Alcanivorax hongdengensis sp. nov., an alkane-degrading bacterium isolated from surface seawater of the straits of Malacca and Singapore, producing a lipopeptide as its biosurfactant

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A taxonomic study was carried out on strain A-11-3T, which was isolated from an oil-enriched consortium from the surface seawater of Hong-Deng dock in the Straits of Malacca and Singapore. Cells were aerobic, Gram-negative, non-spore-forming irregular rods. The strain was catalase- and oxidase-negative. It grew on a restricted spectrum of organic compounds, including some organic acids and alkanes. 16S rRNA gene sequence comparisons showed that strain A-11-3T was most closely related to the type strains of Alcanivorax jadensis (96.8 % sequence similarity), Alcanivorax borkumensis (96.8 %), Alcanivorax dieselolei (94.8 %), Alcanivorax venustensis (94.2 %) and Alcanivorax balearicus (94.0 %). The predominant fatty acids were C۱۶:۰ (31.2 %), C۱۸:۱۰۷c (24.8 %), C۱۸:۰ (9.6 %), C۱۲:۰ (8.3 %), C۱۶:۱۰۷c (8.3 %) and C۱۶:۰ 3-OH (5.1 %). The G+C content of the genomic DNA was 54.7 mol%. Moreover, the strain produced lipopeptides as its surface-active compounds. According to physiological and biochemical tests, DNA–DNA hybridization results and sequence comparisons of the 16S–23S internal transcribed spacer, the gyrB gene and the alkane hydroxylase gene alkB1, strain A-11-3T was affiliated with the genus Alcanivorax but could be readily distinguished from recognized Alcanivorax species. Therefore strain A-11-3T represents a novel species of the genus Alcanivorax for which the name Alcanivorax hongdengensis sp. nov. is proposed. The type strain is A-11-3T (=CGMCC 1.7084T=LMG 24624T=MCCC 1A01496T).

Alcanivorax-like bacteria have now been detected in oil-impacted environments across the globe (Head et al., 2006). The genus Alcanivorax currently includes five species, Alcanivorax borkumensis, A. jadensis, A. venustensis, A. dieselolei and A. balearicus, all of which were isolated from marine environments except A. balearicus, which was isolated from a subterranean saline lake (Yakimov et al., 1998; Bruns & Berthe-Corti, 1999; Fernández-Martínez et al., 2003; Liu & Shao, 2005a; Rivas et al., 2007). In this study, we isolated a novel strain, designated A-11-3T, from an oil-enriched consortium. Characterization using a polyphasic approach and classification of strain A-11-3T showed that it was phylogenetically related to members of the genus Alcanivorax.

Surface seawater was sampled while passing through the Straits of Malacca and Singapore on the research vessel Da-Yang Yi-Hao in December 2005, from the site S74 (102° 25.81’ E 1° 48.15’ N) near the Hong-Deng dock of Singapore. Enrichment was started on-board immediately after sampling. About 100 ml of the sample was loaded into sterile tubes and supplemented with 1 ml sterilized NH4NO3 (100 g l–1) and KH2PO4 (50 g l–1), 100 μl FeSO4.7H2O (2.8 g l–1) and 0.5 ml diesel fuel. The cultures were incubated at 28°C for 1 week. In the laboratory, bacteria were isolated by using the plate-screening method on PTA...
were incubated in a rotary shaker at 180 r.p.m. and 28 °C. Strain A-11-3 T indicated that it belonged to the class Gammaproteobacteria based on the putative alkane hydroxylase gene (alkB1) amplified by PCR using primers that have been described previously (Liu & Shao, 2005a). The gyrB gene was sequenced using the method described by Yamamoto et al. (2000). Sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 4 (Tamura et al., 2007) after multiple alignment of data by DNAMAN (version 5.1; Lynnon Biosoft). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method of Saitou & Nei (1987) and minimum-evolution method (data not shown because the result was similar to that obtained with the neighbour-joining method) of Rzhetsky & Nei (1992, 1993) were determined by using bootstrap values based on 1000 replications.

Genomic DNA was prepared according to the method of Ausubel et al. (1995) and the 16S rRNA gene, 16S–23S internal transcribed spacer (ITS) regions and the fragment of the putative alkane hydroxylase gene (alkB1) were amplified by PCR using primers that have been described previously (Liu & Shao, 2005a). The gyrB gene was sequenced using the method described by Yamamoto et al. (2000). Sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 4 (Tamura et al., 2007) after multiple alignment of data by DNAMAN (version 5.1; Lynnon Biosoft). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method of Saitou & Nei (1987) and minimum-evolution method (data not shown because the result was similar to that obtained with the neighbour-joining method) of Rzhetsky & Nei (1992, 1993) were determined by using bootstrap values based on 1000 replications.

A nearly full-length 16S rRNA gene sequence (1504 nt) of strain A-11-3 T was determined. Phylogenetic analysis of strain A-11-3 T indicated that it belonged to the class Gammaproteobacteria, forming a robust clade within the genus Alcanivorax (Fig. 1). The strains with the highest sequence similarities were A. jadensis T9 T (96.8 %), A. borkumensis SK2 T (96.8 %), A. dieselolei B-5 T (94.8 %), A. venustensis ISO4 T (94.2 %) and A. balearicus MACL04 T (94.0 %); other species shared <94 % sequence similarity. In general, 16S rRNA gene sequence divergence greater than 2 % is accepted as a criterion for delineating different species (Stackebrandt & Goebel, 1994). Thus the data support the view that strain A-11-3 T represents a novel species.

For comparison of 16S–23S ITS sequences, PCR was conducted and two-band products were yielded in strain A-11-3 T, with sizes 504 and 322 nt, respectively. The sequences of the large and small ITS regions showed the highest similarities with the counterparts of A. borkumensis SK2 T (85.7 % and 75.8 %, respectively). Phylogenetic analysis based on the ITS sequences showed similar groupings to that based on the 16S rRNA gene sequences. The ITS sequences of strain A-11-3 T and Alcanivorax species had a mean similarity of 23.2 ± 14.8 % (SD), which was within the limit of a single and well-defined genus (Garciá-Martínez & Rodríguez-Valera, 2000; Fernández-Martínez et al., 2003) (Supplementary Fig. S1, available in IJSEM Online).

With respect to the gyrB gene, a fragment of 1115 nt was obtained from strain A-11-3 T, which showed the highest similarity with sequences of A. venustensis ISO4 T (82.8 %), A. jadensis T9 T (82.4 %), A. borkumensis SK2 T (80.6 %), A. dieselolei B-5 T (79.9 %) and A. balearicus MACL04 T (78.3 %) (Supplementary Fig. S2). This indicated that strain A-11-3 T satisfied the threshold criterion (10 % nucleotide substitution rate; Venkateswaran et al., 1999) of sequence diversity as distinct species. Phylogenetic trees based on this housekeeping gene sequence showed that strain A-11-3 T and other species of the genus Alcanivorax formed independent monophyletic clusters that were similar to those based on the 16S rRNA gene sequences.

A fragment of the alkB1 gene (426 nt) was amplified from strain A-11-3 T. It showed the highest similarity to the corresponding regions of alkB in Burkholderia xenovorans LB400 T (69 %). The phylogenetic tree based on the alkB gene was different to those based on the 16S rRNA, ITS and gyrB genes (Supplementary Fig. S3). The alkB1 genes of strain A-11-3 T and A. borkumensis SK2 T formed a group that was phylogenetically separate from the other species of the genus Alcanivorax.

DNA–DNA hybridization experiments were performed with genomic DNA from strain A-11-3 T and recognized Alcanivorax species (except A. balearicus) as described previously (Liu & Shao, 2005a). Genomic DNA from Escherichia coli DH5α was used as an external sample. Each value is the mean of at least two independent hybridization experiments. DNA–DNA relatedness values between strain A-11-3 T and the type strains of Alcanivorax species were highest with A. venustensis ISO4 T (52 %), A. borkumensis SK2 T (45 %), A. dieselolei B-5 T (42 %) and A. jadensis T9 T (32 %); the DNA–DNA relatedness value with E. coli DH5α was 8 %. This suggested that the novel isolate may be categorized as representing a novel species of the genus Alcanivorax, according to the criteria (70 %) for the delineation of bacterial species (Wayne et al., 1987).

General cell morphology was studied under an Olympus inverted microscope using a 3 day culture of strain A-11-3 T.

![Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain A-11-3 T and phylogenetically related species of the genus Alcanivorax based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.01 nucleotide substitution rate (Ksub).](http://ijsem.sgmjournals.org)
grown on PTA medium. For electron microscopy, exponential phase cells were harvested, subsequently suspended and absorbed on a Formvar-carbon-coated grid, then stained with phosphotungstic acid (Supplementary Fig. S4). Gram reaction, catalase and oxidase (Oxidase Reagent; bioMérieux) activities, lipase (Twee 80), amylase, indole production, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase were carried out according to Dong & Cai (2001). The optimal temperature for growth was determined over the temperature range 4–55 °C on PTA medium. The pH for growth was examined with a range from pH 3.0 to 11.0 as the final pH of the medium. Tolerance of NaCl was examined at 0, 0.5, 1, 3, 5, 7, 8, 10, 15 and 20 % (w/v). The chain-length range of n-alkane oxidized by strain A-11-3T was determined according to Smits et al. (2002). The medium used for alkane degradation tests was artificial seawater medium (ASM) as described by Liu & Shao (2005b). Other biochemical tests were carried out using API 20NE and API ZYM strips (bioMérieux) and GN2 MicroPlates (Biolog), according to the manufacturers’ instructions, with the adjustment of the NaCl concentration to 3.0 %. The five type strains of genus Alcanivorax were tested by using API 20NE and API ZYM strips at the same time as strain A-11-3T. The results are given in the species description and Table 1.

Cellular fatty acid analysis of strain A-11-3T was carried out as described by Mrozik et al. (2004) (Supplementary Table S1, in IJSEM Online). The predominant fatty acids of strain A-11-3T were C₁₆:₀ (31.2 %), C₁₈:₁₀7c (24.8 %), C₁₈:₀ (9.6 %), C₁₂:₀ (8.3 %) and C₁₆:₁₀7c (8.3 %). Fatty acids present in smaller amounts were C₁₀:₀ (3.1 %), C₁₂:₁ (0.3 %), C₁₄:₀ (2.6 %), C₁₅:₀ 3-OH (0.9 %), iso-C₁₆:₁ (0.5 %), C₁₆:₀ 3-OH (5.1 %), C₁₇:₀ (0.7 %), C₁₈:₁₀9c (1.1 %), C₂₀:₀ (0.8 %) and an unknown fatty acid (2.2 %). In all of the other Alcanivorax species, the content of C₁₈:₀ was low and C₁₆:₀ 3-OH was absent. C₁₂:₀ was absent in A. dieselolei B-5T and A. borkumensis SK2T. Active growth of strain A-11-3T occurred with C₈–C₂₂ n-alkanes; those shorter than C₈ or longer than C₃₂ resulted in weak growth.

To analyse the components of the surfactant produced by strain A-11-3T, cells were enriched by using the method of Peng et al. (2007), in which n-hexadecane was used as the sole carbon source and cultured by shaking at 150 r.p.m. at 28 °C for 5 days. Isolation and purification of the surfactant were done according to Peng et al. (2007). The purified surfactant was hydrolysed with 6 M HCl at 110 °C for 24 h. The resulting solution was then extracted with ether at least three times (Maneerat et al., 2006). The solvent and aqueous phases, which contained fatty acids and amino acids, respectively, were separated and collected. The solvent phase was subjected to methanol treatment before gas chromatography mass spectrometry analysis. The aqueous fraction containing the amino acids was subjected to amino acid analysis. When C₁₆ was the sole carbon source, strain A-11-3T produced a surfactant that contained lipopeptide-like compounds, in contrast with A. borkumensis SK2T, which produces glucolipid-like compounds (Yakimov et al., 1998). The major fatty acids in the lipopeptide were C₁₅:₀ (46.3 %) and C₁₇:₀ (40.2 %); other fatty acids were present in smaller amounts, including C₁₃:₀ (3.3 %), C₁₇:₁ (6.5 %) and C₁₇:₂ (3.8 %). In the case of the amino acid analysis, two peaks were detected, one of which was identified as tyrosine. The other was not clear.

Thus, according to the phylogenetic analysis of 16S rRNA, ITS, gyrB and alkB gene sequence comparisons, DNA–DNA hybridization data and phenotypic traits, strain A-11-3T belongs to the genus Alcanivorax and represents a novel species, for which the name Alcanivorax hongdengensis sp. nov. is proposed.

**Description of Alcanivorax hongdengensis sp. nov.**

Alcanivorax hongdengensis (hong.deng.en sis. N.L. masc. adj. hongdengensis pertaining to Hong-Deng, isolated from surface seawater near the Hong-Deng dock in the Straits of Malacca and Singapore).

Cells are irregular rods, 0.5–0.8 μm wide and 0.9–3.2 μm long, non-motile, non-flagellated and non-spor-forming. Positive for gelatinase, lipase (Twee 80) and reduction of nitrate to nitrite, but negative for Gram reaction, oxidase, catalase, indole production, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, denitrification and glucose fermentation. On PTA medium after 72 h incubation at 28 °C, produces smooth non-pigmented, white–cream-coloured colonies that are 1–2 mm in diameter, with regular edges, slightly raised in the centre, semi-translucent at the edge, non-halo-like periphery. Moderately halophilic. Grows in 0.5–15 % NaCl (optimum, 3–8 %) and pH 4.0–10.0 (optimum, pH 7.0–8.0) at 10–42 °C (optimum, 18–35 °C), but not at 4 or 45 °C. Principal fatty acids are C₁₆:₀, C₁₈:₁₀7c, C₁₈:₀ 3-OH, C₁₆:₀ 3-OH and C₁₆:₀ 3-OH. With API ZYM, positive for esterase (C4),

C₁₆:₁₀7c, C₁₈:₁₀7c, C₁₈:₁₀9c, C₁₈:₁₀9c and C₁₈:₁₀9c.
Table 1. Physiological characteristics of strain A-11-3<sup>T</sup> and recognized species of the genus *Alcanivorax*

Strains: 1, A-11-3<sup>T</sup> (*A. hongdengensis* sp. nov.); 2, *A. balearicus* LMG 22508<sup>T</sup> (Rivas et al., 2007); 3, *A. dieselolei* B-5<sup>T</sup> (Liu & Shao, 2005a); 4, *A. venustensis* ISO4<sup>T</sup> (Fernández-Martínez et al., 2003); 5, *A. borkumensis* SK2<sup>T</sup> (Yakimov et al., 1998); 6, *A. jadensis* T9<sup>T</sup> (Bruns & Berthe-Corti, 1999; Fernández-Martínez et al., 2003). Data for catalase and oxidase activities, API 20NE and API ZYM tests and antibiotic susceptibility for all six type strains were done at the same time in this study. With API 20NE, all strains were negative for denitrification, indole production, d-glucose fermentation, arginine dihydrolase, urease, β-glucosidase, β-galactosidase and utilization of d-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose and potassium gluconate. With API ZYM, 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-galactosidase, α-glucosidase, α-mannosidase, β-galactosidase, β-glucosidase or β-glucuronidase. +, Positive; 2, negative; V, variable; W, weakly positive; ND, no data available.

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<tr>
<td>Ceftriaxone (30)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
esterase lipase (C8), lipase (C14) and leucine aminopeptidase; weakly positive for acid phosphatase, naphthol-AS-BI-phosphoamidase and valine aminopeptidase; negative for alkaline phosphatase, cystine aminopeptidase, trypsin, \( \alpha \)-chymotrypsin, \( \alpha \)-fucosidase, \( \alpha \)-galactosidase, \( \alpha \)-glucosidase, \( \alpha \)-mannosidase, \( \beta \)-galactosidase, \( \beta \)-glucosidase, \( \beta \)-glucuronidase and \( \beta \)-galactosaminidase. Among the 95 carbon sources in the Biolog GN2 system, utilizes Tweens 40 and 80, from C8 to C36. Sensitive to (sebacic acid, but not citric acid or succinic acid. Grows in ASM with n-alkane as carbon source with chain lengths from C\( \text{C}_{10} \) to C\( \text{C}_{36} \). Sensitive to (\mu g/per disc unless otherwise stated): ampicillin (10), carbenicillin (100), ceftazidime (30), cefazolin (30), cefoperazone (30), cephradin (30), chloramphenicol (30), ciprofloxacin (5), co-trimoxazole (25), erythromycin (15), gentamicin (10), kanamycin (30), minocycline (30), neomycin (10), norfloxacin (10), ofloxacin (5), penicillin G (10), pipercillin (100), polymyxin B (30 IU), rifampicin (5), ceftriaxone (30), streptomycin (10), tetracycline (30) and doxycycline (30); but resistant to tetracycline (30), cefalexin (30), ceftriaxone (30), streptomycin (10), minocycline (30), neomycin (10), norfloxacin (10), ofloxacin (5), penicillin G (10), pipercillin (100), polymyxin B (30 IU), rifampicin (5), ceftriaxone (30), streptomycin (10), tetracycline (30) and doxycycline (30); but resistant to clindamycin (2), furazolidone (15), lincomycin (2), metronidazole (5), oxacillin (1) and vancomycin (30). Characteristics used to distinguish strain A-11-3\( \text{T} \) from related species are shown in Table 1. The G+C content of the type strain is 54.7 mol\%.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin (10)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tetracycline (30)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Doxycycline (30)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>54.7</td>
<td>62.8</td>
<td>62.1</td>
<td>66.4</td>
<td>53–54</td>
<td>63–64</td>
</tr>
</tbody>
</table>

\( o \), Ophoticrous flagella; \( p \), polar or subpolar flagellum.

Acknowledgements

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The type strain, A-11-3\( \text{T} \) (=CGMCC 1.7084\( \text{T} \)=LMG 24624\( \text{T} \)=MCCC 1A01496\( \text{T} \)), was isolated from surface seawater near Hong-Deng dock (102° 25.81′ E 1° 48.15′ N), in the Straits of Malacca and Singapore.

References


