Description of *Kushneria aurantia* gen. nov., sp. nov., a novel member of the family *Halomonadaceae*, and a proposal for reclassification of *Halomonas marisflavi* as *Kushneria marisflavi* comb. nov., of *Halomonas indalinina* as *Kushneria indalinina* comb. nov. and of *Halomonas avicenniae* as *Kushneria avicenniae* comb. nov.

Cristina Sánchez-Porro,¹ Rafael R. de la Haba,¹ Nelis Soto-Ramírez,² M. Carmen Márquez,¹ Rafael Montalvo-Rodríguez² and Antonio Ventosa¹

Correspondence
Antonio Ventosa
ventosa@us.es

¹Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

²Biology Department, Box 9012, University of Puerto Rico, Mayagüez 00681, Puerto Rico

An aerobic, moderately halophilic, Gram-negative, motile, non-sporulating rod-shaped bacterium, designated strain A10^T, was isolated from the surface of leaves of the black mangrove *Avicennia germinans* and was subjected to a polyphasic taxonomic study. Strain A10^T was able to grow at NaCl concentrations in the range 5–17.5% (w/v) with optimum growth at 10% (w/v) NaCl. Growth occurred at temperatures of 20–40 °C (optimal growth at 37 °C) and pH 5.5–8.5 (optimal growth at pH 7.0–8.0). The major respiratory quinone was ubiquinone 9. The major fatty acids were C_{16:0}, C_{18:1}ω7c, C_{19:0} cyclo ω8c and C_{12:0} 3-OH. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, and unidentified phospholipids, glycolipids and an aminoglycolipid. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain A10^T is closely related to *Halomonas avicenniae* MW2a^T (95.7% sequence similarity), *Halomonas marisflavi* SW32^T (95.2%) and *Halomonas indalinina* GC2.1^T (95.0%). Strain A10^T formed a coherent phylogenetic branch with these three species, separated from other species of *Halomonas* and closely related genera (with 16S rRNA gene sequence similarities below 94.0%). A complete 23S rRNA gene sequence comparison of strain A10^T with closely related species confirmed the phylogenetic position of the novel isolate, forming a branch with the species *Halomonas avicenniae*, *Halomonas indalinina* and *Halomonas marisflavi*, separated from other species of the genera belonging to the family *Halomonadaceae* (showing sequence similarities below 91.7%). DNA–DNA hybridization studies between strain A10^T and *Halomonas avicenniae* MW2a^T, *Halomonas marisflavi* DSM 15357^T and *Halomonas indalinina* CG2.1^T were 21, 17 and 10%, respectively. These levels of DNA–DNA relatedness were low enough to classify strain A10^T as representing a genotypically distinct species. Overall, the phenotypic, genotypic, chemotaxonomic and phylogenetic results demonstrated that strain A10^T represents a new genus and species. The name *Kushneria aurantia* gen. nov., sp. nov. is proposed, with strain A10^T (=CCM 7415^T=CECT 7220^T) as the type strain. This is the type species of the new proposed genus, which belongs to the family *Halomonadaceae*. In addition, our data support the placement of the species *Halomonas marisflavi*, *Halomonas indalinina* and *Halomonas avicenniae* within this new genus, as *Kushneria marisflavi* comb. nov., *Kushneria indalinina* comb. nov. and *Kushneria avicenniae* comb. nov., respectively.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A10^T is AM941746, and those for the 23S rRNA gene sequences of strains A10^T, *Halomonas avicenniae* MW2a^T, *Halomonas indalinina* CG2.1^T and *Halomonas marisflavi* DSM 15357^T are AM941756, AM941755, AM941754 and AM941753, respectively.

A table showing the 23S rRNA gene amplification and sequencing primers is available as supplementary material with the online version of this paper.

The family *Halomonadaceae*, in the class *Gammaproteobacteria*, was proposed by Franzmann *et al.* (1988) and, at the time of writing, includes a large number of species within seven genera: *Halomonas* (type genus), *Carnimonas*, *Chromohalobacter*, *Cobetia*, *Halotalea*, *Modicisalibacter* and *Zymobacter* (Vreeland *et al.*, 1980; Ventosa *et al.*, 1989; Okamoto *et al.*, 1993; Garriga *et al.*, 1998; Arahal *et al.*, 2002a; Arahal & Ventosa, 2005; Ntougias *et al.*, 2007; Ben Ali Gam *et al.*, 2007). Most genera have a single species, and only *Chromohalobacter* (9 species) and *Halomonas* (49 species) have more than one species. The genus *Halomonas* includes Gram-negative bacteria mainly with a respiratory type of metabolism, and most species that have been isolated from marine or hypersaline environments (salt-terns, saline lakes, saline soils, salted foods, etc.) are halophilic (Arahal & Ventosa 2005; Arahal *et al.*, 2007; Vreeland, 2005). This genus is very heterogeneous, as reflected by the broad range of phenotypic features of the species that it includes (Mata *et al.*, 2002), or the wide range of G+C content of their DNA (52–68 mol%; Vreeland, 2005). Arahal *et al.* (2002b) carried out a phylogenetic study of the family *Halomonadaceae*, based on a comparison of the complete 16S rRNA and 23S rRNA gene sequences, and they established that the genus *Halomonas* is not monophyletic; it comprises two main clusters of species as well as several species that do not cluster consistently.

Recently, we have investigated the microbiota of *Avicennia germinans*, a mangrove that is able to secrete salt crystals onto the surface of its leaves when the salt concentration is in excess of that normally present in the vascular system (Lugo & Snedaker, 1975). As a result we described two novel bacterial species, *Halomonas avicenniae* (Soto-Ramírez *et al.*, 2007) and *Halobacillus mangrovi* (Soto-Ramírez *et al.*, 2008). In the present study, we determined the taxonomic position of strain A10^T, a novel organism isolated from the surface of leaves of the black mangrove, which is closely related to the species *Halomonas marisflavi* (Yoon *et al.*, 2001), and the recently described species *Halomonas indalinina* (Cabrera *et al.*, 2007) and *Halomonas avicenniae* (Soto-Ramírez *et al.*, 2007). Our results show that the novel bacterium constitutes a novel species of a new genus, for which we propose the name *Kushneria aurantia* gen. nov., sp. nov. In addition, we propose the reclassification within this new genus of these three closely related species previously assigned to the genus *Halomonas*.

Strain A10^T was isolated from the surface of leaves of the black mangrove. For isolation, a total of 40 leaves were collected randomly from four *A. germinans* trees growing near the solar salterns of Cabo Rojo, Puerto Rico. These leaves were placed in sterile bags (Whirl Pak) containing 1 % phosphate buffer (pH 7.0) with 15 % (w/v) NaCl and mixed. This procedure allowed the suspension of the micro-organisms present in the salt crystals and on the surface of the leaves. Ten millilitres of the mix were then used for serial dilutions. The dilutions were poured onto

agar plates containing Sehgal-Gibbons medium at 10 % (w/v) NaCl (Sehgal & Gibbons, 1960). The inoculated plates were incubated at 30 °C. After 3 days of incubation, colonies were selected and purified by using the quadrant streak plate method.

The following reference strains were used in this study: *Halomonas avicenniae* MW2a^T, *Halomonas marisflavi* DSM 15357^T, *Halomonas indalinina* CG2.1^T and *Halomonas elongata* ATCC 33173^T. They were cultivated in SW10 medium with 10 % (w/v) total salts [8.1 % NaCl, 0.7 % MgCl₂, 0.96 % MgSO₄, 0.036 % CaCl₂, 0.2 % KCl, 0.006 % NaHCO₃, 0.0026 % NaBr, 0.5 % yeast extract (Difco)] (Nieto *et al.*, 1989). The pH was adjusted to 7.2 with 1 M KOH. Where necessary, solid media were prepared by adding 2.0 % (w/v) Bacto-agar (Difco).

The optimal conditions for growth were determined by growing strain A10^T in SW medium at 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25 and 30 % (w/v) total salts as well as in this medium but with only NaCl instead of the salt mixture described above, and at temperatures of 4, 15, 20, 25, 30, 37, 40 and 42 °C. The pH range for the isolate was tested in SW10 medium adjusted to the following pH values: 4.0, 5.0, 6.0, 7.0, 8.0, 8.5 and 9.0, with the addition of the appropriate buffering capacity to each medium. The cells were cultivated with constant agitation (180 r.p.m.) and growth was monitored by measuring absorbance at 600 nm. Cells of strain A10^T were Gram-negative, non-spore-forming rods. The cells were 2.0–5.0 µm long and 1.0 µm wide at the exponential phase of growth in SW10 medium at 37 °C, and were motile. Optimal growth conditions occurred at 10 % (w/v) total salts or at 10 % (w/v) NaCl, 37 °C and a pH of 7.0–8.0. Thus, strain A10^T is a moderately halophilic micro-organism (Ventosa *et al.*, 1998).

To characterize the isolate phenotypically, we followed the recommended minimal standards for describing new taxa of the family *Halomonadaceae* (Arahal *et al.*, 2007). The phenotypic tests included Gram reaction (Dussault, 1955), cell morphology, motility, catalase and oxidase production, as well as other tests included in the species description. Macroscopic properties were determined using the classical characterization of colony appearance. All biochemical tests were carried out at 10 % total salts and 37 °C, unless stated otherwise. Catalase activity was determined by adding 1 % (w/v) H₂O₂ solution to colonies on SW10 agar medium. The oxidase test was performed using the Dry Slide Assay (Difco). Hydrolysis of starch, gelatin, tyrosine and Tween 80, and production of urease and phosphatase were determined as described by Cowan & Steel (1965), with the addition of 10 % total salts to the medium. Citrate utilization was determined on Simmons' citrate medium supplemented with SW10. Acid production from carbohydrates was determined using a phenol red base supplemented with 1 % of the carbohydrate and SW10 medium. Growth under anaerobic conditions was determined by incubating strain A10^T in an anaerobic chamber

in SW10 medium. Further tests for acid production from carbohydrates and enzymes were carried out using API 20 NE and API ID32E (bioMérieux), inoculated according to the manufacturer's instructions using inoculated fluid at 10 % NaCl and incubation at 37 °C.

To determine the range of substrates used as carbon and energy sources, the classical medium of Koser (1923) as modified by Ventosa *et al.* (1982) was used, containing (l^{-1}): 75 g NaCl, 2 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g KNO_3 , 1 g $(NH_4)_2HPO_4$, 0.5 g KH_2PO_4 and 0.05 g yeast extract (Difco). Substrates were added as filter-sterilized solutions 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 carbon, nitrogen and energy sources, and the basal medium was therefore prepared without KNO_3 and $(NH_4)_2HPO_4$. Additional nutritional features of the isolate were determined using Biolog MicroPlates. Strain A10^T was grown on SW10 medium at 37 °C for 48 h, and suspended in 10 % (w/v) NaCl solution, within the density range specified by the manufacturer with a Biolog photometer model 21101. Immediately after suspending the cells in the saline solution, the suspensions were transferred into sterile multichannel pipetter reservoirs (Biolog) and the Biolog GN Microplates were inoculated with 125 µl of the cell suspension per well by means of an 8-channel repeating pipetter. The inoculated Biolog plates were incubated at

37 °C for 7 days and the results were read with a MicroPlate Reader using Microlog 3.59 computer software to perform automated reading. Antibiotic susceptibility was determined according to the conventional Kirby-Bauer method (Bauer *et al.*, 1966). Other characterization methods used have been described in detail by Ventosa *et al.* (1982) and Quesada *et al.* (1984). The phenotypic characteristics of strain A10^T are given in Table 1 and in the species description.

Chromosomal DNA was isolated and purified according to the method described by Marmur (1961). The 16S rRNA gene was amplified using the universal primers 16F27 and 16R1488 as described by Mellado *et al.* (1995). An almost complete 16S rRNA nucleotide sequence of strain A10^T was determined by NBT-Newbiotechnics (Sevilla, Spain) using an automated DNA sequencer model 3130XL (Applied Biosystems) and was compared with 16S rRNA reference gene sequences retrieved from GenBank and EMBL databases by BLAST search. Subsequent sequence analysis was conducted using the ARB program package (Ludwig *et al.*, 2004). Following the recommendations of Ludwig *et al.* (1998), alternative treeing methods (maximum-parsimony, neighbour-joining and maximum-likelihood) were used. A comparison using 16S rRNA gene sequences from databases revealed that the 16S rRNA gene sequence (1428 bp) of strain A10^T displayed a high level of similarity to those of *Halomonas* species. The closest relatives were

Table 1. Characteristics that differentiate strain A10^T from the type strains of *Halomonas marisflavi*, *Halomonas indalinina*, *Halomonas avicenniae* and *Halomonas elongata*

Strains: 1, A10^T (*Kushneria aurantia* gen. nov., sp. nov.); 2, *Halomonas marisflavi* KCCM 80003^T; 3, *Halomonas indalinina* CG2.1^T; 4, *Halomonas avicenniae* MW2a^T; 5, *Halomonas elongata* ATCC 33173^T. Data are from Yoon *et al.* (2001), Cabrera *et al.* (2007), Soto-Ramírez *et al.* (2007), Arahal & Ventosa (2005) and this study. +, Positive; –, negative; ±, weak reaction.

Characteristic	1*	2	3	4	5
Cell morphology	Rods	Rods or oval cells	Short rods	Rods or oval cells	Short rods
Colony pigmentation	Orange	Yellow	Orange	Orange	Cream
NaCl range (% w/v)	5–17.5	0.5–27	3–25	0–25	0–20
NaCl optimum (% w/v)	10	0.5–12	7.5–10	5	3–8
pH range	5.5–8.5	5.0–10.0	5.0–9.0	5.0–9.0	5.0–10.0
Temperature range (°C)	20–40	4–37	15–40	12–40	4–45
Nitrate reduction	–	–	+	–	+
H ₂ S production	–	–	–	+	±
Acid production from:					
L-Arabinose	–	+	+	+	+
D-Fructose	+	+	+	+	–
Maltose	+	+	–	±	+
Mannitol	–	±	–*	+	+
Trehalose	–	+	–	+	–
Xylose	+	+	–	+	–
Hydrolysis of:					
Aesculin	+	+	–	+	–
Urea	–	–	–	–	+
DNA G + C content (mol%)	61.7	59.0	60.9	61.5	60.5

*Data from this study.

Halomonas avicenniae MW2a^T, *Halomonas marisflavi* DSM 15357^T and *Halomonas indalinina* CG2.1^T, with 95.7, 95.2 and 95.0 % sequence similarities, respectively. Sequence similarity with respect to other *Halomonas* species was equal to or less than 92.6 %; the similarities obtained when strain A10^T was compared with other species of the genera *Halomonas*, *Chromohalobacter*, *Modicisalibacter*, *Halotalea*, *Zymobacter*, *Carnimonas* or *Cobetia* were 92.6–90.7, 93.6–92.0, 92.5, 91.3, 91.1, 91.0 and 90.9 %, respectively. The phylogenetic tree obtained by using the maximum-parsimony method showed strain A10^T within a cluster constituted by the species *Halomonas avicenniae* MW2a^T, *Halomonas marisflavi* SW32^T and *Halomonas indalinina* CG2.1^T (Fig. 1). The tree topology was similar when we used other algorithms (neighbour-joining and maximum-likelihood). Strain A10^T and these three *Halomonas* species were phylogenetically related to *Chromohalobacter* species (94.0–92.0 % sequence similarity) and to other species of *Halomonas* (93.8–90.7 %). The similarity values with respect to the genera *Modicisalibacter*, *Halotalea*, *Zymobacter*, *Carnimonas* and *Cobetia* were 92.8–92.5 %, 92.8–91.3 %, 93.4–91.1 %, 91.8–91.0 % and 92.6–90.9 %, respectively.

In order to confirm the phylogenetic position of these species, we carried out a phylogenetic study based on a comparison of 23S rRNA gene sequences. The 23S rRNA gene was amplified using primers designed in this study and others described by Arahal *et al.* (2002b). An almost complete 23S rRNA nucleotide sequence was determined by NBT-Newbiotechnics (Sevilla, Spain) using an automated DNA sequencer model 3130XL (Applied Biosystems). 23S rRNA gene amplification and sequencing primers used are shown in Supplementary Table S1

(available in IJSEM Online). The 23S rRNA gene sequences of strain A10^T, *Halomonas avicenniae* MW2a^T, *Halomonas marisflavi* DSM 15357^T and *Halomonas indalinina* CG2.1^T were compared with reference 23S rRNA gene sequences retrieved from the GenBank and EMBL databases by BLAST search. Subsequently, sequence analysis was performed using the ARB program package (Ludwig *et al.*, 2004). Alternative treeing methods (neighbour-joining, maximum-parsimony and maximum-likelihood) were used for these analyses. Similar to the 16S rRNA gene sequence analysis, the 23S rRNA gene sequence (2914 bp) of strain A10^T displayed a high level of similarity to those of *Halomonas avicenniae* MW2a^T, *Halomonas indalinina* CG2.1^T and *Halomonas marisflavi* DSM 15357^T, with 94.5, 93.6 and 93.5 % gene sequence similarities, respectively. The sequence similarities of strain A10^T and other *Halomonas* species were 90.2–87.6 %, whereas when we compared the sequence of strain A10^T with those of other related genera the values were 90.1–89.3 % with *Chromohalobacter*, 89.8 % with *Cobetia*, 88.8 % with *Zymobacter* and 88.3 % with *Carnimonas*. The phylogenetic tree obtained using the maximum-parsimony method showed that strain A10^T fell within a cluster constituted by the species *Halomonas marisflavi* DSM 15357^T, *Halomonas indalinina* CG2.1^T and *Halomonas avicenniae* MW2a^T (Fig. 2). When we used the neighbour-joining or maximum-likelihood algorithms the trees obtained were very similar to each other, confirming the stability of the tree topologies. These data confirm that strain A10^T and the species *Halomonas marisflavi*, *Halomonas indalinina* and *Halomonas avicenniae* constitute a robust and stable phylogenetic branch within the family *Halomonadaceae*, sufficiently separated from other species of the genera of

Fig. 1. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences of strain A10^T and closely related species. Sequence accession numbers used are shown in parentheses. Bootstrap values greater than 50 % are indicated at branch points. *Zymobacter palmarum* ATCC 51623^T was used as an outgroup. Bar, 2 % sequence divergence.

Fig. 2. Maximum-parsimony phylogenetic tree, based on 23S rRNA gene sequences of strain A10^T and closely related species. Sequence accession numbers used are shown in parentheses. Bootstrap values greater than 50% are indicated at branch points. *Zymobacter palmae* ATCC 51623^T was used as an outgroup. Bar, 2% sequence divergence.

this family as to warrant their placement in a new genus. In fact, when we compared the 23S rRNA gene sequences of strain A10^T and those of *Halomonas marisflavi* DSM 15357^T, *Halomonas indalinina* CG2.1^T and *Halomonas avicenniae* MW2a^T with respect to other species of the most closely related genera, we obtained the following similarity values: 91.5–87.6% for other species of the genus *Halomonas*, 91.7–89.3% for species of *Chromohalobacter*, 91.7–89.8% for *Cobetia*, 90.4–88.8% for *Zymobacter* and 89.8–88.3% for *Carnimonas*.

The G+C content of the genomic DNA was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979), as previously described in detail (Ventosa *et al.*, 1999). The DNA G+C content of strain A10^T was 61.7 mol%, which is within the broad range described for species belonging to the genus *Halomonas* (52–68 mol%) (Arahal & Ventosa, 2005; Vreeland, 2005). However, this G+C value was quite similar to those of the three closely related species of *Halomonas*, *Halomonas marisflavi*, *Halomonas indalinina* and *Halomonas avicenniae* (Table 1).

DNA–DNA hybridization studies were performed by using the competition procedure of the membrane method (Johnson, 1994), described in detail by Arahal *et al.* (2001a, b). The hybridization temperature used was 56.9 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970) and the percentage hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate. The levels of DNA–DNA relatedness between strain A10^T and *Halomonas avicenniae* MW2a^T, *Halomonas marisflavi* DSM 15357^T and *Halomonas indalinina* CG2.1^T were 21, 17 and 10%, respectively. These levels of DNA–DNA relatedness were low enough to classify strain A10^T as

representing a genotypically distinct species (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

Analysis of quinones and polar lipids was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The polar lipids detected for strain A10^T were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, three different phospholipids of unknown structure, two glycolipids and an aminoglycolipid. This pattern was essentially the same as those found for *Halomonas avicenniae* MW2a^T, *Halomonas marisflavi* DSM 15357^T and *Halomonas indalinina* CG2.1^T. Strain A10^T contained ubiquinone 9 (Q9) as the major respiratory quinone (95%), as well as ubiquinone 8 (Q8) (5%) and traces of ubiquinone 10 (Q10). The major respiratory quinones detected for *Halomonas avicenniae* MW2a^T were Q9 (99%) and traces of Q8 and Q10, for *Halomonas marisflavi* DSM 15357^T Q9 (92%), Q8 (6%) and Q10 (2%) and for *Halomonas indalinina* CG2.1^T Q9 (93%), Q8 (4%) and Q10 (3%). Thus, the polar lipids and respiratory quinones of strain A10^T were almost identical to those of the other three species; however, Q9 is typically also found in other members of the genus *Halomonas* (Arahal & Ventosa, 2005; Vreeland, 2005; Arahal *et al.*, 2007).

Fatty acids were analysed using GC (Kämpfer & Kroppenstedt, 1996; Miller, 1982) at the BCCM/LMG, Gent, Belgium. Cells were cultured on SW10 medium at pH 7.0 and 37 °C for 24 h. The predominant fatty acids of strain A10^T were C_{16:0}, C_{18:1}ω7c, C_{19:0} cyclo ω8c and C_{12:0} 3-OH. This composition was very similar to those that we obtained under the same conditions for *Halomonas avicenniae* MW2a^T, *Halomonas marisflavi* DSM 15357^T and *Halomonas indalinina* CG2.1^T, but some differences were observed with respect to *Halomonas elongata* ATCC 33173^T (Table 2). However, this is a differential feature of the new

Table 2. Cellular fatty acid compositions (%) of strain A10^T and closely related *Halomonas* species

Strains: 1, A10^T (*Kushneria aurantia* gen. nov., sp. nov.); 2, *Halomonas avicenniae* MW2a^T; 3, *Halomonas indalinina* CG2.1^T; 4, *Halomonas marisflavi* DSM 15357^T; 5, *Halomonas elongata* ATCC 33173^T. —, Not detected.

Fatty acid	1	2	3	4	5
C _{10:0}	1.1	1.0	1.6	2.4	4.7
C _{10:0} 3-OH	—	—	—	—	3.6
C _{12:0}	0.8	0.8	0.8	—	4.7
C _{12:0} 2-OH	0.4	3.2	3.6	3.2	—
C _{12:0} 3-OH	8.8	11.3	12.1	11.0	15.3
C _{14:0}	1.9	0.4	0.7	0.5	—
C _{16:1} ω7c*	3.5	2.6	4.2	3.5	4.5
C _{16:0}	40.4	37.4	40.7	33.4	25.6
C _{16:0} 3-OH	—	—	—	—	1.0
C _{17:0} cyclo	0.9	3.2	2.2	2.6	1.6
C _{18:1} ω7c	28.4	16.6	20.0	25.7	25.6
C _{18:0}	1.1	1.2	1.0	2.1	0.6
C _{19:0} cyclo ω8c	11.8	22.3	12.9	15.7	10.6

*Included in summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH).

genus with respect to other genera of the *Halomonadaceae* (Table 3).

Overall, the phylogenetic data based on both the 16S rRNA and 23S rRNA gene sequence comparisons, the chemotaxonomic and genotypic results and the phenotypic features show clearly that strain A10^T constitutes a novel species and genus, separated from the genus *Halomonas* and other genera belonging to the *Halomonadaceae*, for which we propose the name *Kushneria aurantia* gen. nov., sp. nov. In addition, on the basis of the results reported we also propose to transfer the three species *Halomonas marisflavi*, *Halomonas indalinina* and *Halomonas avicenniae* to this new genus as *Kushneria marisflavi* comb. nov., *Kushneria indalinina* comb. nov. and *Kushneria avicenniae* comb. nov., respectively. The species of this new genus have all the 16S rRNA signature nucleotides characteristic of the family *Halomonadaceae* (Ben Ali Gam *et al.*, 2007). Table 1 shows the differential features between the four species of *Kushneria* gen. nov. Some features that serve to differentiate this new genus from other genera of the family *Halomonadaceae* are shown in Table 3.

Description of *Kushneria* gen. nov.

Kushneria (Kush.ne'ri.a. N.L. fem. n. *Kushneria* from the name Kushner, honouring Dr Donn J. Kushner, a Canadian microbiologist who carried out pioneering studies on halophilic micro-organisms).

Cells are Gram-negative, motile rods. Endospores are not formed. Strictly aerobic. Catalase-positive and oxidase-negative. Halophilic; Na⁺ is required for growth. Mesophilic. Chemo-organotrophic. The major respiratory

quinone is Q9. Major fatty acids are C_{16:0}, C_{18:1}ω7c, C_{19:0} cyclo ω8c and C_{12:0} 3-OH. Polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and unidentified phospholipids and glycolipids. The DNA G+C content is 59.0–61.7 mol%. The genus *Kushneria* is a member of the family *Halomonadaceae*, phylogenetically related to *Chromohalobacter* and *Halomonas*. The type species is *Kushneria aurantia*.

Description of *Kushneria aurantia* sp. nov.

Kushneria aurantia (au.ran'ti.a. N.L. adj. *aurantius* orange; N.L. fem. adj. *aurantia* orange-pigmented).

Exhibits the following properties in addition to those given in the genus description. Cells are 1.0 × 2.0–5.0 µm in size and occur singly or in pairs. Does not produce exopolysaccharide and does not accumulate poly-hydroxyalkanoate. Colonies on SW10 medium after 2 days incubation at 37 °C are 0.5–1.0 mm in diameter, circular, smooth, regular and convex with an entire margin, with an orange pigmentation. Moderately halophilic, requires NaCl; grows at NaCl concentrations in the range 5–17.5 % (w/v), with an optimum at 10 % (w/v) NaCl. Growth occurs at temperatures of 20–40 °C (optimally at 37 °C) and at pH 5.5–8.5 (optimally at pH 7.0–8.0). Respiration with fumarate, nitrate and nitrite is negative. Gelatin and aesculin are hydrolysed. Positive for methyl red, phosphatase and Simmons' citrate tests. Starch, casein, Tween 80, tyrosine and DNA are not hydrolysed. Negative for H₂S production, Voges–Proskauer, indole production, nitrate and nitrite reduction, urease, arginine dihydrolase, phenylalanine deaminase, lysine decarboxylase, α-galactosidase, α-maltosidase, N-acetyl-β-glucosaminidase and L-aspartic acid arylamidase but positive for ornithine decarboxylase, β-galactosidase, α-glucosidase and β-glucuronidase. Acid is produced from D-glucose, D-galactose, D-fructose, maltose, D-mannose and xylose, but not from arabinose, glycerol, mannitol, trehalose or sorbitol. Does not grow on MacConkey agar with 10 % (w/v) salts. The following compounds are used as sole carbon and energy sources: D-fructose, D-glucose, DL-malate, D-ribose, D-tartrate and D-xylose. The following compounds are not used as sole carbon and energy sources: aesculin, benzoate, ethanol, fumarate, hippurate, D-lactose, melezitose, salicin and starch. L-Cysteine is used as sole carbon, nitrogen and energy sources. The following compounds are not used as sole carbons, nitrogen and energy sources: L-isoleucine, DL-lysine, L-methionine, L-tryptophan and L-valine. The following compounds are used (oxidized) as sole carbon and energy sources (Biolog): dextrin, glycogen, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, D-galactose, gentiobiose, α-D-glucose, maltose, D-mannitol, D-mannose, D-psicose, D-sorbitol, trehalose, turanose, xylitol, succinic acid monomethyl ester, acetic acid, cis-aconitic acid, citric acid, D-galacturonic acid, α-ketoglutaric acid, DL-lactic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, L-glutamic acid,

Table 3. Characteristics that differentiate *Kushneria* gen. nov. from other genera of the family *Halomonadaceae*

Genera: 1, *Kushneria*; 2, *Halomonas*; 3, *Chromohalobacter*; 4, *Cobetia*; 5, *Modicisalibacter*; 6, *Halotalea*; 7, *Carrimonas*; 8, *Zymobacter*. Data are from Arahah *et al.* (2002a), Arahah & Ventosa (2005), Ben Ali Gam *et al.* (2007), Garriga *et al.* (1998), Ntougias *et al.* (2007), Okamoto *et al.* (1993), Ventosa *et al.* (1989), Vreeland (2005) and this study. +, Positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8
Cell morphology	Rods	Straight or curved rods (coccoid)	Straight or slightly curved rods	Straight rods	Rods	Rods	Straight or slightly curved rods	Rods
NaCl requirement	Moderately halophilic	Moderately halophilic/ slightly halophilic/ halotolerant	Moderately halophilic	Slightly halophilic	Moderately halophilic	Halotolerant	Halotolerant	Non-halophilic
Oxygen requirement	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Facultatively anaerobic
Oxidase	–	+	–	–	–	+	+	–
Major fatty acids	C _{16:0} , C _{18:1} ω7c, C _{19:0} cyclo ω8c, C _{12:0} 3-OH	C _{16:1} , C _{17:0} cyclo, C _{16:0} , C _{18:1} , C _{19:0} cyclo	C _{16:0} , C _{19:0} cyclo ω8c, C _{17:0} , C _{18:1} ω7c, C _{12:0} 3-OH	C _{16:0} , C _{16:1} ω7c, C _{18:1} ω7c, C _{17:0} , C _{12:0} 3-OH	C _{16:0} , C _{18:1} ω7c, C _{16:1} ω7c, C _{19:0} cyclo ω8c, C _{12:0} 3-OH, C _{16:1} ω7c/iso-C _{15:0} 2-OH	C _{18:1} ω7c, C _{16:0} , C _{19:0} cyclo ω8c, C _{12:0} 3-OH, C _{16:1} ω7c/iso-C _{15:0} 2-OH	C _{16:0} , C _{19:0} cyclo, C _{18:1} ω7c, C _{16:1} ω7c, C _{12:0} 3-OH, C _{18:1} ω9c, C _{12:0}	C _{16:0} , C _{19:0} cyclo, C _{12:0} 3-OH, C _{18:1} ω9c, C _{12:0}
DNA G+C content (mol%)	59.0–61.7	52–68	62.1–64.9	62–64	53.7	64.4	56.0	55.4–56.2

*Some species are oxidase-negative.

L-proline, L-pyrogutamic acid, L-serine, L-threonine, urocanic acid, inosine, uridine, thymidine, 2,3-butanediol, glycerol and D-glucose 6-phosphate. The following compounds are not used (oxidized) as sole carbon and energy sources (Biolog): α-cyclodextrin, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-fucose, myo-inositol, α-D-lactose, lactulose, D-melibiose, methyl α-D-glucoside, D-raffinose, L-rhamnose, sucrose, pyruvic acid methyl ester, formic acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, sebacic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, D-serine, DL-carnitine, γ-aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol, DL-α-glycerol phosphate and α-D-glucose 1-phosphate. Resistant to (μg per disc, unless specified otherwise): bacitracin (10 U), cephalothin (30), erythromycin (15), kanamycin (30), nalidixic acid (30) and streptomycin (10). Susceptible to chloramphenicol (30), novobiocin (30), penicillin G (10 U), polymyxin B (300 U), rifampicin (5), sulfamethazole/trimethoprim (23.75/1.25) and vancomycin (30). The DNA G+C content of the type strain is 61.7 mol%.

The type strain, A10^T (=CCM 7415^T=CECT 7220^T), was isolated from the leaf surface of *A. germinans* (black mangrove).

Description of *Kushneria marisflavi* comb. nov.

Kushneria marisflavi (ma.ris fla'vi. L. gen. neut. n. *maris* of the sea; L. neut. adj. *flavum* yellow; L. gen. neut. n. *marisflavi* of the Yellow Sea, Korea).

Basonym: *Halomonas marisflavi* Yoon *et al.* 2001.

The description is the same as that for *Halomonas marisflavi* (Yoon *et al.* 2001), with the addition that the polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, aminoglycolipid and an unidentified phospholipid and a glycolipid.

The type strain is SW32^T (=DSM 15357^T=JCM 10873^T=KCCM 80003^T).

Description of *Kushneria indalinina* comb. nov.

Kushneria indalinina (in.da.li.ni'na. N.L. n. *indalo* -inis a prehistoric magical symbol; L. suff. -inis-a-um suffix used in the sense of 'belonging to'; N.L. fem. adj. *indalinina* pertaining to the Indalo, the symbol of the province of Almería, Spain, from where the type strain was isolated).

Basonym: *Halomonas indalinina* Cabrera *et al.* 2007.

The description is the same as that for *Halomonas indalinina* (Cabrera *et al.*, 2007), with the addition that

the polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, aminoglycolipid and an unidentified phospholipid and a glycolipid. The major respiratory quinone is Q9.

The type strain is CG2.1^T (=CECT 5902^T=CIP 109528^T=LMG 23625^T).

Description of *Kushneria avicenniae* comb. nov.

Kushneria avicenniae [a.vi.cen.ni'ae. N.L. n. *Avicennia* scientific name of a botanical genus which encompasses *Avicennia germinans* (the black mangrove); N.L. gen. n. *avicenniae* of *Avicennia*, isolated from leaves of *Avicennia germinans*].

Basonym: *Halomonas avicenniae* Soto-Ramírez *et al.* 2007.

The description is the same as that for *Halomonas avicenniae* (Soto-Ramírez *et al.* 2007), with the addition that the polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, aminoglycolipid and an unidentified phospholipid and a glycolipid. The major respiratory quinone is Q9.

The type strain is MW2a^T (=CECT 7193^T=CIP 109711^T=CCM 7396^T).

Acknowledgements

This study was supported by grants from the Spanish Ministerio de Educación y Ciencia (project BIO2006-006927), from the Junta de Andalucía (project P06-CVI-01829), and by the Dean of Arts and Sciences Seed Money Program at the University of Puerto Rico-Mayagüez Campus and the University of Puerto Rico AlaCima Program.

References

- Arahal, D. R. & Ventosa, A. (2005). The family *Halomonadaceae*. In *The Prokaryotes: an Evolving Electronic Resource for the Microbiological Community*, 3rd edn, release 3.20. Edited by M. Dworkin. New York: Springer.
- Arahal, D. R., García, M. T., Ludwig, W., Schleifer, K. H. & Ventosa, A. (2001a). Transfer of *Halomonas canadensis* and *Halomonas israelensis* to the genus *Chromohalobacter*, as *Chromohalobacter canadensis* comb. nov. and *Chromohalobacter israelensis* comb. nov. *Int J Syst Evol Microbiol* 51, 1443–1448.
- Arahal, D. R., García, M. T., Vargas, C., Cánovas, D., Nieto, J. J. & Ventosa, A. (2001b). *Chromohalobacter salexigens* sp. nov., a moderately halophilic species that includes *Halomonas elongata* DSM 3043 and ATCC 33174. *Int J Syst Evol Microbiol* 51, 1457–1462.
- Arahal, D. R., Castillo, A. M., Ludwig, W., Schleifer, K. H. & Ventosa, A. (2002a). Proposal of *Cobetia marina* gen. nov., comb. nov., within the family *Halomonadaceae*, to include the species *Halomonas marina*. *Syst Appl Microbiol* 25, 207–211.
- Arahal, D. R., Ludwig, W., Schleifer, K. H. & Ventosa, A. (2002b). Phylogeny of the family *Halomonadaceae* based on 23S and 16S rDNA sequence analyses. *Int J Syst Evol Microbiol* 52, 241–249.
- Arahal, D. R., Vreeland, R. H., Litchfield, C. D., Mormile, M. R., Tindall, B. J., Oren, A., Bejar, V., Quesada, E. & Ventosa, A. (2007). Recommended minimal standards for describing new taxa of the family *Halomonadaceae*. *Int J Syst Evol Microbiol* 57, 2436–2446.
- Bauer, A. W., Kirby, W. M. M., Sherris, J. C. & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 45, 493–496.
- Ben Ali Gam, Z., Abdelkafi, S., Casalot, L., Tholozan, J. L., Oueslati, R. & Labat, M. (2007). *Modicisalibacter tunisiensis* gen. nov., sp. nov., an aerobic, moderately halophilic bacterium isolated from an oilfield-water injection sample, and emended description of the family *Halomonadaceae* Franzmann *et al.* 1989 emend Dobson and Franzmann 1996 emend. Ntougias *et al.* 2007. *Int J Syst Evol Microbiol* 57, 2307–2313.
- Cabrera, A., Aguilera, M., Fuentes, S., Incerti, C., Russell, N. J., Ramos-Cormenzana, A. & Monteoliva-Sánchez, M. (2007). *Halomonas indalini* sp. nov., a moderately halophilic bacterium isolated from a solar saltern in Cabo de Gata, Almería, southern Spain. *Int J Syst Evol Microbiol* 57, 376–380.
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- De Ley, J. & Tijtgat, R. (1970). Evaluation of membrane filter methods for DNA-DNA hybridization. *Antonie van Leeuwenhoek* 36, 461–474.
- Dussault, H. P. (1955). An improved technique for staining red-halophilic bacteria. *J Bacteriol* 70, 484–485.
- Franzmann, P. D., Wehmeyer, U. & Stackebrandt, E. (1988). *Halomonadaceae* fam. nov., a new family of the class *Proteobacteria* to accommodate the genera *Halomonas* and *Deleya*. *Syst Appl Microbiol* 11, 16–19.
- Garriga, M., Ehrmann, M. A., Arnau, J., Hugas, M. & Vogel, R. F. (1998). *Carnimonas nigrificans* gen. nov., sp. nov., a bacterial causative agent for black spot formation on cured meat products. *Int J Syst Bacteriol* 48, 677–686.
- Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–681. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* 42, 989–1005.
- Koser, S. A. (1923). Utilization of the salts of organic acids by the colon-aerogenes group. *J Bacteriol* 8, 493–520.
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenernegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K.-H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* 19, 554–568.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* 32, 1363–1371.
- Lugo, A. E. & Snedaker, S. C. (1975). Properties of a mangrove forest in southern Florida. In *Actas, International Symposium on Biological Management of Mangroves*, pp. 170–212. Edited by G. Walsh, S. C. Snedaker & H. Teas. Gainesville: University of Florida.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 3, 208–218.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 5, 109–118.
- Mata, J. A., Martínez-Cánovas, J., Quesada, E. & Béjar, V. (2002). A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Syst Appl Microbiol* 25, 360–375.

- Mellado, E., Moore, E. R. B., Nieto, J. J. & Ventosa, A. (1995). Phylogenetic inferences and taxonomic consequences of 16S ribosomal DNA sequence comparison of *Chromohalobacter marismortui*, *Volcaniella eurihalina*, and *Deleya salina* and reclassification of *V. eurihalina* as *Halomonas eurihalina* comb. nov. *Int J Syst Bacteriol* **45**, 712–716.
- Miller, L. T. (1982). Single derivation method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxyl acids. *J Clin Microbiol* **16**, 584–586.
- Nieto, J. J., Fernández-Castillo, R., Márquez, M. C., Ventosa, A., Quesada, E. & Ruiz-Berraquero, F. (1989). Survey of metal tolerance in moderately halophilic eubacteria. *Appl Environ Microbiol* **55**, 2385–2390.
- Ntougias, S., Zervakis, G. I. & Fasseas, C. (2007). *Halotalea alkalilenta* gen. nov., sp. nov., a novel osmotolerant and alkalitolerant bacterium from alkaline olive mill wastes, and emended description of the family *Halomonadaceae* Franzmann *et al.* 1989, emend. Dobson and Franzmann 1996. *Int J Syst Evol Microbiol* **57**, 1975–1983.
- Okamoto, T., Taguchi, H., Nakamura, K., Ikenaga, H., Kuraishi, H. & Yamasato, K. (1993). *Zymobacter palmae* gen. nov., sp. nov., a new ethanol-fermenting peritrichous bacterium isolated from palm sap. *Arch Microbiol* **160**, 333–337.
- Owen, R. J. & Hill, L. R. (1979). The estimation of base compositions, base pairing and genome sizes of bacterial deoxyribonucleic acids. In *Chemical Methods for Microbiologists*, pp. 277–296. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.
- Quesada, E., Ventosa, A., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1984). *Deleya halophila*, a new species of moderately halophilic bacteria. *Int J Syst Bacteriol* **34**, 287–292.
- Sehgal, S. N. & Gibbons, N. E. (1960). Effect of some metal ions on the growth of *Halobacterium cutirubrum*. *Can J Microbiol* **6**, 165–169.
- Soto-Ramírez, N., Sánchez-Porro, C., Rosas, S., González, W., Quiñones, M., Ventosa, A. & Montalvo-Rodríguez, R. (2007). *Halomonas avicenniae* sp. nov., isolated from the salty leaves of the black mangrove *Avicennia germinans* in Puerto Rico. *Int J Syst Evol Microbiol* **57**, 900–905.
- Soto-Ramírez, N., Sánchez-Porro, C., Rosas-Padilla, S., Almodóvar, K., Jiménez, G., Machado-Rodríguez, M., Zapata, M., Ventosa, A. & Montalvo-Rodríguez, R. (2008). *Halobacillus mangrovi* sp. nov., a moderately halophilic bacterium isolated from the black mangrove *Avicennia germinans*. *Int J Syst Evol Microbiol* **58**, 125–130.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stackebrandt, E., Fredericksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.
- Ventosa, A., Quesada, E., Rodríguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J Gen Microbiol* **128**, 1959–1968.
- Ventosa, A., Gutierrez, M. C., Garcia, M. T. & Ruiz-Berraquero, F. (1989). Classification of *Chromobacterium marismortui* in a new genus, *Chromohalobacter* gen. nov., as *Chromohalobacter marismortui* comb. nov., nom. rev. *Int J Syst Bacteriol* **39**, 382–386.
- Ventosa, A., Nieto, J. J. & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* **62**, 504–544.
- Ventosa, A., Gutierrez, M. C., Kamekura, M. & Dyal-Smith, M. L. (1999). Proposal to transfer *Halococcus turkmenicus*, *Halobacterium trapanicum* JCM 9743 and strain GSL-11 to *Haloterrigena turkmenica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 131–136.
- Vreeland, R. H. (2005). Genus I. *Halomonas*. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, pp. 300–313. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.
- Vreeland, R. H., Litchfield, C. D., Martin, E. L. & Elliot, E. (1980). *Halomonas elongata*, a new genus and species of extremely salt tolerant bacteria. *Int J Syst Bacteriol* **30**, 485–495.
- Yoon, J.-H., Choi, S. H., Lee, K.-C., Kho, Y. H., Kang, K. H. & Park, Y.-H. (2001). *Halomonas marisflavae* sp. nov., a halophilic bacterium isolated from the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **51**, 1171–1177.