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Vallitalea guaymasensis gen. nov., sp. nov., isolated from marine sediment

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Abstract:

A novel obligately anaerobic, non-spore-forming, rod-shaped mesophilic, halophilic, Gram-stain-negative bacterium, was isolated from sediments of Guaymas Basin. The strain, designated Ra1766G1^T, grew at 20–40 °C (optimum, 30–35 °C) and at pH 6.0–8.0 (optimum, pH 6.5–7.5). It required 0.5–7.5% NaCl (optimum, 2–3%) for growth. Sulfate, thiosulfate, elemental sulfur, sulfite, fumarate, nitrate and nitrite were not used as terminal electron acceptors. Strain Ra1766G1^T used cellobiose, glucose, mannose, maltose, arabinose, raffinose, galactose, ribose, sucrose, pyruvate and xylose as electron donors. The main fermentation product from glucose metabolism was acetate. The predominant cellular fatty acids were anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso DMA-$C_{15:0}$ and $C_{16:0}$. The main polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, iso-DMA-$C_{15:0}$ glycolipids and phospholipids. The G+C content of the genomic DNA was 31.2 mol%. The closest phylogenetic relatives of strain Ra1766G1^T were *Natranaerovirga pectinivora* AP3^T (92.4% 16S rRNA gene sequence similarity), *Natranaerovirga hydrolytica* APP2^T(90.2%) and *Defluviitalea saccharophila* 6LT2^T (88.9%). On the basis of phylogenetic inference and phenotypic properties, strain Ra1766G1^T represents a novel species of a new genus for which the name *Vallitalea guaymasensis* is proposed. The type strain of the type species is Ra1766G1^T (=DSM 24848^T=JCM17997^T).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ra1766G1^T is HE805640.

The Guaymas Basin hydrothermal site at 2000 m depth in the Gulf of California is a unique hydrothermal system. At Guaymas Basin, the microbial recycling of fossil organic matter occurs in highly active sediments on a scale of less than a meter, which facilitates sampling, rate measurements and microbial analyses. Molecular analysis based on rRNA, functional genes and lipid biomarkers have revealed a wide range of uncultured bacteria, archaea and eukarya in Guaymas Basin (Edgcomb *et al*., 2002, Teske *et al*., 2002). In this paper, we describe a novel mesophilic, anaerobic bacterium, designated as strain Ra1766G1^T, isolated from the Guaymas Basin, displaying phenotypic traits that allowed its assignment to a novel species of a novel genus, representing a novel phylogenetic lineage at the family level.

Sediments were collected on the 29 of June 2010 from the Guaymas basin, at the Southern Trough site (27°00'44''N, 111°24'53''W, at 2002 m depth), during the BIG 2010 cruise (RV *Atalante*) using the submersible *Nautile* (IFREMER, La Seyne-sur-Mer, France). Strain Ra1766G1^T was isolated from white mats on the sediments surface (Supplementary Figure 1), at an *in situ* temperature of 25°C. For deployment and recovery, a hermetic tube was used, to avoid washing and mixing. Using the port manipulator of the submersible *Nautile*, the samples were placed in the submersible insulated basket for the trip to the surface. On board, sediments were stored anaerobically and frozen at -20°C in 10 % DMSO, or directly inoculated in basal medium.

For enrichment and isolation, the basal medium contained (per liter of 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 Laboratories), 1 ml trace mineral element solution (Widdel and Pfennig, 1981) and 1 ml of 0.1 % resazurin (Sigma). The pH was adjusted to 7.2 with a 10 M KOH solution. The medium was boiled under a stream of O_2-free N_2 gas and cooled to 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 of 10 % (w/v) $NaHCO_3$, 0.1 ml of 2 % (w/v) $Na_2S.9\ H_2O$, 0.1 ml of 150 g/l $MgCl_2$, $6H_2O$ and 20 mM of glucose from sterile stock solutions were injected into the tubes. The Hungate technique (Hungate, 1969) was used throughout the study.

A 0.5-ml aliquot of the sample was inoculated 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 and 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; their morphology and metabolic profiles were similar and the same phylogenic inference was obtained for all of them. One strain, designated Ra1766G1^T, was selected and used for further metabolic and physiological characterization.

Methods for purification of the DNA, PCR amplification and sequencing of the 16S rRNA gene were described previously (Ben Dhia Thabet *et al.,* 2004). A sequence of 1444 nucleotides was obtained and deposited in the GenBank database under accession number HE805640. The most closely related sequences were retrieved from the Ribosomal Database Project (release 10) and the GenBank (version 178) using BLAST (Altschul *et al.,* 1990), then aligned using the Muscle program (Edgar, 2004). Phylogenetic analyses were conducted in MEGA5 (Tamura *et al,* 2011) and computed using the Maximum Likelihood method (Tamura *et al*, 2004) (Fig. 1). The analysis involved 75 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1869 positions in the final dataset. Tree topology was re-examined by the bootstrap method (500 replications) of resampling

(Felsenstein, 1985). Its topology was also supported using the neighbor-joining and maximum-parsimony algorithms.

Strain Ra1766G1^T shared 92.4% similarity with the type strain *Natranaerovirga pectinovirga* isolated from sediments of hypersaline soda lakes from the Kulunda Steppe (Sorokin *et al*, 2012) and 88.9% similarity with *Defluviitalea saccharophila* isolated from an up flow anaerobic filter treating abattoir wastewaters in Tunisia (Jabari *et al*., 2012).

Growth experiments were performed in duplicate, using Hungate tubes containing the basal medium. The pH, temperature, and NaCl concentration ranges for growth were determined using basal medium. The pH of the medium was adjusted by injecting in Hungate tubes aliquots of anaerobic stock solutions of 1M HCl (acidic pHs), 10 % $NaHCO_3$ or Na_2CO_3 (alkaline pHs) (to test pH between 5 and 8.6) and checked after autoclaving. Water baths were used for incubating bacterial cultures from 20 °C to 55 °C, with increments of 5°C. To study the requirement of NaCl, NaCl was weighed directly in the tubes (0-10%) before the medium was dispensed. The strain was subcultured at least twice under the same experimental conditions before the growth rates were determined. Arabinose, cellobiose, glucose, fructose, galactose, ribose, glycerol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, saccharose, xylose, peptone, casaminoacids, acetate, succinate, butyrate, formate, lactate and pyruvate, were tested as electron donors. Each substrate was added to the basal medium at a final concentration of 20 mM whereas H_2/CO_2 (80/20), or H_2/CO_2 (80/20) 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), Na sulfite (2 mM), fumarate (20mM), sodium nitrate (10 mM) and sodium nitrite (2 mM), were tested as terminal electron acceptors. 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 high pressure liquid chromatography (HPLC) and gas chromatography of the gases released after 2 weeks of incubation at 37 °C (Fardeau *et al.,* 2000).

Cellular morphology and 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). The presence of spores was checked by microscopic observation of cultures and pasteurization tests performed at 80, 90 and 100 °C for 10 and 20 min.

Cells of strain Ra1766G1^T stained Gram-negative (Gram staining reaction) and had a multilayered cell-wall and an outer membrane typical of Gram-negative bacteria (Gram type based on cell wall analysis). The cells were non motile, non-spore-forming rods (Supplementary Figure 2 A, B, C). The phenotypic characteristics of strain Ra1766G1^T are listed in the genus and species descriptions and Table 1. The isolate required yeast extract for growth which could be replaced by biotrypcase or vitamins of Balch (Balch *et al.,* 1979). However, growth was better in presence of yeast extract.

Analysis of the respiratory quinones and polar lipid analyses were carried out by Dr Brian Tindall at the Identification Service of the DSMZ (Braunschweig. Germany). For fatty acid analysis, the biomass of strain Ra1766G1^T was standardized for its physiological age at the point of harvest according to Technical Note 101 of MIDI (http://www.microbialid.com/PDF/TechNote_101.pdf). 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, TSBA40). DNA was isolated and purified by

chromatography on hydroxyapatite using the procedure of Cashion *et al.* (1977) and the G+C content was determined by using HPLC (Mesbah *et al.,* 1989).

The major fatty acids were: anteiso-$C_{15:0}$ (22.7%), iso-$C_{15:0}$ (13.7%), anteiso DMA-$C_{15:0}$ (10.8%) and $C_{16:0}$ (7.0%). No quinones were detectable and the polar lipid profile of strain Ra1766G1^T consisted of diphosphatiglycerol, phosphatidylglycerol, two glycolipids, two phospholipids, and a range of unidentified glyco and phospholipids. (Table 2; Supplementary Figure 3). The G + C content of the genomic DNA of strain Ra1766G1^T was 31.2% (Table 1). Based on an inferred phylogenetic tree, the closest cultured relatives of strain Ra1766G1^T are *Defluviitalea saccharophila* (Jabari *et al.*, 2012), a member of the family *Defluviitaleacea* and *Natranaerovirga pectinivorga,* not classified in a family. But the two strains differ of strain Ra1766G1^T by several points (Tables 1 and 2). *D. saccharophila* is Gram positive and grows at 50-55 °C. *Natranaerovirga pectinivorga* is also Gram positive and grows at 43°C, is alkaliphilic and presents a very narrow substrate spectrum for growth, restricted to galacturonic acid and its polymer (*e.g*. pectin). Strain Ra1766G1^T is Gram negative and grows at 30-35 °C. The G+C% is also different and the profiles of polar lipids and of fatty acids differ too. The association of the novel isolate with the genus *Natranaerovirga* could be evident from the RDP classifier, which gave 92.8 % probability with the clustering. Nevertheless, there are too many differences between the strains and the position of the novel isolate will remain uncertain until more sequences of related organisms appear in the database.

Therefore, on the basis of phylogenetic inference and phenotypic characteristics, we propose that strain Ra1766G1^T represents a novel species of a novel genus and was proposed as *Vallitalea guaymasensis .*

Description of *Vallitalea* gen. nov.

Vallitalea: Val.li.ta'le.a. L. n. vallis, a valley, vale; L. fem. n. talea, a rod; N.L. fem. n. Vallitalea, a rod isolated from a vale.

Cells stain Gram-negative (Gram staining reaction). Non-motile, non-spore-forming, mesophilic, rods with a fermentative and obligately anaerobic type of metabolism. No quinones detectable, and the major fatty acids are : anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso DMA-$C_{15:0}$ and $C_{16:0}$. The DNA G+C content of the type strain of the type species is 31.2 mol%
The type species is *Vallitalea guaymasensis*.

Description of *Vallitalea guaymasensis* sp. nov.

guaymasensis: guay.mas.en'sis. N.L. fem. adj. *guaymasensis*, of or belonging to Guaymas.

Displays the following characteristics in addition to those listed in the genus description. Cells are rods approximately 2 to 10 µm in length and 0.5 to 1 µm in diameter, occurring singly or in pairs. Growth occurs at 20-40 °C, (optimum, 30-35 °C), at pH 6.0-8.0 (optimum, pH 6.5-7.5) and at NaCl concentration 0.5%-7.5% (optimum, 2%-3%). Yeast extract is required for growth. Cellobiose, glucose, mannose, maltose, arabinose, raffinose, galactose, ribose, saccharose, pyruvate and xylose are used as electron donors, but glycerol, mannitol, casaminoacids, acetate, lactate, fructose, succinate, butyrate, peptone, lactose, rhamnose, formate, H_2/CO_2, or H_2/CO_2 in the presence of acetate are not. The main fermentation product from glucose metabolism was acetate. Sulfate, thiosulfate, elemental sulfur, sulfite, nitrate and nitrite were not used as terminal electron acceptors. Contains significant amounts of anteiso-$C_{15:0}$, iso-$C_{15:0}$, anteiso DMA-$C_{15:0}$ and $C_{16:0}$.The G+C content of the genomic DNA of the type strain is 31.2 mol%. The type strain, Ra1766G1^T (= DSM 24848^T = JCM 17997^T) was isolated from sediments of Guaymas basin.

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Figure 1. Phylogenetic position of strain Ra1766G1^T within the Clostridiales based on 16S rRNA gene sequence analysis using the Neighbor-Joining method. The analysis involved 75 nucleotide sequences. There were a total of 1869 positions in the final dataset. Numbers at node indicate bootstrap values above 60% (bootstrap test from 500 replicates). Scale bar: 0.01 substitutions per site.

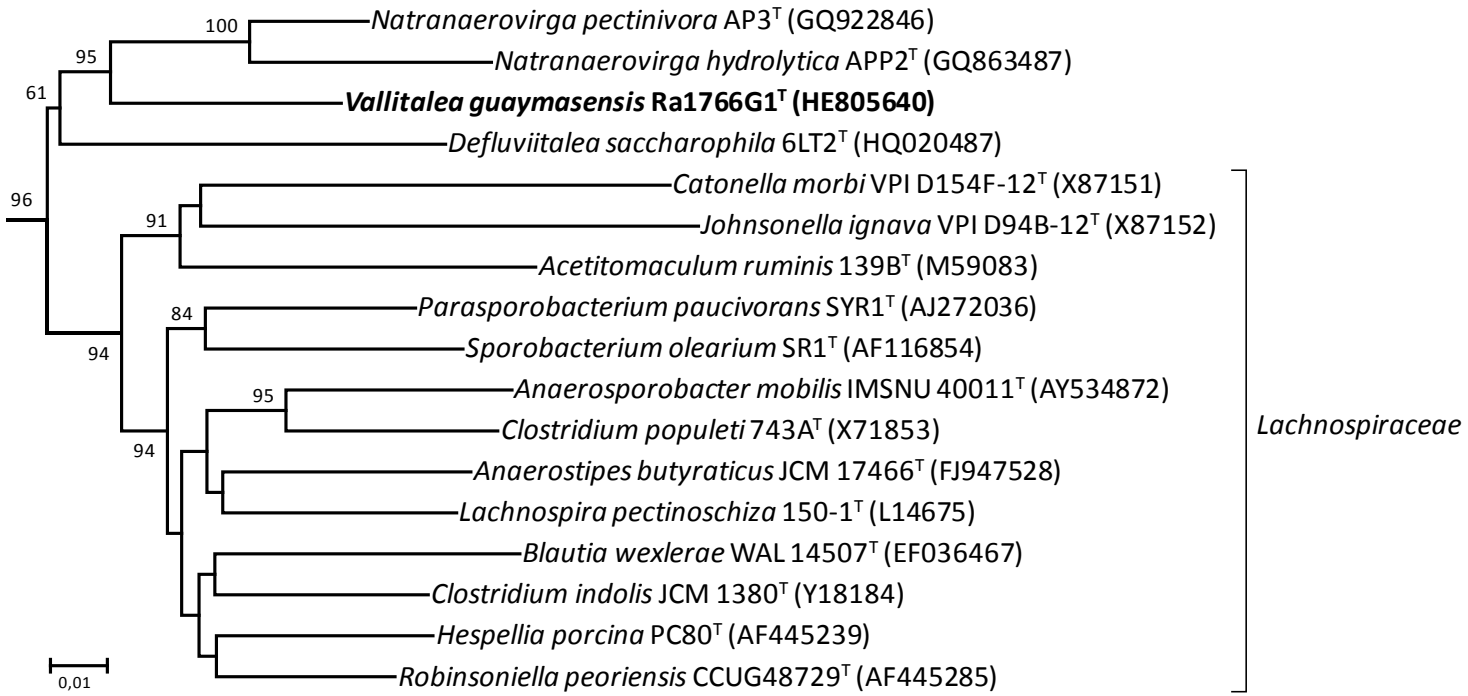


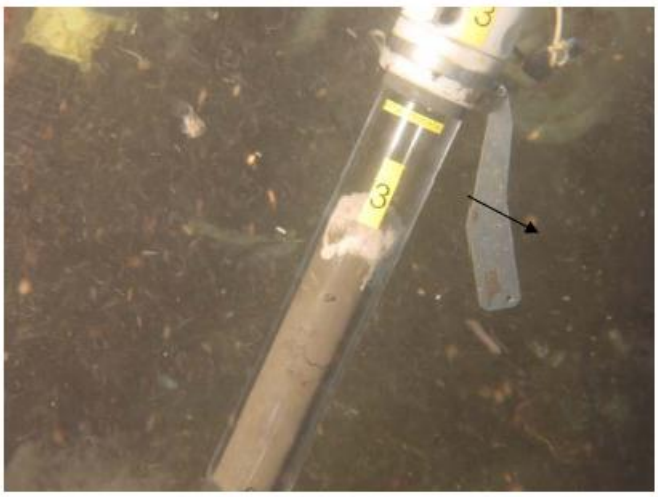
Table 1. Differential characteristics between strain Ra1766G1^T, *Defluviitalea saccharophila*^{T}, and *Natranaerovirga pectinivorga*^{T}.

Characteristics	Strain Ra1766G1^T	*Defluviitalea saccharophila*^{T}	*Natranaerovirga pectinivorga*^{T}
Gram stain	-	+	**+**
Optimum temperature (°C)	30-35	50-55	43
Optimum pH	6.5-7.5	7-7.5	9.5-9.7
Optimum NaCl (%)	2-3	0.5	2
G + C content (mol%)	31.2	35.2	30.7
Main substrates used	Carbohydrates	Carbohydrates	Galacturonic acid and pectin
Cellobiose,	+	+	**-**
arabinose,	+	-	**-**
raffinose	+	-	**-**
glucose,	+	+	**-**
mannose,	+	+	**-**
maltose,	+	+	**-**
mannitol,	+	+	**-**
saccharose,	+	+	
xylose,	+	+	**-**
pyruvate	+	-	**-**
End products of fermentation	acetate	formate, acetate, butyrate, isobutyrate	acetate, formate
Polar lipids	PG, DPG, PL, GL	PG, DPG, PL, GL, PGL	PG, DPG, PL, GL, APL

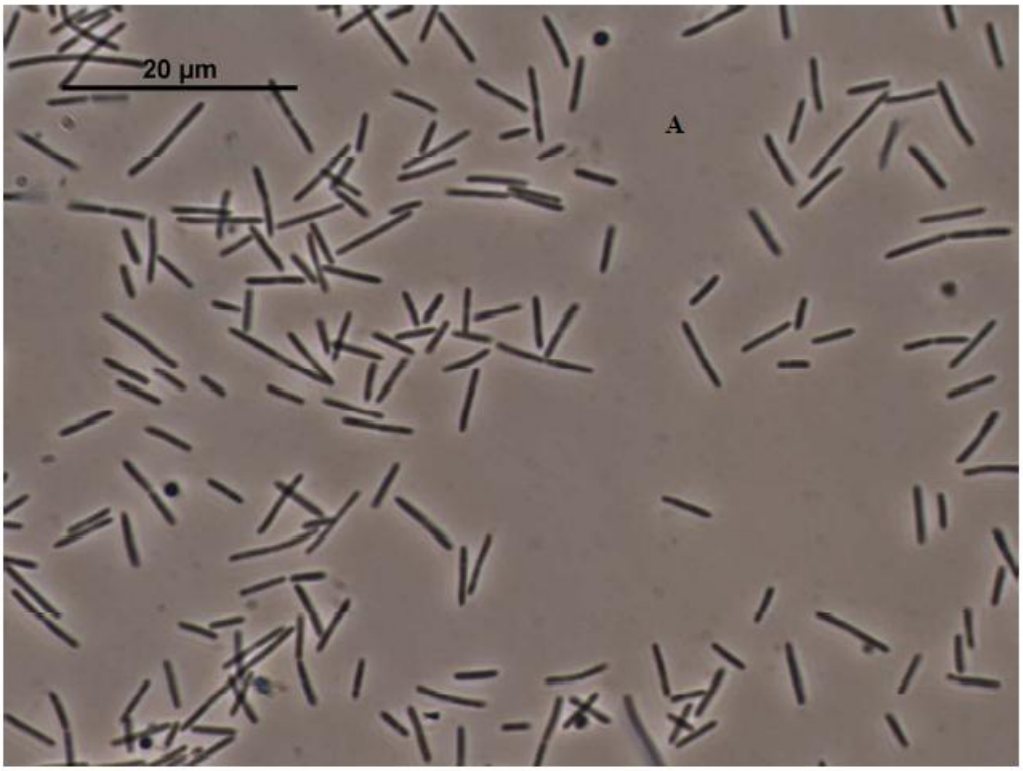
Table 2. Cellular fatty acid profile (%) of strains Ra1766G1^T , *Defluviitalea saccharophila*^{T}, and *Natranaerovirga pectinivorga*^{T}
Fatty acids amounting to less than 1% are not shown.
The summed feature 13 consisted of $C_{15:0}$ anteiso DMA.

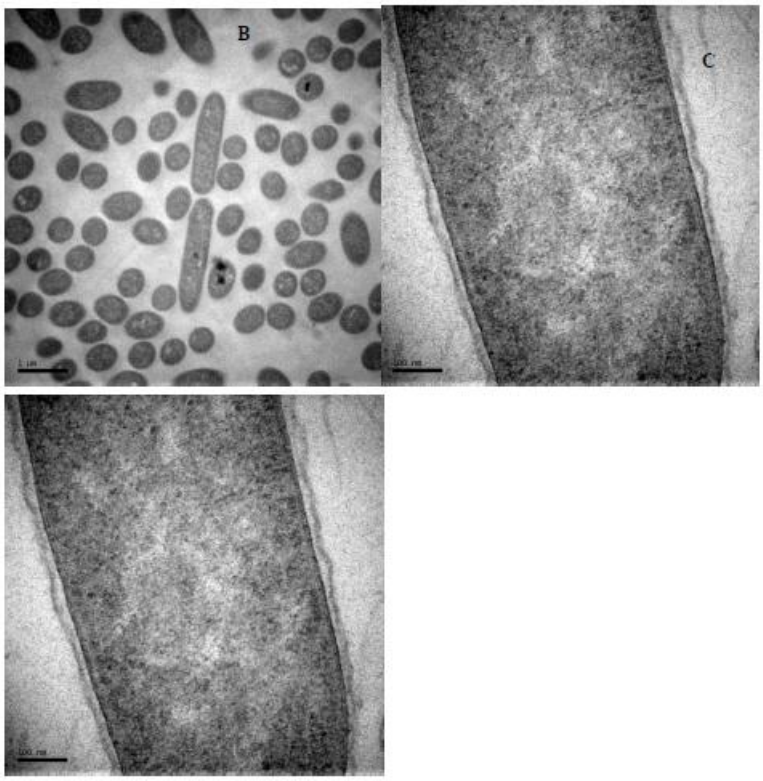
Cellular fatty acid	Strain Ra1766G1^T	*Defluviitalea saccharophila*^{T}	*Natranaerovirga pectinivorga*^{T}
$C_{13:0\ iso}$ 3 OH	1.5		
$C_{14:0}$ DMA	2.2		
$C_{14:0\ iso}$	2.7		
$C_{14:0}$	2.6	8.3	-
$C_{15:0}$ iso DMA	10.0		
$C_{15:0}$ anteiso	22.7		
$C_{16:0}$	7.0	68.4	46.9
$C_{16:0}$ iso	2.4		
Sum in feature 13	10.8		
$C_{18:0}$		7.3	
$C_{16:1}$ cis9		5.3	
C18:1 w7c		4.1	17.5
$C_{16:0\ DMA}$	5.5		
$C_{15:0}$ iso	13.7		

Supplementary Figure 1. Sediment sampling at the Southern trough site, on the 29 of June 2010, at the Guaymas basin, by the submersible *Nautile*. Arrow: white mats at the sediments surface.



Supplementary Figure 2. Cell morphology of strain Ra1766G1^T. A, phase-contrast micrograph (bar, 20 µm); B (bar, 1 µm) and C (bar, 0.2 µm): thin-section electron micrographs showing the Gram-negative type of cell wall.





Supplementary Figure 3. Total polar lipids of strain Ra1766G1^T after two-dimensional TLC and detection with molybdatophosphoric acid and heating at 200 °C for 10 minutes. DPG, diphosphatidylglycerol, PG phosphatidylglycerol, PL1-4, phospholipids, GL1-2, glycolipids, PGL phosphoglycolipids, APL aminophospholipids.