Alcanivorax pacificus sp. nov., isolated from a deep-sea pyrene-degrading consortium

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A taxonomic study was carried out on a novel bacterial strain, designated W11-5^T, which was isolated from a pyrene-degrading consortium enriched from deep-sea sediment of the Pacific Ocean. The isolate was Gram-reaction-negative and oxidase- and catalase-positive. Growth was observed in 0.5–12 % (w/v) NaCl and at 10–42 °C. On the basis of 16S rRNA gene sequence analysis, strain W11-5^T was shown to belong to the genus *Alcanivorax* with a close relation to *A. dieselolei* B-5^T (93.9 % 16S rRNA sequence similarity), *A. balearicus* MACL04^T (93.1 %), *A. hongdengensis* A-11-3^T (93.1 %), *A. borkumensis* SK2^T (93.0 %), *A. venustensis* ISO4^T (93.0 %) and *A. jadensis* T9^T (92.9 %). Similarities between the *gyrB* gene sequences of W11-5^T and other species of the genus *Alcanivorax* were between 76.8 and 80.8 %. The principal fatty acids were $C_{12:0}$ 3-OH (8.0 %), $C_{16:0}$ (29.1 %) and $C_{18:1}\omega7c$ (27.4 %). The G+C content of the chromosomal DNA was 60.8 mol%. Based on its morphology, physiology and fatty acid composition as well as the results of 16S rRNA and *gyrB* gene sequence analyses, strain W11-5^T (=MCCC 1A00474^T =CCTCC AB 208236^T =LMG 25514^T) represents a novel species of the genus *Alcanivorax*, for which the name *Alcanivorax pacificus* sp. nov. is proposed.

The genus *Alcanivorax*, a group of marine hydrocarbon-degrading bacteria, is recognized as part of the obligate hydrocarbonoclastic bacteria (OHCB) due to the capacity of its members to utilize a variety of hydrocarbons. Since the genus was first proposed by Yakimov *et al.* (1998), >250 *Alcanivorax*-affiliated bacteria, based on 16S rRNA gene sequences, have been isolated or detected in various types of marine environment, such as surface, shallow and deep seawater, sediments and hydrothermal vents, and even a few terrestrial environments (Yakimov *et al.*, 2007). At the time of writing, the genus *Alcanivorax* comprised six recognized species, *A. borkumensis* (Yakimov *et al.*, 1998), *A. jadensis* (Bruns & Berthe-Corti, 1999), *A. venustensis* (Fernández-Martínez *et al.*, 2003), *A. dieselolei* (Liu & Shao, 2005a), *A. balearicus* (Rivas *et al.*, 2007) and *A. hongdengensis* (Wu *et al.*, 2009), all of which were isolated from marine environments except *A. balearicus*, which was isolated from a subterraneous saline lake in Mallorca, Spain. In this study, we describe a novel strain, designated W11-5^T, which was isolated from a pyrene-degrading consortium enriched from a deep-sea sediment sample collected from the western Pacific Ocean (Wang *et al.*, 2008). Three novel species have, so far, been isolated from this pyrene-degrading consortium and described: *Roseovarius pacificus* (Wang *et al.*, 2009), *Oceanicola pacificus* (Yuan *et al.*, 2009) and *Bowmanella pacifica* (Lai *et al.*, 2009). The type strains of these three species, along with strain W11-5^T, were, however, unable to degrade pyrene. Strain W11-5^T was characterized and classified by using a polyphasic approach and was found to be phylogenetically related to members of the genus *Alcanivorax*. Routine cultivation of the strain and most of the phenotypic tests were carried out on 216L marine agar medium (MAM), comprising (g l^{-1} seawater) CH_3COONa (1), tryptone (10), yeast extract (2), sodium citrate (0.5), NH_4NO_3 (0.2) (pH 7.5), according to (Yuan *et al.*, 2009) unless stated otherwise.

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995) and the 16S rRNA gene was amplified by PCR using primers that have been described previously (Liu & Shao, 2005a). 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 4 (Tamura *et al.*, 2007) after multiple sequence alignment

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Abbreviation: OHCB, obligate hydrocarbonoclastic bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, partial *gyrB* and partial *alkB* gene sequences of strain W11-5^T are DQ659451, GU395984 and GU395983, respectively.

Four supplementary figures and two supplementary tables are available with the online version of this paper.

using DNAMAN version 5.1 (Lynnon Biosoft). Distances (distance options according to the Kimura two-parameter model; Kimura, 1980) and clustering with the neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Rzhetsky & Nei, 1992, 1993) methods were determined by using bootstrap support based on 1000 replications.

The nearly full-length 16S rRNA gene sequence (1506 nt) of strain W11-5^T was determined. Phylogenetic analysis of strain W11-5^T indicated that it was a member of the class *Gammaproteobacteria*, forming a robust clade within the genus *Alcanivorax* (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). The closest related neighbours to strain W11-5^T were *A. dieselolei* B-5^T, *A. balearicus* MACL04^T, *A. hongdengensis* A-11-3^T, *A. venustensis* ISO4^T, *A. borkumensis* SK2^T and *A. jadensis* T9^T with 16S rRNA gene sequence similarities of 93.9, 93.1, 93.1, 93.0, 93.0 and 92.9 %, respectively; type strains of other species shared <92 % sequence similarity with strain W11-5^T. As a 16S rRNA gene sequence divergence greater than 3 % is generally accepted as the recommended criterion for the delineation of bacterial species (Stackebrandt & Goebel, 1994), these similarity values supported the view that strain W11-5^T represented a novel species of the genus *Alcanivorax*.

A 1058 nt fragment of the housekeeping gene *gyrB* (encoding DNA gyrase subunit B) was obtained from strain W11-5^T by using the method described by Yamamoto *et al.* (2000) and showed sequence similarities of 80.8, 80.7, 78.9, 77.8, 76.9 and 76.8 % to *A. venustensis* ISO4^T, *A. hongdengensis* A-11-3^T, *A. jadensis* T9^T, *A. dieselolei* B-5^T, *A. borkumensis* SK2^T and *A. balearicus* MACL04^T, respectively. This indicated that strain W11-5^T satisfied the recommended threshold criterion (10 % nucleotide substitution rate; Venkateswaran *et al.*, 1999) of sequence diversity to distinguish it as a novel species. The phylogenetic tree based on *gyrB* gene sequences (Supplementary Fig. S2) showed that strain W11-5^T formed an independent monophyletic cluster with other species of the genus *Alcanivorax*, which was similar to that seen in the tree based on 16S rRNA gene sequences.

A 549 nt fragment of the alkane hydroxylase gene *alkB* was amplified from strain W11-5^T by PCR using previously described primers (Wang *et al.*, 2010) and alignment of deduced amino acid sequences showed that strain W11-5^T had the highest similarity to *A. venustensis* ISO4^T (82.5 %). Based on amino acid sequences deduced from the *alkB* gene, strain W11-5^T clustered with *A. dieselolei* B-5^T, *A. balearicus* MACL04^T and *A. venustensis* ISO4^T (Supplementary Fig. S3), forming a separate group with similarities of 78.7–82.5 %; however, compared to phylogenetic positions based on 16S rRNA gene sequences, there was no clear grouping of species, as previously described by Van Beilen *et al.* (2003). For example, *A. dieselolei* B-5^T and *A. borkumensis* SK2^T contain multiple *alkB* gene sequences, therefore, phylogenetic analysis of their deduced amino acid sequences placed them on completely different branches, unlike analyses based on 16S rRNA and *gyrB* gene sequences (Supplementary Fig. S1 and S2).

Cell morphology was determined under an Olympus inverted microscope using two-day-old cultures of strain W11-5^T grown on 216L MAM according to (Yuan *et al.*, 2009). For electron microscopy, exponential-phase cells were harvested, suspended and subsequently absorbed on to a Formvar–carbon-coated grid followed by staining with phosphotungstic acid (Supplementary Fig. S4). Gram-staining and activities of catalase, oxidase, amylase and gelatinase were determined according to standard methods (Dong & Cai, 2001). The optimal temperature for growth was determined by growth at 4–45 °C on 216L MAM. Tolerance of NaCl was determined by using 216L MAM, with seawater replaced by distilled water, supplemented with 0, 0.5, 1, 3, 5, 7, 10, 12, 15, 17 and 20 % (w/v) NaCl. Utilization of *n*-alkanes as carbon sources was determined by growing cells of strain W11-5^T in 0.1 % (w/v) ASM medium as described by Liu & Shao (2005b) with chain-lengths ranging from C_{10} to C_{36}. Other biochemical tests were carried out by using API 20 NE and API ZYM test kits (bioMérieux) and a Biolog GN2 MicroPlate panel, according to the manufacturer's instructions but with the media adjusted to contain 3 % (w/v) NaCl, the results of which are given in the species description and in Table 1.

The DNA G+C content of strain W11-5^T was determined using reversed-phase HPLC according to the methods described by Mesbah & Whitman (1989). The DNA G+C content of strain W11-5^T was 60.8 mol%, which was within the range previously reported for species of the genus *Alcanivorax* (53.4–66.4 mol%; Table 1).

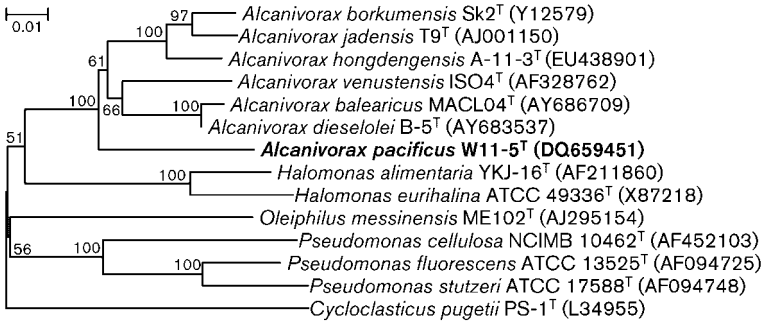

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain W11-5^T with those of type strains of closely related taxa. Bootstrap values >50 % (based on 1000 replications) are shown at branch points. Bar, 0.01 substitutions per nucleotide position (K_{nuc}).

Table 1. Characteristics of strain W11-5^T and closely related members of the genus *Alcanivorax*

Taxa: 1, W11-5^T (data from this study); 2, *A. dieselolei* B-5^T (data from Liu & Shao, 2005a); 3, *A. balearicus* MACL04^T (Rivas *et al.*, 2007); 4, *A. hongdengensis* A-11-3^T (Wu *et al.*, 2009); 5, *A. venustensis* ISO4^T (Fernández-Martínez *et al.*, 2003); 6, *A. borkumensis* SK2^T (Yakimov *et al.*, 1998); 7, *A. jadensis* T9^T (Bruns & Berthe-Corti, 1999; Fernández-Martínez *et al.*, 2003). Tests for catalase and oxidase activities and tests in the API 20 NE and API ZYM systems were performed in parallel for all seven type strains. In the API 20 NE system, all strains were negative for denitrification, indole production, arginine dihydrolase and β-galactosidase activities and for the utilization of D-mannose, D-mannitol, maltose and potassium gluconate. In the API ZYM system, all strains were positive for esterase (C4), esterase lipase (C8), lipase (C14), leucine aminopeptidase, weakly positive for valine aminopeptidase and negative for cystine aminopeptidase, *N*-acetyl-β-glucosaminidase, trypsin, α-chymotrypsin, α-fucosidase, α- and β-galactosidase, α-glucosidase, α-mannosidase and β-glucuronidase. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4	5	6	7
Catalase	+	+	+	−	+	+	−
Oxidase	+	W	W	−	+	W	+
Motility, flagella arrangement	−	+*	+†	−	+*	−	−
Ionic requirements	Na^+	Na^+	−	Na^+	Complex	Complex	Na^+
Growth in 17 % NaCl	−	W	−	−	+	−	−
Growth at 42 °C	+	+	−	+	+	−	+
Growth at 45 °C	−	+	−	−	+	−	+
DNA G+C content (mol%)	60.8	62.1	62.8	54.7	66.4	53.4	63.6
API 20 NE:							
Nitrate reduction	+	−	W	+	−	−	−
D-Glucose fermentation	W	−	−	−	−	−	−
Urease, β-glucosidase, D-glucose, L-arabinose, *N*-acetylglucosamine	+	−	−	−	−	−	−
Gelatin hydrolysis	−	−	−	+	−	+	−
Capric acid, phenylacetic acid	−	+	+	−	−	−	−
Adipic acid	W	+	+	−	+	−	−
Malic acid	+	+	−	−	−	−	−
Trisodium citrate	−	+	+	−	−	−	−
API ZYM:							
Acid phosphatase, naphthol-AS-B1-phosphoamidase	W	+	+	W	+	+	+
Alkaline phosphatase	W	+	+	−	W	+	+
β-Glucosidase	+	−	−	−	−	−	−

*Lophotrichous flagella.
†Polar or subpolar flagellum.

Antibiotic susceptibility tests were performed according to Lai *et al.* (2009) by using the disc diffusion method with Oxoid discs. Strain W11-5^T and the six type strains of species of the genus *Alcanivorax* were tested in parallel. All the strains were sensitive to (μg per disc unless otherwise indicated) polymyxin B (30) but resistant to clindamycin (2), furazolidone (15), lincomycin (2), metronidazole (5), oxacillin (1) and vancomycin (30). The sensitivity of the seven strains to 23 other kinds of antibiotics was also determined (Supplementary Table S1).

Fatty acids of cells grown aerobically on Marine Agar (BD) supplemented with 1.0 g CH_3COONa l^{-1} and 0.5 g trisodium citrate l^{-1} (pH 7.5) at 28 °C for 72 h were extracted, saponified and esterified and the fatty acid methyl esters were analyzed by GC according to the instructions of the MIDI system (Sasser, 1990). The fatty acids profiles of the six type strains of species of the genus *Alcanivorax* were also determined by using the same method with cells grown under the same conditions. As shown in Supplementary Table S2, the predominant fatty acids of all seven type strains were $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), which accounted for ~47–76 % of the total fatty acids. In addition, strain W11-5^T contained summed feature 9 (iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl) (9 %), which was absent in the other strains.

As shown in Table 1, there are some significant characteristics that distinguish strain W11-5^T from closely related species of the genus *Alcanivorax*. Therefore, on the basis of its morphological, physiological and chemotaxonomic characteristics, together with the results of phylogenetic analyses based on the 16S rRNA and *gyrB* gene

sequences, strain W11-5^T represents a novel species of the genus *Alcanivorax*, for which the name *Alcanivorax pacificus* sp. nov. is proposed.

Description of *Alcanivorax pacificus* sp. nov.

Alcanivorax pacificus (pa.ci′fic.us. L. masc. adj. *pacificus* pacific, pertaining to the Pacific Ocean).

Cells are Gram-reaction-negative, short non-motile rods, 1.7–2.3 μm × 0.3 μm. Oxidase- and catalase-positive. Positive for nitrate reduction, urease and *β*-glucosidase activities and D-glucose fermentation (weakly) but negative for indole production, denitrification and gelatinase, *β*-galactosidase and arginine dihydrolase activities. Produces smooth, grey–white, non-pigmented colonies with regular edges that are 1–2 mm in diameter and slightly raised in the centre after 72 h incubation at 28 °C on 216L MAM. Moderately halophilic. Grows in 0.5–12 % (w/v) NaCl (optimum 3–5 %) and at 10–42 °C (optimum 25–28 °C) but not at 4 °C or 45 °C within a week. The principal fatty acids were $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{18:1}\omega7c$, summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) and summed feature 9 (iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl). In the API ZYM system, tests are positive for esterase (C4), esterase lipase (C8), leucine aminopeptidase, *β*-glucosidase and lipase (C14) activities, weakly positive for acid phosphatase, alkaline phosphatase, naphthol-AS-B1-phosphoamidase and valine aminopeptidase activities and negative for cystine aminopeptidase, *N*-acetyl-*β*-glucosaminidase, trypsin, α-chymotrypsin, α-fucosidase, α- and *β*-galactosidase, α-glucosidase, α-mannosidase and *β*-glucuronidase activities. In the API 20 NE system, tests are positive for utilization of adipic acid (weakly), D-glucose, L-arabinose, malic acid and *N*-acetylglucosamine but negative for utilization of capric acid, maltose, D-mannitol, D-mannose, phenylacetic acid, potassium gluconate and trisodium citrate. Among the 95 carbon sources in the Biolog system (GN2 plate), tests are positive for utilization of Tweens 40 and 80, L-arabinose and methyl pyruvate and weakly positive for utilization of propionic acid. Sensitive to (μg per disc unless otherwise indicated) kanamycin (30), minomycin (30), ciprofloxacin (5), rifampicin (5), chloromycetin (30) and polymyxin B (30 IU) but resistant to ampicillin (10), carbenicillin (100), cefalexin (30), cefazolin (30), Cefobid (30), cephradin (30), clindamycin (2), Cotrimoxazole (25), erythromycin (15), furazolidone (15), gentamicin (10), lincomycin (2), metronidazole (5), neomycin (10), norfloxacin (10), ofloxacin (5), oxacillin (1), penicillin G (10), piperacillin (100), rocephin (30), streptomycin (10), tetracycline (30), vancomycin (30) and vibramycin (30). Grows well in ASM with *n*-alkane as a carbon source with chain-lengths from C_{14} to C_{28}.

The type strain, W11-5^T (=MCCC 1A00474^T =CCTCC AB 208236^T =LMG 25514^T), was isolated from sediment of the Pacific Ocean. The DNA G+C content of the type strain is 60.8 mol%.

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