Crassaminicella profunda gen. nov., sp. nov., an anaerobic marine bacterium isolated from deep-sea sediments

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A novel, anaerobic, chemo-organotrophic bacterium, designated strain Ra1766H^T, was isolated from sediments of the Guaymas basin (Gulf of California, Mexico) taken from a depth of 2002 m. Cells were thin, motile, Gram-stain-positive, flexible rods forming terminal endospores. Strain Ra1766H^T grew at temperatures of 25–45 °C (optimum 30 °C), pH 6.7–8.1 (optimum 7.5) and in a salinity of 5–60 g l^{-1} NaCl (optimum 30 g l^{-1}). It was an obligate heterotrophic bacterium fermenting carbohydrates (glucose and mannose) and organic acids (pyruvate and succinate). Casamino acids and amino acids (glutamate, aspartate and glycine) were also fermented. The main end products from glucose fermentation were acetate, butyrate, ethanol, H_2 and CO_2. Sulfate, sulfite, thiosulfate, elemental sulfur, fumarate, nitrate, nitrite and Fe(III) were not used as terminal electron acceptors. The predominant cellular fatty acids were $C_{14:0}$, $C_{16:1}\omega7$, $C_{16:1}\omega7$ DMA and $C_{16:0}$. The main polar lipids consisted of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and phospholipids. The G+C content of the genomic DNA was 33.7 mol%. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain Ra1766H^T was affiliated to cluster XI of the order *Clostridiales,* phylum *Firmicutes.* The closest phylogenetic relative of Ra1766H^T was *Geosporobacter subterraneus* (94.2 % 16S rRNA gene sequence similarity). On the basis of phylogenetic inference and phenotypic properties, strain Ra1766H^T (=DSM 27501^T=JCM 19377^T) is proposed to be the type strain of a novel species of a novel genus, named *Crassaminicella profunda.*

Guaymas basin in the Gulf of California, consists of deep, semi-enclosed basins formed by the 5–6 cm year^{-1} separation of Baja California from mainland Mexico. Hydrothermal vent systems in the south rift of the Guaymas basin were discovered after studying conductive heat flow (Williams *et al.*, 1979) and bottom-water helium in the troughs that mark the spreading axes of this basin (Lupton, 1979). These vents systems are located in an area of high sediment loading with high biological productivity in the overlying surface waters resulting in deposition of abundant organic-rich sediments with total organic carbon concentrations of up to 4 %. Petroleum is actively produced within the basin sediments as a result of magmatic heating of the thick, organic-rich sediment cover (Simoneit, 1985). Thus, this environment is prone to bacterial activity and hydrocarbon degradation or recycling (Pearson *et al.*, 2005). White or yellow microbial biofilms are common features found at sediment-water interfaces (Gundersen *et al.*, 1992). Molecular studies, based on 16S rRNA gene sequences and lipid biomarkers, have shown that Guaymas basin sediments harbour a great diversity of cultured and non-cultured *Bacteria*, *Archaea* and *Eukarya* (Teske *et al.*, 2002; Dick & Tebo, 2010). Amongst *Bacteria*, the presence of members of the *Firmicutes* has been established (Dick *et al.*, 2006; Lakhal *et al.*, 2013). Here, the taxonomic characterization of a novel bacterium isolated from sediments of the Guaymas basin is presented. The bacterium was found to show phenotypic and phylogenetic traits that led to its assignment to a novel species of a novel genus within the phylum *Firmicutes*, order *Clostridiales*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ra1766H^T is KC329523.

Sediments were collected from the Guaymas basin at a depth of 2002 m on 29 June 2010, at the Southern Trough site (27° 00′ 44″ N 111° 24′ 53″ W), during the BIG 2010 cruise (RV *Atalante*) using the submersible *Nautile* (Ifremer, France). Strain Ra1766H^T was isolated from white mats on the sediments surface at an *in situ* temperature of 25 °C (Lakhal *et al.*, 2013). Sediment sampling was performed with a push core using the port manipulator of the submersible *Nautile*. Samples were then placed in the submersible's insulated basket for the trip to the surface. On board, sediments were directly inoculated in basal medium. For enrichment and isolation, the basal medium contained (l^{-1} distilled water) 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1 g NH_4Cl, 23 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2 . 2H_2O$, 0.5 g cysteine-HCl, 1 g yeast extract (Difco), 1 ml trace mineral element solution (Widdel & Pfennig, 1981) and 1 ml of 0.1 % (w/v) resazurin (Sigma). The pH was adjusted to 7.2 with 10 M KOH. The medium was boiled under a stream of O_2-free N_2 gas and cooled at room temperature. Aliquots of 5 ml were dispensed into Hungate tubes, degassed under N_2/CO_2 (80 : 20, v/v) and subsequently sterilized by autoclaving at 120 °C for 20 min. Prior to culture inoculation, 0.1 ml 10 % (w/v) $NaHCO_3$, 0.1 ml 2 % (w/v) $Na_2S . 9H_2O$, 0.1 ml 150 g l^{-1} $MgCl_2 . 6H_2O$ and 20 mM glucose from sterile stock solutions were injected into the tubes. The Hungate technique (Hungate, 1969) was used throughout the study. A 0.5 aliquot of the sample was used as inoculum into the Hungate tubes that were subsequently incubated at 37 °C. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in roll tubes (Miller & Wolin, 1974) containing the same culture medium supplemented with 2 % agar (w/v). Several colonies developed after incubation at 37 °C and were picked separately. Colonies were white and circular with diameters ranging from 1.0 to 2.0 mm after 3–5 days of incubation at 37 °C. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several strains were isolated and had high similarities (>99 %) in 16S rRNA genes. One strain, designated Ra1766H^T, was selected for further taxonomic characterization.

Methods for purification of the DNA, PCR amplification and sequencing of the 16S rRNA gene have been described previously (Thabet *et al.*, 2004). The partial sequences generated were assembled using BioEdit v. 5.0.9. (Hall, 1999) and the consensus sequence of 1456 nt was corrected manually for errors and deposited in the GenBank database under accession number KC329523. The sequence was compared with available sequences in GenBank, using a

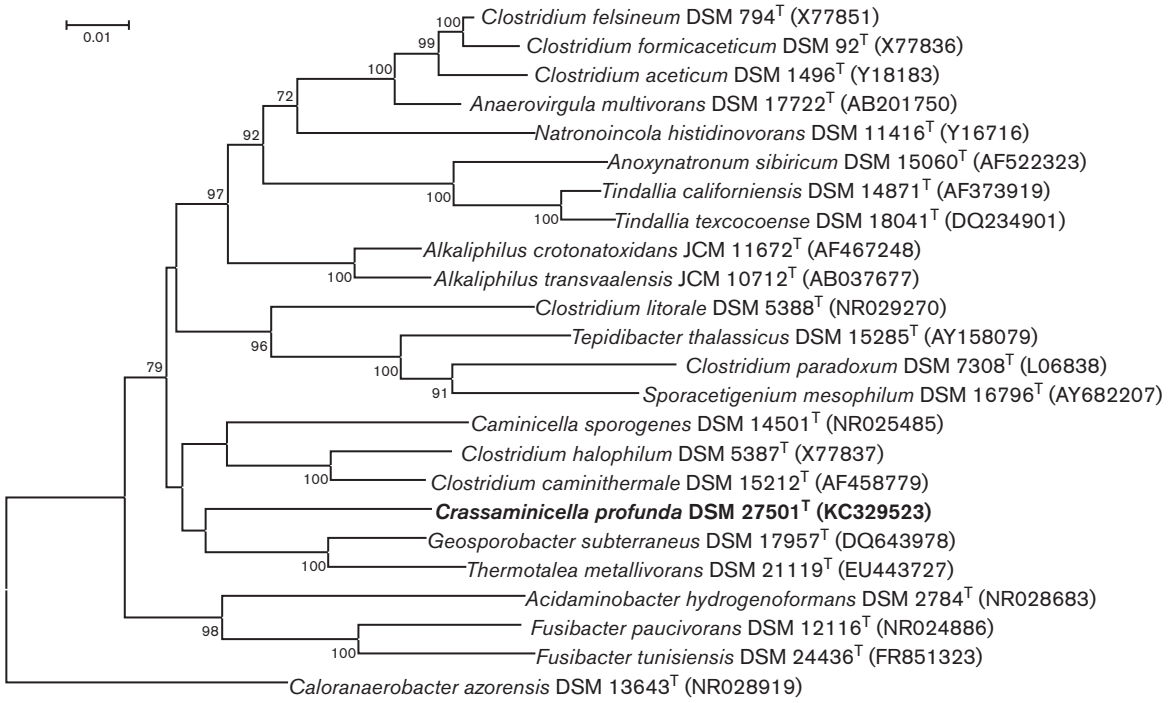

Fig. 1. Neighbour-joining phylogenetic tree of 16S rRNA gene sequence showing the relationships between strain Ra1766H^T and related type strains amongst cluster XI of the order *Clostridiales*. *Caloranaerobacter azorensis* MV1087^T belonging to the cluster XII was used as the outgroup. Bootstrap values >70 % based on 1500 replicates are shown. Bar, 1 change per 100 nucleotide positions.

BLAST search (Altschul *et al.*, 1990). Nucleotide ambiguities were omitted and evolutionary distances were calculated using the maximum composite likelihood model (Tamura *et al.*, 2004). Phylogenetic tree topology determined using the neighbour-joining method is shown in Fig. 1 (Saitou & Nei, 1987). Support for internal nodes was assessed by bootstrap analysis (1500 replicates) (Felsenstein, 1985). These analyses were conducted in MEGA6 (Tamura *et al.*, 2013). The position of strain Ra1766T related to *Geosporobacter subterraneus* and *Thermotalea metallivorans* was also confirmed using the minimum evolution method with the maximum composite likelihood model (Rzhetsky & Nei, 1992), and the maximum-likelihood method with the general time reversible model (Nei & Kumar, 2000). The 16S rRNA sequence analysis indicated that strain Ra1766H^T was a member of the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales* and family *Clostridiaceae* (Collins *et al.*, 1994). The most closely related species phylogenetically were members of cluster XI of the order *Clostridiales*. They included Geosporobacter subterraneus VNs68T isolated from a deep geothermal aquifer (Klouche *et al.*, 2007) with 94.2 % identity, Clostridium caminithermale DVird3T isolated a deep-sea hydrothermal vent chimney (Brisbarre *et al.*, 2003) with 93.7 % identity, Thermotalea metallivorans B2-1T isolated from a borehole in the Great Artesian Aquifer (Ogg & Patel 2009) with 92.4 % identity and Caminicella sporogenes AM1114T (Alain *et al.*, 2002) isolated from deep-sea hydrothermal vent chimneys with 91.9 % identity.

Experiments on the optimum growth conditions were performed in duplicate, using Hungate tubes containing the basal medium. We also performed experiments in parallel on the two most closely related strains, *G. subterraneus* VNs68^T and *Clostridium caminithermale* DVird3^T. The pH, temperature and NaCl concentration ranges for growth were determined using basal medium. Water baths were used for incubating bacterial cultures at between 20 °C and 55 °C, with increments of 5 °C. The pH of the medium was adjusted by injecting into Hungate tubes aliquots of anaerobic stock solutions of 1 M HCl (for acidic pH values), 10 % $NaHCO_3$ or 8 % Na_2CO_3 (for alkaline pH values), checked after autoclaving and used to test for growth at pH 5 to 8.6. To study the salinity requirement, NaCl was weighed directly into tubes (0–10 %, w/v) before the medium was dispensed. Strains Ra1766H^T, VNs68^T and DVird3^T were subcultured at least twice under the same experimental conditions before growth rates were determined. Arabinose, cellobiose, sorbose, glucose, fructose, galactose, ribose, glycerol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, sucrose, xylose, peptone, Casamino acids, acetate, succinate, butyrate, formate, lactate and pyruvate were tested as growth substrates during strain Ra1766H^T cultivation. Each substrate was added to the basal medium at a final concentration of 20 mM, while H_2/CO_2 (80 : 20, v/v) in the presence of acetate (2 mM acetate) was tested at 2 bars. Elemental sulfur (1 %, w/v), sodium sulfate (20 mM), sodium thiosulfate (20 mM), sodium sulfite (2 mM), fumarate (20 mM), sodium nitrate (10 mM), sodium nitrite (2 mM) and $Fe(OH)_3$ (13 mM) were tested as terminal electron acceptors in the presence of glucose (20 mM) and yeast extract (1 g l^{-1}). H_2S production was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). Bacterial growth was monitored by measuring the increase in turbidity at 580 nm by insertion of Hungate tubes into the cuvette holder of a spectrophotometer (Cary 50, Varian). End products of metabolism were measured by HPLC and gas chromatography of the gases released after 2 weeks of incubation at 37 °C (Fardeau *et al.*, 2000). Cellular morphology and the purity of the strains were assessed under an Optiphot (Nikon) phase-contrast microscope. For transmission electron microscopy studies, cells were negatively stained with sodium phosphotungstate, as previously described (Fardeau *et al.*, 1997) and observed under a Zeiss EM912 microscope. The presence of spores was checked from the microscopic observation of cultures and pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min. Cells of strain Ra1766H^T stained Gram-positive (Gram staining reaction) and they were motile. Strain Ra1766H^T produced thin, long (0.5–1 μm × 6–10 μm), straight or curved rods. The presence of terminal endospores was observed only in old cultures (incubations over one month old) (Fig. 2). Ultrathin sections were obtained as described by Fardeau *et al.* (1997) and revealed a thin stratified, Gram-stain-positive type of cell wall (Fig. 2). The phenotypic characteristics of strain

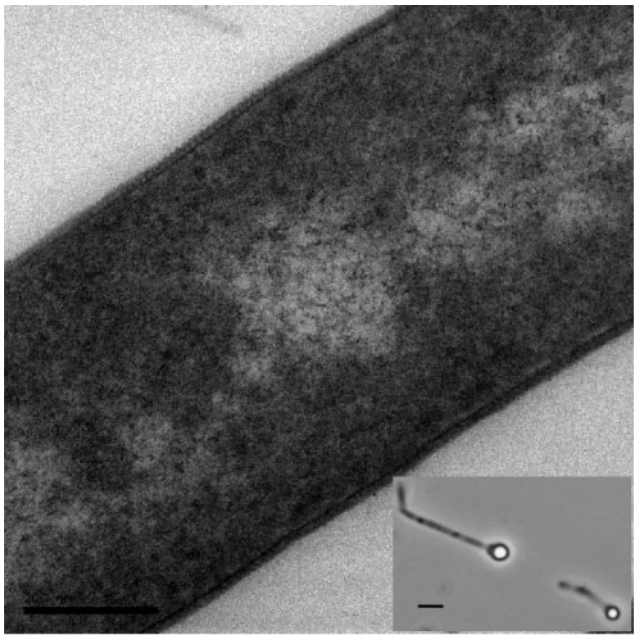

Fig. 2. Thin-section electron microphotograph showing the cell morphology of strain Ra1766HT and Gram-positive type of cell wall. Bar: 0.2 μm. Insert: optical microphotograph of a strain Ra1766HT culture showing the morphology of the bacterium and the presence of terminal endospore. Bar: 2 μm.

Table 1. Differential characteristics between strain Ra1766H^T and related members of cluster XI of the order *Clostridiales* (Collins *et al.*, 1994)

Taxa: 1, Strain Ra1766H^T; 2, *G. subterraneus* VNs68^T; 3, *C. caminithermale* DVird3^T; 4, *C. sporogenes* AM1114^T; 5, *T. metallivorans* B2-1^T. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5
Presence of spores	+	+	+	+	−
Gram-stain	+	+	+	−	−
Temperature range for growth (optimum) (°C)	25–45 (30)	25–55 (45)*	20–60 (45)*	45–65 (60)	30–55 (50)
pH range for growth (optimum)	6.7–8.1 (7.5)	5.6–8.5 (7.3)*	5.8–8.6 (6.6)*	4.5–8 (7.5)	6.5–9 (8)
NaCl range for growth (g l^{-1})	5–60 (30)	0–45 (20)*	10–45 (35)*	15–46 (19–23)	0–40 (0–10)
Motility	+	−	+	+	+
Substrates used					
Cellobiose	−	+	−	ND	+
Fructose	−	+	+	+	+
Succinate	+	−	+	+	−
Propionate	−	−	−	ND	−
Pyruvate	+	−	+	−	−
Ribose	−	+	+	ND	−
Mannose	+	−	+	ND	+
Reduction of Fe(III)	−	+*	−*	−	ND
End products of glucose fermentation (trace products)	Acetate, butyrate, ethanol, H_2, CO_2	Acetate, formate, H_2, CO_2 (ethanol)	Acetate, butyrate, propionate, H_2, CO_2	Butyrate, acetate, ethanol, H_2, CO_2	Ethanol, acetate
DNA G+C content (mol%)	33.7	42.2	33.1	24.2	48

*Data from the present study.

Ra1766H^T are listed in Table 1 and in the genus and species descriptions. Strain Ra1766H^T required yeast extract for growth (1 g l^{-1}), which could be replaced by biotrypcase, but not by Balch vitamins solution (Balch *et al.*, 1979). It grew at temperatures between 25 °C and 45 °C, with an optimum at 30 °C (with no growth at 20 °C and 50 °C). Growth occurred at salinities of between 5 g l^{-1} and 60 g l^{-1} NaCl, with an optimum at 30 g l^{-1} (with no growth at 0 g l^{-1} or 65 g l^{-1}). During cultivation of strain Ra1766H^T, the following carbohydrates were used as carbon and energy sources: glucose, mannose, acetate, propionate, succinate and pyruvate. Fructose, cellobiose, sorbose, ribose, xylose, methanol, formate, lactate, ethanol and H_2/CO_2 (80 : 20, v/v) in the presence of 2 mM acetate were not used. The main fermentation products from glucose were acetate, butyrate, ethanol, H_2 and CO_2. They were acetate, butyrate, H_2 and CO_2 from pyruvate, and acetate, butyrate, propionate, H_2 and CO_2 from succinate. Casamino acids and amino acids (glutamate, aspartate and glycine) were also fermented, whereas alanine, phenylalanine, leucine, glutamine, tyrosine, cysteine, lysine, arginine, proline, serine, isoleucine, asparagine, valine, histidine, methionine and tryptophan were not. Fatty acids were extracted using the method of Miller (1982) with the modifications of Kuykendall *et al.* (1988) and analysed by gas chromatography (model 6890N, Agilent Technologies) using the Microbial Identification System (MIDI, Sherlock Version 6.1; database, ANAER6). Analyses of the respiratory quinones and polar lipids were carried out at the Identification Service of the DSMZ (Braunschweig, Germany). The G+C content was determined by HPLC (Mesbah *et al.*, 1989) after DNA had been isolated and purified by chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977).

The major fatty acids were $C_{14:0}$ (32.5 %), $C_{16:1}\omega7$ (21.7 %), $C_{16:1}\omega7$ DMA (10.9 %) and $C_{16:0}$ (10.3 %). No quinones were detectable and the polar lipid profile of strain Ra1766H^T consisted of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, and phospholipids (Fig. S1, available in the online Supplementary Material). The G+C content of the genomic DNA of strain Ra1766H^T was 33.7 %.

The phylogenetic characteristics of strain Ra1766H^T pertaining to cluster XI of the *Clostridiales* suggests that this isolate represents a novel genus within this cluster. This is strongly supported by some genetic and phenotypic traits, as detailed in Table 1. For example, strain Ra1766H^T had a DNA G+C content (33.7 mol%) far from the values exhibited by *G. subterraneus* (44.2 mol%), *T. metallivorans* (48 mol%) or *Caminicella sporogenes* (24.2 mol%). In contrast to *Clostridium caminithermale*,

which exhibits a similar DNA G+C content (33.1 mol%), strain Ra1766H^T used a different range of substrates and differed in its metabolism, as determined by the end products of glucose fermentation (Table 1). There are also some differences between all these isolates with respect to the optimum growth temperatures, with strain Ra1766H^T exhibiting the lowest value (Table 1). It is notable that strain Ra1766H^T is slightly halophilic, possibly confirming its marine origin. However, its ecological significance in the Guaymas basin needs further investigation, as the most closely related strains, *G. subterraneus*, *Clostridium caminithermale, Caminicella sporogenes* and *T. metallivorans* share a deep origin. On the basis of the 16S rRNA gene similarity values between strain Ra1766H^T and the most closely related strains of species with validly published names, as well as the phenotypic differences, we propose that strain Ra1766H^T should be assigned to a novel genus and species within cluster XI of the subphylum *Clostridium,* with the name *Crassaminicella profunda* gen. nov., sp., nov.

Description of *Crassaminicella* gen. nov.

Cras.sa.mi.ni.cel'la. L.n crassamen -inis sediment; L. fem. n. *cella* a storeroom, chamber and, in biology, a cell; N.L. fem. n. *Crassaminicella* a cell isolated from sediments.

Gram- stain-positive, motile, straight or curved rods occurring singly forming terminal endospores. Mesophilic, neutrophilic and slightly halophilic. Anaerobic and heterotrophic, fermenting carbohydrates and proteinaceous substrates. The end products from glucose fermentation are acetate, butyrate and ethanol with H_2 and CO_2. The genus is included phylogenetically in cluster XI of the order *Clostridiales.*

The type species is *Crassaminicella profunda.*

Description of *Crassaminicella profunda* gen. nov., sp. nov.

Crassaminicella profunda. (pro.fun'da. L. fem. adj. *profunda* from the deep). Isolated from sediments of the Guaymas basin.

Description is as for the species. Rods (0.5–1 μm × 6–10 μm) grow at NaCl concentrations ranging from 0.5 to 6 % (w/v), with an optimum at 3 % (w/v). Optimal growth occurs at pH values of 7.5 (range 6.7–8.1; no growth at pH 6.5 and 8.2). The optimal temperature for growth is 30 °C (range 25–45 °C, no growth at 20 or 50 °C). Carbohydrates (glucose and mannose) and organic acids (pyruvate and succinate) are fermented. Casamino acids and some amino acids (glutamate, aspartate and glycine) serve as growth substrates. The main end products of glucose catabolism are acetate, butyrate, ethanol, H_2 and CO_2. Fructose, cellobiose, sorbose, ribose, xylose, methanol, formate, lactate, fumarate, ethanol, a gas mixture of H_2/CO_2 (80 : 20, v/v) supplemented with acetate, alanine, phenylalanine, leucine, glutamine, tyrosine, cysteine, lysine, arginine, proline, isoleucine, asparagine, valine, histidine, methionine, tryptophan, threonine and serine are not used. Sulfate, sulfite, thiosulfate and elemental sulfur are not utilized as electron acceptors. Yeast extract is required for growth.

The type strain Ra1766H^T (=DSM 27501^T=JCM 19377^T) was isolated from sediments of the Guaymas basin. The G+C content of the genomic DNA is 33.7 mol%.

Acknowledgements

We thank Dr Jean Euzéby for checking the Latin etymology of the genus name. This BIG 2010 cruise (A.G and Daniel Prieur, chief scientists) was funded by Ifremer (France) and has benefited from a work permit in Mexican waters (DAPA/2/281009/3803, 28 October 2009). We also thank Manon Joseph (IRD) for help with transmission electron microscopy studies.

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