Marinitoga litoralis sp. nov., a thermophilic, heterotrophic bacterium isolated from a coastal thermal spring on Île Saint-Paul, Southern Indian Ocean

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A novel thermophilic, anaerobic and organotrophic bacterium, designated strain MC3^T, was isolated from a coastal thermal spring on Île Saint-Paul in the Southern Indian Ocean. Cells of strain MC3^T were motile rods, 0.8–1.0 µm wide and 1.0–2.4 µm long during exponential phase and up to 7.0 µm long during stationary phase. Strain MC3^T was an anaerobic organotroph able to use diverse organic compounds. It was also able to reduce sulfur to sulfide. Growth was observed at temperatures ranging from 45 to 70 °C (optimum at 60 °C), between pH 5.5 and 7.5 (optimum at pH 6) and from 8 to 46 g NaCl l⁻¹ (optimum at 26 g l⁻¹). The total G + C content of the genomic DNA was 26.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparisons indicated that strain MC3^T was affiliated with the genus *Marinitoga* within the order *Thermotogales*. It shared 94.4–95.7 % 16S rRNA gene sequence similarity with strains of other *Marinitoga* species; *Marinitoga hydrogenitolerans* was found to be the most closely related organism. Based on the data from the phylogenetic analysis and the physiological properties of the novel isolate, strain MC3^T should be classified as a representative of a novel species, for which the name *Marinitoga litoralis* sp. nov. is proposed; the type strain is MC3^T (=DSM 21709^T =JCM 15581^T).

Members of the order *Thermotogales* are thermophilic and heterotrophic bacteria that grow under anaerobic conditions. They consume diverse organic substrates and may reduce sulfur compounds. At the time of writing, the order *Thermotogales* included six genera: *Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Petrotoga*, *Geotoga* and *Marinitoga*. Members of these genera have been isolated from various extreme environments: *Geotoga* and *Petrotoga* species have been found only in oil reservoirs (Ollivier & Cayol, 2005), whereas *Thermotoga* and *Thermosipho* species have been isolated from hot ecosystems throughout the world (e.g. terrestrial thermal springs, oceanic hydrothermal vents). Within the genus *Marinitoga*, four species have been characterized to date: *Marinitoga camini*, *M. piezophila*, *M. hydrogenitolerans* and *M. okinawensis* (Wery

et al., 2001a; Alain *et al.*, 2002; Postec *et al.*, 2005; Nunoura *et al.*, 2007). They all originated from deep oceanic hydrothermal vents and one of them has been described as piezophilic (Alain *et al.*, 2002). Here, the isolation and characterization are described of a novel bacterium belonging to the genus *Marinitoga*, the first to be isolated from a coastal thermal spring. Considering its phenotypic and genetic characteristics, the novel strain is proposed as a representative of a novel species of the genus *Marinitoga*.

A thermal spring was sampled on Île Saint-Paul (38° 43' S 77° 31' E), located in the South Indian Ocean and belonging to the French Southern and Antarctic Lands. Île Saint-Paul is a 6.5 km² island corresponding to the emerged part of a partially sunk volcano. The central crater is 1.6 km in diameter and forms a protected lagoon. In the intertidal zone, water mixed with sediment was collected from a thermal spring. The pH measured *in situ* was 6.2, the temperature was 54–57 °C and conductivity was 51 µS cm⁻¹. Samples were stored anaerobically in sterile glass bottles at 4 °C and later used as inocula at 2 % (v/v). Enrichment cultures were performed at 60 °C on the following medium, modified from Ravot *et al.* (1995) (l⁻¹): 0.3 g NH₄Cl, 0.5 g MgCl₂ · 6H₂O, 0.1 g CaCl₂ · 2H₂O, 0.5 g

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Transmission and scanning electron micrographs of cells of strain MC3^T are available as supplementary material with the online version of this paper.

KCl, 0.83 g sodium acetate trihydrate, 2 g yeast extract (Difco), 2 g Biotrypcase (Difco), 2 g maltose, 30 g sea salts, 3.3 g PIPES, 1 ml polyvitamin solution (Balch *et al.*, 1979) and 1 mg resazurin (all compounds from Sigma unless otherwise indicated). The medium was prepared anaerobically and the gas phase was 100% N₂. The pH was adjusted to 6.0 before autoclaving for 20 min at 121 °C. The medium was then reduced with 0.5 g Na₂S · 9H₂O and sterile sulfur was added. Cell growth was observed and isolation was performed by repeated dilution-to-extinction in series. Single colonies were obtained by streaking on the same medium solidified with 15 g Gelrite l⁻¹. One isolate, referenced as strain MC3^T, is described in this study. Purity of the strain was routinely confirmed by microscopic examination.

Morphological characteristics of strain MC3^T cells were determined by optical microscopy (Olympus CX40), transmission electron microscopy (JEOL JEM 100 CX II) and scanning electron microscopy (FEI Quanta 200). Cells were Gram-negative, motile rods; no spore formation was detected. During exponential phase, they appeared singly or in pairs with a polar flagellum and they divided by constriction. Cells were 0.8–1.0 × 1.0–2.4 µm. During stationary phase, cells became elongated and reached 7.0 µm long (Supplementary Fig. S1, available in IJSEM Online). Chains containing up to five cells were observed and togas were visible.

Physiological characterization of the novel isolate was carried out using the medium modified from Ravot *et al.* (1995). Methods for determination of growth parameters were as described previously (Wery *et al.*, 2001b). All experiments were performed in triplicate. Bacterial growth was monitored in culture tubes by spectrometry at 600 nm. Cell count correlations with optical density measurements were determined using a Thoma chamber. The temperature range for growth at pH 6.0 was 45–70 °C, with optimum growth at 60 °C. The pH range for growth at 60 °C was 5.5–7.5, with optimum growth at pH 6. Growth was not observed at temperatures ≤37 or ≥75 °C, or at pH ≤4.8 or ≥8.1. The NaCl range for growth at pH 6 and 60 °C was 8–46 g NaCl l⁻¹, with optimum growth at 26 g l⁻¹. No growth was observed in the absence of NaCl or at NaCl concentrations ≥52 g l⁻¹. Under optimal growth conditions, the maximum cell density reached 5.6 × 10⁸ cells ml⁻¹ and the maximal growth rate was 0.48 h⁻¹, corresponding to a mean generation time of 1.44 h.

Strain MC3^T is an obligate chemo-organotroph. No growth was detected on minimal medium with H₂/CO₂ (80/20) as a gas phase. The ability of the isolate to use various carbon sources was tested using the mineral base of the modified Ravot medium described above with 0.2 g yeast extract l⁻¹ as sole organic carbon. The following carbohydrates were tested at 5 g l⁻¹: cellobiose, fructose, galactose, glucose, glycogen, glycerol, lactose, maltose, ribose and starch. The following organic substrates were tested at 2 g l⁻¹: acetate,

brain heart infusion, Casamino acids, casein, formate, peptone, propionate, pyruvate, succinate, tryptone and yeast extract. A cell pellet of a preculture was washed twice with modified Ravot medium depleted in carbon source and was then used as inoculum. Each substrate was tested in duplicate. Positive cultures were confirmed by subcultures on the same medium. Medium without added organic compound was used as a negative control. Cultures were incubated at 60 °C and growth was monitored by microscopic observation after 24 h of cultivation. Compared with the negative control, growth was enhanced by cellobiose, galactose, glucose, glycogen, lactose, maltose, ribose, starch, brain heart infusion, Casamino acids, casein, peptone, pyruvate, tryptone and yeast extract.

Nitrogen sources were tested in duplicate on modified Ravot medium lacking yeast extract, Biotrypcase and NH₄Cl. Compared with a negative control that lacked any nitrogen sources, growth occurred in the presence of gelatin (2 g l⁻¹), glutamate (2 g l⁻¹), urea (2 g l⁻¹), NaNO₃ (20 mM) and NH₄Cl (20 mM).

The use of several sulfur compounds by strain MC3^T was tested in duplicate using modified Ravot medium. Elemental sulfur (5 g l⁻¹), L-cystine (20 mM) and thio-sulfate (20 mM) slightly enhanced growth of strain MC3^T. In the presence of elemental sulfur, H₂S production was detected, using a method described elsewhere (Cord-Ruwisch, 1985).

Growth of strain MC3^T cultivated on modified Ravot medium was tested separately with and without elemental sulfur in the presence of various gas phases: N₂ (100%), H₂/CO₂ (80/20) and H₂ (100%). Growth was not affected by H₂ concentration and similar maximal cell densities were obtained in each case. Strain MC3^T tolerated up to

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain MC3^T among members of the order *Thermotogales*. A total of 1204 sites were used for the phylogenetic analysis. Accession numbers are indicated in parentheses. The topology corresponds to an unrooted tree obtained by the neighbour-joining method. Numbers at nodes indicate bootstrap values above 70% from 500 replications. Bar, 0.05 substitutions per nucleotide position.

2 % oxygen in the gas phase, but growth was inhibited in 4 % oxygen.

Genomic DNA of strain MC3^T was extracted as described by Wery *et al.* (2001a). The G + C content of the genomic DNA was determined as 26.2 mol% at the DSMZ (Braunschweig, Germany) using HPLC (Shimadzu) as described by Mesbah *et al.* (1989).

The 16S rRNA gene was selectively amplified and sequenced as described by Wery *et al.* (2001b). The sequence was then compared with available sequences in GenBank using a BLAST search (Altschul *et al.*, 1990). A

multiple sequence file was generated using the program BioEdit. Alignments and similarity levels were obtained by the CLUSTAL W method (Thompson *et al.*, 1994). Alignments were manually refined using BioEdit and the phylogenetic reconstruction was performed using PHYLO_WIN (Galtier *et al.*, 1996) with the following algorithms: neighbour-joining method with Jukes–Cantor corrections (Saitou & Nei, 1987), and maximum-parsimony and maximum-likelihood methods (Felsenstein, 1981). Bootstrap values were determined as described by Felsenstein (1985). Strain MC3^T was affiliated phylogenetically to the genus *Marinitoga*, order *Thermotogales*

Table 1. Characteristics of members of the genus *Marinitoga*

Strains: 1, *Marinitoga litoralis* sp. nov. MC3^T (data from this study); 2, *M. hydrogenitolerans* AT1271^T (Postec *et al.*, 2005); 3, *M. okinawensis* TFS10-5^T (Nunoura *et al.*, 2007); 4, *M. camini* MV1075^T (Wery *et al.*, 2001a); 5, *M. piezophila* KA3^T (Alain *et al.*, 2002). +, Positive; –, negative; (+), weakly positive; ND, no data available.

Characteristic	1	2	3	4	5
Strain isolation					
Origin*	SIO (Île Saint-Paul)	MAR (Rainbow)	SOT (Yonaguni Knoll IV)	MAR (Menez-Gwen)	EPR (Grandbonum)
Latitude	38° 43' S	36° 13' N	24° 51' N	37° 51' N	12° 48' N
Longitude	77° 31' E	33° 54' W	122° 42' E	31° 31' W	103° 56' W
Depth (m)	0	2275	1365	980	2630
pH for growth					
Range	5.5–7.5	4.5–8.5	5.0–7.4	5–9	5–8
Optimum	6	6	5.5–5.8	7	6
Temperature for growth (°C)					
Range	45–70	35–65	30–70	25–65	45–70
Optimum	60	60	55–60	55	65
NaCl concentration for growth (% w/v)					
Range	0.8–4.6	1.0–6.5	1.0–5.5	1.0–4.5	1.0–5.0
Optimum	2.6	3.0–4.0	3.0–3.5	2.0	3.0
Maximal doubling time (h)	1.44	1.9	0.8	1.7	0.35
Utilization of:					
Casamino acids	+	–	–	–	+
Casein	+	+	–	–	+
Cellobiose	+	–	–	(+)	(+)
Galactose	+	–	–	–	(+)
Glucose	+	–	+	+	(+)
Pyruvate	+	+	–	+	–
Starch	+	–	+	+	(+)
Tryptone	+	–	+	+	+
Growth without electron acceptor under 100 % H ₂	+	+	+†	–	–
Tolerance of O ₂	<4 %	<4 %	Obligate anaerobe	ND	ND
Sulfur compounds that stimulate growth	S ⁰ , S ₂ O ₃ ^{2–} , L-Cys	S ⁰ , S ₂ O ₃ ^{2–} , L-Cys	S ⁰ , L-Cys	S ⁰ , L-Cys	S ⁰ , S ₂ O ₃ ^{2–} , L-Cys
DNA G + C content (mol%)	26.2	28	28	29	29
16S rRNA gene sequence similarity (%)‡	(100)	95.7	95.5	95.6	94.4

*EPR, East-Pacific Rise; MAR, Mid-Atlantic Ridge; SIO, Southern Indian Ocean; SOT, Southern Okinawa Trough.

†Slight inhibition was observed.

‡16S rRNA gene sequence similarities were calculated with reference to the sequence of the novel isolate MC3^T.

(Fig. 1); the most closely related strains were the type strains of *M. hydrogenitolerans* and *M. camini*, with 16S rRNA gene sequence similarities of 95.7 and 95.6 %, respectively. The positioning of strain MC3^T was supported by the results of the three algorithms used.

The characteristics of strain MC3^T are consistent with its assignment to the order *Thermotogales*. On the basis of the results of 16S rRNA gene sequence analysis, the isolate is most closely related to the type strain of *M. hydrogenitolerans*. The two strains can be distinguished based on phylogenetic, genomic and phenotypic criteria: they share 95.7 % 16S rRNA gene sequence similarity. The genomic DNA G+C content of strain MC3^T is lower than that of *M. hydrogenitolerans* (26.2 and 28.0 mol%, respectively). Moreover, compared with *M. hydrogenitolerans*, the maximum temperature for growth of the isolate was slightly higher and the NaCl concentrations for growth, both range and optimum, were lower (Table 1). Furthermore, in contrast to *M. hydrogenitolerans*, strain MC3^T used Casamino acids, cellobiose, galactose, glucose, starch and tryptone. Strain MC3^T is also unique regarding its isolation site. Indeed, it is the first species of the genus *Marinitoga* to be isolated from the Southern hemisphere, from the Indian Ocean and from a coastal thermal spring. The four previously described *Marinitoga* species were isolated from deep oceanic hydrothermal vents. Given the low levels of 16S rRNA gene sequence similarity between strain MC3^T and the other recognized *Marinitoga* species and the differences in physiological features (Table 1), the isolate represents a novel species of the genus *Marinitoga*, for which the name *Marinitoga litoralis* sp. nov. is proposed.

Description of *Marinitoga litoralis* sp. nov.

Marinitoga litoralis (li.to.ra'lis. L. fem. adj. *litoralis* of or belonging to the seashore).

Cells are rod-shaped, motile, Gram-negative and surrounded by a sheath-like structure. Growth occurs at 45–70 °C (optimum 60 °C), pH 5.5–7.5 (optimum pH 6) and 8–46 g NaCl l⁻¹ (optimum 26 g l⁻¹). The doubling time under optimal conditions is 1.44 h and the maximum cell yield is 5.6 × 10⁸ cells ml⁻¹. Anaerobic; resistant to concentrations of oxygen below 4 % and hydrogen up to 100 %. Chemo-organotrophic; able to ferment cellobiose, galactose, glucose, glycogen, lactose, maltose, ribose, starch, brain heart infusion, Casamino acids, casein, peptone, pyruvate, tryptone and yeast extract. Sulfur, cystine and thiosulfate slightly stimulate growth and H₂S production is observed in the presence of sulfur. 16S rRNA gene sequence analysis indicated that the species is classified in the genus *Marinitoga*, order *Thermotogales* of the bacterial domain.

The type strain is MC3^T (=DSM 21709^T =JCM 15581^T), isolated from a coastal thermal spring on Île Saint-Paul (38° 43' S 77° 31' E) in the Southern Indian Ocean. The G+C content of the genomic DNA of the type strain is 26.2 mol%. 16S rRNA gene sequence similarity between

strain MC3^T and the type strain of *M. hydrogenitolerans* is 95.7 %. Strain MC3^T is available by request from the 'Souchothèque de Bretagne' culture collection, strain no. 3067 (<http://www.ifremer.fr/souchotheque/>).

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