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Novel haloalkaliphilic methanotrophic bacteria: An attempt for enhancing methane bio-refinery

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Highlights

1. First proof of concept of CH_4 bioconversion into ectoine and hydroxyectoine.
2. The haloalkaliphilic consortium used efficiently converted CH_4 into ectoines
3. Two novel species capable of using CH_4 as the sole carbon source were isolated.
4. *Halomonas* sp. strain PGE1 accumulated high ectoine yields using CH_4.
5. This article boosts the competitiveness of industrial CH_4-based ectoine production.

Novel haloalkaliphilic methanotrophic bacteria: An attempt for enhancing methane bio-refinery

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Abstract

Methane bioconversion into products with a high market value, such as ectoine or hydroxyectoine, can be optimized via isolation of more efficient novel methanotrophic bacteria. The research here presented focused on the enrichment of methanotrophic consortia able to co-produce different ectoines during CH_4 biodegradation. Four different enrichments (Cow3, Slu3, Cow6 and Slu6) were carried out in basal media supplemented with 3 and 6 % NaCl, and using methane as the sole carbon and energy source. The highest ectoine accumulation (~20 mg ectoine g biomass^{-1}) was recorded in the two consortia enriched at 6 % NaCl (Cow6 and Slu6). Moreover, hydroxyectoine was detected for the first time using methane as a feedstock in Cow6 and Slu6 (~5 mg g biomass^{-1}). The majority of the haloalkaliphilic bacteria identified by 16S rRNA community profiling in both consortia have not been previously described as methanotrophs. From these enrichments, two novel strains (representing novel species) capable of using methane as the sole carbon and energy source were isolated: *Alishewanella* sp. strain RM1 and *Halomonas* sp. strain PGE1. *Halomonas* sp. strain PGE1 showed higher ectoine yields (70 - 92 mg ectoine g biomass^{-1}) than those previously described for other methanotrophs under continuous cultivation mode (~37 - 70 mg ectoine g biomass^{-1}). The results here obtained highlight the potential of isolating novel methanotrophs in order to boost the competitiveness of industrial CH_4-based ectoine production.

Keywords: *Alishewanella*, CH_4 biorefinery, Ectoine, *Halomonas*, Methane treatment.

1. Introduction

Methanotrophic bacteria are receiving an increasing scientific and industrial interest due to their ability to cost-effectively convert methane (CH_4), the second most important greenhouse gas (GHG), into less harmful products (Hanson and Hanson, 1996) or even into products with a high profit margin (Khmelenina et al., 2015; Strong et al., 2016). In recent years, methanotrophic bacteria have been used as a microbial platform for the production of bioplastics, single cell proteins (SCP) and lipids. Companies such as Mango Materials, Calista Inc. and UniBio A/S, VTT Ltd. have started to distribute commercial SCP for animal feeding as well as polyhydroxyalkanoates for bioplastic production using methane as a feedstock (Petersen et al., 2017; Pieja et al., 2017; Ritala et al., 2017). However, there is still a large portfolio of methane-based bioproducts unexploited, ectoines (hydroxyectoine and ectoine) being undoubtedly one of the most profitable commercial bioproducts synthesized by methanotrophs (Cantera et al., 2016b).

Ectoines provide osmotic balance to a wide number of halotolerant and halophilic bacteria (Lang et al., 2011; Pastor et al., 2010; Strong et al., 2016). Due to their high effectiveness as stabilizers of enzymes, DNA-protein complexes and nucleic acids, ectoines are used in medicine, cosmetology, dermatology and nutrition (Poli et al., 2017). Hydroxyectoine, despite being almost chemically identical to ectoine, is considered a more powerful bioprotectant due to its key role in heat stress protection (Pastor et al., 2010). In this regard, ectoines currently retail in the pharmaceutical industry at approximately US$1000 kg^{-1} and account for a global consumption of 15000 tones $year^{-1}$ (Strong et al., 2016).

Since 1997, some species of the genus *Methylomicrobium* (i.e. *M. alcaliphilum, M. buryatense, M. kenyense* or *M. japanense*), as well as *Methylobacter marinus* and *Methylohalobius cremeensis,* have been shown to synthesize ectoine during CH_4 biodegradation (Goraj and Stępniewska, 2016; Reshetnikov et al., 2011; Stępniewska et al., 2014). Based on its higher accumulation capacity in both batch and continuous bioreactors, *M. alcaliphilum* has been the most studied species (Cantera et al., 2017a, 2016b). However, up to date, hydroxyectoine accumulation by methanotrophs has never been reported, and ectoine productivities in this species cannot compete with those from heterotrophic bacteria such as *Halomonas elongate* and *Halomonas salina.* These *Halomonas* spp. achieved

productivities ranging from 5.3 to 7.9 g ectoine L^{-1} day^{-1}, while methanotrophic bacteria achieved productivities in the range of 7.5 to 9.4 mg ectoine L^{-1} day^{-1} (Pastor et al., 2010; Salar-García et al., 2017). Moreover, the halophilic methanotrophs discovered to date are sensitive to mechanical stress, which entails the need for low agitation rates in the bioreactor, thus hampering the mass transfer of CH_4 to the microbial community (Cantera et al., 2016c).

The aim of this research was to enrich, isolate and identify novel methanotrophic microorganisms able to produce high quantities of ectoine and hydroxyectoine combined with high methane removal rates in order to tackle with the current mentioned limitations in the biological CH_4-based ectoine production.

2. Materials and Methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) used during the enrichment and isolation of haloalkaliphilic methanotrophs presented a high pH (9.0) and high-salt content according to a previous isolation medium of methane oxidizing bacteria from soda lakes (Kalyuzhnaya et al., 2008). During culture enrichments NaCl was added to the MSM up to 3 and 6 %, while 1.5 % (g/v) agar was added to the MSM medium for solid media preparation. All chemicals and reagents were obtained from PANREAC S.A (Barcelona, Spain) with a purity higher than 99.0 %. CH_4 (purity of at least 99.5 %) was purchased from Abello-Linde, S.A (Barcelona, Spain).

2.2 Microorganisms and enrichment procedure

Fresh settled activated sludge (≈ 6 g dry weight L^{-1}) from a denitrification-nitrification wastewater treatment plant with seawater intrusion (Cantabria, Spain) and fresh cow manure and soil from a dairy farm on the coastline of Cantabria (Spain) were used as inoculum for the enrichment of halophilic methanotrophs. The cow manure and soil were dissolved in 10 mL of MSM to a final concentration of 10 g dry weight L^{-1}. Culture enrichment was carried in four gas-tight glass bottles (1.2 L) containing 190 mL of MSM at the two concentrations of salt tested (3 and 6 % NaCl). The bottles were closed with butyl septa and plastic screw caps. CH_4 was injected to obtain a CH_4 headspace concentration of 55 ±7 g CH_4 m^{-3}. After sterilization, two of the bottles with different salinities were inoculated with 10 mL of the

cow manure and soil mixture solution (Cow3 and Cow 6), and the other two bottles were inoculated with 10 mL of activated raw sludge (Slu3 and Slu6). The enrichments were transferred 7 times to fresh medium bottles upon CH_4 depletion using 10 % inoculum aliquots. The magnetic agitation rate was set in 600 rpm and the temperature used was 25º C. The CO_2 and CH_4 headspace concentrations were daily monitored along with ectoines and biomass concentration (estimated from optical density), which were measured by drawing 5 mL of cultivation broth in the last enrichment cycle.

2.3 Enrichments community analysis

Aliquots of 5 mL of cultivation broth from the last enrichments were centrifuged at 9000 g for 10 min. The resting pellet was used for DNA extraction with the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's procedure. DNA was quantified with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). DNA was added as template at a final concentration of 10−20 ng μL^{-1} for PCR amplification. PCR was carried out as described by Feng et al. (2017) for both bacterial and archaeal 16S rRNA genes. The purified PCR products were then cloned into *Escherichia coli* XL1-Blue Competent Cells (Agilent Technologies, Santa Clara, CA) by using the p-GEM Easy Vector Systems (Promega, Madison, WI). Sanger sequencing was performed by GATC Biotech (Konstanz, Germany) using SP6 (5-ATTTAGGTGACACTATAGAA-3) as the sequencing primer (Florentino et al., 2015). Vector contamination was removed from the sequences with the DNA Baser software (version 4.20.0. Heracle BioSoft SRL, Pitesti, Romania). Sequences were then aligned with SINA and merged using the Silva SSU Ref database (Heracle BioSoft, 2013; Pruesse et al., 2012; Quast et al., 2013). Phylogenetic trees were constructed in the ARB software package (v. 6) by using the maximum likelihood, neighbor-joining and maximum parsimony algorithms as implemented in the ARB package (Ludwig, 2004). Sequences were deposited in NCBI under the accession numbers MG956950 - MG957094. Finally, metabolic analyses were carried out with the KEGG and the Biocyc databases of metabolic pathways (Caspi et al., 2016; Kanehisa et al., 2016) using the available genomes of the platform.

2.4 Isolation and identification of novel haloalkaliphilic methanotrophs

Aliquots of 100 µL of each enriched culture were spread onto petri plates (5 replicates) and incubated in separate aerobic jars pressurized under an air/CH_4 (80:20, v/v) headspace at 30 °C until colony growth was observed. A total of 400 individual colonies were transferred to sterile hungate tubes filled with 10 mL of MSM under an air/CH_4 (80:20, v/v) headspace. Tubes where growth was observed were selected for further isolation by consecutive streaking on agar plates. Amplification and sequencing of the 16S rDNA gene was carried out with the primers 27-F (5- AGAGTTTGATCCTGGCTCAG-3) and 1492-R (5- GYTACCTTGTTACGACTT- 3) for each isolate. A total of 15 methanotrophic isolates belonging to three different genera were obtained and phylogenetically identified. The 16S rRNA gene sequences from the isolates were deposited in the NCBI database under accession numbers MH042736-50. Two of those isolates were selected for further characterization based on their novelty and ability of use methane as the sole carbon and energy source. The 16S rRNA gene sequences from the isolates were deposited in the NCBI database under the accession numbers MG958593-MG958594 (Table 1).

2.5 Physiological Tests.

Substrate utilization of both strains was tested using *API 50 CH* and *API 20 A* kits (bioMerieux SA, Lyon, France) according to the manufacturer's procedure. *API 50 CH* is a standardized kit that involves 50 biochemical tests for the study of the microbial carbohydrate metabolism. On the other hand, the *API 20 A* kit enables 21 tests to be carried out quickly and easily for the biochemical identification of anaerobes. The optimum temperature, pH and NaCl concentration of the isolates was assessed in the ranges 20-37 °C, 6-11 and 0-12 %, respectively, in serum bottles containing 50 ml of MSM under an air:CH_4 (80:20,v/v) headspace. The analyses were carried out in triplicate and the results provided as the average and standard deviation.

2.6 Analytical procedures

The concentration of intra-cellular ectoine and hydroxyectoine was determined using 2 mL of cultivation broth according to Cantera et al. 2016b (Cantera et al., 2016b; Tanimura et al., 2013). The specific intra-cellular concentration (mg ectoine/hydroxyectoine g biomass^{-1}) was calculated using the total suspended solids (TSS) values (g L^{-1}) of the corresponding cultivation broth. The measurement was carried out by high performance liquid

chromatography in a HPLC 717 plus auto-sampler (Waters, Bellefonte, USA) coupled with a UV Dual λ Absorbance detector (Waters, Bellefonte, USA detector) at 210 nm and 40 ºC using a LC-18 AQ + C Supelcosil column (Waters, Bellefonte, EEUU) and a C18 AQ + pre-column (Waters, Bellefonte, EEUU). A phosphate buffer consisting of 0.8 mM K_2HPO_4 and 6.0 mM Na_2HPO_4 at a pH of 7.6 was used as a mobile phase at 40 ºC and a flow rate of 1 mL min^{-1} (Tanimura et al., 2013). Ectoine and hydroxyectoine quantification was carried out using external standards of commercially available ectoine ((S)-b-2-methyl-1,4,5,6-tetrahydro-pyrimidine-4-carboxylic acid, purity 95 %, Sigma Aldrich, Spain) and hydroxyectoine (4S,5S)-5-Hydroxy-2-methyl-1,4,5,6-tetrahydropyrimidine-4 carboxylic acid, purity 95 %, Sigma Aldrich, Spain). The detection and quantification limits (DL and QL) were calculated via determination of the signal-to-noise ratio performed by comparing the measured signals from samples with known low concentrations of the analyte with those of blank samples (ICH, 2005).

CH_4 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A (15 m × 0.53 µm × 15 µm) and a CP-PoraBOND Q (25 m × 0.53 µm × 10 µm) column. The oven, injector and detector temperatures were maintained at 45 ºC, 150 ºC and 200 ºC, respectively. Helium was used as the carrier gas at 13.7 mL min^{-1}.

Microbial growth was estimated from culture absorbance measurements at 650 nm using a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). TSS concentration was measured according to standard methods (American Water Works Association, 2012).

3. Results and discussion

3.1 Enrichment of halotolerant methanotrophic bacteria

CH_4 degradation in the enrichments using fresh sludge and manure-soil at a NaCl concentration of 6 % (Slu6 and Cow6) was characterized by a longer lag phase compared to Slu3 and Cow3. However, the degradation rates obtained in the exponential phase were similar regardless of the salinity (1.08, 1.09, 1.10 and 1.12 g CH_4 m^{-3} h^{-1} in Cow3, Slu3,

Cow6 and Slu6, respectively). The maximum ectoine production was observed on the first days of growth (2-4) in both saline conditions (Figure 1).

<Figure 1>

Maximum values of 9.1 ± 0.1 mg ectoine g biomass^{-1} were found when using the manure-soil inoculum at 3 % NaCl (Cow3), similar to those obtained (8.8 ± 0.0 mg ectoine g biomass^{-1}) when using fresh activated sludge at a similar salinity (Slu3). The enrichments at 6 % NaCl showed concentrations of ectoine 2× times higher than at 3 % NaCl: 19.9 ± 0.3 and 20.9 ± 0.4 mg ectoine g biomass^{-1} in Slu6 and Cow6, respectively. Moreover, 6 % NaCl promoted the accumulation of maximum hydroxyectoine concentrations of 4.3 ± 0.7 mg and 4.5 ± 0.1 mg hydroxyectoine g biomass^{-1} in Slu6 and Cow6, respectively. In the case of 3 % NaCl, hydroxyectoine was not detected regardless of the inocula tested.

Although the ectoine yields obtained with the methanotrophic consortia enriched in this study were lower than the ones obtained with the pure strain *M. alcaliphilum* 20 Z (30.4-66.9 mg ectoine g biomass^{-1}) (Cantera et al., 2017a, 2016b; Khmelenina et al., 2015), these consortia were also able to synthesize hydroxyectoine. To the best of our knowledge, no hydroxyectoine accumulation has been detected to date using methanotrophs grown at salinities ranging from 0 up to 9 % NaCl (Khmelenina et al., 2015; Reshetnikov et al., 2011). This compatible solute is more commonly produced by Gram-positive halophilic/halotolerant bacteria such as *Marinococcus* M52 (134.8 mg hydroxyectoine g biomass^{-1}), but it is often synthesized at lower concentrations together with ectoine in many other ectoine-producing species, such as *Halomonas boliviensis* DSM 15516 (36.8 mg hydroxyectoine g biomass^{-1}) and *Halomonas elongata* KS3 (45 mg hydroxyectoine g biomass^{-1}) (Fallet et al., 2010; Salar-García et al., 2017; Van-Thuoc et al., 2010). Moreover, the use of a consortium instead of a pure strain could be beneficial for industrial ectoines production. In this context, *M. alcaliphilum* 20 Z typically achieves low average biomass concentrations (1.0 g L^{-1}), which results in ectoine productivities and CH_4 removal efficiencies 4 times lower than the ones obtained with other methanotrophic bacteria (CH_4 removal efficiencies of 43 to 67 %) (Cantera et al., 2017b, 2016a). Furthermore, the purer a culture is, the higher are the restrictions to grow under environmental stress factors, while a higher population richness and diversity promotes higher resilience and therefore, better reactor performance and bio-product recovery (Cabrol et al., 2012). Despite this was, to the best of our knowledge, the

first attempt to enrich for ectoine producers, adaptive laboratory evolution could be also carried out to gain insights into the adaptive changes that experience microbial populations during long term selection: better ectoines productivities and higher methane degradation rates (Dragosits and Mattanovich, 2013). In this context, the methane abatement combined with the co-production of hydroxyectoine and ectoine by mixed methanotrophic communities reported in this study opened up new opportunities for the development of novel and more profitable methane biorefineries based on extremophile methanotrophic consortia.

The microbial community analysis of the 4 enrichments yielded a total of 362 16S rDNA bacterial gene sequences, from which 144 passed the NCBI quality control with an average length of 1380 nucleotides. The individual phylotypes were clustered (identity criteria of 0.97) mainly into 5 known phyla: *Proteobacteria*, *Bacteroidetes*, *Spirochaetes*, *Firmicutes* and *Planctomycetes*. No archaeal communities were detected in any of the enrichments. At the genus level, the sequences clustered into 14 genera (Figure 2). About 6.7 % of all the sequences could not be identified at the genus level and were classified at the next highest possible resolution level. Some of the sequences analyzed belonged to known methane oxidizers such as *Methylomicrobium* spp. (36.8, 16.3, 2.3 and 4.7 % in Cow3, Slu3 Cow6 and Slu6, respectively), while others were identified as *Methylophaga* (26.3, 60.5 2.7 and 3.8 % in Cow3, Slu3 Cow6 and Slu6, respectively), which are methylotrophic bacteria able to oxidize methanol, monomethylamines, dimethylsulfides, etc.

<Figure 2>

Overall, the structure of the microbial population enriched was more determined by the salinity used during enrichment than by the source of inoculum, likely due to the highly selective conditions imposed by a high salinity and high pH. Thus, methane metabolism related microorganisms were the most abundant at 3 % NaCl regardless of the inoculum source. Marine chemoorganotrophic microorganisms such as *Marinobacter* (15.7 and 4.0 % in Cow3 and Slu3, respectively) and *Wandonia* (4.6 and 4.6 % in Cow3 and Slu3, respectively) were detected, as well as other ubiquitous bacteria belonging to the orders *Clostridiales, Sphirochaetales* and *Alteromonadales* (representing up to 10.9 and 5.2 % of the total bacteria in Cow3 and Slu3, respectively).

On the other hand, the majority of microorganisms identified at 6 % NaCl belonged to *Proteobacteria* such as *Xanthomas* spp. (77.8 and 34.2 % in Cow6 and Slu6, respectively), *Brevundimonas* spp. (31.8 % in Slu6), *Pannonibacter* spp. (4.6 % in Cow6) and *Caulobacter* spp. (2.8 and 11.5 % in Cow6 and Slu6, respectively). It has been previously observed that non-methanotrophic methylotrophs and oligotrophic heterotrophic bacteria such as *Brevundimonas, Pannonibacter* or *Caulobacter* are present on primary isolation agar plates when enriching methanotrophic bacteria (Butterbach-Bahl et al., 2011). Although some species of the genera *Brevundimonas, Pannonibacter*, *Caulobacter* and *Xanthomonas* are identified as haloalkalotolerant aerobic bacteria, to the best of our knowledge, none of them have been described as methanotrophs. Moreover, no proteins related to methane metabolism are present in the genomes of the mentioned genera according to KEGG (Kanehisa et al., 2016) and Biocyc (Caspi et al., 2016) database resources. Some known ectoine producers such as *Methylomicrobium* (2.3 and 4.7 % in Cow6 and Slu6, respectively), the aforementioned methylotrophic *Methylophaga* (2.7 and 3.8 % in Cow6 and Slu6, respectively) and some marine microorganisms belonging to the orders *Oceanospirillales* (14.8 and 13.4 % in Cow6 and Slu6, respectively) were identified at 6 % NaCl.

3.2 Isolation of new haloalkaliphilic strains

Following the streaking procedure on agar plates, a total of 15 methanotrophic isolates were obtained and phylogenetically identified (Table 1).

<Table 1>

All of the isolates obtained belonged to the *Proteobacteria* phylum (more specifically to the *Gamma-proteobacteria* class), while the original enrichments were composed of the phyla *Planctomycetes, Bacteroidetes, Proteobacteria, Firmicutes* and *S*pirochaetes (Figure 3). Within the isolates, 66.7 % were affiliated with *Methylomicrobium alcaliphilum* 20Z (99 % similarity). However, two other cluster of isolates belonged to the genera *Alishewanella* and *Halomonas,* from which, one isolate was chosen per genera. The isolate affiliated with *Alishewanella* sp., designated as strain RM1 (MG958594), was isolated from the enrichment Slu3 at pH 9. The closest phylogenetic relatives were *A. aestuari* (Roh et al., 2009) (98 % 16S rRNA gene similarity) and *A. agri* (Kim et al., 2010) (98 % 16S rRNA gene similarity) isolated from a tidal flat soil and a landfill cover soil, respectively (Figure 3). The other phylotype found, isolated from enrichment Slu6, was designated as *Halomonas* sp. strain

PGE1 (MG958593). This strain was related with *H. ventosae* and *H. salina* with 98 % 16S rRNA gene similarity on both cases, both species isolated from hypersaline soils (Figure 3). According to the species definition of stablishing the cut-off for new species in 98.7 % of 16S rRNA gene sequence identity (Yarza et al., 2014), *Alishewanella sp.* strain RM1 and *Halomonas* sp. strain PGE1 represent new species.

<Figure 3>

***Alishewanella sp.* strain RM1**

The genus *Alishewanella* was described for the first time by Vogel et al. (2000) (Vogel et al., 2000) and emended independently by Roh et al. (2009), Kim et al. (2010) and Sisinthy et al. (2017)(Kim et al., 2010; Roh et al., 2009; Sisinthy et al., 2017; Vogel et al., 2000). It belongs to the family *Alteromonadaceae* order *Alteromonadales* of the class *Gammaproteobacteria* (Xia et al., 2016). According to Sisinthy et al. (2017), *Alishewanella* genus contains a total of 8 species: *A. aestuarii*, *A. agri*, *A. fetalis*, *A. jeotgali*, *A. solinquinati*, *A. tabrizica*, *A. longhuensis* and *A. alkalitolerans* (Sisinthy et al., 2017). This genus is widely distributed and appears in sediments, saline environments or fresh water. Cells stain Gram-negative. Cells of *Alishewanella sp.* strain RM1 are coccoid-shaped occurring either singly or in pairs (Table 2). Growth of strain RM1 in CH_4 occurred at temperatures ranging from 20 to 37 °C, with an optimum at 30°C. The described members of this genus are mesophyles, with a temperature growth range of 4 - 44 °C (Kim et al., 2010; Roh et al., 2009). Growth of *Alishewanella* sp. strain RM1 in CH_4 occurred at pHs ranging from 7 to 9, with an optimum growth at pH 8. *Alishewanella* spp. typically has an optimum pH in the range of 6.0-8.0, although they are able to grow in broader pH ranges (i.e. 5.5 to 12) (Kim et al., 2010; Roh et al., 2009) (Table 2). On the other hand, the optimum salt concentration was 3 % NaCl, although the strain was able to grow in the range of 0 to 6 %. The ability of Allishewanella to grow in the presence of high concentrations of NaCl is species specific. For instance *A. fetalis* can grow at 8 % NaCl, *A. agri* at 6 % NaCl, *A. alkalitolerants* at 3 % NaCl), while *A. aestuarii* is not able to grow at NaCl concentrations > 1 % NaCl (Kim et al., 2010; Roh et al., 2009; Sisinthy et al., 2017; Vogel et al., 2000).

Alishewanella sp. strain RM1 is able to use glycerol, erytritol, L-Arabinose, D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, esculin, cellobiose, raffinose and glycogen as the sole carbon and energy source. No growth was detected under anaerobic

conditions. A phenotypic comparison with their closest relatives is shown in Table 2. The ability of *Alishewanella sp.* strain RM1 to use CH_4 as the sole carbon and energy source has not been reported for members of the *Alishewanella* genus. Specific CH_4 biodegradation rates during the exponential growth phase under optimal growth conditions (pH 8, 3 % NaCl and 25 ºC) were 24.2 ± 3.1 mg CH_4 g biomass^{-1} h^{-1}, while CO_2 production rates accounted for 10 ± 0.5 mg CO_2 g biomass^{-1} h^{-1}. These values are similar to the ones described for other pure methanotrophic strains such as *Methylomicrobium alcaliphilum* 20Z (Cantera et al., 2016b). The *Alishewanella* genus is constituted by versatile microorganisms, known for their heterotrophic aerobic and anaerobic respiratory metabolism of a broad range of substrates (Sisinthy et al., 2017), which makes feasible their ability to grow on methane as the sole carbon and energy source (Vogel et al., 2000). Ectoine or hydroxyectoine production by *Alishewanella sp*. strain RM1 was not observed regardless of the condition tested, although a wide range of halotolerant bacteria including other genera within the same order (e.g *Marinobacter aquaeolei* and *Marinobacter hidrocarbonoclasticus*) (86-85 % 16S rRNA gene similarity to *Alishewanella sp*. strain RM1) have been found to produce ectoine in the presence of salt (Pastor et al., 2010). In this regard, it is probable that *Alishewanella sp.* strain RM1 accumulates low molecular weight osmolites different from ectoine such as polyols, sugars, amino acids or betaines to survive at high salt concentrations (Roberts, 2005).

<Table 2>

***Halomonas sp.* strain PGE1**

The family *Halomonadaceae,* which belongs to the *Oceanospirillales* order within the *Gammaproteobacteria* phylum, contains 13 genera (Vahed et al., 2018). *Halomonas* was defined as a genus in 1980 and includes ~100 different validly defined species (Vahed et al., 2018; Vreeland et al., 1980). *Halomonas* cells stain Gram-negative and are able to grow in a wide range of salt concentrations (0 to 25 % NaCl) and in multiple habitats. *Halomonas* spp. perform aerobic and anaerobic respiration, and they are able to use a great range of carbon sources (Mata et al., 2002; Tan et al., 2014; Vahed et al., 2018). *Halomonas* sp. strain PGE1 cells are rod-shaped and moderately halophilic. Indeed, this strain is able of growing at salt concentrations of 3–10 % NaCl (w/v), whereas no growth occurs at 1 % NaCl (Table 3). Strain PGE1 is an aerobic microorganism capable of growing in CH_4 within the temperature range 20–37 °C and at pH values between 6 and 11. *Halomonas* sp. strain PGE1 can use esculin, D-glucose, melibiose, raffinose and glycogen as the sole carbon and energy source. A phenotypic comparison with their closest relatives is shown in table 3.

<Table 3>

Despite no member of *Halomonas* genus has been reported as a methane oxidizer, members of this genus possess a highly versatile metabolism and are typically found in methane rich environments such as hydrocarbon reservoirs, methane seeps or methane rich sediments ((Niederberger et al., 2010; Piceno et al., 2014)). Under optimal growth conditions (pH 9, 6 % NaCl and 30 ºC), maximum specific methane removal rates of 25.3 ± 1.2 mg CH_4 g biomass^{-1} h^{-1} were achieved during the exponential growth phase, values similar to those recorded in other methanotrophic bacteria (Cantera et al., 2016; Gebert et al., 2003). However, methane degradation and oxygen consumption stopped by day 12. This inhibition on methanotrophic activity was likely due to the accumulation of a toxic intermediate of the methane oxidation pathway such as formic acid or methanol, as described in other methanotrophic bacteria (Hou et al., 1979; Strong et al., 2015). Indeed, the growth of the strain and CH_4 degradation resumed following medium replacement.

Hydroxyectoine production by *Halomonas sp.* strain PGE1 was not observed in the range of tested conditions. However, ectoine yields of 70.2-91.7 mg ectoine g biomass^{-1} were achieved when *Halomonas sp.* strain PGE1 was cultivated at 6 % of NaCl and pH 8, 9 and 10. A large number of *Halomonas* species are able to produce ectoine. Of them, *H. elongate* strain IH15 ^{T} is the main strain used in industry for ectoine production via a fed-batch sugar fermentation process called *biomilking* (total duration ~120 h)(Pastor et al., 2010; Sauer and Galinski, 1998). However, this process is costly due to the high quality of the substrates required as a carbon feedstock (Kunte et al., 2014; Lang et al., 2011; Pastor et al., 2010). Other *Halomonas* species such as *H. salina* and *H. boliviensis* are able to synthesise high quantities of ectoine and excrete it naturally to the cultivation broth, thus supporting a more efficient and less costly biotechnological process. In this context, members of the genus *Halomonas* typically exhibit high ectoine production yields (170, 154 and 358 mg ectoine g biomass^{-1} in *H. boliviensis*, *H. elongate* and *H. salina*, respectively) and high biomass productivities (3.4, 9.1 and 7.9 g ectoine L^{-1} day^{-1} in *H. boliviensis*, *H. elongate* and *H. salina*, respectively). The ectoine yields obtained in this study by *Halomonas sp.* strain PGE1 are lower than those obtained by other *Halomonas* species likely due to the limited CH_4 mass transfer rates from the headspace (Chen et al., 2018). However, the ectoine yields here obtained are higher than those recorded in *M. alcaliphilum* 20Z under the similar cultivation conditions (30.4-66.9 mg g biomass^{-1}). Furthermore, *Halomonas sp.* strain PGE1 likely supported higher productivities

than *M. alcaliphilum* as a result of its superior resistance to shear stress and environmental stress. The enzyme used for the oxidation of methane could be different than methane monooxygenase, which typically limits CH_4 biodegradation due to the high requirements of reducing power (in the form of $NADPH^+$) to activate the otherwise inert methane molecule (James C. Liao, Luo Mi, 2016). However, the use of *Halomonas sp.* strain PGE1 for industrial ectoine production will be eventually limited by the production of a secondary metabolite toxic for the bacterial growth. In this context, the use of continuous high-mass transfer bioreactors with biomass retention operated at high dilution rates could overcome the above mentioned limitation and boost the production of ectoine coupled to climate change mitigation.

4. Conclusions

New species of alkaliphilic and halophilic methanotrophic bacteria were enriched and isolated in this research with the aim of enhancing the cost-competitiveness of CH_4 bioconversion into ectoines. First, several consortia were enriched using methane as the sole carbon and energy source. The consortia enriched at 6 % NaCl were able to degrade methane efficiently and were able to co-produce, for the first time, both ectoine and hydroxyectoine using methane as the only feedstock. Furthermore, two novel species of haloalkaliphilic methanotrophic bacteria, *Halomonas sp.* strain PGE1 and *Alishewanella sp.* strain RM1, were isolated in this research, both of them from genera not considered to date able to use methane as energy and carbon source. From these strains, special attention should be given to *Halomonas sp.* strain PGE1 since it was able to degrade methane with higher ectoine yields than those reported for the already known ectoine producer methanotrophs.

5. Acknowledgements

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Figure

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Figure 1:

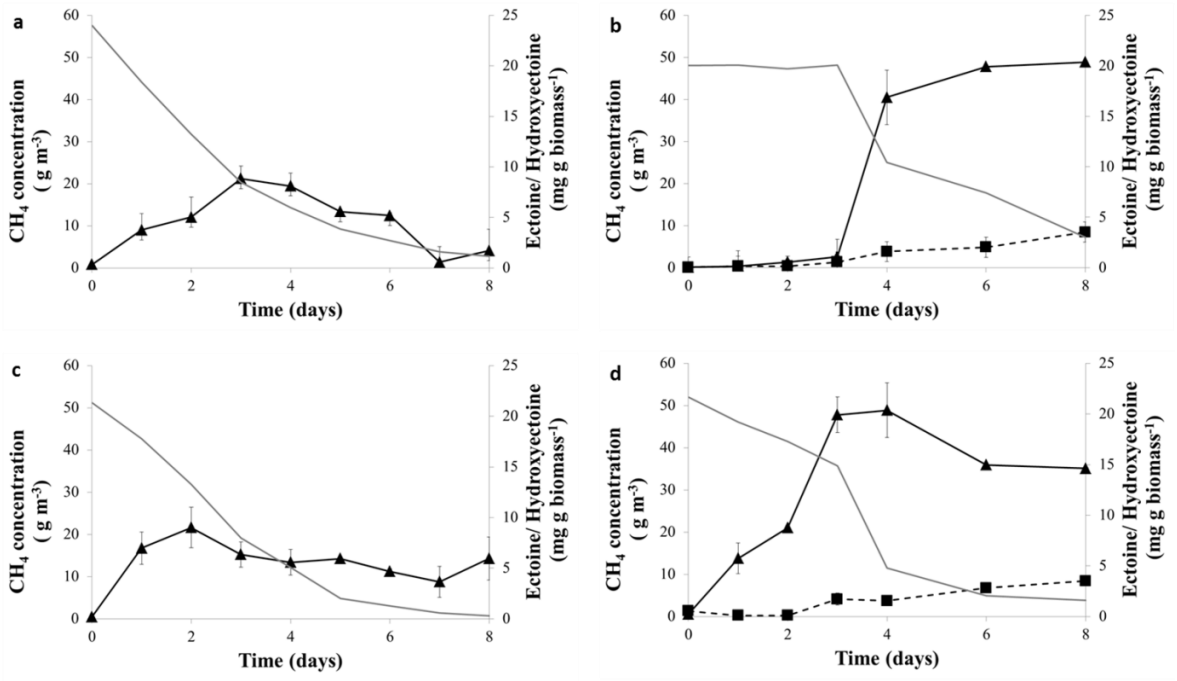

Figure 1. Time course of the concentration of CH_4 (continuous grey line), ectoine (▲) and hydroxyectoine (■) in the enrichment a) Cow3; b) Cow6; c) Slu3; d) Slu6 .

Figure 2:

TAXA	Cow3	SLu3	Cow6	Slu6	Phylum
Thiotricales (Methylophaga)	26.5	60.5	2.7	3.8	*Proteobacteria*
Methylococcales (Methylomicrobium)	36.8	16.3	2.3	4.7	
Chromatiales (Uncultured)	ND	2.1	ND	ND	
Alteromonadales (Marinobacter)	15.7	4	ND	ND	
Alteromonadales (Alishewanella)	ND	5.2	ND	ND	
Unclassified Bacterium	5.5	2.7	ND	ND	
Xanthomonadales(Xanthomonas)	ND	ND	77.8	34.2	
Caulobacterales (Caulobacter)	ND	ND	2.8	11.5	
Caulobacterales (Brevundimonas)	ND	ND	ND	31.8	
Rhodobacterales (Pannonibacter)	ND	ND	4.6	ND	
Oceanospirilales (Uncultured)	ND	ND	9.8	4.9	
Rhizobiales (Aliihoeflea)	ND	ND	ND	9.1	
Sphirochaetales (Sphirochaeta)	5.2	ND	ND	ND	*Spirochaetes*
Clostridiales(Uncultured)	5.7	ND	ND	ND	*Firmicutes*
Flavobacteriales (Wandonia)	4.6	4.6	ND	ND	*Bacteroidetes*
Planctomycetales (Planctomyces)	ND	4.6	ND	ND	*Planctomycetes*

Figure 2. Heat-map of the main orders and genera determined in the four enrichments. ND: Not detected.

Figure 3:

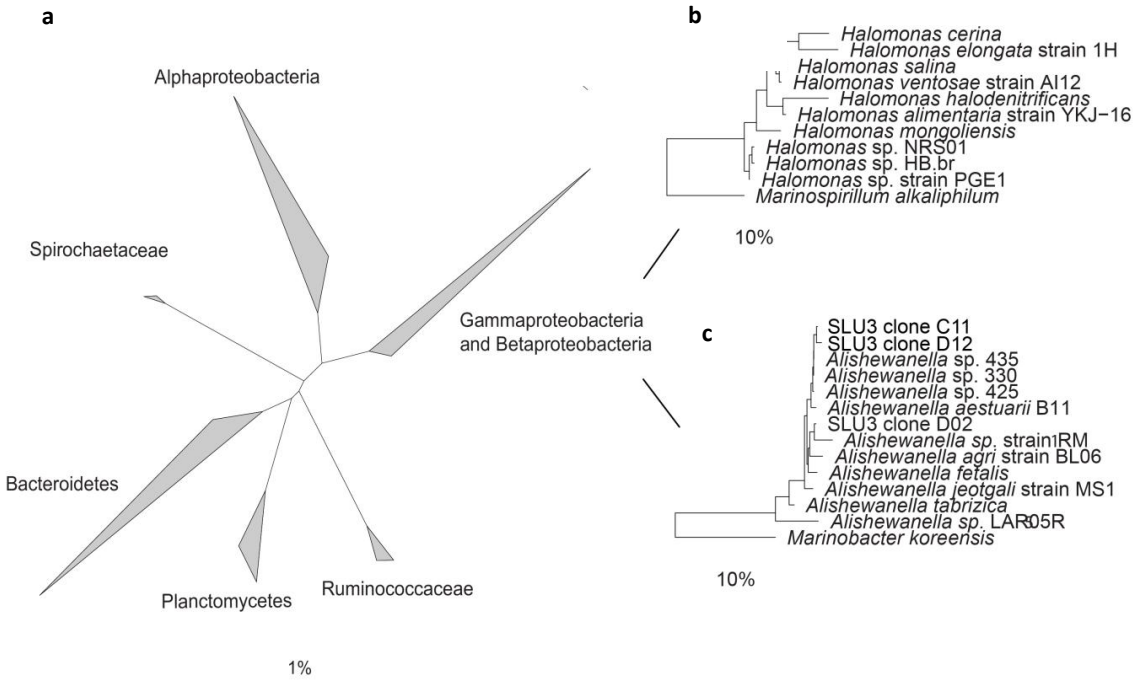

Figure 3. Phylogenetic affiliations of the 16S rDNA sequences obtained (a) in the four enrichments obtained, (b) from the isolate of *Halomonas* sp. strain PGE1 and its closest phylogenetic relatives and (c) *Alishewanella sp.* strain RM1 and its closest phylogenetic relatives. The trees display a consensus from neighbor-joining, maximum likelihood and maximum parsimony algorithms. Bars represent 10 (in b and c) changes per site or 100 % divergence in sequence.

Table

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Table 1. Characteristics of the 15 isolated strains and the two novel strains selected for further studies.

Phylogenetic affiliation	Identity (%)	Accession nº of closest organism	Abundance within the isolates (%)	Salinity of the source of inocula (% NaCl)	Accession number
Methylomicrobium alcaliphilum	99	NR_074649	66.7	3-6	MH042736-45
Alishewanella sp.	98	NR_116499	20.0	3	MH042746-48
Halomonas sp.	97	NR_042812	13.3	6	MH042749-50
*Alishewanella sp.*strain RM1	*98*	NR_116499	Selected for phenotype description	3	MG958594
Halomonas sp. strain PGE1	98	NR_042812	Selected for phenotype description	6	MG958593

Table 2: Phenotypic characteristics of strain RM1 and the closest phylogenetic relatives of *Alishewanella genus.*

Parameter	*Alishewanella* sp. strain RM1	*Alishewanella aestuarii* strain B11^T	*Alishewanella agri* strain BL06 ^{T}
Optimum:			
Temperature (ºC)	30	37	30
NaCl (%)	3	3	2
pH	8	NR	6-8
Ectoine production	-	NR	NR
Substrates			
CH_4	+	NR	NR
Glycerol	+	-	-
Erythritol	+	NR	-
L-Arabinose	+	-	-
D-ribose	-	NR	-
D-xylose	+	NR	-
L-xylose	-	NR	-
D-adonitol	-	NR	-
Methyl D-Xylopyranoside	-	NR	-
D-galactose	-	NR	-
D-glucose	+	-	+
D-fructose	+	+	-
D-mannose	+	-	-
L-sorbose	-	NR	-
L-rhamnose	+	NR	-
Dulcitol	-	NR	-
D-mannitol	+	-	-
D-sorbitol	-	NR	-
Methyl D-Mannopyranoside	-	NR	-
Methyl D-Xylopyranoside	-	NR	-
Amygdalin	-	NR	-
Arbutín	-	NR	-
Esculin	+	-	+
Salicin	-	NR	-
Cellobiose	+	NR	-
Maltose	-	+	+
D-lactose	-	NR	-
Melibiose	-	NR	-
Sucrose	-	NR	+
Insulin	-	NR	-
Melezitose	-	NR	-
Raffinose	+	-	-
Starch	-	-	+
Glycogen	+	-	-
Xylitol	-	NR	-
Gentiobiose	-	NR	-

Turanose	-	NR	-
D-lyxose	-	NR	-
D-tagatose	-	NR	-
D-fucose	-	NR	-
L-fucose	-	NR	-
D-Arabitol	-	NR	-
L-Arabitol	-	NR	-
2-ketogluconate	-	NR	-
5-ketogluconate	-	NR	-

Strains: 1 ***Alishewanella aestuarii***, otherwise indicated data from (Roh et al., 2009); and, ***Alishewanella agri***, otherwise indicated data from (Kim et al., 2010). + supported growth, - did not support growth, NR- not reported. All strains are rods, Gram-negative bacteria. None of the strains were capable of using D-Arabinose, myo-inositol, N-acetylglucosamine, trehalose and potassium gluconate.

Table 3: Phenotypic characteristics of the strain PGE1 and the closest *Halomonas* species.

Parameter	*Halomonas sp. strain* **PGE1**	*Halomonas ventosae* **AI12** ^{T}	*Halomonas elongate* **IH15** ^{T}
Optimum:			
Temperature (ºC)	30	15-50	30
NaCl (%)	6	6-9	1
pH	9	3-15	5-9
Substrates:			
CH_4	+	NR	NR
D-galactose	-	+	-
D-glucose	+	+	+
D-mannose	-	-	+
D-sorbitol	-	+	-
Esculin	+	-	+
Cellobiose	-	-	+
Maltose	-	+	-
D-lactose	-	-	+
Melibiose	+	-	-
Sucrose	-	-	+
Raffinose	+	-	-
Glycogen	+	-	-
Potassium Gluconate	-	+	+

Strains: 1 ***Halomonas ventosae*** **AI12** ^{T}, otherwise indicated data from (Martínez-Cánovas et al., 2004); and, 2. ***Halomonas elongate*** **IH15** ^{T}, otherwise indicated data from (Vreeland et al., 1980). + supported growth, - did not support growth, NR- not reported. Eg: All strains are rods, Gram-negative bacteria able to produce ectoine and use glucose as carbon source. None of the strains were capable of using erythritol, D-Arabinose, L-Arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl D-Xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, myo-inositol, D-mannitol, methyl D-mannopyranoside, methyl D-Xylopyranoside, N-acetylglucosamine, amygdalin, arbutín, salicin, trehalose, insulin, melezitose, starch, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-Arabitol, L-Arabitol, 2-ketogluconate, 5-ketogluconate.