.6	2.2	0.9
C _{18:1} ω7c	6.4	16.6	25.7	20.0	28.4
C _{18:1} ω9c	1.4	—	—	—	—
C _{18:0}	3.7	1.2	2.1	1.0	1.1
C _{19:0} cyclo ω8c	33.9	22.3	15.7	12.9	11.8

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C_{16:1} ω7c and/or iso-C_{15:0} 2-OH.

Description of *Kushneria sinocarnis* sp. nov.

Kushneria sinocarnis (si.no.car'nis. M.L. n. *Sina* China; L. n. *caro*, *carnis* flesh, meat; N.L. gen. n. *sinocarnis* of a Chinese meat, relating to the Chinese traditional cured meat from which the organism was first isolated).

Cells are Gram-negative, non-motile rods or oval shapes (0.5–0.8 × 1.4–3.5 μm). Colonies on MH medium are yellow, circular, smooth and convex after incubation for 2 days. Aerobic, moderately halophilic. Growth occurs at 1–20% (w/v) NaCl (optimum 10%) and 5–30% (w/v) total salts (optimum 12.5%). No growth in the absence of NaCl: KCl cannot substitute NaCl. Growth occurs at 4–42 °C (optimum 37 °C), but not at 0 or 45 °C, and pH 4.5–8.5 (optimum pH 7.0). Respiration with fumarate and nitrate is positive but with nitrite is negative. No production of exopolysaccharide or poly-β-hydroxybutyrate. Grows on MacConkey agar supplemented with 10% (w/v) artificial seawater. Oxidase-negative, catalase-positive and negative for fermentation of D-glucose. Nitrate is reduced to nitrite but not to nitrogen. Indole and H₂S are not produced. Methyl red and Voges–Proskauer tests are negative. ONPG and PNPG test results are positive. Aesculin and Tween 20 are hydrolysed, but starch, casein, gelatin, tyrosine, urea, DNA and Tween 80 are not hydrolysed. Lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase and arginine dihydrolase are not produced. Acid is produced from adonitol, L-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-man-

nitro, D-mannose, L-rhamnose, D-salicin, D-sorbitol, sorbose, trehalose and D-xylose, but not from *myo*-inositol, melezitose, raffinose, ribose or sucrose. The following substrates are utilized as sole carbon and nitrogen sources: L-arabinose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, melibiose, ribose, sucrose, trehalose, D-xylose, citrate, lactate, pyruvate, gluconate, succinate, glycerol, sorbitol, L-glutamate, L-proline, DL-isoleucine and L-serine. The following substrates are not utilized: aesculin, cellobiose, lactose, melezitose, raffinose, L-rhamnose, D-salicin, starch, acetate, formate, fumarate, malonate, propionate, caprylate, benzoate, hippurate, malate, tartrate, adonitol, ethanol, 1,2-propanediol, *myo*-inositol, D-mannitol, methanol, *N*-acetylglucosamine, DL- β -hydroxybutyrate, L-alanine, L-histidine, L-lysine, L-methionine, L-valine, L-arginine, L-aspartate, creatine, L-cysteine, L-leucine, L-ornithine, L-phenylalanine, L-threonine and L-tryptophan. Resistant to ($\mu\text{g ml}^{-1}$ unless otherwise stated) amoxicillin (20), ampicillin (10 U), cefotaxime (30), clavulanic acid (10), cefoxitin (30), chloramphenicol (30), kanamycin (30), nalidixic acid (30), polymyxin B (25), streptomycin (10), tobramycin (10) and trimethoprim/sulphamethoxazol (5). Sensitive to erythromycin (15) and nitrofurantoin (300). The quinone system consists of Q-9 and small amounts of Q-8 and Q-10. The major fatty acids (>5%) are C_{19:0} cyclo ω 8c, C_{16:0}, C_{17:0} cyclo, C_{18:1} ω 7c and C_{12:0} 3-OH. The DNA G+C content of the type strain is 59.1 mol% (HPLC).

The type strain, Z35^T (=CCTCC AB 209027^T =NRRL B-59197^T =DSM 23229^T), was isolated from Chinese traditional cured meat in Wuhan, Hubei Province, PR China.

Acknowledgements

We would like to thank Dr Rafael Montalvo-Rodríguez (University of Puerto Rico) for kindly providing *K. avicenniae* MW2a^T. This work was supported by the National Science Foundation of China (30671140) and the Returning Overseas Scientist Fund of the Ministry of Education of China for G. W.

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