Marinobacter algicola sp. nov., isolated from laboratory cultures of paralytic shellfish toxin-producing dinoflagellates

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Phylogenetic and phenotypic analysis of cultivable marine bacteria isolated from laboratory cultures of two paralytic shellfish toxin-producing dinoflagellates, Gymnodinium catenatum and Alexandrium tamarense, showed the presence of a novel group of Gram-negative, aerobic, moderately halophilic and hydrocarbon-degrading bacteria, related to the genus Marinobacter. The strains, designated DG893T, DG1136 and ATAM407-13, grew optimally in media with 3–6 % NaCl and at 25–30 °C, and all could utilize n-hexadecane and n-tetradecane as the sole carbon source. The strains had a 16S rRNA gene sequence similarity of 94–94.3 % to Marinobacter hydrocarbonoclasticus ATCC 27132, and a similarity of 97.5–97.8 % to the closest phylogenetically related type strain, Marinobacter flavimaris DSM 16070T. DNA–DNA hybridization levels to M. flavimaris and other Marinobacter type strains were ≤42 %, while DNA–DNA reassociation values among DG893T, DG1136 and ATAM407-13 were ≥83 %. The DNA G+C content was 54–55 mol% and the major isoprenoid quinone was ubiquinone-9. On the basis of phenotypic, chemotaxonomic, DNA–DNA hybridization and phylogenetic analysis, it is proposed that these three strains represent a novel species, Marinobacter algicola sp. nov. The type strain is DG893T (≡ DSM 16394T = NCIMB 14009T).

The genus Marinobacter was first proposed in 1992 to accommodate a novel Gram-negative, aerobic, halophilic gammaproteobacterium capable of degrading a variety of hydrocarbons (Gauthier et al., 1992). The type species, Marinobacter hydrocarbonoclasticus, was isolated from coastal waters, and since then a further 12 species of Marinobacter have been recognized. These were isolated from various locations such as an offshore oil-well head (Huu et al., 1999), a coastal thermal spring (Shieh et al., 2003), temperate (Yoon et al., 2004) and Antarctic (Shivaji et al., 2005) sea water, saline soils (Martin et al., 2003) and marine sediments (Gorshkova et al., 2003; Romanenko et al., 2005).

To date, only Marinobacter bryozoorum 50-11T has an identifiable biological source, having been isolated from homogenized bryozoan tissue (Romanenko et al., 2005).

The bacterial flora associated with the dinoflagellates Gymnodinium catenatum and Alexandrium tamarense (Lebour) Balech have been proposed to be involved in producing a potent suite of neurotoxins, the paralytic shellfish toxins (PSTs), produced by these algae (Gallacher et al., 1997; Kodama et al., 1990). As a part of the work to understand the relationship between the bacterial flora of PST-producing dinoflagellates and PST production, we identified bacterial isolates with a phylogenetic affiliation to M. hydrocarbonoclasticus in laboratory cultures of these dinoflagellates that formed a discrete phylogenetic cluster (Green et al., 2004; Hold et al., 2001). The phylogenetic clustering and frequency of occurrence in laboratory cultures was suggestive of a specific association between the dinoflagellate and the M. hydrocarbonoclasticus-like isolates (Green et al., 2004; Hold et al., 2001). Bacterial isolates...
DG893T and DG1136 were isolated from G. catenatum YC499B15 (recovered from the Yellow Sea, Korea) and G. catenatum GC21V (recovered from the Ria de Vigo, Spain), respectively, by serial dilution on a low-concentration marine agar (ZM/10; Green et al., 2004). ATAM407-13 was isolated from Alexandrium tamarense NEPCC 407 (recovered from Jericho Beach, Vancouver, Canada) by culture on marine 2216 agar (Difco). The isolates were grown aerobically at 18–20 °C for 1–3 weeks and then stored frozen at −80 °C in marine broth (Difco) supplemented with 20 % glycerol (v/v).

Phenotypic examination of the isolates typically used colonies grown for 48–72 h on marine 2216 agar at 30 °C, streaked from ZM/10 marine agar slopes. M. hydrocarbonoclasticus NCIMB 1967 (= ATCC 27132), Marinobacter aquaeolei NCIMB 13596T, Marinobacter flavigenis DSM 16070T and Marinobacter lipolyticus NCIMB 13907T were used for comparison, all maintained as above. Gram reaction and cell morphology of the strains were observed by light microscopy, and negatively stained whole cells were viewed by transmission electron microscopy on a JEOL-100SX (Beveridge et al., 1994). Catalase and oxidase activity, utilization of Tween 40 and 80 and casein and starch hydrolysis were also determined as described by Smibert & Krieg (1994). Nitrate reduction, indole production, arginine dihydrolase, urease, gelatin liquefaction, β-glucosidase and β-galactosidase activity were examined using API 20NE strips (Vitek-bioMérieux) inoculated with colonies suspended in 3 % NaCl (w/v). The ability of the isolates to utilize a range of organic substrates was determined using 96-well Biolog GN MicroPlates as described by Smith et al. (2002). The ability of the strains to utilize aliphatic hydrocarbons was determined by growing the strains on a synthetic sea-water agar (SM1; Yakimov et al., 1998), varying only in that NH4NO3 was used as the nitrogen source. Bacteria were streaked onto the agar, and hydrocarbon was supplied in the vapour phase by adding 200 μl of either n-tetradecane (Sigma) or n-hexadecane (Sigma) as the sole carbon source to a sterile piece of Whatman Qualitative 1 filter paper placed in the lid of the Petri dish. Growth was assessed after 3 weeks incubation at 25 °C. Utilization of hydrocarbon was assessed based on the amount of growth relative to cells streaked onto the same agar but without any added carbon source.

Strains DG893T, DG1136 and ATAM407-13 were all similar in terms of their cell morphology, growth and phenotypic characteristics (see the species description, Table 1 and Supplementary Fig. S1 available in IJSEM Online). The three novel strains all demonstrated an ability to utilize a comparatively wide range of organic compounds as their sole carbon and energy source compared with those utilized by the other Marinobacter type strains examined. Discriminatory phenotypic characteristics are given in Table 1. DG893T, DG1136 and ATAM407-13 were all positive for the utilization of Tweens 40 and 80, glycerol, dextrin, glycerogen, glucose, methyl pyruvate, acetate, citrate, β-hydroxybutyrate, DL-lactate, propionate, succinate, bromosuccinate, D-alanine, L-glutamate, L-leucine, L-proline and L-pyroglutamate. All strains were negative for the utilization of α-cyclodextrin, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, erythritol, L-fucose, D-galactose, gentiobiose, myo-inositol, α-lactose, α-D-lactose lactulose, D-mannose, D-melibiose, methyl β-D-glucoside, psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, p-hydroxyphenylacetic acid, itaconic acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, glucuronamide, alaninamide, L-α-lactyl glycine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-D-L-proline, L-ornithine, DL-serine, L-threonine, DL-carnitine, γ-amino butyric acid, uronic acid, inosine, uridine, thymidine, phenyl ethylamine, putrescine, z-DL-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate.

Consistent with all recognized Marinobacter species, Na+ was essential for growth, and K+ would not suffice in its place. Of the NaCl concentrations tested [0, 1, 3, 6, 9, 12, 15, 20, 25 % (w/v)], growth was only observed at ≥ 1 % NaCl (w/v), and at up to 9 % for DG1136 and ATAM407-13, and up to 12 % for DG893T. The range of pH over which all strains grew was consistent with other Marinobacter species. Growth was observed between pH 5 and 10 with an observed optimum of pH 7.5 for all strains. Anaerobic growth was observed to occur in all strains in the presence of nitrate and acetate, but not glucose. The temperatures examined for growth were 5, 10, 18, 25, 30, 37, 40 and 45 °C. The upper temperature at which growth was observed was 40 °C, while growth, albeit slow, occurred at 5 °C (Table 1). The optimal temperature range for growth of all strains was 25–30 °C.

Strains DG893T, DG1136 and ATAM407-13 were capable of growth on n-tetradecane and n-hexadecane as their sole carbon source. Growth of ATAM407-13 on these two hydrocarbon sources was weaker than that of DG893T and DG1136, and all three strains showed a preference for n-tetradecane. All strains showed an ability to grow on synthetic sea-water agar without any added carbon source, and formed shallow (< 1 mm), colony-sized depressions in the agar; subsurface growth could be seen radiating out from beneath each colony. However, there was no evidence of softening of the agar surrounding the colony. This observation indicates the capacity for oligotrophic growth and/or the ability to utilize agar as a carbon source. Agarolytic activity was not otherwise observed in any of the strains when grown on more complex media, marine 2216 agar or the more dilute version, ZM/10 agar.

The 16S rRNA genes were sequenced as described by Green et al. (2004) and Hold et al. (2001). Taxonomic assignment based on the 16S rRNA gene sequences was initially performed using the Sequence Match facility of the Ribosomal Database Project II (Cole et al., 2003). Multiple alignments were performed using CLUSTAL_X (Thompson et al., 1997).
Alignments were manually corrected and ambiguous regions were masked from the analysis. The program PAUP4.0* (Swofford, 2001) was used to construct the distance tree based on the neighbour-joining algorithm (Saitou & Nei, 1987) according to the maximum-likelihood model, and bootstrap support for the inferred tree was established by resampling 1000 datasets by the neighbour-joining method (Saitou & Nei, 1987). Alcanivorax borkumensis SK2T (GenBank accession no. Y12579) and Alcanivorax venustensis ISO4T (GenBank accession no. AF328762) were used as the outgroup.

16S rRNA gene sequence analysis showed that DG893T, DG1136 and ATAM407-13 were most closely related to M. flavimaris SW-145T (97.8, 97.5 and 97.5 %, respectively) and M. lipolyticus SM19T (97.1, 97.2 and 97.3, respectively); lower similarities were observed to the type strains of other Marinobacter species, such as Marinobacter sediminum R65T (96.4–96.6 %), M. hydrocarbonoclasticus ATCC 27132 (94.2–94.3 %) and M. aquaeolii NCIMB 13596T. Additional data for type strains were taken from Gauthier et al. (1992), Huu et al. (1999), Martín et al. (2003), Yoon et al. (2004) and Romanenko et al. (2005). °, Positive; –, negative; v, variable response.

Table 1. Differential phenotypic characteristics between Marinobacter algicola sp. nov. and the type strains of selected Marinobacter species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<th>5</th>
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<td>Nitrate reduced to nitrite</td>
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<td>Growth:</td>
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<td>Minimum temperature (°C)</td>
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<td>15</td>
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<td>4</td>
<td>10</td>
<td>13</td>
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<td>Maximum temperature (°C)</td>
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<td>45</td>
<td>40</td>
<td>42</td>
<td>42</td>
<td>45</td>
<td>50</td>
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<td>NaCl range (%)</td>
<td>1–12*</td>
<td>1–20</td>
<td>1–15</td>
<td>1–18</td>
<td>0–5–18</td>
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<td>Amylase</td>
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<td>Urease</td>
<td>v†</td>
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<td>Arginine dihydrolase</td>
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<td>D-Fructose</td>
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<td>D-Mannitol</td>
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<td>Sucrose</td>
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<td>cis-Aconitic acid</td>
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<td>l-Alanine</td>
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<td>l-Phenylalanine</td>
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<tr>
<td>l-Proline</td>
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<td>–</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<td>DNA G+C content (mol%)</td>
<td>54–55</td>
<td>58</td>
<td>57</td>
<td>59-6</td>
<td>56-5</td>
<td>53</td>
<td>56</td>
</tr>
</tbody>
</table>

*NaCl tolerance was 1–12 % for strain DG893T and 1–9 % for strains DG1136 and ATAM407-13.
†DG893T and DG1136 were both positive and ATAM407-13 was negative.
‡DG893T was positive whereas DG1136 and ATAM407-13 were negative.
§Huu et al. (1999) reported citrate utilization as positive.

http://ijs.sgmjournals.org
Marinobacter hydrocarbonoclasticus ATCC 49840\(^T\) (X67022)
Marinobacter aquaeolei V78\(^T\) (AJ000728)
Marinobacter daniofoetis SW-159\(^T\) (AY517633)
Marinobacter alkaliphilus ODP1200D-0.1\(^T\) (AB125941)
Marinobacter litoralis SW-45\(^T\) (AF479689)
Marinobacter excellens KMM 3809\(^T\) (AY180101)
‘Marinobacter arcticus’ (AF148811)
Marinobacter lipolyticus SM19\(^T\) (AY147906)
Marinobacter fruvimaris SW-145\(^T\) (AY157632)
Marinobacter algicola DG893\(^T\) (AY258110)
Marinobacter algicola DG1136 (A028116)
Marinobacter algicola 407-13 (AJ294359)
Marinobacter sediminis R65\(^T\) (AJ609270)
Marinobacter bryozooicum 50-11\(^T\) (AJ609271)
Marinobacter lutaeonisi T505\(^T\) (AF281157)
Marinobacter aquaeolivensis 2aad4\(^T\) (AJ439500)
Marinobacter maritimus CK 47\(^T\) (AJ704395)

0·01 substitutions per site

Fig. 1. Phylogenetic dendrogram based on the nearly complete 16S rRNA gene sequences of *Marinobacter algicola* sp. nov. and the type strains of other *Marinobacter* species (*Alcanivorax borkumensis* and *A. venustensis* were used as the outgroup; not shown). The tree was constructed by neighbour-joining using the maximum-likelihood model and bootstrap support (1000 replicates each) based on neighbour-joining. Bar, 0·01 nucleotide substitutions per site.

supported phylogenetic cluster separate from *M. flavimaris* SW-145\(^T\) and *M. lipolyticus* SM19\(^T\), the two closest phylogenetic relatives. It also shows that the three strains comprise a coherent phylogenetic cluster within the genus *Marinobacter*.

Whole-genome DNA–DNA hybridization studies were conducted between DG893\(^T\), DG1136, ATAM407-13, *M. hydrocarbonoclasticus* NCIMB 1967, *M. aquaeolei* NCIMB 13596\(^T\), *M. lipolyticus* NCIMB 13907\(^T\) and *M. flavimaris* DSM 16070\(^T\). Genomic DNA was extracted from washed, harvested cells grown in marine 2216 broth for 24 h at 30 °C according to the method described by Marmur (1961). Analysis of the G + C content and DNA–DNA hybridization were both performed essentially as described by Bowman et al. (1998). DG893\(^T\) showed DNA–DNA reassociation values of 35, 39, 42 and 35 % with *M. hydrocarbonoclasticus*, *M. aquaeolei*, *M. lipolyticus* and *M. flavimaris*, respectively. As these values were all < 70 %, this delineates DG893\(^T\) from the other *Marinobacter* type strains, and supports the proposal that DG893\(^T\) represents a novel species (Stackebrandt & Goebel, 1994). The DNA–DNA reassociation values of DG893\(^T\) with DG1136 and ATAM407-13 were 95 and 83 %, respectively, and the reassociation value of DG1136 to ATAM407-13 was 90 %. These DNA–DNA hybridization levels (> 70 %) support the proposal that DG893\(^T\), DG1136 and ATAM407-13 represent a single species (Stackebrandt & Goebel, 1994). The DNA G + C content of DG893\(^T\), DG1136 and ATAM407-13 was 55, 55 and 54 mol %, respectively, consistent with values reported for the type strains of other *Marinobacter* species (Table 1).

Cellular fatty acid and isoprenoid quinone content were determined for cell mass grown on marine 2216 agar and broth, respectively, for 48 h at 30 °C. Fatty acid methyl esters were extracted and prepared according to standard protocols as described for the MIDI Microbial Identification System (Sasser, 1990). Cellular fatty acid content was analysed with the MIDI Microbial Identification system. Results are available as Supplementary Table S1 in IJSEM Online. The fatty acid composition was generally consistent with that of other *Marinobacter* type strains, although DG893\(^T\), DG1136 and ATAM407-13 differed in having increased abundances of \(C_{16,1}ω7c/iso-C_{15,0}2\)-OH (summed feature MIDI fatty acid methyl ester analysis), \(C_{16,0}\) 10-methyl and \(C_{18,1}ω7c\) and a reduced abundance of \(C_{16,1}ω9c\) (Supplementary Table S1). The isoprenoid quinone content of the three novel strains, with *M. hydrocarbonoclasticus* NCIMB 1967 and *M. flavimaris* DSM 16070\(^T\) as reference strains, was determined by reversed-phase HPLC using an Eclipse XDB-C18 column (Agilent Technologies), essentially as described by Komagata & Suzuki (1987). The principal isoprenoid quinone was shown to be ubiquinone-9 (Q-9), consistent with other *Marinobacter* type strains except *Marinobacter lutaeonis*, which contains ubiquinone-8 (Shieh et al., 2003).

On the basis of 16S rRNA gene sequence analysis, DNA–DNA hybridization, phenotypic characteristics and chemotaxonomic properties, DG893\(^T\), DG1136 and ATAM407-13 are proposed as three related strains of a novel species within the genus *Marinobacter*, for which the name *Marinobacter algicola* sp. nov. is proposed.

**Description of *Marinobacter algicola* sp. nov.**

*Marinobacter algicola* [al.gi’co.la. L. n. alga -ae an alga (seaweed, dinoflagellate etc.); L. suff. -cola from L. n. incola an inhabitant; N.L. fem. n. algicola an inhabitant of algae].

Gram-negative, oxidase- and catalase-positive, non-spore-forming rods, 1·6–2·5 × 0·45–0·55 μm, occurring as single cells, in pairs or as short chains of cells. Motile by means of a single, non-sheathed, polar flagellum. Colonies on marine 2216 agar at 25–30 °C after 72 h are cream, circular (1–2 mm), smooth, convex and shiny with entire edges. Older colonies change in shape from convex to umbonate and become light beige in the centre with pale margins. Growth requires Na\(^+\) and occurs across a range of total NaCl (w/v) of 1–9 % for DG1136 and ATAM407-13 and 1–12 % for DG893\(^T\); optimal growth is between 3 and 6 % (w/v) NaCl. Growth occurs at 5–40 °C, with an optimal temperature of 25–30 °C, and over a pH range of 5–10, with an optimum of pH 7·5. Nitrate and nitrite are not reduced. Growth is aerobic, but anaerobic growth in the presence of nitrate and acetate, but not glucose, is observed. Tweens 40 and 80 and starch are hydrolysed, but gelatin and casein are not. Strains DG893\(^T\) and DG1136 are urease-positive, but ATAM407-13 is not. All strains identified to date are positive for arginine.
dihydrolase activity, but negative for indole, β-glucosidase and β-galactosidase activity. All strains utilize n-hexadecane and n-tetradecane and other carbon sources as sole carbon and energy sources, as indicated in Table 1 and the main text. The predominant cellular fatty acids are C16:0 (22.0–25.5 %), C16:1ω7c/iso-C15:0 2-ОH (19.5–20.5 %), C18:1ω9c (9.7–11.5 %) and C12:0 3-ОH (8.7–10.0 %). Q-9 is the principal isoprenoid quinone.

The type strain is DG893T (= DSM 16394T = NCIMB 14009T). The DNA G+C content of the type strain is 55 mol%. Strains DG893T and DG1136 were isolated from laboratory cultures of the dinoflagellate Gymnodinium catenatum (from Korea and Spain, respectively) and ATAM407-13 from the dinoflagellate Alexandrium tama-reense (from Canada).

Acknowledgements

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References


