International Journal of Systematic and Evolutionary Microbiology (2003), **53**, 331–338 DOI 10.1099/ijs.0.01923-0

Description of *Alcanivorax venustensis* sp. nov. and reclassification of *Fundibacter jadensis* DSM 12178^T (Bruns and Berthe-Corti 1999) as *Alcanivorax jadensis* comb. nov., members of the emended genus *Alcanivorax*

Javier Fernández-Martínez,[1] María J. Pujalte,[2,3] Jesús García-Martínez,[1]† Manuel Mata,[1] Esperanza Garay[2,3] and Francisco Rodríguez-Valera[1]

Correspondence
Jesús García-Martínez
jesus.garcia@ua.es

[1]División de Microbiología, Universidad Miguel Hernández, Campus de San Juan, Ctra. de Valencia km 87, 03550 San Juan, Alicante, Spain

[2]Departamento de Microbiología, Universitat de València, Campus de Burjassot, 46100 Burjassot, Valencia, Spain

[3]Instituto Cavanilles de Biodiversidad y Biologia Evolutiva, Universitat de València, Spain

Two strains of a novel bacterium were isolated independently of each other, from different depths in the Mediterranean Sea, within a time period of 7 months, using two different isolation approaches that were focused on different objectives. Both strains, designated ISO1 and ISO4^T, were halophilic, Gram-negative, strictly aerobic, straight rods that were oxidase- and catalase-positive. Both strains produced mucoid colonies in some defined minimal media and were able to grow with organic acids and some alkanes; they were also able to accumulate intracellular poly-β-hydroxybutyrate granules. The G+C content of the DNA of strain ISO4^T was 66 mol%. Comparative analysis of 16S rRNA gene sequences showed that the closest described species to the novel strains were *Alcanivorax borkumensis* and *Fundibacter jadensis*, both of the γ-*Proteobacteria*. Both of these recognized species were originally isolated from North Sea waters and are able to degrade aliphatic compounds, a property shared with strains ISO1 and ISO4^T. However, strains ISO1 and ISO4^T were different from *A. borkumensis* and *F. jadensis*, not only in their 16S rDNA sequences but also in the motility of their cells (by polar flagella) and by the presence of $C_{19:0cyclo}$ in their cellular fatty acids, among other differential features. On the basis of biochemical and molecular data, it is suggested that strains ISO1 and ISO4^T be recognized as a novel species of the genus *Alcanivorax*, for which the name *Alcanivorax venustensis* (ISO4^T = DSM 13974^T = CECT 5388^T) is proposed. On the basis of its high phenotypic similarity and close phylogenetic relatedness to *A. borkumensis*, it is also proposed that *F. jadensis* (DSM 12178^T) be reclassified as *Alcanivorax jadensis* in the genus *Alcanivorax*, and that the description of the genus *Alcanivorax* be emended.

INTRODUCTION

The so-called PCR approach has shown that marine waters are vast reservoirs of previously unrecognized micro-organisms (Giovannoni *et al.*, 1995). Although the number of novel taxa that have been brought into pure culture has not increased dramatically, the use of molecular techniques to characterize micro-organisms and the development of more sophisticated culture media and retrieval strategies (trying to match natural conditions) have certainly contributed to increasing the success of isolating novel, previously uncultivated prokaryotes from a given environment (Button

†Present address: Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Ctra. de San Vicente s/n. Ap. 99-03080, Alicante, Spain.

Abbreviations: ITS, internal transcribed spacer; MA, marine agar; MB, marine broth; PHB, poly-β-hydroxybutyrate.

The GenBank accession numbers for the nucleotide sequences reported in this study are AF328761 (*Alcanivorax venustensis* ISO1, 16S rRNA gene), AF328762 (*A. venustensis* ISO4^T, 16S rRNA gene), AF328763 (ISO1, large ITS), AF328764 (ISO1, small ITS), AF328765 (ISO4^T, large ITS), AF328766 (ISO4^T, small ITS), AF328767 (*Fundibacter jadensis* T9^T, large ITS) and AF328768 (T9^T, small ITS).

et al., 1998). Therefore, we have tried to improve our chances of isolating novel marine micro-organisms by using extremely oligotrophic enrichment conditions and size fractionation of samples (i.e. inoculation after filtering the sample through absolute membrane filters of different pore sizes). As a result of two independent sampling campaigns in the Mediterranean, a variety of bacterial strains were isolated using these two different approaches. Of these strains, two (designated ISO1 and ISO4^T) showed marked similarity in their 16S rDNA sequences (99·7 %) and were not related to any other strains/species in the databases above a similarity level of 94 %. Both strains were able to utilize several aliphatic compounds. Hydrocarbon-degrading bacteria are of potential importance in bioremediation processes (Chayabutra & Ju, 2000; Wilson *et al.*, 1999; Banat *et al.*, 2000; Nocentini *et al.*, 2000) and many of these micro-organisms have, in fact, been isolated from polluted areas in the Mediterranean (Bertrand *et al.*, 1983). Here, we characterize strains ISO1 and ISO4^T and, on the basis of their phenotypic and phylogenetic characteristics, propose they that be classified as a novel species within the genus *Alcanivorax*.

METHODS

Isolation and cultivation. Strain ISO1 was retrieved from a sea-water sample obtained, with a Niskin bottle, off the coast of Alicante (Mediterranean Sea, southeastern Spain; at 38°06·970′N, 0°27·626′W) from a depth of 5 m (bottom depth 45 m) during December 1999. The sample was filtered through 5 μm and 0·22 μm pore-diameter filters (Durapore; Millipore), respectively, to remove larger particles, eukaryotes and larger prokaryotes. Initial enrichments were prepared in duplicate by inoculating 1 ml of the filtered sample into 9 ml of medium containing aged Mediterranean sea water filtered through a 0·22 μm pore-size membane and supplemented with 0·1 g FRV l^{-1} [FRV = a mix of equal parts of *Spirulina* (Sigma), *Artemia salina* (the kind provided by pet shops for aquaria) and fish-meal (animal-feed grade), resuspended in filtered sea water for 4 h and then passed through filter paper to remove larger particles]. The medium was adjusted to pH 7·4 and autoclaved. Enrichments were incubated in the dark at 14 °C and at room temperature (22–23 °C) under light for 1 week. After this period, enrichments were again filtered through a 0·22 μm pore-size membrane; they were then diluted into aged sea water with 3×10^{-3} g FRV l^{-1}, and incubated again at 14 °C in the dark and at room temperature under light for 1 month. After this incubation, the enrichments were plated onto solid FRV (3×10^{-3} g l^{-1}). This enrichment strategy was intended to select for ultramicrobacteria, which are able to pass through 0·2 μm pore-size filters, or bacteria producing smaller starvation forms under oligotrophic conditions. Single colonies were then transferred serially to obtain pure cultures and to produce biomass for further analyses.

Strain ISO4^T was isolated from a sea-water sample obtained 22 miles off the coast of Santa Pola (Alicante, Spain; at 37° 55·020′N, 0° 17·014′W) at a depth of 200 m (bottom depth 280 m), using a Niskin bottle, during June 1999. The sample was submitted to enrichment in continuous culture at extremely low nutrient concentrations and dilution rates, to retrieve bacteria able to grow under oligotrophic conditions. The sea-water sample was passed through a 5 μm pore-size filter to remove large particles. The small bioreactor (150 ml) was completely filled up with the sample and maintained at 14 °C (*in situ* temperature). After incubation without any supplement for 24 h, a flow rate was established with a medium made with sterile sea water supplemented with 3×10^{-3} g FRV l^{-1}. The dilution rate was set at 120 h^{-1}. Aliquots were taken from the bioreactor at regular intervals for up to 3 months after the beginning of the experiment. These aliquots were immediately plated onto medium (sterile sea water supplemented with 3×10^{-3} g FRV l^{-1}) containing 1·5 % agar. After 2 months incubation, the smallest detectable colonies on the agar were selected and re-isolated to obtain pure cultures.

*Alcanivorax borkumens*is DSM 11573^T (=SK2^T =CECT 5355^T) and *Fundibacter jadensis* DSM 12178^T (=T9^T =CECT 5356^T) were obtained from the Colección Española de Cultivos Tipo (CECT) (Universitat de València, Valencia, Spain) and grown as recommended (Yakimov *et al.*, 1998; Bruns & Berthe-Corti, 1999).

DNA extraction, amplification and sequencing. Single colonies from pure cultures were picked up with autoclaved toothpicks, resuspended in 150 μl sterile distilled water and boiled for 10 min. The suspensions were then centrifuged at maximum speed. Five microlitres of the resulting supernatant were used to perform PCRs in a final volume of 50 μl (*Taq* DNA polymerase, Recombinant; GIBCO). The ribosomal 16S and 23S primers used for amplifications were ANT1 (16S; forward; positions 7–26, *Escherichia coli* numbering; 5′-AGAGTTTGATCATGGCTCAG-3′; García-Martínez *et al.*, 1999), 16S14F (16S; forward; 1389–1407; 5′-CTTGTACACACCG-CCCGTC-3′; García-Martínez *et al.*, 1996) and 23S1R (23S; reverse; 110–130; 5′-GGGTTTCCCCATTCGGAAATC-3′; García-Martínez *et al.*, 1999). The primer pair ANT1/23S1R was used to amplify the complete 16S rRNA genes plus the 16S–23S internal transcribed spacers (ITSs) of strains ISO1 and ISO4^T, whereas primer pair 16S14F/23S1R was used to amplify the ITS region of *F. jadensis* T9^T. Amplification products were run on 1 % low-melting-point agarose gels (Bio-Rad), and the resulting bands were purified using the GENECLEAN II kit (BIO 101). Sequencing of the fragments was carried out on a model 377 automated DNA sequencer (Applied Biosystems).

Phylogenetic analysis. BLAST similarity searches in the databases (Altschul *et al.*, 1997) were conducted with the sequences generated for strains ISO1, ISO4^T and T9^T. The presence/absence of tRNA genes within the ITS regions was confirmed using the tRNAScan-SE search server (Lowe & Eddy, 1997). Alignments were carried out using CLUSTAL W in the MEGALIGN program (DNASTAR), while phylogenetic analyses were carried out using MEGA (version 1.02; Pennsylvania State University, USA).

Determination of G+C content and cellular fatty acid analysis. DNA G+C content was determined only for strain ISO4^T, whereas analysis of fatty acids was performed on strains ISO1 and ISO4^T. All tests were carried out at the identification service laboratories of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cells were grown as recommended by Yakimov *et al.* (1998).

Automated ribotyping. Automated ribotyping was performed using the RiboPrinter microbial characterization system according to the manufacturer's instructions (Qualicon). Restriction enzymes used were *Eco*RI, *Pst*I and *Pvu*II. A large DNA probe harbouring the genes for both the small- and large-subunit rRNA of *E. coli* was employed. Each set of data was normalized, using an adjacent standard marker set, by the RiboPrinter integrated software.

Phenotypic characterization. The strains were tested for growth on marine agar (MA; Difco) and marine broth (MB), both at standard nutrient concentration and as 1/10 dilutions of the standard nutrient concentration, at 13 and 25 °C. Diluted versions of MA and MB were obtained by dissolving 1/10 of the usual amount of MA or MB powder in half-strength artificial sea water (Baumann & Baumann, 1981), to maintain the salinity of the medium, with the

addition of purified agar (final concn 1·2 %, w/v; Oxoid) to MA. After obtaining growth on MA and in MB, routine cultivation was done on MA or in MB at 23–25 °C.

Morphology and motility of the cells were examined on wet-mounts by phase-contrast microscopy (Leica; DAS Mikroskop LeiztDMR), using 3-day-old cultures of the strains grown on MA. Flagellar arrangement was observed after staining the same cells used in the phase-contrast microscopy, following the method of Heimbrook *et al.* (1989). The cells were also examined by scanning and transmission electron microscopy, according to Acinas *et al.* (1999) and Cole & Popkin (1981). Methods used to determine most of the phenotypic traits of the strains have been described previously (Baumann & Baumann, 1981; Ortigosa *et al.*, 1994), except for sodium, magnesium and calcium requirements and salinity tolerance, which were determined in salt tolerance broth (STB 1/10: 0·5 g tryptone l^{-1}; 0·2 g yeast extract l^{-1}; 0–200 g NaCl l^{-1}; 0–1 g $MgCl_2$ l^{-1}; 0–1 g $CaCl_2$ l^{-1}; pH 7·2) and MB plus NaCl (from normal to 20 %, w/v, total NaCl). The use of nitrate as sole nitrogen source by the strains was tested on basal medium agar with $NaNO_3$ instead of NH_4Cl, and 1 g sodium pyruvate l^{-1} as the carbon source. Tests for hydrolases, anaerobic growth with glucose or nitrate, growth at 4 °C and growth in the presence of sole carbon sources were incubated for up to 28 days; other tests were determined after 14 days incubation. The presence of intracellular poly-β-hydroxybutyrate (PHB) accumulation was examined as described by Burdon (1946). Susceptibility to antimicrobial agents was determined by the disc-diffusion test on MA plates, after 7 days incubation.

The filterability of strain ISO1 was examined by plating successive dilutions (from 10^{-1} to 10^{-6}) of a cell suspension from a colony on solid medium onto MA, before and after filtration through 0·22 μm pore-size membrane filters.

RESULTS AND DISCUSSION

Isolation and phenotypic characterization

Strains ISO1 and ISO4^T were Gram-negative, straight rods (0·9–1·8 × 0·3–0·5 μm wide), when observed on wet-mounts by optical microscopy and by scanning electron microscopy (Fig. 1). The fact that they were isolated after two passes through 0·22 μm membrane filters might be an indication of either an extremely flexible cell wall or the hypothetical presence of some smaller, filterable forms. However, no such filterability was observed under laboratory conditions, i.e. no colonies were detected after filtration of as many as 10^4 cells (c.f.u.) through 0·22 μm membrane filters. Strains ISO1 and ISO4^T were motile by at least one polar flagellum, as observed for a few cells by transmission electron microscopy. Flagella were abundant in mounts prepared by the method of Heimbrook *et al.* (1989) on actively moving cells, but it was difficult to observe the number of flagella per pole. When grown on glucose-containing media, the cells showed some PHB accumulation (granules).

Colonies of strains ISO1 and ISO4^T were small (<1 mm), regular-shaped, translucent and non-pigmented when grown on MA. Swarming and luminescence were not observed for either strain. When grown on propionate-, acetate-, pyruvate- or β-hydroxybutyrate-containing basal medium, colonies of the strains were opaque, mucoid and larger than the MA colonies, suggesting the presence of a thick capsule or slime layer around the cells.

Strains ISO1 and ISO4^T were able to grow at temperatures ranging from 4 to 40 °C, but not at 45 °C. Both strains were strictly halophilic, since no growth was observed without the addition of marine salts to the media. Furthermore, media containing NaCl alone or a combination of NaCl, $MgCl_2$ and $CaCl_2$ did not support growth, but good growth was observed in normal MB and in MB with NaCl added up to 10 %. Weak growth of the strains was also observed in MB supplemented with NaCl up to 15 % and even up to 20 %, although growth on the latter was very weak. This suggests that strains ISO1 and ISO4^T have more complex ionic requirements than other marine bacteria, which often only require the addition of NaCl to media.

Strains ISO1 and ISO4^T were aerobic chemoheterotrophs that were unable to ferment glucose or to develop with nitrate in anaerobic conditions. They did not reduce nitrate to nitrite or gas. They used both ammonium and nitrate as a nitrogen source in basal medium containing pyruvate. No extracellular hydrolysis was detected on casein, gelatin, starch, agar, alginate or DNA. Tween 80 was readily hydrolysed by the strains. Both strains were oxidase- and catalase-positive, but no activities were observed for arginine dihydrolase (in both Thornley and Moeller's

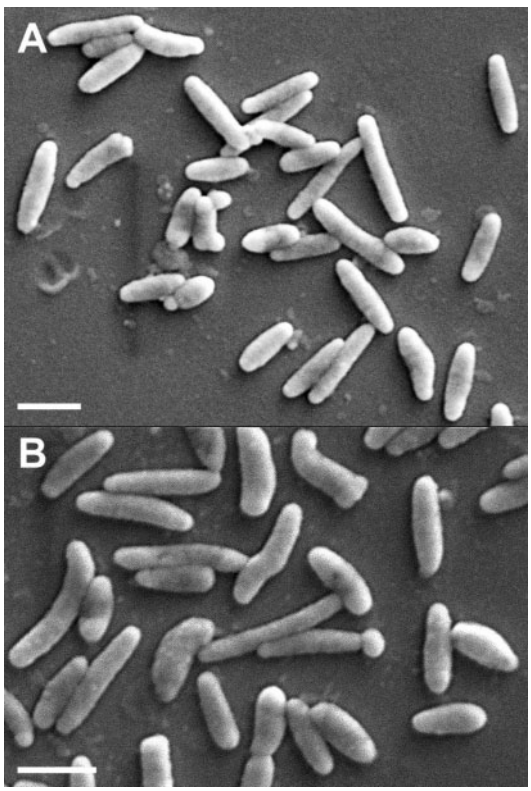

Fig. 1. Scanning electron micrographs of cells of (A) strain ISO1 and (B) strain ISO4^T. Bars, 1 μm.

media) or lysine decarboxylase. Neither strain produced indole from tryptophan.

The spectrum of carbon sources used by the bacteria was narrow. Out of the 62 compounds tested with strains ISO1 and ISO4^T, good growth was obtained only with some organic acids (propionate, pyruvate, acetate and 3-hydroxybutyrate) and the alkane tetramethylpentadecane. The strains also grew, to a lesser extent, on hexadecane and tetradecane. Variable results were obtained on DL-lactate, D-mannose and glycerol. Some carbohydrates rendered slight growth of the strains (D-ribose, D-glucose, D-fructose, D-trehalose, sucrose, *N*-acetylglucosamine and D-mannitol), but these results were not reproducible. None of the following substrates was used by either strain: L-arabinose; D-xylose; D-galactose; L-rhamnose; maltose; D-cellobiose; lactose; D-melibiose; raffinose; salicin; amygdalin; D-gluconate; D-glucuronate; D-galacturonate; glucosamine; D-sorbitol; *m*-inositol; D-glycerate; saccharate; citrate; *trans*-aconitate; 2-oxoglutarate; succinate; fumarate; malate; *p*-hydroxybenzoate; n-eicosane; phenanthrene; glycine; L-leucine; L-serine; L-threonine; L-arginine; L-tyrosine; L-glutamate; L-alanine; GABA; L-ornithine; L-citrulline; L-aspartate; L-glutamine; L-lysine; L-histidine; sarcosine; putrescine.

Antimicrobial susceptibility testing revealed that strain ISO1 was sensitive to augmentin (30 μg ml^{-1}), cefotaxime (30 μg ml^{-1}), cefuroxime (30 μg ml^{-1}), cephalotin (30 μg ml^{-1}), ciprofloxacin (1 μg ml^{-1}) and rifampicin (2 μg ml^{-1}), whereas strain ISO4^T was only sensitive to cefotaxime (30 μg ml^{-1}) and cephalotin (30 μg ml^{-1}).

Table 1 shows characteristics useful for distinguishing strains ISO1 and ISO4^T from related marine bacterial taxa.

Cellular fatty acid composition

The fatty acid profiles of strains ISO1 and ISO4^T are shown in Table 2.

Table 1. Characteristics useful for distinguishing strains ISO1 and ISO4^T from related marine γ-*Proteobacteria*

Strains/species: 1, strains ISO1/ISO4^T (this study); 2, *A. borkumensis* (Yakimov *et al.*, 1998); 3, *F. jadensis* (Bruns & Berthe-Corti, 1999); 4, *Marinobacter* spp. (Gauthier *et al.*, 1992; Huu *et al.*, 1999); 5, *Neptunomonas naphthovorans* (Hedlund *et al.*, 1999); 6, *Halomonas* spp. (Vreeland, 1992; Kersters, 1992); 7, *Oceanospirillum* spp. (Krieg, 1984). ND, No data; +, positive reaction or growth; −, no reaction or growth; W, weak reaction; V, variable reaction.

Characteristic	1	2	3	4	5	6	7
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Spirilla
Motility/flagella arrangement	+, Polar	−	−	+, Polar	+, Polar	V, peritrichous or polar	+, Bipolar
PHB accumulation	+	−	+	−	+	+	+
Exopolymer	+	+					
Pigment	−	−	−	−/Yellow	Brown	−	V
Fermentative	−	−	−	−	+	−	−
NO_3^- reduction	−	+	+	+	−	V	−
Ionic requirements	Complex	Complex (Na^+, Mg^{2+})	Na^+	Na^+/−	Na^+	−/Na^+	Sea water
Utilization of carbohydrates	−/W	−	−	−	+	+	−
Utilization of alkanes:							
Tetradecane	+	ND	+	+	ND	ND	ND
Hexadecane	+	+	+	+	ND	−/ND	ND
Utilization of organic acids:							
Pyruvate	+	+	+	ND	+	V	V
Propionate	+	+	ND	−	−	+	V
Acetate	+	+	+	+	+	+	V
Succinate	−	−	−	+	+	+	V
2-Oxoglutarate	−	+	ND	−	ND	V	−
3-Hydroxybutyrate	+	−	ND	−	+	+	−
Utilization of glutamate	−	−	−	+	+	+	+
Fatty content (percentage of total):							
$C_{18:1\omega9c}$	−/1·2	−	−	20–30	ND	ND	11–30*
$C_{19:0cyclo}$	19/10	−	−	−	ND	ND	ND
DNA G+C content (mol%)	ND/66·4	53–54	63–64	53–58	46	56–68	45–50

*Position of double bond not specified (Sakane & Yokota, 1994).

Table 2. Fatty acid compositions of strains ISO1 and ISO4^T, and *A. borkumensis* and *F. jadensis*

Strains/species: 1, ISO1/ISO4^T (this study); 2, *A. borkumensis* (Yakimov *et al.*, 1998); 3, *F. jadensis* (Bruns & Berthe-Corti, 1999).

Fatty acid	1	2	3
$C_{10:0}$	2·7/3·2	–	–
$C_{12:0}$	4·9/5·1	–	5·2
2-OH $C_{12:0}$	–	–	1·1
3-OH $C_{12:0}$	3·6/10·7	–	4·9
$C_{14:0}$	–/1·4	1·1	1·7
$C_{15:1\omega6c}$	–/1·1	–	–
$C_{15:0}$	–/2·0	–	–
anteiso-$C_{15:0}$	–	–	0·2
$C_{16:1\omega7c}$	8·1/15·4	17·1*	13·5
$C_{16:0}$	35·4/20·2	31·5	23·4
$C_{17:1\omega8c}$	–/0·5	–	–
$C_{17:0cyclo}$	4·5/1·4	–	–
$C_{17:0}$	3·2/2·8	–	–
$C_{18:1\omega9c}$	–/1·2	–	–
$C_{18:1\omega7c}$	10·5/19·9	47·1†	20·7
$C_{18:1\omega5}$	–	0·2	–
$C_{18:0}$	–/0·9	2·0	–
anteiso-$C_{18:1\omega7c}$	–/1·2	–	–
$C_{19:0cyclo}$	19·8/10·1	–	–
$C_{20:2\omega6,9c}$	1·1/–	–	–
Unknown	7·4/1·5	0·9	29·2

*Sum of $C_{16:1\omega7c}$/$C_{16:1\omega9t}$ (Yakimov *et al.*, 1998).
†Sum of $C_{18:1\omega7c}$/$C_{18:1\omega7t}$/$C_{18:1\omega9t}$/$C_{18:1\omega12}$ (Yakimov *et al.*, 1998).

G+C content

The G+C content of the DNA of strain ISO4^T was 66 mol%, as determined by HPLC.

Automated ribotyping

The ribotype patterns of strains ISO1 and ISO4^T were always identical, whatever the enzyme used, and were different from the ones produced from *A. borkumensis* DSM 11573^T and *F. jadensis* DSM 12178^T. *A. borkumensis* DSM 11573^T and *F. jadensis* DSM 12178^T also produced identical patterns to each other when digested with the three enzymes. Fig. 2 shows the profiles obtained with *Eco*RI.

Fig. 2. Ribotype patterns obtained with *Eco*RI for strains ISO1 and ISO4^T (lanes 3 and 4, respectively), and *A. borkumensis* DSM 11573^T (lane 1) and *F. jadensis* DSM 12178^T (lane 2). M, Molecular size markers.

Comparative analysis of 16S rDNA sequences

Comparative analysis of the almost-complete 16S rDNA sequences of strains ISO1 and ISO4^T showed a high similarity between them (>99 %), while their most similar sequences found among recognized species corresponded to *A. borkumensis* DSM 11573^T (94·7 %) and *F. jadensis* DSM 12178^T (94·6 %) (Fig. 3).

Comparative analysis of ITS sequences

A close similarity was found between the alignable regions of the 16S–23S ITS sequences of strains ISO1 and ISO4^T (small ITS, about 308 bp; large ITS, about 580 bp, with tRNA–Ile and tRNA–Ala genes), *F. jadensis* DSM 12178^T (333 bp; 547 bp, with tRNA–Ile and tRNA–Ala) and *A. borkumensis* strains SK2^T (=DSM 11573^T) and LE4 (about 330 bp; 520 bp, with tRNA–Ile and tRNA–Ala), with a mean variation of 20·52±13·30 %. This variation is very similar to that found in other groups belonging to the *γ-Proteobacteria*, such as *Vibrio* [22·54±19·66 %, with sequence data from Chun *et al.* (1999) and Heidelberg *et al.* (2000)] and *Pseudomonas* [27·89±20·68 %, with

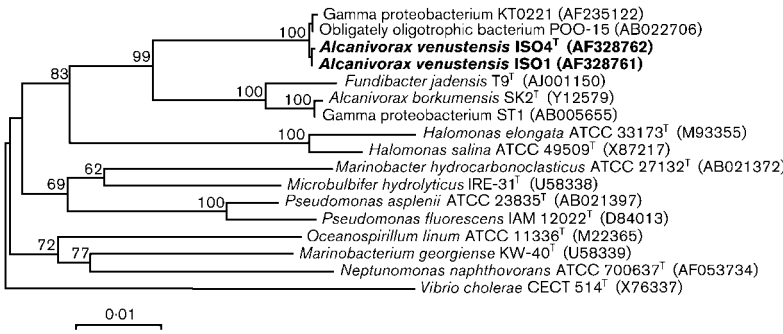

Fig. 3. Phylogenetic tree showing relationship of strains ISO1 and ISO4^T with other *γ-Proteobacteria*, as inferred from a comparison of aligned 16S rDNA sequences (1197 bp; Kimura two-correction parameter, neighbour-joining method). Only significant bootstrap values are shown at the nodes.

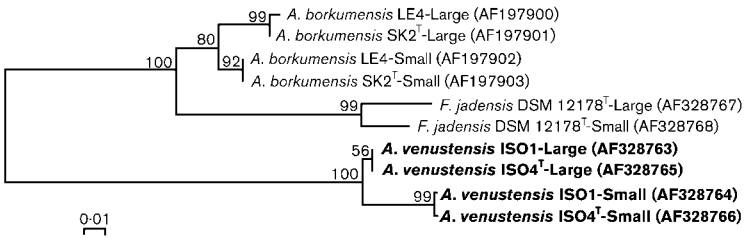

Fig. 4. Genetic relationships inferred using the alignable regions of the 16S–23S rRNA ITS sequences (large and small, 225 bp in total) from two strains of *A. borkumensis*, SK2^T and LE4, *F. jadensis* DSM 12178^T and strains ISO1 and ISO4^T (Kimura two-correction parameter, neighbour-joining method). Bootstrap values are shown at the nodes.

sequence data from Guasp *et al.* (2000) and Koike *et al.* (1999)]. Fig. 4 shows the phylogenetic relationships inferred from this set of data.

The data presented here support the conclusion that strains ISO1 and ISO4^T represent a novel species of marine bacteria. This was confirmed by the phylogenetic relationships the two strains had with recognized bacterial species (<97 % sequence similarity with 16S rDNA sequences from other *γ-Proteobacteria*; Stackebrandt & Goebel, 1994) and by their distinctive phenotypic traits, which included their fatty acid composition and physiological and biochemical features (Tables 1 and 2). The analysis of ITS sequences and the identical and unique patterns obtained for both strains by riboprinting with three different enzymes also reinforce this conclusion.

However, the assignment of the novel species to a genus is problematic. The two recognized species most closely related to the novel strains in the phylogenetic analysis were *A. borkumens*is and *F. jadensis*, two species that share several key physiological characteristics with strains ISO1 and ISO4^T, such as their ability to use alkanes as carbon and energy sources, their requirement for marine salts and their major fatty acids. The phylogenetic distance, based on 16S rDNA sequence analysis, of strains ISO1 and ISO4^T from both recognized species was on the borderline of the 95 % level suggested to define genera (Rosselló-Mora & Amann, 2000), and analyses of differences in ITS sequences were also compatible with the inclusion of the novel species in the genus *Alcanivorax* or *Fundibacter*: the differences were within the mean differences characteristic for a single and well-defined genus (García-Martínez *et al.*, 1999; García-Martínez & Rodríguez-Valera, 2000). In addition, the 16S rRNA gene sequences of *A. borkumens*is DSM 11573^T and *F. jadensis* DSM 12178^T displayed 97·2 % similarity, which was higher than the 16S rRNA gene sequence similarity observed between the ISO strains and these recognized species. The ribotypes of *A. borkumensis* and *F. jadensis* were always coincident and few phenotypic traits allowed the discrimination of these supposedly different genera. For example, PHB accumulation, exopolymer production and fimbriae synthesis are traits that are usually considered to be strain- or culture-dependent. The only feature that allowed discrimination of the two recognized genera was DNA G+C content. From our analyses, it appears that *A. borkumens*is DSM 11573^T and *F. jadensis* DSM 12178^T are, in fact, more similar to each other than to strains ISO1 and ISO4^T, with the exception of G+C content and PHB accumulation, which were more similar between *F. jadensis* DSM 12178^T and the ISO strains. The fatty acid pattern of strains ISO1 and ISO4^T showed the same principal components as *A. borkumens*is DSM 11573^T and *F. jadensis* DSM 12178^T, but it also included a specific component, $C_{10:0cyclo}$ (Table 2). Nevertheless, the unusually high percentage of undetermined fatty acids of *F. jadensis* DSM 12178^T (Bruns & Berthe-Corti, 1999) makes it difficult to establish any definitive conclusion. In contrast to *A. borkumens*is (DSM 11573^T) and *F. jadensis* (DSM 12178^T), which were non-motile, strains ISO1 and ISO4^T were motile by polar flagella.

Thus, from the above data, it is our opinion that the maintenance of the genera *Alcanivorax* and *Fundibacter* and the description of a novel genus to accommodate strains ISO1 and ISO4^T is unjustified. The global phenotypic similarity of the three species and the monophyly of the group in the phylogenetic analyses done here (16S rRNA and ITS) have led us to emend the description of the genus *Alcanivorax* to accommodate the transfer of *F. jadensis* DSM 12178^T to the genus, and to describe a novel species, represented by strains ISO1 and ISO4^T, for which the name *Alcanivorax venustensis* is proposed.

Emended description of the genus *Alcanivorax* (Yakimov *et al.* 1998)

Species are Gram-negative, aerobic, straight rods. Non-motile and non-flagellated, or motile by polar flagella. Strictly respiratory type of metabolism. Do not ferment carbohydrates. Some species may use nitrate as an alternate electron acceptor, but none denitrify nitrogen compounds. Oxidase- and catalase-positive. Halophilic, requiring (at least) Na^+ ions for growth: some species have more complex ionic requirements. Able to grow in the presence of up to 12 % NaCl. Chemo-organotrophic, using short-chain fatty acids and some alkanes as sole or principal carbon sources. Acetate, pyruvate and hexadecane are used as carbon sources. Principal fatty acids are $C_{16:0}$, $C_{18:1\omega7c}$ and $C_{16:1\omega7c}$. DNA G+C content of species ranges from 53 to 66 mol%. Isolated from marine habitats. On the basis of

16S-rDNA-based phylogenetic analyses, the genus belongs to the *γ-Proteobacteria*. Type species is *Alcanivorax borkumensis* (SK2^T = DSM 11573^T = CECT 5355^T = ATCC 700651^T = CIP 105606^T) (Yakimov *et al.*, 1998).

Description of *Alcanivorax jadensis* comb. nov. (basonym *Fundibacter jadensis* Bruns and Berthe-Corti 1999)

The description is identical to the one given by Bruns & Berthe-Corti (1999). The type strain of the species is T9^T (= DSM 12178^T = CECT 5356^T = ATCC 700854^T).

Description of *Alcanivorax venustensis* sp. nov.

Alcanivorax venustensis (ve.nus.ten′sis. M.L. adj. *venustensis* from 'Portus Venustus', Elegant Port, one of the ancient Latin names of Santa Pola in Roman times, a coastal town south of Alicante, the nearest town to the sites from which the two strains of the species were retrieved. It might also refer to the elegant and slender aspects of the rods).

Aerobic. Gram-negative, straight rods (0·9–1·8 × 0·3–0·5 μm). Motile by means of polar flagella. Grows well on MA (Difco), as small, regular-shaped, non-pigmented colonies. Growth at extremely low nutrient concentrations is also possible (facultative oligotroph). Strain ISO1 was isolated from filtered sea water (0·22 μm pore size), but no other filterable forms have been detected in the laboratory. Temperature range for growth is 4–40 °C. Marine salts are required for growth and are not replaceable by NaCl at an equivalent concentration. Grows in the presence of up to 15 % (w/v) NaCl in the medium. Degrades Tween 80, but casein, gelatin, starch, alginate, agar and DNA are not hydrolysed. Nitrate is not reduced to nitrite or gas. Sugars are not fermented. Catalase- and oxidase-positive. Carbon sources that support good growth are pyruvate, propionate, acetate, 3-hydroxybutyrate and tetramethylpentadecane. Amino acids are not used. Growth on minimal media with organic acids is accompanied by the formation of mucous colonies. Principal fatty acids are $C_{16:0}$, $C_{16:1w7c}$, $C_{18:1w7c}$ and $C_{19:0cyclo}$, with minor amounts of 3OH-$C_{12:0}$, $C_{12:0}$ and $C_{10:0}$. DNA G+C content of the type strain is 66 mol%. Isolated from sea water. The type strain is ISO4^T (= DSM 13974^T = CECT 5388^T). Strain ISO1 has also been deposited in the CECT as CECT 5389.

Distribution and ecological aspects

Although both strains of *A. venustensis* were obtained from two independent samples and enrichment approaches, the sampling sites were geographically quite close (within a few miles of each other). However, it is very likely that related organisms might also be retrieved from rather widespread locations (as was the case with *A. borkumensis* DSM 11573^T). Analysis of the complete 16S rDNA sequences of strains ISO1 (1530 bp) and ISO4^T (1532 bp) showed that both strains had more than 99 % similarity with the 16S rDNA sequences of unnamed strains isolated in Japan and the North Sea [1302 bp (Yoshinaga *et al.*, 1999) and 1331 bp (Eilers *et al.*, 2000), respectively]. However, the Japanese strain was primarily characterized as being obligately oligotrophic, a trait that is not shared with the proposed novel species, a facultative oligotroph and psychrophile. The most closely related recognized species to *A. venustensis*, based on 16S rDNA sequence analysis, were *A. borkumensis* and *F. jadensis* (Yakimov *et al.*, 1998; Bruns & Berthe-Corti, 1999). The global distribution of *A. borkumensis* and *F. jadensis* and the fact that they are essentially non-fastidious micro-organisms that are relatively easy to culture raises some questions regarding their late isolation. One possibility is, of course, that the recent use and development of molecular techniques for large-scale screening of environmental samples is only now revealing the full scale of potentially novel micro-organisms. Another possibility is the likely limited numbers of these species within the total microflora of their respective habitats, making their isolation from mixed populations more difficult. It is possible that the contribution of the oligotrophic conditions in the FRV medium used for the enrichments favoured the growth of strains ISO1 and ISO4^T, even in the case of ISO1 after two filtration steps. Some authors have suggested for hydrocarbon-degrading bacteria such as *F. jadensis* and *Marinobacter hydrocarbonoclasticus* (Bruns & Berthe-Corti, 1999) that their abundance in certain locations might be associated with polluted waters. However, strains ISO1 and ISO4^T were isolated from open, apparently pristine, sea waters, with one of the sample sites being 20 miles off the coast and at a depth of 280 m (ISO4^T), with no evidence of hydrocarbon pollution. Nevertheless, it should be noted that the pick-up coordinates for strain ISO4^T were close to a busy shipping route, and occasional oil spillages might occur at this site. If this were the case, members of the genus *Alcanivorax* and other closely related *γ-Proteobacteria* could become useful micro-organisms as bio-indicators of waters contaminated with low to high levels of long-chain hydrocarbons.

ACKNOWLEDGEMENTS

We thank Stuart Ingham for graphics and image processing, Carmela Belloch from CECT for her technical assistance in the ribotyping and H.-J. Busse for his help with fatty acid data interpretation. This work was supported by MIDAS (European Commission MAST Grant MAS3-CT-97-0154).

REFERENCES

Acinas, S. G., Antón, J. & Rodríguez-Valera, F. (1999). Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of genes encoding 16S rDNA. *Appl Environ Microbiol* **65**, 514–522.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Banat, I. M., Makkar, R. S. & Cameotra, S. S. (2000). Potential commercial applications of microbial surfactants. *Appl Microbiol Biotechnol* **53**, 495–508.

Baumann, P. & Baumann, L. (1981). The marine gram-negative eubacteria: genera *Photobacterium*, *Beneckea*, *Alteromonas*, *Pseudomonas* and *Alcaligenes*. In *The Prokaryotes*, vol. 1, pp. 1302–1331. Edited by M. P. Starr, H. Stolp, H. G. Trüper, A. Balows & H. Schlegel. Berlin: Springer-Verlag.

Bertrand, J. C., Rambeloarisoa, E., Rontani, J. F., Giusi, G. & Mattei, G. (1983). Microbial degradation of crude oil in sea water in continuous culture. *Biotechnol Lett* **5**, 567–572.

Bruns, A. & Berthe-Corti, L. (1999). *Fundibacter jadensis* gen. nov., sp. nov., a new slightly halophilic bacterium, isolated from intertidal sediment. *Int J Syst Bacteriol* **49**, 441–448.

Burdon, K. L. (1946). Fatty material in bacterial and fungi revealed by staining dried, fixed slide preparations. *J Bacteriol* **52**, 665–678.

Button, D. K., Robertson, B. R., Lepp, P. W. & Schmidt, T. M. (1998). A small, dilute-cytoplasm, high-affinity, novel bacterium isolated by extinction culture and having kinetic constants compatible with growth at ambient concentrations of dissolved nutrients in seawater. *Appl Environ Microbiol* **64**, 4467–4476.

Chayabutra, C. & Ju, L. K. (2000). Degradation of n-hexadecane and its metabolites by *Pseudomonas aeruginosa* under microaerobic and anaerobic denitrifying conditions. *Appl Environ Microbiol* **66**, 493–498.

Chun, J., Huq, A. & Colwell, R. R. (1999). Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus*. *Appl Environ Microbiol* **65**, 2202–2208.

Cole, R. M. & Popkin, T. J. (1981). Electron Microscopy. In *Manual of Methods for General Bacteriology*, pp. 34–51. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

Eilers, H., Pernthaler, J., Glockner, F. O. & Amann, R. (2000). Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66**, 3044–3051.

García-Martínez, J. & Rodríguez-Valera, F. (2000). Microdiversity of uncultured marine prokaryotes: the SAR11 cluster and the marine *Archaea* of Group I. *Mol Ecol* **9**, 935–948.

García-Martínez, J., Martínez-Murcia, A. J., Rodríguez-Valera, F. & Zorraquino, A. (1996). Molecular evidence supporting the existence of two major groups in uropathogenic *Escherichia coli*. *FEMS Immunol Med Microbiol* **14**, 231–244.

García-Martínez, J., Acinas, S. G., Antón, A. I. & Rodríguez-Valera, F. (1999). Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity. *J Microbiol Methods* **36**, 55–64.

Gauthier, M. J., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P. & Bertrand, J.-C. (1992). *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int J Syst Bacteriol* **42**, 568–576.

Giovannoni, S. J., Mullins, T. D. & Field, K. G. (1995). Microbial diversity in oceanic systems: rRNA approaches to the study of unculturable microbes. In *Molecular Ecology of Aquatic Microbes*, NATO ASI Series, vol. G 38, pp. 217–248. Edited by I. Joint. Berlin: Springer-Verlag.

Guasp, C., Moore, E. R. B., Lalucat, J. & Bennasar, A. (2000). Utility of internally transcribed 16S–23S rDNA spacer regions for the definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. *Int J Syst Evol Microbiol* **50**, 1629–1639.

Hedlund, B. P., Geiselbrecht, A. D., Bair, T. J. & Staley, J. T. (1999). Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. *Appl Environ Microbiol* **65**, 251–259.

Heidelberg, J. F., Eisen, J. A., Nelson, W. C. & 23 other authors (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**, 477–483.

Heimbrook, M. E., Wang, W. L. L. & Campbell, G. (1989). Staining bacterial flagella easily. *J Clin Microbiol* **27**, 2612–2615.

Huu, N. B., Denner, E. B. M., Ha Dang, T. C., Wanner, G. & Stan-Lotter, H. (1999). *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from Vietnamese oil-producing well. *Int J Syst Bacteriol* **49**, 367–375.

Kersters, K. (1992). The genus *Deleya*. In *The Prokaryotes*, vol. 4, pp. 3189–3197. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.

Koike, S. T., Barak, J. D., Henderson, D. M. & Gilbertson, R. L. (1999). Bacterial blight of leek: a new disease in California caused by *Pseudomonas syringae*. *Plant Dis* **83**, 165–170.

Krieg, N. R. (1984). Genus *Oceanospirillum* Hylemon, Wells, Krieg and Jannasch 1973, 361^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol 1, pp. 104–110. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Lowe, T. & Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* **25**, 955–964.

Nocentini, M., Pinelli, D. & Fava, F. (2000). Bioremediation of a soil contaminated by hydrocarbon mixtures: the residual concentration problem. *Chemosphere* **41**, 1115–1123.

Ortigosa, M., Garay, E. & Pujalte, M. J. (1994). Numerical taxonomy of aerobic, Gram-negative bacteria associated to oysters and surrounding seawater of the Mediterranean coast. *Syst Appl Microbiol* **17**, 589–600.

Rosselló-Mora, R. & Amann, R. (2000). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Sakane, T. & Yokota, A. (1994). Chemotaxonomic investigation of heterotrophic, aerobic and microaerophilic spirilla, the genera *Aquaspirillum*, *Magnetospirillum* and *Oceanospirillum*. *Syst Appl Microbiol* **17**, 128–134.

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.

Vreeland, R. H. (1992). The family *Halomonadaceae*. In *The Prokaryotes*, vol. 4, pp. 3181–3188. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.

Wilson, V. L., Tatford, B. C., Yin, X., Rajki, S. C., Walsh, M. M. & LaRock, P. (1999). Species-specific detection of hydrocarbon-utilizing bacteria. *J Microbiol Methods* **39**, 59–78.

Yakimov, M. M., Golyshin, P. N., Lang, S., Moore, E. R., Abraham, W. R., Lünsdorf, H. & Timmis, K. N. (1998). *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int J Syst Bacteriol* **48**, 339–348.

Yoshinaga, I., Katanozaka, N. & Ueno, Y. (1999). VBNC (viable but nonculturable) marine bacteria on the molecular aspects. *Microb Environ* **14**, 123–129.