Marinitoga hydrogenitolerans sp. nov., a novel member of the order *Thermotogales* isolated from a black smoker chimney on the Mid-Atlantic Ridge

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A novel, thermophilic, anaerobic bacterium that is able to tolerate hydrogen was isolated from a deep-sea hydrothermal chimney collected at the Rainbow field on the Mid-Atlantic Ridge. Cells were rod-shaped and surrounded by a sheath-like outer structure (toga); they were weakly motile by means of a polar flagellum. They appeared singly, in pairs or in short chains. They grew at 35–65 °C (optimum 60 °C), pH 4·5–8·5 (optimum pH 6·0) and 10–65 g sea salts l^{-1} (optimum 30–40 g l^{-1}). The isolate was organotrophic, and able to grow on various carbohydrates or complex proteinaceous substrates. Growth was not inhibited under 100 % hydrogen or in the presence of 2 % oxygen in the gas phase. The isolate reduces sulfur, although sulfur reduction is not required for growth. The fermentation products identified on glucose were acetate, ethanol, formate, hydrogen and CO_2. The G+C content of the genomic DNA was 28 ± 1 mol%. Phylogenetic analysis of the 16S rRNA gene placed the strain within the genus *Marinitoga*, order *Thermotogales*, in the bacterial domain. On the basis of the 16S rRNA gene sequence comparisons and physiological characteristics, the isolate is considered to represent a novel species, for which the name *Marinitoga hydrogenitolerans* sp. nov. is proposed. The type strain is AT1271^T (=DSM 16785^T=JCM 12826^T).

The order *Thermotogales* comprises rod-shaped, Gram-negative, non-sporulating bacteria that have a loose surrounding membrane or 'toga'. They are anaerobic, organotrophic and thermophilic. These organisms have been isolated from a variety of environments, such as oil reservoirs, hot springs and marine hydrothermal vents (Antoine *et al.*, 1997; Lien *et al.*, 1998; Windberger *et al.*, 1989). The order *Thermotogales* includes six genera: *Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Petrotoga*, *Geotoga* and *Marinitoga* (Davey *et al.*, 1993; Huber *et al.*, 1986, 1989; Patel *et al.*, 1985; Wery *et al.*, 2001a). To date, only two species belonging to the genus *Marinitoga* have been described, *Marinitoga camini* (Wery *et al.*, 2001a) and *Marinitoga piezophila* (Alain *et al.*, 2002), both originating from deep-sea hydrothermal vents. Members of the order *Thermotogales* are fermentative bacteria that produce hydrogen, the accumulation of which has been widely reported to inhibit their growth (Van Ooteghem *et al.*, 2002). This inhibition could be overcome by addition of elemental sulfur or thiosulfate (Alain *et al.*, 2002; Antoine *et al.*, 1997; Jeanthon *et al.*, 1995; Lien *et al.*, 1998). Here we report the description of a novel thermophilic bacterium belonging to the genus *Marinitoga* that is able to tolerate 100 % hydrogen in the headspace when growing on organic substrates.

Samples were collected by the ROV *Victor* in 2001 during the ATOS cruise on the Mid-Atlantic Ridge. The deep-sea vent field Rainbow (36° 13′ N 33° 54′ W) at 2275 m depth was explored and part of an active black smoker chimney was brought to the surface in a decontaminated insulated box. On board, eight subsamples representative of the whole chimney were crushed in an anaerobic chamber and stored in sterile serum vials filled with sterile sea water containing 0·5 mg Na_2S l^{-1}. The subsamples were pooled and used as inoculum at 2 % (v/v). A continuous enrichment culture was performed on a modified SME medium (Postec *et al.*, 2005; Sharp & Raven, 1997) in a 2 litre gas-lift bioreactor (Godfroy *et al.*, 2000; Raven *et al.*, 1992) at 60 °C at

Published online ahead of print on 7 January 2005 as DOI 10.1099/ijs.0.63550-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of AT1271^T is AJ786363.

atmospheric pressure. A culture sample collected on day 7 permitted the isolation of several strains by three serial dilutions to extinction (Baross, 1995). Single colonies were obtained by streaking on the same medium solidified with 15 g Gelrite l^{-1} (Scott Laboratories). Plates were incubated for 2 days at 60 °C in anaerobic jars (Godfroy *et al.*, 1997). Colonies were subsequently picked and streaked twice under the same conditions. One isolate was referenced as strain AT1271^T and was chosen for further characterization.

Microscopic observations indicated that cells of isolate AT1271^T were weakly motile rods surrounded by a 'toga', an outer sheath-like structure. Cells were approximately 0·6 μm wide and 1·5 μm long during the exponential phase and appeared single or in short chains within the envelope. Cells became elongated, forming long chains, during stationary phase (Fig. 1). Cells were negatively stained for examination under a transmission electron microscope (Raguénès *et al.*, 1997): a polar flagellum and the presence of a toga were observed (Fig. 1). The Ryu KOH reaction (Powers, 1995) leading to immediate cell lysis, as confirmed by phase-contrast microscopy, was positive, indicating that cells of strain AT1271^T were Gram-negative.

The new isolate was routinely grown on GYPS medium containing (per litre): 5 g (+)-D-glucose (Sigma), 0·5 g yeast extract (Difco), 1 g bacto-peptone (Difco), 30 g sea salts (Sigma), 3·9 g MES buffer (Sigma) and 0·1 % (v/v) resazurin solution. The pH was adjusted to 6·0 before autoclaving for 20 min at 121 °C. The medium was reduced by addition of 0·5 g Na_2S before inoculation. Cultures were incubated under anaerobic conditions, $N_2/H_2/CO_2$ (90 : 5 : 5), at atmospheric pressure. All experiments were performed in triplicate. Methods for the determination of growth parameters was as described by Wery *et al.* (2001b). Bacterial growth was directly monitored in culture tubes by spectrometry at 600 nm (a Spectronic 401 instrument; Milton Roy). Previous correlation between the optical density at 600 nm and cell numeration was simultaneously established by direct counting of the cells using a Thoma chamber viewed with an Olympus model BH-2 microscope. Isolate AT1271^T grew at 35–65 °C; the optimum temperature was 60 °C. Growth occurred at pH 4·5–8·5, the optimum being around pH 6. No growth was observed at pH 4 or pH 9. The strain grew at sea salt concentrations ranging from 10 to 65 g l^{-1} (corresponding to 8–50 g NaCl l^{-1}). The optimum sea salt concentration for growth was approximately 35 g l^{-1} (corresponding to 27 g NaCl l^{-1}). Under optimal conditions, the maximal cell density obtained was $6{\cdot}0 \times 10^8$ cells ml^{-1} and the maximal observed growth rate was 0·37 h^{-1} (equivalent to a doubling time of 1·9 h).

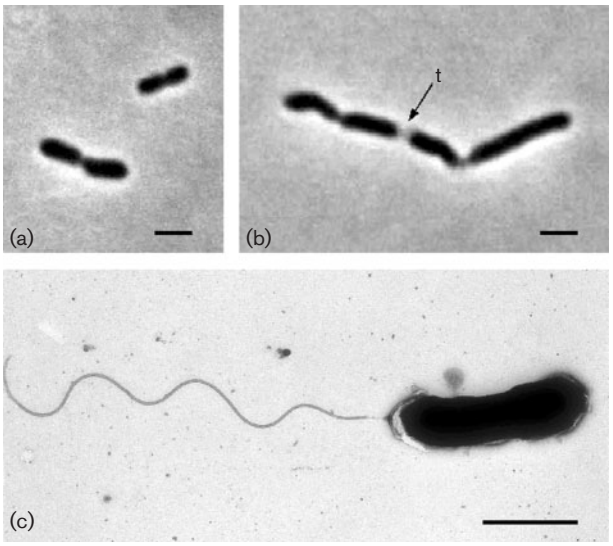

Fig. 1. (a, b) Phase-contrast micrographs of strain AT1271^T. (a) Rod-shaped cells in the mid-exponential phase of growth, dividing by constriction; bar, 1 μm. (b) Cells in a chain surrounded by a sheath-like structure or 'toga' (t); bar, 1 μm. (c) Electron micrograph of a negatively stained cell showing the polar flagellum; bar, 1 μm.

Strain AT1271^T is an obligate chemo-organotroph. No growth was detected on mineral media complemented with a H_2/CO_2 (80 : 20) headspace. The ability of the isolate to use various carbon sources was tested in medium containing (per litre): 30 g sea salts, 3·9 g MES buffer, 10 ml vitamin solution (Balch *et al.*, 1979), 10 ml mineral solution (Balch *et al.*, 1979), 0·1 g yeast extract as the nitrogen source and complex carbon sources. The following carbohydrates were tested at 0·5 % (w/v): cellobiose, glucose, maltose, fructose, galactose, ribose, starch, cellulose, glycogen, chitin, pectin and carboxymethylcellulose. Ethanol was tested at 0·5 % (v/v). The following organic acids and proteinaceous substrates were tested at 0·2 % (w/v): acetate, pyruvate, yeast extract, brain heart infusion, peptone, tryptone, casein, Casamino acids and keratin. A solution of 20 amino acids, each at 0·1 g l^{-1}, was also tested. Positive cultures were confirmed by subcultures on the same medium. Compared with growth on the medium without added carbon source, growth was enhanced by addition of glucose, starch, glycogen, chitin, yeast extract, brain heart infusion, peptone and casein. To extend the investigation of carbohydrate consumption, cellobiose, glucose, maltose, fructose, galactose, ribose, acetate, pyruvate and rhamnose were tested at 1 g l^{-1} in the presence 1 g yeast extract l^{-1}. A negative control was performed without added sugar. Growth was monitored by spectrometry and carbohydrate concentrations were estimated by HPLC after 6 and 13 h of culture. At these conditions, growth of strain AT1271^T was enhanced when pyruvate or maltose was added. Nitrogen sources were tested in medium containing (per litre): 30 g sea salts, 3·9 g MES buffer, 10 ml Balch vitamins, 10 ml mineral solution and glucose (5 g l^{-1}). Growth occurred in the presence of yeast extract (0·2 g l^{-1}), whereas urea (0·2 g l^{-1}) and NH_4Cl (1 g l^{-1}) alone did not support growth.

The end-products of glucose metabolism were acetate, ethanol, formate, hydrogen and CO_2. In the presence of elemental sulfur, no hydrogen was detected. Growth in the presence of different electron acceptors was tested on GYPS medium. A small increase in the final cell concentration was obtained when thiosulfate (20 mM), cystine (50 mM) or sulfur (10 g l^{-1}) was added. Addition of sulfate (20 mM), sulfite (1 mM), nitrate (20 mM) or nitrite (1 mM) did not enhance growth. Strong production of H_2S was revealed by using lead acetate strips (Whatman) on the culture with cystine and sulfur, indicating the use of these electron acceptors.

Various gas phases were tested in the presence and absence of sulfur (10 g l^{-1}) on GYPS medium: $N_2/H_2/CO_2$ (90 : 5 : 5), H_2/CO_2 (80 : 20), N_2/CO_2 (80 : 20) and N_2 (100 %). Cell density was above 1×10^8 ml^{-1} in the absence of sulfur, and above 4×10^8 ml^{-1} in the presence of sulfur, regardless of the gas phase.

The effect of H_2 in the gas phase was tested using the following N_2/H_2 ratios: 100 : 0, 80 : 20, 60 : 40, 40 : 60, 20 : 80 and 0 : 100. *M. camini*, *M. piezophila* and strain AT1271^T were grown for comparison in triplicate on GYPS at 50, 65 and 60 °C, respectively. The initial gas phase of the culture medium ($N_2/H_2/CO_2$ 90 : 5 : 5) was replaced and 10 cycles of vacuum extraction/addition of the different calibrated mixtures of N_2/H_2 were performed. Cell densities were estimated after 4, 8, 12 and 24 h of incubation by counting. No significant difference appeared between controls made with $N_2/H_2/CO_2$ (90 : 5 : 5). Maximal cell concentrations for *M. camini* and *M. piezophila* were obtained with 0 % H_2. Congruently, no growth was observed for either species with 80 % H_2 (Alain *et al.*, 2002; Wery *et al.*, 2001a). *M. camini* and *M. piezophila* displayed a linear decrease in maximal cell concentration with H_2 increase, and total inhibition of growth at 40 and 60 % H_2, respectively. In contrast, strain AT1271^T grew at up to 2–4 $\times 10^8$ cells ml^{-1} regardless of H_2 concentration. Strain AT1271^T tolerates up to 2 % oxygen in the gas phase. Growth was inhibited in the presence of 4 % oxygen.

Genomic DNA was extracted as described by Wery *et al.* (2001a). The G+C content was determined by thermal denaturation (Marmur & Doty, 1962) with the modifications described by Raguénès *et al.* (1997). The G+C content of the genomic DNA of strain AT1271^T was 28 ± 1 mol%. The 16S rRNA gene was selectively amplified as described by Wery *et al.* (2001b), and the PCR product was sequenced with the primers described by Raguénès *et al.* (1996). The sequence was then compared with available sequences in the GenBank database using a BLAST search (Altschul *et al.*, 1990). A multiple sequence file was obtained by using the Wisconsin Package version 10.3 (Accelrys Inc.). Alignments and similarity levels were obtained by the CLUSTAL W method with weighted residues (Thompson *et al.*, 1994). Alignments were manually refined using the multiple sequence alignment editor SEAVIEW and the phylogenetic reconstruction was produced using PHYLO_WIN (Galtier *et al.*, 1996) with the following algorithms: Jukes–Cantor distance matrix and successively the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony and maximum-likelihood methods (Felsenstein, 1981). Bootstrap values (Felsenstein, 1985) were determined. Strain AT1271^T was phylogenetically affiliated to the genus *Marinitoga* (Fig. 2), the nearest recognized relatives being *M. camini* and *M. piezophila* with respective 16S rRNA gene sequence similarities of 96 and 94 %. The positioning of strain AT1271^T was supported by the results of the three algorithms used.

The characteristics of strain AT1271^T are consistent with its assignment to the order *Thermotogales*. On the basis of the results of 16S rRNA gene sequence analysis, the new isolate is most closely related to *M. camini*, the first species described among the genus *Marinitoga*. *M. camini* and strain AT1271^T both originated from the Mid-Atlantic Ridge. However, strain AT1271^T can be distinguished from *M. camini* based on phylogenetic and phenotypic criteria: the two share 96 % 16S rRNA gene sequence similarity. Also, in comparison with *M. camini*, the pH range and pH optimum for the new isolate were lower and optimum NaCl concentration for growth was higher (30–40 versus 20 g l^{-1}) (Table 1). Moreover, unlike *M. camini*, strain AT1271^T does not use sucrose, fructose, cellobiose, cellulose, carboxymethylcellulose or pectin, and it does ferment casein. It is noteworthy that strain AT1271^T tolerates 100 % hydrogen in the gas phase whereas the growth of other *Marinitoga* species is inhibited at lower hydrogen concentrations. This tolerance to hydrogen has never been reported to date among the order *Thermotogales*, and could be related to the high hydrogen concentrations measured (16 mmol kg^{-1}) in all vent fluids at the Rainbow field,

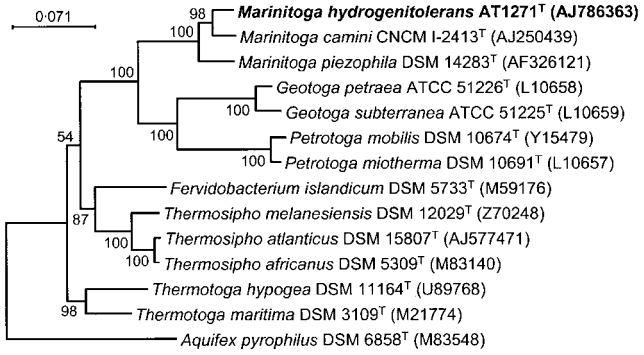

Fig. 2. Phylogenetic position of strain AT1271^T within the order *Thermotogales* based on 16S rRNA gene sequences. A total of 1351 sites were used for the phylogenetic analysis. Accession numbers and type strains are indicated. The topology shown corresponds to an unrooted tree obtained by a neighbour-joining algorithm (Jukes–Cantor corrections) established using PHYLO_WIN and manually refined using SEAVIEW. Bootstrap values are displayed on their respective branches. Bar, 7·1 nucleotide substitutions per 100 nucleotides.

Table 1. Characteristics used to differentiate members of the genus *Marinitoga*

+, Positive, −, negative; (+), weakly positive; ND, not determined. Substrates were tested in the presence of 0·1 g yeast extract l^{-1}, except *M. piezophila* (0·2 g l^{-1}). All three type strains were isolated from hydrothermal vents: EPR, East-Pacific Ridge; MAR, Mid-Atlantic Ridge.

Characteristic	*M. camini* MV1075^T	*M. piezophila* KA3^T	AT1271^T
Reference	Wery *et al.* (2001a)	Alain *et al.* (2002)	This study
Origin	MAR, Menez-Gwen site (37° 51′ N 31° 31′ W)	EPR, Grandbonum site (12° 48′ N 103° 56′ W)	MAR, Rainbow site (36° 13′ N 33° 54′ W)
Motility	+	+	(+)
pH for growth			
Range	5–9	5–8	4·5–8·5
Optimum	7	6	6
Temperature for growth (°C)			
Range	25–65	45–70	35–65
Optimum	55	65	60
NaCl concentration for growth (g l^{-1})			
Range	10–45	10–50	10–65
Optimum	20	30	30–40
Optimal doubling time (min)	102	21 (at 40 MPa)	112
Utilization of:			
Casein	−	+	+
Tryptone	+	+	−
Casamino acids	−	+	−
Fructose	(+)	+	−
Cellobiose	(+)	+	−
Galactose	−	+	−
Ribose	−	(+)	−
Carboxymethylcellulose	+	ND	−
Pectin	+	ND	−
Cellulose	+	ND	−
Glycogen	ND	−	+
Pyruvate	+	−	+
H_2 inhibition	From 40 % H_2	From 60 % H_2	No inhibition
Products of glucose fermentation	Acetate, H_2, CO_2	ND	Acetate, ethanol, formate, H_2, CO_2

the site from where strain AT1271^T was recovered (Charlou *et al.*, 2002).

On the basis of its genetic and phenotypic characteristics, we propose that strain AT1271^T should be assigned to a novel species of the genus *Marinitoga* belonging to the *Thermotogales*. On the basis of its high tolerance to hydrogen, the name *Marinitoga hydrogenitolerans* sp. nov. is proposed.

Description of *Marinitoga hydrogenitolerans* sp. nov.

Marinitoga hydrogenitolerans (hy.dro.ge.ni.to′le.rans. N.L. *hydrogenum* hydrogen; L. pres. part. *tolerans* tolerating; N.L. adj. *hydrogenitolerans* hydrogen-tolerating, referring to its ability to tolerate a high level of hydrogen in the headspace).

Rod-shaped, weakly motile, Gram-negative bacteria surrounded by a sheath-like structure. Growth occurs at 35–65 °C (optimum 60 °C), pH 4·5–8·5 (optimum pH 6·0) and 10–65 g sea salts l^{-1} (optimum 30–40 g l^{-1}). The doubling time under optimal conditions is 1·9 h and the maximum cell yield is $6{\cdot}0 \times 10^8$ cells ml^{-1}. Anaerobic; resistant to concentrations of oxygen up to 2 % and hydrogen up to 100 %. Chemo-organotrophic; able to ferment glucose, maltose, pyruvate, starch, glycogen, chitin, yeast extract, brain heart infusion, peptone and casein. Fermentation products identified on glucose are acetate, ethanol, formate, hydrogen and CO_2. Sulfur, cystine and thiosulfate stimulate growth slightly and H_2S production is observed with sulfur and cystine. The G+C content of the genomic DNA of the type strain is 28 mol%. 16S rRNA gene sequence analysis indicates that the species is classified within the genus *Marinitoga*, order *Thermotogales* in the bacterial domain. 16S rRNA gene sequence similarity of the type strain to *M. camini* MV1075^T is 96 %.

The type strain, AT1271^T (=DSM 16785^T=JCM 12826^T), was isolated from a sample collected on the Rainbow hydrothermal site on the Mid-Atlantic Ridge (36° 13′ N 33° 54′ W).

Acknowledgements

We wish to thank P. M. Sarradin, chief scientist of the ATOS cruise, as well as the captain and crew of the *Atalante* and the *Victor* team. We thank Mélusine Gaillard and Gérard Sinquin for their technical support with transmission electron microscopy. We thank Laurent Urios for helpful discussions. This work was supported by Ifremer, European VENTOX Program (EVK3-CT1999-2003) and Région Bretagne.

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