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

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A novel Gram-negative, motile, nonsporulating, rod-shaped bacterium isolated from alkaline sludge-like wastes ('alpeorujo' or 'alperujo') of two-phase olive oil extraction is described. The strain, designated AW-7^T, is an obligate aerobe that is halotolerant (tolerating up to 15% w/v NaCl), sugar-tolerant (tolerating up to 45% and 60% w/v (+)-D-glucose and maltose respectively; these are the highest concentrations tolerated by any known members of the *Bacteria* domain) and alkalitolerant (growing at a broad pH range of 5–11). Strain AW-7^T is chemo-organotrophic. Ubiquinone-9 was detected in the respiratory chain of strain AW-7^T. The major fatty acids present are C_{18:1}ω7c, C_{16:0}, C_{19:0} cyclo ω8c, C_{12:0} 3-OH and C_{16:1}ω7c/iso-C_{15:0} 2-OH. Based on 16S rRNA gene sequence analysis, strain AW-7^T showed almost equal phylogenetic distances from *Zymobacter palmae* (95.6% similarity) and *Carnimonas nigrificans* (95.4% similarity). In addition, low DNA–DNA relatedness values were found for strain AW-7^T against *Carnimonas nigrificans* CECT 4437^T (22.5–25.4%) and *Z. palmae* DSM 10491^T (11.9–14.4%). The DNA G + C content of strain AW-7^T is 64.4 mol%. Physiological and chemotaxonomic data further confirmed the differentiation of strain AW-7^T from the genera *Zymobacter* and *Carnimonas*. Thus, strain AW-7^T represents a novel bacterial genus within the family *Halomonadaceae*, for which the name *Halotalea* gen. nov. is proposed. *Halotalea alkalilenta* sp. nov. (type strain AW-7^T=DSM 17697^T=CECT 7134^T) is proposed as the type species of the genus *Halotalea* gen. nov. A reassignment of the descriptive 16S rRNA signature characteristics of the family *Halomonadaceae* permitted the placement of the novel genus *Halotalea* into the family; in contrast, the genus *Halovibrio* possessed only 12 out of the 18 signature characteristics proposed, and hence it was excluded from the family *Halomonadaceae*.

The family *Halomonadaceae* was proposed by Franzmann *et al.* (1988) initially to accommodate the genera *Deleya* and *Halomonas*. Dobson *et al.* (1993) stated that the genus *Halovibrio* was related to the genera *Halomonas* and *Deleya* and later Dobson & Franzmann (1996) unified the genera *Halomonas*, *Deleya* and *Halovibrio*, together with the

misclassified species *Paracoccus halodenitrificans*, into the genus *Halomonas* (Vreeland *et al.*, 1980). Based on 16S rRNA gene sequence and DNA–DNA hybridization analyses, the genus *Chromohalobacter* (Ventosa *et al.*, 1989) was proposed to be a member of the family *Halomonadaceae* (Kerstens, 1992; Mellado *et al.*, 1995). Dobson & Franzmann (1996) included the genus *Chromohalobacter*, together with the genus *Zymobacter* (Okamoto *et al.*, 1993), in the emended description of the family *Halomonadaceae*. The genus *Carnimonas* was initially excluded from this family (Garriga *et al.*, 1998), but Arahall *et al.* (2002b) argued that it should be classified within the family *Halomonadaceae*. In addition, Arahall *et al.* (2002a) transferred the species *Halomonas marina* into the genus *Cobetia*. The majority of the members of the family

Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AW-7^T is DQ421388.

Figures showing the morphology of cells of strain AW-7^T, as examined by negative-staining electron microscopy, and the phylogenetic position of the novel strain, calculated by the maximum-parsimony and maximum-likelihood methods, are available as supplementary figures with the online version of this paper.

Halomonadaceae are either halotolerant or moderately halophilic bacteria that mainly originate from marine and/or saline environments (Ventosa *et al.*, 1998). However, bacteria of the genera *Carnimonas* and *Zymobacter* are halotolerant and osmotolerant and have been isolated from processed meat products and palm sap, respectively (Okamoto *et al.*, 1993; Garriga *et al.*, 1998).

Olive oil mills equipped with two-phase centrifugation systems are now widespread in most olive oil-producing countries. Their operation results in the production (apart from olive oil) of a moderately acidic and viscous liquid-solid waste, termed 'alpeorujo' in Spain (Jones *et al.*, 2000). To prevent the release of odours, $\text{Ca}(\text{OH})_2$ is added to alpeorujo, creating a moderately alkaline-saline secondary waste (Ntougias *et al.*, 2006). A limited number of studies on bacteria originating from alpeorujo (including alkaline alpeorujo) have been performed. In a study of the osmoregulatory responses of bacteria from alpeorujo, two isolates, which tolerated high concentrations of salt and sucrose, were identified by partial 16S rRNA gene sequencing as *Bacillus amyloliquefaciens* and *Salmonella* sp. (Jones *et al.*, 2000). In addition, several halotolerant alkaliphiles and moderate to extreme halotolerant and/or alkalitolerant bacteria have been previously identified from alkaline alpeorujo (Ntougias *et al.*, 2006). *Olivibacter sitiensis* is the only non-halotolerant and non-alkalitolerant bacterium to have been isolated from this type of wastes (Ntougias *et al.*, 2007).

In this paper, we describe the morphological, physiological, chemotaxonomic and phylogenetic characteristics of a novel halotolerant and alkalitolerant bacterium, isolated from alkaline alpeorujo. Due to its unique taxonomic position, we conclude that this isolate represents a new bacterial genus and novel species.

Strain AW-7^T was isolated from alkaline alpeorujo, a waste originally produced by the two-phase centrifugal decanter system of an olive mill, which was further treated with 0.7 % w/w $\text{Ca}(\text{OH})_2$. Alkaline alpeorujo was disposed of in an open-air settling pond (from which our sample was obtained) next to an olive mill, located at the premises of the Toplou Monastery, Sitia (26° 6' 6" N 35° 13' 12" E), NE Crete, Greece. The pH and electrical conductivity (EC) of the sample (diluted in water at 1:1 v/v) were 8.7 and 16.2 mS cm⁻¹, respectively.

To isolate strain AW-7^T, 10 g of alkaline alpeorujo was added to 100 ml of 8.5 g l⁻¹ NaCl solution and tenfold dilution series were prepared. Extract from alkaline alpeorujo (100 g in 1 l of distilled water, mixed for 20 min, filtered and adjusted to pH 7) solidified with agar was used as the isolation medium (Ntougias *et al.*, 2006). After incubation at 25 °C for a week, strain AW-7^T was selected and subcultured on solid medium consisting of 10 g glucose l⁻¹, 5 g yeast extract l⁻¹, 5 g peptone l⁻¹, 0.1 mM MgSO_4 and phosphate buffer at pH 7 (Ntougias *et al.*, 2006). The experimental temperature for growth was 32 °C (optimum temperature). Growth of strain AW-7^T

was assessed on the medium described above, unless otherwise specified.

The pH range for growth (pH 4–11 in steps of one full pH unit) was investigated using the appropriate buffer solution (Ntougias & Russell, 2000). The pH was checked before inoculation and immediately after the end of the experiment. Salt tolerance was examined in presence of 0, 30, 50, 80, 100, 150 and 200 g NaCl l⁻¹. Sugar tolerance was tested by substituting glucose in the medium described above (in the absence of agar) with 0, 100, 200, 300, 450, 500, 600 and 700 g (+)-D-glucose or maltose l⁻¹. To investigate bacterial substrate utilization, nutrient media (pH 7, the pH was adjusted when it was necessary) containing a specific sugar, amino acid or other substrate (see species description), 0.1 mM MgSO_4 and 0.05 g yeast extract l⁻¹ were prepared (no growth was observed in the medium containing the above concentration of yeast extract as the sole carbon source). The concentration of each substrate tested was 0.05 M, unless the medium was saturated in a lower concentration and therefore a concentration up to the saturation point was used (Ntougias *et al.*, 2007). The concentration of ethanol and glycerol was 0.5 % v/v, and 0.5 g phenol l⁻¹ was also tested. Salts, metabolic substrates, yeast extract and the buffer were autoclaved separately and mixed aseptically. The sensitive compounds were filter-sterilized. Incubations were performed at 3, 5, 10, 15, 20, 25, 28, 32, 37, 40, 45 and 50 °C to examine the temperature range for growth. Anaerobic growth was tested using the Anaerocult A system (Merck). Antibiotic susceptibility was investigated on solid medium containing 50 µg ml⁻¹ of the antibiotic tested (Ntougias *et al.*, 2006). Acid production from various carbohydrates was examined in phenol red broth (18 mg phenol red l⁻¹, pH 7.4) containing 10 g l⁻¹ of the substrate tested (Chaturvedi & Shivaji, 2006). Reduction of nitrate, the methyl red reaction, activities of arginine dihydrolase, lysine and ornithine decarboxylases and phenylalanine deaminase, and production of H₂S from L-cysteine and indole from tryptophan were tested following the methods described in Barrow & Feltham (1993). Urease, catalase and oxidase activities, spore-formation, Gram-staining, Voges-Proskauer test, and hydrolysis of gelatin, starch, Tweens 20 and 80 were examined according to Smibert & Krieg (1994). An alternative Gram test with KOH was also performed (Powers, 1995). Casein hydrolysis was tested on skim milk agar (10 % w/v skim milk). DNase test agar with toluidine blue (Sigma-Aldrich Chemie GmbH) was used to assay DNase activity.

For transmission electron microscopy (TEM), suspensions of bacterial cells were placed on pyroxyline-coated, copper grids (AEI) and negatively stained with 1 % w/v phosphotungstic acid (pH 7.0) for 10 s. Specimens were examined and photographed with a TEM (model 9S; Zeiss).

DNA from pelleted cells was extracted as described by Wilson (1992). The nearly full-length 16S rRNA gene was amplified by using the two universal primers A_{for}

5'-GGAGAGTTAGATCTTGGCTCAG-3' (sense; positions 6 to 27 according to the *Escherichia coli* numbering system) and C_{rev} , 5'-AGAAAGGAGGTGATCCAGCC-3' (anti-sense; positions 1542 to 1525 according to *E. coli* numbering). A reaction mixture (50 μ l) containing 1 μ l (50 ng μ l⁻¹) genomic DNA, 10 $\times$ PCR buffer (Finnzymes OY), 2 mM MgCl₂, 200 μ M each of dATP, dTTP, dCTP and dGTP, 0.5 μ M each of primers A_{for} and C_{rev} and 1 U DNA polymerase (Dynazyme EXT; Finnzymes OY) was prepared (Ntougias & Russell, 2001). Genomic DNA was amplified using a PTC-200 thermocycler (MJ Research Inc.) with a denaturation step of 2 min at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 55 °C and 1 min DNA chain extension at 72 °C. The PCR was completed by a 5 min DNA chain extension at 72 °C.

Three independent clones were obtained by cloning the respective amplicons in the pGEM-T Easy Vector (Promega) before insertion into DH5a-competent cells. Plasmid DNA was purified using the NucleoSpin plasmid Quick Pure kit (Macherey-Nagel). PCR sequencing using the fluorescence-labelled primers SP6 and T7 (Promega) and additional primers (5'-GGCGGCCTTCTGGACTG-3' and 5'-TCGCTGGCAAATAAGGATAGG-3') was performed at the Institute of Molecular Biology and Biotechnology (IMBB), Heraklion, Greece, via a LI-COR Long ReadIR2 4200 automated sequencer (LI-COR).

The 16S rRNA gene sequences were assembled using DNASTAR software (DNASTAR Inc.). Similarity searches were performed using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and SeqMatch (Ribosomal Database Project II, http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and its closest relatives were obtained for further phylogenetic analyses. Alignment of sequences and construction of the phylogenetic tree based on the distance-matrix method were carried out using CLUSTAL W (<http://www.ebi.ac.uk/clustalw/>) and TREECON for Windows (version 1.3b) (Van de Peer & de Wachter, 1993), respectively. Evolutionary distances were calculated using the method of Jukes & Cantor (1969) and topology was inferred using the neighbour-joining method (Saitou & Nei, 1987) based on bootstrap analysis of 1000 trees. Phylogenetic trees were also generated by maximum-likelihood (DNAML; Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (DNAPARS; Kluge & Farris, 1969) analyses using the PHYLIP version 3.6 phylogenetic package (Felsenstein, 2004), which includes the SEQBOOT program for bootstrap analysis. Phylogenetic trees calculated by maximum-parsimony and maximum-likelihood methods were drawn by TreeView software, version 1.6.6 (Page, 1996). Only almost full-length sequences were used for tree constructions. 16S rRNA secondary structures were predicted by the RNAstructure program (version 4) using the Zuker algorithm based on free energy minimization (Mathews *et al.*, 2004).

Chemotaxonomic analyses (quinone and FAME analyses), DNA–DNA hybridization and DNA G + C content

determination were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. DNA for spectroscopic DNA–DNA hybridization and G + C content determination was isolated as described by Cashion *et al.* (1977). DNA–DNA hybridization was performed as described by De Ley *et al.* (1970) and modified as suggested by Huß *et al.* (1983). DNA G + C content determination was carried out according to Tamaoka & Komagata (1984) and Mesbah *et al.* (1989). Fatty acid methyl esters were obtained as previously described (Kroppenstedt, 1985; Kämpfer & Kroppenstedt, 1996) using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988). Respiratory lipoquinone analyses were performed by Dr B. J. Tindall and the Identification Service of the DSMZ using a standard procedure (Tindall, 1990a, b).

Morphological, cultural, physiological and biochemical characteristics of strain AW-7^T are given in the species description, Table 1 and in Supplementary Fig. S1 (available in IJSEM Online).

Ubiquinone-9 was the only quinone detected in the respiratory chain of strain AW-7^T. The detailed fatty acid profile of strain AW-7^T consisted of C_{10:0} (0.9 %), C_{12:0} (0.6 %), C_{12:0} 2-OH (3.2 %), C_{12:0} 3-OH (9.1 %), C_{14:0} (0.3 %), iso I C_{16:1} and/or C_{14:0} 3-OH (2.4 %), C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH (6.2 %), C_{16:0} (28.5 %), C_{17:0} cyclo (0.4 %), C_{18:1} ω 7c (35.6 %), C_{18:0} (0.4 %) and C_{19:0} cyclo ω 8c (12.4 %).

Strain AW-7^T tolerated the greatest (+)-D-glucose and maltose concentrations (45 and 60 % w/v, respectively) ever reported for recognized members of the domain *Bacteria*. Members of the genera *Zymomonas* and *Saccharibacter* can tolerate up to 40 % w/v (+)-D-glucose (Swings & De Ley, 1983; Jojima *et al.*, 2004), while strains of the genus *Zymobacter* can tolerate up to 20 and 50 % w/v (+)-D-glucose and maltose concentrations, respectively (Okamoto *et al.*, 1993).

Construction of phylogenetic trees using distance-matrix, character-based (parsimony) and maximum-likelihood methods all placed strain AW-7^T in a phylogenetic position related to *Z. palmae* and *Carnimonas nigrificans* (see Fig. 1 and Supplementary Figs S2 and S3 in IJSEM Online). Based on 16S rRNA gene sequence analysis, strain AW-7^T was associated, but not closely related, to *Z. palmae* (95.6 % similarity) and *Carnimonas nigrificans* (95.4 % similarity).

The DNA G + C content of strain AW-7^T was 64.4 mol%. DNA–DNA relatedness values for strain AW-7^T with *Carnimonas nigrificans* CECT 4437^T and *Z. palmae* DSM 10491^T were 22.5–25.4 % and 11.9–14.4 % respectively.

Bacterial strain AW-7^T possessed 13 out of the 18 base signature characteristics proposed by Dobson *et al.* (1993) (Table 2), while it formed a 7 bp instead of 6 bp stem in its 16S rRNA secondary structure (referring to positions 76–93 in the *E. coli* numbering system) (Fig. 2).

Table 1. Differential physiological and chemotaxonomic characteristics of strain AW-7^T and members of the genera *Carnimonas* and *Zymobacter*

Taxa: 1, Strain AW-7^T; 2, *Carnimonas* (data from Garriga *et al.*, 1998); 3, *Zymobacter* (Okamoto *et al.*, 1993). +, Growth/presence/positive reaction; –, no growth/absence/negative reaction; NR, not reported.

Characteristic	Strain AW-7 ^T	<i>Carnimonas</i>	<i>Zymobacter</i>
Colour of colony	Pale yellow	White	Milky white
Motility	Motile	Non-motile	Motile
Flagella	Peritrichous	–	Peritrichous
Oxygen requirement	Strict aerobe	Strict aerobe	Facultative anaerobe
pH range	5–11	NR	3–10
Temperature range (°C)	5–45	NR	21–39
Optimum temperature (°C)	32–37	28–30	30
Growth at 5 °C	+	–	–
Growth at 37 °C	+	–	+
NaCl tolerance (% w/v)	0–15	0–8	NR
(+)-D-glucose tolerance (% w/v)	0–45	NR	0–20
Maltose tolerance (% w/v)	0–60	NR	0–50
Utilization of citrate	+	+	–
Utilization of D-mannitol	+	–	+
Oxidase	+	+	–
Starch hydrolysis	–	+	–
Phenylalanine deaminase	–	+	–
Methyl red reaction	–	NR	+
Voges–Proskauer	–	–	+
Acid production from:			
D-mannitol	–	+	+
(+)-D-mannose	+	+	–
Sorbitol	–	NR	+
Sucrose	–	+	+
(+)-D-xylose	–	+	–
Detection of:			
C _{10:0}	+ (0.9 %)	–	–
C _{10:0} 3-OH	–	–	+ (<0.5 %)
C _{12:0}	+ (0.6 %)	–	+ (5.0–6.0 %)
C _{12:0} 2-OH	+ (3.2 %)	–	–
C _{14:0}	+ (0.3 %)	–	+ (3.0–4.0 %)
C _{15:0}	–	–	+ (<0.5 %)
C _{16:0} 3-OH	–	+ (0.5 %)	–
C _{17:0}	–	–	+ (<1.0 %)
cyclo-C _{17:0}	+ (0.4 %)	–	–
C _{18:0}	+ (0.4 %)	–	+ (4.0–6.0 %)
C _{18:1} ω9	–	–	+ (7.0–21.0 %)
C _{18:1} t9	–	+ (7.5 %)	–
C _{18:1} (c11/t9/t6)*	+ (35.6 %)†	+ (12.9 %)	–
Major fatty acids (in order)	C _{18:1} ω7c (35.6 %), C _{16:0} (28.5 %), C _{19:0} cyclo ω8c (12.4 %), C _{12:0} 3-OH (9.1 %), C _{16:1} ω7c/iso-C _{15:1} 2-OH (6.2 %)	C _{16:0} (40.0 %), C _{19:0} cyclo (21.1 %), C _{18:1} t9 (7.5 %), C _{16:1} (6.7 %)	C _{16:0} (46.0–54.0 %), C _{19:0} cyclo (21.0–26.0 %), C _{12:0} 3-OH (8.0–13.0 %), C _{18:1} ω9 (7.0–21.0 %), C _{12:0} (5.0–6.0 %)
DNA G + C content (mol%)	64.4	56.0 ± 0.3	55.4–56.2

*Summed feature C_{18:1} (c11/t9/t6).

†Presence of C_{18:1}ω7 (C_{18:1}c11).

Bacterial strain AW-7^T showed distinctive phenotypic, chemotaxonomic and phylogenetic features when compared with its closest relatives *Z. palmae* and *Carnimonas nigrificans* (Table 1).

Bacterial strain AW-7^T is an obligate aerobe which formed pale yellow colonies (not white), utilized citrate and grew at temperatures between 5 and 45 °C. In addition, cells of strain AW-7^T were oxidase-positive and produced acid

Fig. 1. Phylogenetic position of strain AW-7^T isolated from alkaline alpeorajo, presented in association with members of the genera *Halomonas*, *Chromohalobacter*, *Zymobacter*, *Carnimonas*, *Cobetia* and *Halovibrio*. Evolutionary distances were calculated using the method of Jukes & Cantor (1969), and the topology was inferred using the neighbour-joining method (Saitou & Nei, 1987) based on bootstrap analysis of 1000 trees. Bar, 0.02 substitutions per site. The 16S rRNA gene sequence of *E. coli* ATCC 11775^T was arbitrarily chosen as the outgroup.

from (+)-D-mannose. In contrast, *Z. palmae* is a facultative anaerobe that grows over a relatively narrow temperature range (21–39 °C), gives positive reactions in

Table 2. 16S rRNA sequence base signature characteristics of strain AW-7^T and members of the genera *Halomonas*, *Chromohalobacter*, *Zymobacter*, *Carnimonas*, *Cobetia* and *Halovibrio*

Taxa: 1, *Halomonas*; 2, *Chromohalobacter*; 3, *Cobetia*; 4, *Zymobacter*; 5, *Carnimonas*; 6, Strain AW-7^T; 7, *Halovibrio*. The positions are according to the *E. coli* numbering system.

Position	1	2	3	4	5	6	7
484	A	A	A	A	G	G	G
486	C	C	C	C	U	U	U
640	G	G	G	G	G	G	A
660	A	A	A	A	A	A	A
745	U	U	U	U	U	U	U
668	A	A	A	A	A	A	G
738	U	U	U	U	U	U	C
669	A	A	A	A	A	A	C
737	U	U	U	U	U	U	G
776	U	U	U	U	U	U	U
1124	U	U or G	U	U	U	U	G
1297	U	U	U	U	U	U	G
1298	C	C	C	C	C	C	U
1423	A	A	A	A	A	A	A
1424	C	C	C	U	U	U	C
1439	U	U	U	C	C	C	U
1462	A	A	A	G	G	G	A
1464	C	C	C	U	C	C	C

methyl red and Voges–Proskauer tests and produces acid from D-mannitol, sorbitol and sucrose (Okamoto *et al.*, 1993). The presence of *C*_{18:1ω7c} and the lack of *C*_{18:1ω9} in the fatty acid profile of strain AW-7^T, compared with that of *Z. palmae*, indicates significant chemotaxonomic differences. DNA–DNA relatedness between strain AW-7^T and *Z. palmae* was low (11.9–14.4 %). Moreover, significant divergence between strain AW-7^T and *Z. palmae* was detected in their respective DNA G+C contents. The G+C content of strain AW-7^T was 8.2 mol% higher than that of *Z. palmae* (55.4–56.2 mol%) (Okamoto *et al.*, 1993). Strain AW-7^T showed 95.6 % 16S rRNA gene sequence similarity with *Z. palmae*.

Strain AW-7^T also showed an almost equal phylogenetic distance to *Carnimonas nigrificans* (95.4 % 16S rRNA gene sequence similarity). Strain AW-7^T and *Carnimonas nigrificans* showed significant differences in their DNA G+C contents. The DNA G+C content of strain AW-7^T was 8.4 mol% higher than that of *Carnimonas nigrificans* (56.0 ± 0.3 mol%) (Garriga *et al.*, 1998). In addition, low DNA–DNA relatedness (22.5–25.4 %) was found between strain AW-7^T and *Carnimonas nigrificans*. Strain AW-7^T utilized D-mannitol and did not hydrolyse starch. Colonies of strain AW-7^T were pale yellow and the cells were motile. The novel strain grew at 5 °C and at 37 °C (optimum temperature of 32–37 °C) and tolerated up to 15 % w/v NaCl. In contrast, *Carnimonas nigrificans* was a non-motile bacterium forming white colonies which failed to grow at 5 °C and 37 °C and at a salt concentration greater than 8 % w/v NaCl. The optimum growth temperature of *Carnimonas nigrificans* was lower (28–30 °C) than that of the novel strain. In addition, *Carnimonas nigrificans* did not utilize D-mannitol, but was able to hydrolyse starch

Fig. 2. 16S rRNA secondary structures (referring to positions 61–106 in the *E. coli* numbering system) of strain AW-7^T and the genera *Zymobacter*, *Carnimonas*, *Cobetia*, *Halomonas*, *Chromohalobacter* and *Halovibrio*. Nucleotide sequences (positions 61–106) of *Z. palmae* IAM 14233^T (GenBank accession number D14555), *Carnimonas nigrificans* CTCBS1^T (Y13299), *Cobetia marina* DSM 4741^T (AJ306890), *Halomonas elongata* ATCC 33173^T (X67023), *Chromohalobacter marismortui* A-492 (X87222) and *Halovibrio denitrificans* DSM 15503^T (DQ072718) were folded to predict their respective secondary structures. Numbers correspond to the *E. coli* numbering system.

(Garriga *et al.*, 1998). Furthermore, *Carnimonas nigrificans* was positive for phenylalanine deaminase activity and produced acid from D-mannitol, sucrose and (+)-D-xylose. In the fatty acid profiles of the strains examined, the lack of *trans* isomers and the presence of significant amounts of hydroxylated fatty acids were a distinctive feature of strain AW-7^T when compared to *Carnimonas nigrificans*.

Strains of the genus *Cobetia*, as compared to strain AW-7^T, are oxidase-negative halophiles, that utilize *myo*-inositol, ethanol, L-lysine, and D-cellobiose, but not sorbitol or D-xylose. Moreover, they form cream-pigmented colonies, contain *trans* monounsaturated fatty acids, mainly C_{16:1}9 and to a lesser extent C_{18:1}9, as well as C_{18:1}ω9. Ubiquinone-8 is also present as a minor component in the respiratory chain of members of the genus *Cobetia* (Arahal *et al.*, 2002a; Yumoto *et al.*, 2004). In addition, members of the genus *Cobetia* are motile by means of polar flagella, while strain AW-7^T is motile by peritrichous flagella. Strain AW-7^T showed 93.0% 16S rRNA gene sequence similarity with *Cobetia marina*.

In contrast to strain AW-7^T, members of the genus *Chromohalobacter* are halophiles growing at salt concentrations of up to 30% w/v NaCl. In addition, members of the genus *Chromohalobacter* produce pigments and oxidase activity is not present in all species (Arahal *et al.*, 2001). In comparison with species of the genus *Chromohalobacter*, strain AW-7^T contains the fatty acids C_{10:0}, C_{12:0}, C_{12:0} 2-OH and a significant amount of C_{12:0} 2-OH. Hydroxylated-C_{12:0} fatty acids have never been detected

in any member of the genus *Chromohalobacter* so far tested (Mutnuri *et al.*, 2005; Vargas *et al.*, 2005). In comparison with strain AW-7^T, members of the genus *Chromohalobacter* showed 16S rRNA gene sequence similarities of less than 91.8%. The closest relative of strain AW-7^T from the genus *Chromohalobacter* was *Chromohalobacter israelensis* (91.8% gene sequence similarity).

In contrast to strain AW-7^T, species of the genus *Halomonas* produce pigments and many of them have extreme upper salt concentrations for growth (Mata *et al.*, 2002). In addition, the majority of the type strains of *Halomonas* species can utilize ethanol, inositol, L-isoleucine and L-lysine and they are susceptible to ampicillin and chloramphenicol (Mata *et al.*, 2002). Due to the large number of species included in the genus *Halomonas*, differences in fatty acid contents depend on the specific species that is compared with strain AW-7^T. As the DNA G + C content of species of the genus *Halomonas* ranges widely from 51.4 to 74.3 mol% (Martinez-Canovas *et al.*, 2004; Quillaguan *et al.*, 2004), this characteristic cannot be used as a taxonomic tool. Concerns about the taxonomy of the genus *Halomonas* should be expressed as there is a significant variability in phenotypic, chemotaxonomic and molecular characteristics among species of the genus *Halomonas*. A study by Mata *et al.* (2002) showed that none of 205 phenotypic and physiological characteristics tested were common for all type strains of species of the genus *Halomonas*. When compared with members of the genus *Halomonas*, strain AW-7^T showed 16S rRNA gene sequence similarities lower than 92.2%. Its closest relative from the genus *Halomonas* was *Halomonas pacifica* (92.1% gene sequence similarity).

Dobson *et al.* (1993) reported 19 characteristic signatures (18 characteristic bases and a 6 bp stem located in the 76–93 positions, *E. coli* numbering system) in the 16S rRNA secondary structures of members of the genera *Deleya*, *Halomonas* and *Halovibrio* in order to accommodate these three genera within the family *Halomonadaceae*. These genera were unified into a single genus by Dobson & Franzmann (1996), the genus *Halomonas*, and the number of signature characteristics was reduced to 15 in order to include the genus *Zymobacter* in the family. Arahall *et al.* (2002b) placed the genus *Carnimonas* in the family *Halomonadaceae*, reporting 13 elements common to all members plus six residues with two possible bases. When the presence of a 6 bp stem in positions 76–93 according to the *E. coli* numbering system was excluded from the analysis, we found that all species of the genera *Halomonas*, *Chromohalobacter* and *Cobetia* possess all the remaining characteristics (18 out of 19) proposed by Dobson *et al.* (1993) (Table 2), with the exception of *Chromohalobacter marismortui* (it possesses G instead of U in position 1124 according to *E. coli* numbering) and *Halomonas muralis* (C and U instead of U and C in positions 1297 and 1298, respectively). The above differences in the 16S rRNA secondary structure of *H. muralis* may be due to sequence misreading (a reverse order was observed for positions 1297–1298). Taking into account the 18 characteristic base positions described by Dobson *et al.* (1993), *Z. palmarum*, *Carnimonas nigrificans* and strain AW-7^T possessed 14, 13 and 13 signature characteristics, respectively. When the bases located in the 61–106 positions (including positions 76–93) were folded, only members of the genera *Carnimonas*, *Chromohalobacter* and *Cobetia* formed a 6 bp stem, while members of the genus *Zymobacter* and strain AW-7^T formed a 7 bp stem (Fig. 2). However, no formation of a 6 bp stem was observed for the type species of the genera of the family *Halomonadaceae* when almost complete 16S rRNA sequences were folded using the standard conditions of the RNAstructure program (Version 4.2). Based on the above data, we propose the exclusion of the 6 bp stem from the list of signature characteristics of the family *Halomonadaceae*. In contrast to previous results (Garriga *et al.*, 1998; Arahall *et al.*, 2002b), we detected a G instead of the C reported in position 1462 (*E. coli* numbering) in members of the genera *Zymobacter* and *Carnimonas* and a 7 bp instead of a 6 bp stem in the 16S rRNA secondary structure of the genus *Zymobacter*. The genus *Halovibrio*, consisting of the species *Halovibrio denitrificans* and *Halovibrio variabilis* (*Pseudomonas halophila* DSM 3050^T) (Sorokin & Tindall, 2006; Sorokin *et al.*, 2006), possessed only 8 signature characteristics (Table 2 and Fig. 2) from the 19 reported by Dobson *et al.* (1993). This observation, in conjunction with the distinct phylogenetic (showing 16S rRNA gene sequence similarities with the type species of the family *Halomonadaceae* and with strain AW-7^T that are lower than 87.5 %) and chemotaxonomic position (Sorokin *et al.*, 2006), lead us to propose the exclusion of the genus *Halovibrio* from the family *Halomonadaceae*. We also

propose the reassignment of the family *Halomonadaceae* (see emended description), based on its 16S rRNA sequence signature characteristics, to accommodate the previously described genera *Halomonas*, *Chromohalobacter*, *Zymobacter*, *Cobetia* and *Carnimonas* and a new genus *Halotalea* gen. nov.

On the basis of phenotypic, chemotaxonomic and phylogenetic data, we conclude that strain AW-7^T represents a new bacterial genus and novel species of the family *Halomonadaceae*, for which we propose the name *Halotalea alkalilenta* gen. nov., sp. nov.

Description of *Halotalea* gen. nov.

Halotalea (Ha.lo.ta.le'a. Gr. n. *hals halos* salt; L. fem. n. *talea* a staff, rod; N.L. fem. n. *Halotalea* rod-shaped cells living in saline conditions).

Cells are Gram-negative, rods, motile by peritrichous flagella and forming small, non-pigmented pale yellow colonies. Endospores are not formed. Strictly aerobic. Halotolerant and alkalitolerant. Sugar-tolerant. Oxidase- and catalase-positive. Chemo-organotrophic. Ubiquinone-9 is present in the respiratory chain. The major fatty acids are C_{18:1}ω7c, C_{16:0}, C_{19:0} cyclo ω8c, C_{12:0} 3-OH and C_{16:1}ω7c/iso-C_{15:0} 2-OH. The DNA G+C content is 64.4 mol%. The type species is *Halotalea alkalilenta*.

Description of *Halotalea alkalilenta* sp. nov.

Halotalea alkalilenta (al.ka.li.len.ta. N.L. n. *alkali* from Arabic *al qaliy*, soda ash; L. adj. *lentus* -a slow; N.L. n. *alkalilenta* slow in alkaline conditions/alkalitolerant).

Strict aerobe that tolerates up to 15 % w/v NaCl, with an optimum salt concentration of 0–3 % w/v NaCl. Tolerates up to 45 and 60 % w/v (+)-D-glucose and maltose, respectively. Grows at pH 5 to 11, with optimum pH at 7. The temperature range for growth is 5 to 45 °C, with an optimum temperature of 32–37 °C. Chemo-organotrophic. Preferentially utilizes L-glutamine and L-proline (OD₆₀₀ > 0.35; growth measured at 32 °C and pH 7; OD₆₀₀ values cited correspond to measurements taken after 72 h), followed by (+)-D-galactose, (+)-D-glucose, glycerol, D-mannitol, protocatechuate, L-serine, succinate, sucrose and (+)-D-xylose (respective OD₆₀₀ values in the range 0.13–0.35). Weak growth on acetate, citrate, (–)-D-fructose, maltose and sorbitol (respective OD₆₀₀ values in the range 0.05–0.12). Grows on gallate (10⁶ cells ml^{–1} in the defined medium). (+)-D-Cellobiose, cinnamate, L-cysteine, ethanol, ferrulate, glycine, L-histidine, inositol, L-isoleucine, lactose, L-lysine, L-methionine, phenol, syringate, L-tryptophan, vanillate and o-vanillin are not utilized. Negative reactions in tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, phenylalanine deaminase, nitrate reduction, H₂S production from L-cysteine, indole production, methyl red and Voges–Proskauer. Acid is produced from (–)-D-fructose, (+)-D-galactose, (+)-D-glucose, maltose, (+)-D-mannose and melibiose, but it is not produced from

(+)-L-arabinose, (+)-D-cellobiose, inositol, lactose, D-mannitol, sorbitol, sucrose or (+)-D-xylose. Hydrolyses Tween 20, but does not hydrolyse casein, DNA, gelatin, starch, Tween 80 or urea. Susceptible to (50 µg ml⁻¹) kanamycin, polymyxin B, rifampicin, streptomycin and tetracycline, but resistant to ampicillin, bacitracin, chloramphenicol, penicillin and trimethoprim. The DNA G+C content of the type strain is 64.4 mol%.

The type strain, AW-7^T (=DSM 17697^T=CECT 7134^T), was isolated from olive mill waste (alkaline alpeorujo) obtained from the premises of the Toplou Monastery in the region of Sitia, Crete, Greece.

Emended description of the family *Halomonadaceae* Franzmann *et al.* 1989, emend. Dobson and Franzmann 1996

The description is as given by Franzmann *et al.* (1988) and Dobson & Franzmann (1996), but an alteration in 16S rRNA sequence signature characteristics. The 16S rRNA sequence signature characteristics of the family *Halomonadaceae* are redefined as follows: position 484 (A or G), position 486 (C or U), position 640 (G), position 660 (A), position 745 (U), position 668 (A), position 738 (U), position 669 (A), position 737 (U), position 776 (U), position 1124 (U or G), position 1297 (U), position 1298 (C), position 1423 (A), position 1424 (C or U), position 1439 (U or C), position 1462 (A or G) and position 1464 (C or U). The family comprises the genera *Carnimonas*, *Chromohalobacter*, *Cobetia*, *Halomonas*, *Halotalea* and *Zymobacter*.

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