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Amphritea atlantica gen. nov., sp. nov., a gammaproteobacterium from the Logatchev hydrothermal vent field

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A novel Gram-negative, motile, aerobic rod-shaped bacterium was isolated from a *Bathymodiolus* sp. specimen collected from the Logatchev hydrothermal vent field at the Mid-Atlantic Ridge. The novel strain, M41^T, was catalase- and oxidase-positive and metabolised various carbohydrates and amino acids. It grew well in marine broth with an optimal growth temperature of 31 °C to 34 °C (range 4–40 °C) and salinity requirement of 3 % (range 0.3–9 %). The pH range for growth was pH 4.6 to 9.5, with an optimum at pH 8.0. The predominant fatty acids were $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}\omega7c$. The DNA G+C content of strain M41^T was 52.2 mol%. The 16S rRNA gene sequence was 94 % similar to that of the type strain of *Oceanospirillum beijerinckii*, the closest cultivated relative. Other related type strains were *Oceanospirillum multiglobuliferum* (93 % gene sequence similarity), *Neptunomonas naphthovorans* (92%) and *Marinobacterium jannaschii* (92 %). According to phylogenetic analysis and physiological characteristics, it is suggested that strain M41^T represents a new genus and novel species for which the name *Amphritea atlantica* gen. nov., sp. nov. is proposed. The type strain is M41^T (=DSM 18887^T=LMG 24143^T).

The deep sea is an extreme habitat in which organisms need to adapt to such factors as high pressure, low nutrient concentrations, low temperatures, darkness and irregular food availability (Jannasch & Taylor, 1984). At deep-sea hydrothermal vent fields, organisms also have to deal with steep physico-chemical gradients such as extremely large ranges in temperature, heavy-metal concentrations, oxygen supply and pH (Kelley *et al.*, 2002). Along these gradients, many microhabitats are formed and a high diversity of micro-organisms can develop. Even though an increasing number of bacterial clone sequences in the databases originate from deep-sea habitats and also from deep-sea hydrothermal fields (Hoek *et al.*, 2003; López-García *et al.*, 2003; Radjasa *et al.*, 2001; Reysenbach *et al.*, 2000), at present only a small number of deep-sea bacteria have been cultivated.

Phylogenetic information derived from 16S rRNA gene sequences does not provide ecological or physiological information that could support the functional characterization of these extreme habitats. Hence, cultivation approaches are necessary and need to be applied. Moreover, micro-organisms from extreme marine environments are considered as a rich source for the detection of new secondary metabolites, new metabolic pathways and new enzymes and therefore have become a special focus of research in recent years (Faulkner, 2000; Haefner, 2003; Jensen & Fenical, 1994; Kelecom, 2002).

As part of a study of the microbial biodiversity of hydrothermal vents in the Mid-Atlantic Ridge, we have isolated numerous bacteria living in habitats influenced by hydrothermal activity. Among these, a novel Gram-negative, mesophilic, rod-shaped gammaproteobacterium, strain M41^T, showed less than 94 % 16S rRNA gene sequence similarity to any other recognized bacterium. In this study, the novel isolate was characterized by phenotypic and phylogenetic analyses and is proposed to be a member of a new genus and novel species.

Strain M41^T was isolated by the dilution-plating method from a *Bathymodiolus* sp. mussel sample collected at the Logatchev hydrothermal vent field at the Mid-Atlantic Ridge (14° 45.19′ N 44° 58.75′ W) at a depth of about 3000 m. The mussels were sampled using a TV-grab (a set of steel jaws with a video camera in the center) on research cruise M60/3, which recovered several mussels and other typical hydrothermal fauna such as crabs, snails and ophiuroids. One of the mussels was stored immediately in sterile seawater at 4 °C for further isolation procedures. Later, the storage water was diluted and plated on TSB medium [l^{-1}; 3.0 g tryptic soy broth (Difco), 15 g agar

Abbreviations: PHB, poly-β-hydroxybutyrate; SAP, shrimp alkaline phosphatase.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Amphritea atlantica* gen. nov., sp. nov. M41^T is AM156910.

(Difco), 25 g NaCl] and incubated at 22 °C for 14 days. Strain M41^T was isolated and maintained at −20 °C using the Cryobank System (Mast Diagnostica GmbH).

Cell morphology was determined by scanning electron microscopy. Strain M41^T was cultivated for 24 h in liquid marine broth (MB; Difco) at 28 °C on a rotary shaker at 100 r.p.m., followed by fixation with a final concentration of 1 % formol and filtration through 0.2 μm polycarbonate filters (Sarstedt). The filters were also applied in a subsequent ethanol dehydration series (50 %, 70 %, 90 % and three times at 100 % for 10 min each; Boyde & Wood, 1969) followed by critical-point drying with CO_2 and sputter-coating with Au/Pb. Samples were examined with a scanning electron microscope (DSM 940; Zeiss).

Strain M41^T was cultivated aerobically on MB. The temperature range for growth was tested by incubation in liquid MB medium at 4, 15, 20, 27, 29, 31, 33, 35, 37, 39, 41, 43 and 45 °C. The salinity requirement of the novel strain was tested at concentrations from 0 to 13 % in liquid medium consisting of different amounts of marine salt (Tropic Marin), 1 g l^{-1} peptone (Difco) and 5 g l^{-1} yeast extract (Difco) at 28 °C. The pH range for growth was tested over the range from pH 3.5–10 in steps of 0.5 pH units. To test whether NaCl could replace the salt requirement of natural seawater, strain M41^T was incubated at 28 °C in a medium consisting of 1 g peptone (Difco), 5 g yeast extract (Difco) and 15 g agar (Bacto) in 1 l of pure water and supplemented with 0−100 g NaCl. The Na^+ requirement was tested in a marine broth in which NaCl was replaced by either $CaCl_2$ or $MgCl_2$.

Gram-staining using KOH (according to Gregersen, 1978), poly-β-hydroxybutyrate (PHB)-staining with Sudan black (according to Smibert & Krieg, 1994) and catalase production (detected with 5 % H_2O_2) were all performed with overnight cultures incubated at 28 °C in MB and determined by phase-contrast microscopy (Axiophot; Zeiss). Luminescence was tested on liquid and solid MB supplemented with 3 % glycerol.

The aerobic oxidation of various compounds was tested using the Biolog GN2 system (Oxoid). Liquid cultures of strain M41^T were diluted in 1 % NaCl solution to obtain an optical density of 0.3–0.35 (OD_{600}). This solution was used as the inoculum for the tests which were performed in triplicate and checked daily for one week. A test result was deemed to be positive on the basis of three positive reactions. Additional tests were performed with the API 20NE test system for Gram-negative bacteria (bioMérieux) and API ZYM (bioMérieux). The inoculum was prepared as described before and incubated for at least 3–7 days at 32 °C. Both tests were run in triplicate.

The DNA G+C content and fatty acid content of strain M41^T were analysed at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) using the HPLC-method (Mesbah *et al.*, 1989) and the MIDI-System (Sasser, 1990).

For phylogenetic analysis, genomic DNA from the culture was extracted as follows: cell material was transferred from an agar plate into 500 μl DNA-free water (Sigma-Aldrich) and homogenized (2 × 6300 r.p.m. for 20 s) in a Precellys 24 homogenizer (PEQLAB Biotechnologie GmbH). After centrifugation (10 min; 8000 ***g***), the supernatant was used directly for PCR. Amplification of the 16S rRNA gene was performed using PuRe*Taq* ready-to-go PCR beads (Amersham Biosciences) with the eubacterial primers 27f and 1492r (Lane, 1991). The PCR conditions were: initial denaturation (2 min at 94 °C) followed by 30 cycles of primer extension (90 s at 72 °C), primer annealing (40 s at 50 °C) and denaturation (40 s at 94 °C) and a final step of primer annealing (1 min at 42 °C) as well as a final extension step (5 min at 72 °C).

Purification of PCR products was carried out with Exonuclease I (Exo I, GE Healthcare) and shrimp alkaline phosphatase (SAP, Roche). For each reaction, 1.5 U Exo I and 0.3 U SAP were added to the PCR product and incubated for 15 min at 37 °C, followed by heat inactivation of the enzymes for 15 min at 72 °C. Sequencing was performed using the BigDye Terminator v1.1 sequencing kit (Applied Biosystems) in a DNA analyzer (3730; Applied Biosystems) as specified by the manufacturer. To obtain sequence information for both DNA strands, sequencing was performed with the primers 27f, 342f (Lane, 1991), 790f 5′-GATACCCTGGTAGTCC-3′, 543r (Muyzer *et al.*, 1993), 907r (Lane *et al.*, 1985) and 1492r. The 16S rRNA gene sequence was submitted to the EMBL/GenBank Database.

The 16S rRNA sequence of strain M41^T was compared to other gene sequences in the NCBI GenBank database using a BLAST search (Altschul *et al.*, 1990), the ribosomal database project II (Cole *et al.*, 2007) using the sequence match tool and the EMBL-EBI online tool FASTA (Pearson, 1990). The sequence was aligned using the ARB software package (Ludwig, 2004). A maximum-parsimony tree and a neighbour-joining tree were calculated within ARB (data not shown). Additionally, a maximum-likelihood tree was calculated with the online version of PHYML (Guindon *et al.*, 2005) using the general time reversible (GTR) model with bootstrap values of 500.

Phenotypical and physiological characteristics

Using solid MB medium, strain M41^T formed flat and circular beige colonies with entire whitish edges. The cells were Gram-negative, rod-shaped, 0.3–0.5 × 0.5–2 μm and possessed a single monopolar flagella in overnight cultures (Fig. 1). In contrast, members of the phylogenetically closest genus, *Oceanospirillum*, formed helical cells with bipolar tufts.

After one week of incubation, coccoid bodies, so called microcysts, occurred in the cultures. The occurrence of microcysts has been described previously for older cultures of '*Spirillum lunatum*' and it was found that the majority of coccoid bodies present in old cultures were viable and

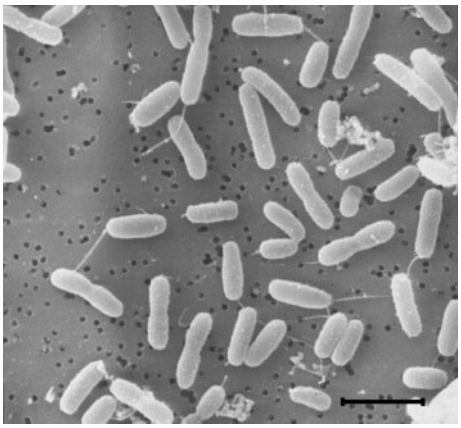

Fig. 1. Scanning electron micrograph of cells of strain M41^T cultivated in MB for 24 h at 28 °C. Bar, 2 μm.

could germinate when placed into fresh medium (Rittenberg & Williams, 1956).

Strain M41^T was able to grow at 4 °C (with an incubation time of more than 7 days) but could not grow at temperatures higher than 40 °C. Optimal growth occurred at between 31 and 34 °C and 3 % salinity. The salinity range for growth was between 0.3 and 9 % if sea salt was used. NaCl could replace the complex mixture of sea salt, but no growth occurred in the absence of NaCl or sea salts. Growth in medium with NaCl as the sole salt source occurred at concentrations from 0.3 to 6 % NaCl. An obligate requirement for the sodium ion was indicated by the inability to replace NaCl with either $CaCl_2$ or $MgCl_2$. The optimum pH for growth was pH 8, the growth range was from pH 4.6 to 9.5. Further detailed phenotypic data are given in Table 2 and in the species description.

Chemotaxonomic characteristics

The fatty acid profile of strain M41^T (Table 1) was characterized by mainly straight-chain saturated and unsaturated fatty acids with approximately 8 % short-chain 3-hydroxy-fatty acids. The predominant fatty acid was $C_{16:1}\omega7c$ and made up 40 % of the total fatty acid profile. Other fatty acids were: $C_{16:0}$ (29 %), $C_{18:1}\omega7c$ (22 %), $C_{12:0}$ (2 %), $C_{18:0}$ (0.8 %) and $C_{10:0}$ (0.3 %). The hydroxy fatty acids comprised approximately 8 % of the total fatty acids, $C_{10:0}$ 3-OH (5 %) and $C_{12:1}$ 3-OH (3 %).

The fatty acid profile was similar to that of members of the closely related genus *Oceanospirillum* (Hylemon *et al.*, 1973) and to other related genera such as *Neptunomonas* (Hedlund *et al.*, 1999) and *Marinobacterium* (Satomi *et al.*, 2002) and to the more distantly related genus *Reinekea* (Romanenko *et al.*, 2004). Predominant fatty acids were $C_{16:1}$, $C_{16:0}$ and $C_{18:1}$. A characteristic feature of strain M41^T was the presence of $C_{12:1}$ 3-OH (Table 1).

The DNA G+C content of strain M41^T was 52.2 mol%.

Table 1. Fatty acid content of strain M41^T and members of closely related genera

Taxa: 1, strain M41^T (data from this study); 2, *Oceanospirillum beijerinckii* IFO 15445^T; 3, *Oceanospirillum linum* IFO 15448^T; 4, *Marinobacterium jannaschii* IFO 15466^T; 5, *Oceanobacter kriegii* IFO 15467^T; 6, *Oceanospirillum multiglobuliferum* IFO 13614^T (data for taxa 2–6 are from Hylemon *et al.*, 1973); 7, *Reinekea marinisedimentorum* KMM 3655^T (data from Romanenko *et al.*, 2004). Percentages of all non-polar and all hydroxy fatty acids are considered separately.

Fatty acids	1	2	3	4	5	6	7
Non-polar							
$C_{10:0}$	0.3						
Unknown	2						
$C_{12:0}$	2	4	3	2	7	3	<1
$C_{12:1}$			2		4	2	
$C_{14:0}$		4	1	1	1	2	2
$C_{14:1}$					1		
$C_{15:0}$		1			2		4
$C_{16:0}$	29	32	16	19	16	28	32
$C_{16:1}$	43	50	48	46	36	44	27
$C_{17:0}$					2		6
$C_{17:1}$					3		3
$C_{18.0}$	0.8			1	1		<1
$C_{18:1}$	22	9	30	31	27	20	19
Hydroxy							
$C_{10:0}$ 3-OH	64	63	100	100	19	100	
$C_{12:0}$ 3-OH					54		
$C_{12:1}$ 3-OH	36						
$C_{14:0}$ 3-OH		30					2
$C_{16:0}$ 3-OH		6			27		

Phylogenetic analysis

Sequence analysis of the 16S rRNA gene and phylogenetic calculations showed that *Oceanospirillum beijerinckii* IFO 13614^T (93.8 %) and *Oceanospirillum multiglobuliferum* IFO 13614^T (93.4 %) were the most closely related cultivated organisms to strain M41^T. The maximum-likelihood tree is shown in Fig. 2 and the topology was supported by the neighbour-joining and the maximum-parsimony trees (data not shown).

The 16S rRNA gene sequence of *Neptunomonas naphthovorans* ATCC 700637^T had 92.6 % similarity to that of strain M41^T. Four uncultivated clone sequences from a sea dyke in Isahaya Bay (Japan) showed 100 % sequence similarity to strain M41^T. One representative of these clone sequences (GenBank accession no. AY910901) is included in Fig. 2. Other uncultivated clone sequences that had equal or higher sequence similarity to strain M41^T than to any of the cultivated bacteria are represented by (i) a sediment clone (AY375069) that originated from a warm pool of the western pacific (95 % gene sequence similarity), (ii) an uncultivated carbazole-degrading bacterium (AB086227) of unknown origin (93.4 % similarity) and (iii) a tributyl-resistant,

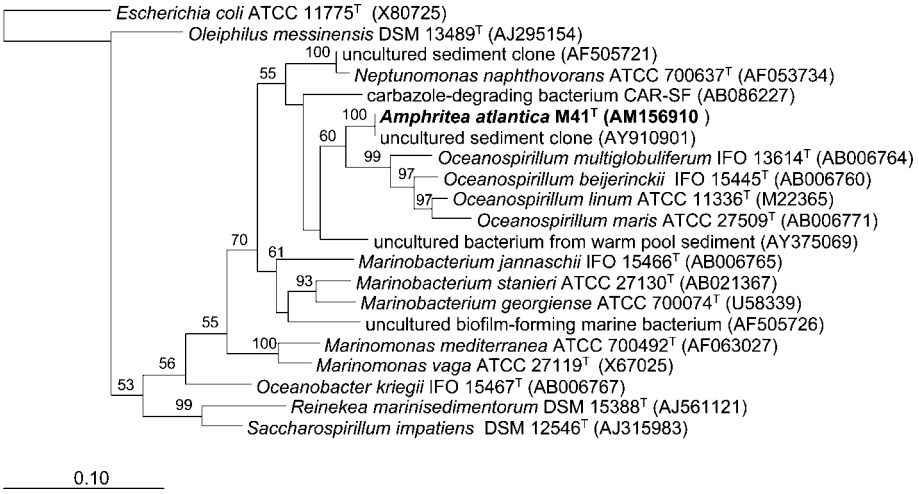

Fig. 2. Phylogenetic maximum-likelihood tree of strain M41^T and related type strains. Some selected uncultivated clone sequences with a close relationship to strain M41^T were also included in the phylogenetic calculation. The tree was calculated with the online version of PHYML with 500 bootstraps using the GTR evolution model. Bootstrap values higher than 50 % (expressed as a percentage of 500 replications) are shown next to the branching points. Bar, 0.1 substitutions per nucleotide position.

biofilm-forming bacterium (AF505721) from Boston harbour surface water (93.1 % similarity).

Consistent with the phylogenetic placement, strain M41^T shared some physiological properties with members of the genus *Oceanospirillum*, such as the formation of coccoid bodies in old cultures, the accumulation of PHB and positive catalase- and oxidase reactions. However, there were also some major phenotypic differences (Table 2). Members of the genus *Oceanospirillum* possess helical cells with bipolar tufts and a DNA G+C content as low as 42–48 mol%. They do not oxidize or ferment sugars (González & Whitman, 2001; Krieg, 1976). In contrast, strain M41^T had a DNA G+C content of 52.2 mol% and formed straight rods with a monopolar flagellum. Furthermore, strain M41^T was able to oxidize some sugars. The low sequence similarity of strain M41^T to representatives of all the related genera of the family *Oceanospirillaceae* (<95 %), in addition to significant physiological differences, necessitates the description of a new genus, for which the name *Amphritea* gen. nov. is proposed.

The physiological properties of strain M41^T suggest that it is a typical marine bacterium with an obligate requirement for sodium, optimum growth at close to seawater salinity and a temperature range for growth from cold deep ocean temperatures up to the elevated mesophilic range found in proximity to the hydrothermal vent systems of the deep sea. In addition, substrate utilization patterns (carbohydrates, amino acids) and enzymic activities characterize this bacterium as a degrader of the organic matter available in the vicinity of hydrothermal mussel fields.

Table 2. Properties of strain M41^T in comparison to related genera

Taxa: 1, strain M41^T (data from this study); 2, *Oceanospirillum* (Hylemon *et al.*, 1973); 3, *Neptunomonas* (Hedlund *et al.*, 1999; Hylemon *et al.*, 1973); 4, *Marinobacterium* (Hylemon *et al.*, 1973; Satomi *et al.*, 2002). ND, Not determined; +, present; −, absent.

Characteristic	1	2	3	4
Morphology	Straight rods	Helical	Straight rods	Straight rods
Cell size (μm)	0.5 × 0.5–2	0.4–1.2 × 2–4	0.7–0.9	0.5–0.7 × 1.6–2.3
Flagella	Single polar	Bipolar tufts	Single polar	1–2 polar
Coccoid body formation	+	+	+	−
Growth at 4 °C	+	−	+	+
DNA G+C content (mol%)	52.2	47–49	46	54.9
PHB	+	+	+	−

Description of *Amphritea* gen. nov.

Amphritea (Am.phri′tea. N.L. fem. n. *Amphritea* from Gr. fem. n. Amphrite, a nymph of the ocean in Greek mythology, referring to the habitat of the bacteria).

Cells are Gram-negative rods, motile by monopolar flagella. Coccoid bodies may be formed in old cultures. Catalase- and oxidase-positive and accumulate PHB. Growth range is from <4 to 40 °C, from 0.3 to 9 % salinity and from pH 4.6 to 9.5. Various sugars and carboxylic acids are oxidized. Predominant fatty acids are $C_{18:1}\omega7c$, $C_{16:1}\omega7c$ and $C_{16:0}$. 16S rRNA gene sequence analysis positions the genus in close proximity to the genera *Oceanospirillum* and *Neptunomonas* within the family *Oceanospirillaceae*. The type species is *Amphritea atlantica*.

Description of *Amphritea atlantica* sp. nov.

Amphritea atlantica (at.lan′ti.ca. L. fem. adj. *atlantica* of or pertaining to the Atlantic Ocean).

Main characteristics are the same as those given for the genus. The mean cell size is 0.5 × 0.5–2.0 μm. Colonies on MB agar are circular beige with entire whitish edges. Optimal growth occurs at 31–34 °C with 3 % salinity and pH 8. The sodium ion of NaCl is required for growth. Tests for luminescence are negative. The main fatty acids are $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}$. Hydroxy fatty acids are represented by $C_{10:0}$ 3-OH and $C_{12:1}$ 3-OH. Carbon sources oxidized (Biolog GN2) are: Tween 80, Tween 40, D-fructose, α-D-glucose, sucrose, methyl pyruvate, monomethyl succinate, *p*-hydroxyphenylacetic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-phenylalanine, L-proline, L-pyroglutamic acid and γ-aminobutyric acid. Enzyme activities are positive for alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Negative reactions are obtained for esterase lipase, lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosamidase, α-mannosidase and α-fucosidase. The API 20NE test system shows strong activities for arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase and urease. Positive in tests for acetoin production and citrate utilization. Negative reactions in tests for indole production, β-galactosidase, H_2S production, tryptophan deaminase and gelatinase. The DNA G+C content is 52.2 mol%.

The type strain, M41^T (=DSM 18887^T=LMG 24143^T), was isolated from warm sediment samples at 3000 m depth from the Mid-Atlantic Ridge.

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