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Phylogenetic diversity of methyl-coenzyme M reductase (*mcrA*) gene and methanogenesis from trimethylamine in hypersaline environments

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Summary. Methanogens have been reported in complex microbial communities from hypersaline environments, but little is known about their phylogenetic diversity. In this work, methane concentrations in environmental gas samples were determined while methane production rates were measured in microcosm experiments with competitive and non-competitive substrates. In addition, the phylogenetic diversity of methanogens in microbial mats from two geographical locations was analyzed: the well studied Guerrero Negro hypersaline ecosystem, and a site not previously investigated, namely Laguna San Ignacio, Baja California Sur, Mexico. Methanogenesis in these microbial mats was suspected based on the detection of methane (in the range of 0.00086 to 3.204 %) in environmental gas samples. Microcosm experiments confirmed methane production by the mats and demonstrated that it was promoted only by non-competitive substrates (trimethylamine and methanol), suggesting that methylotrophy is the main characteristic process by which these hypersaline microbial mats produce methane. Phylogenetic analysis of amino acid sequences of the methyl coenzyme-M reductase (*mcrA*) gene from natural and manipulated samples revealed various methylotrophic methanogens belonging exclusively to the family Methanosarcinaceae. Moderately halophilic microorganisms of the genus *Methanohalophilus* were predominant (>60 % of *mcrA* sequences retrieved). Slightly halophilic and marine microorganisms of the genera *Methanococcoides* and *Methanolobus*, respectively, were also identified, but in lower abundances. [**Int Microbiol** 2012; 15(1):33-41]

Keywords: Methanosarcinaceae · hypersaline environments · microbial mats · trimethylamine · gene *mcrA*

Introduction

Methane-producing anaerobes (methanogens) were first identified by Woese and Fox in 1977 [45] as being phylogenetically distinct from all other cell types, and they are the founding members of the Archaea Domain [11]. Methanobacteria comprise a large and diverse Class whose members are the main constituents of the Kingdom Euryarchaeota [46]. The five Orders recognized thus far (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, and Methanopyrales) have distinctive characteristics [11,24,44]; a novel Order of methanogens, Methanocellales, was proposed recently and is currently represented by a single strain [23,36]. Metha-

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nogenic archaea are vital for the anaerobic microbial degradation of organic waste; the resultant production of methane, a potent greenhouse gas, is valuable as a non-fossil fuel [29].

Methanogenic archaea obtain energy for growth by the oxidation of a limited number of substrates, with the concomitant production of methane gas. In freshwater aquatic environments, methanogens are quantitatively extremely important terminal oxidizers of organic matter. In marine and hypersaline environments, characterized by the presence of high concentrations of sulfate, sulfate-reducing bacteria, not methanogenic archaea, are the primary mediators of terminal anaerobic mineralization. Sulfate reducers [6] utilizing sulfate as terminal electron acceptor outcompete methanogens for CO_2/H_2, formate, and acetate. A limited number of other compounds such as methanol, monomethylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), and dimethylsulfide, and some alcohols, such as isopropanol, isobutanol, cyclopentanol, and ethanol, are also substrates for some methanogens [44]. Of these, MMA, DMA, TMA and dimethylsulfide are unavailable to sulfate reducers, and so are referred to as "non-competitive substrates." The relative lack of information on methanogens from hypersaline environments may stem, in part, from a belief that significant rates of methane production are unlikely to occur when sulfate concentrations exceed many tens of millimoles per liter (> 30 mM), as is commonly found in the brine of these environments [15].

Although sulfate-reducing organisms dominate anaerobic carbon consumption in marine microbial mats, methanogens persist and their activities vary both vertically and temporally in the mat system in response to non-competitive substrates such as TMA [32]. All methanogens that have been isolated to date from hypersaline environments use TMA as a catabolic substrate [44], and all the TMA-degrading methanogens from marine and hypersaline environments belong to the family Methanosarcinaceae.

Culture-dependent and culture-independent techniques targeting 16S rRNA and methyl coenzyme M reductase (*mcrA*) genes have been used to assess the phylogenetic diversity of methanogen assemblages [20,28,29,41]. Methane production has been studied most extensively in microbial mats from the salterns of Exportadora de Sal in Baja California Sur, Mexico. Incubation of the surface layers of microbial mats obtained near the photic zone predominantly yield *Methanolobus* spp., while *Methanococcoides* has been preferentially recovered from incubations of unconsolidated sediments underlying the mat. *Methanohalophilus* sequences in low abundances have been retrieved from samples of 20- to 60-mm depth [33]. Clone libraries from microbial mats maintained in field-like conditions in a long-term greenhouse study consist exclusively of sequences related to methylotrophic members of the genus *Methanolobus*. Increases in pore water methane concentrations under conditions of low sulfate (from 50 to 0 mM), in mats maintained for more than one year in that same greenhouse study, were associated with an increase in the abundance of putative hydrogenotrophic *mcrA* sequences related to *Methanogenium* [37].

Profoundly distinct vertical microenvironments at millimetric and micrometric scales have been recognized in microbial mats [4,6,34]. Differences in microbial community structure have also been observed in the horizontal direction, as a result of either different adaptations to gradients of salinity [5,10,35,38], thereby favoring marine and halophilic methanogens, or by the association of methanogen assemblages with different types of minerals. These observations suggest heterogeneity and the existence of three-dimensional microniches. The study of microbial community structure from as yet unexamined sites could broaden our understanding of the composition of the microbial community.

As such, the aim of the present work was to further our knowledge of methanogenesis in hypersaline environments. To this end, we analyzed several features of both the well-studied Guerrero Negro hypersaline ecosystem and a location not previously investigated, namely, Laguna San Ignacio, Baja California Sur, Mexico. The locations share a physiographic setting and climate [16]. Methane concentrations in environmental gas samples were investigated, together with the methane production rates obtained in microcosm experiments using hypersaline microbial mats samples amended with competitive and non-competitive substrates. Finally, the phylogenetic diversity of the methanogenic community was determined.

Materials and methods

Site description and sample collection. Samples were collected from two locations along the Pacific coast in the state of Baja California Sur, Mexico in March 2009. Location 1, with one site, corresponded to a concentrating pond of Exportadora de Sal (ESSA), a solar salt works located near Guerrero Negro (28°N, 114°W). Location 2, with two studied sites (26° 50′ N, 113° 10′ W), was the evaporitic flats at Laguna San Ignacio (LSI), a natural hypersaline ecosystem. Three types of mats were studied: (i) thick (10 mm), soft, well-laminated, green-black microbial mats found in Area 1 of ESSA (site ESSA-A1); (ii) thick (7 mm), leathery textured, pustular, non-laminated, black microbial mats found at site H7 of LSI (site LSI-H7); and (iii) thick (8 mm), smooth, laminated, orange-pink microbial mats found at site H8 of LSI (site LSI-H8). All three microbial mats were growing on top of

highly sulfidic sediments. Mat cores (1 cm deep × 1 cm diameter) were collected and stored in liquid nitrogen for molecular analysis. Five core samples 1 cm deep × 1cm of diameter (~10–20 g) from LSI sites were placed into 38-ml serum vials, and water samples were saved for further microcosm experiments. Larger cores (8 cm deep × 8 cm diameter) were collected and transported at room temperature for subsequent measurements of sulfate content in the sediments.

Gas bubbles were collected by perturbing the mat and sediments and trapping the released bubbles in a capped, inverted funnel at flooded sites (ESSA-A1). From desiccated sites (LSI-H7 and LSI-H8), gas samples were collected directly from large (2–10 cm) bubble structures overlain by a raised microbial mat (sometimes called pustular mat) using a hypodermic needle fitted to a 5-ml syringe. All bubbles were then transferred with a syringe to an evacuated serum vial for quantification of the methane concentration by gas chromatography.

Microcosm experiment. Ten ml of deoxygenated (N_2-purged) brine from the site was added to the serum vials with the mat samples to make a slurry. The vials were capped with blue butyl rubber stoppers and aluminum crimps, and the headspace was flushed with N_2 to remove any O_2. The slurries from the LSI sites were amended with TMA (15 mM), methanol (20 mM), formate (20 mM), acetate (20 mM), H_2/CO_2 (80/20 %), and sodium molybdate (2 mM). The sodium molybdate was added to specifically inhibit sulfate reduction in these samples. TMA (1 mM) was tested in slurries of ESSA-A1. Samples of ESSA-A1 were incubated at room temperature (ca. 25 °C) for 48 h, while LSI samples were incubated at 30 °C for 33 days. Gas samples were collected at different time points to monitor the methane concentration in the headspace, which allowed the specific production rates per gram of mat sample (nmol g^{-1} day^{-1}) to be calculated. At the end of the experiment, vials of LSI were maintained at room temperature (25 °C) for 1 year and were subsequently re-activated with specific medium for *Methanohalophilus* DSM 525, using TMA (15 mM) and methanol (20 mM) as substrates, with an incubation at 30 °C for one week. Vials with DSM 525 medium that were previously enriched with TMA, and methanol were incubated again with the same substrates at the same concentration. Meanwhile, the vials used for methanogenesis inhibition were further incubated with methanol (data not shown); these new incubations were used for further molecular analyses.

Methane and sulfate determinations. Gas samples collected from all incubations and from environmental bubbles were used to measure the evolved methane and methane concentrations, respectively, by gas chromatography with a flame ionization detector (Shimadzu GC-14 A, Kyoto, Japan) equipped with a 2-m Porapak N column held at 40 °C [3]. Sulfate content in the sediments was analyzed by turbidimetry in an automated spectrophotometer (QuickChem series 8000 FIAS, Lachat Instruments, Hach, Loveland, CO, USA), using Morgan solution as extractant and $BaCl_2$ for the precipitation [7].

DNA extraction. DNA was extracted from natural and enriched samples (PowerBiofilm DNA isolation kit 24000-50, Mo Bio Laboratories, Carlsbad, CA, USA). Extraction was performed according to the manufacturer's protocol, starting from 0.1 g samples and with 45-s bead-beating at speed 5 for cell lysis. DNA was observed in an agarose gel.

PCR of *mcrA* gene. PCRs were carried out in a final volume of 25 µl containing 2 µl of undiluted template DNA, 1 µl of each primer (10mM), and 12.5 µl of GoTaq master mix (Promega M7122, Madison, WI, USA). The *mcrA* gene was amplified using the primers developed by Luton et al. [29]. Amplification consisted of the following steps: 95 °C for 1 min, 35 cycles at 94 °C for 30 s each, 55–54.5 °C (decreasing 0.1 °C for the first five cycles) for 30 s each, 72 °C for 1 min, and a final elongation for 5 min at 72 °C.

Denaturing gradient gel electrophoresis (DGGE). PCR products from DNA extracted from slurries in microcosm experiment with DSM 525 medium were separated using a modification of a previously published protocol [26]. The gels were stained with ethidium bromide and archived with a UV photograph documentor (BioDoc-It® Imaging System, GelDoc-It TS300, UVP, Upland, CA, USA). Representative bands were excised with a sterile scalpel and DNA was eluted in ultra clean, pure water overnight at 4 °C. DNA was re-amplified and sequenced by a commercial service.

Clone libraries. Fresh PCR products from natural samples from ESSA-A1 and LSI-H8 were purified with an extraction kit (QIAquick gel extraction kit 28704, Qiagen, Valencia, CA, USA). One–2 µl of the purified PCR product was ligated into a cloning vector (pCR4-TOPO), which was then used to chemically transform TOP10 *Escherichia coli* competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). White colonies were inoculated into 125 µl LB broth amended with 8 % (v/v) glycerol and carbenicillin (100 mg/ml), then incubated overnight at 37 °C. The inserts were verified by PCR using the M13F and M13R primers. Positive colonies were shipped at room temperature (ca. 23 °C) to a commercial firm for sequencing (Sequetech, Mountain View, CA, USA).

Phylogenetic analysis and nucleotide sequence accession numbers. All sequences were quality-filtered, trimmed, translated into protein sequences, and aligned with a custom database of methyl-coenzyme M reductase alpha protein sequences using bioinformatics software (Geneious 5.3, Biomatters, Auckland, NZ). Inferred amino acid sequences were clustered at 97 % similarity using CD-HIT [22]; representative sequences were queried against the NCBI non-redundant peptide sequence databases [2] to identify closest protein matches for phylogenetic analysis and tree building. Taxonomic assignment of the sequences was based on comparisons with sequences in the gene databases [1] with > 97 % similarity (Table 1).

Phylogenetic analyses were performed on the aligned amino acid sequences using maximum-parsimony and neighbor-joining evolutionary models in PAUP* version 4 (Sinauer Pub., Sunderland, MA, USA). The robustness of inferred tree topologies was evaluated by 1000 bootstrap resamplings of the data. Two topologies from the different analyses were similar and the presented tree was based on a neighbor-joining analysis, with

Table 1. Taxonomic assignment and accession numbers of all the sequences obtained in this study

Genes	Techniques	Accession numbers	Taxonomic assignment/number of sequences
mcrA	DGGE	JF836061 – JF836067	*Methanolobus*/5 *Methanococcoides*/2
mcrA	Clone libraries	HQ131850 - HQ131869	*Methanohalophilus*/17 *Methanolobus*/3

Table 2. Salinity in water samples, methane concentration in environmental gas samples and sulfate concentration in sediment samples from studied sites

Site	Geoposition	Salinity (ppt)	Methane (%)	Sulfate (g/kg)
LSI-H7	26°47.998´N 113°07.649´W	~93	0.00086 (± 0.0003)	5.35 (± 1.36)
LSI-H8	26°45.223´N 113°07.406´W	>100	0.01293 (± 0.02)	8.36 (± 0.86)
ESSA-Area1	27°36.01´N 113°53.46´W	~50	3.204 (± 0.37)	4.12 (± 0.15)

bootstrap support of branch nodes only when supported by the two models. All sequences determined in this study are available in GenBank (accession nos. HQ131850 to HQ131869 and JF836061 to JF836067) (Table 1).

Results and Discussion

Detection and quantification of methane in environmental gas samples. Methane gas was detected from environmental samples from all field sites sampled (two evaporitic flats at Laguna San Ignacio and from area 1 of Exportadora de Sal at Laguna Ojo de Liebre). Methane concentrations ranged from 0.00086 to 3.204 %. Methane production has been observed in a wide variety of hypersaline environments [32], including stratified microbial mats and endoevaporites [8,13,15,39]. Similar results were previously reported for soft microbial mats from hypersaline environments near the field sites described here (Tazaz et al., personal communication).

Site ESSA-A1 had the highest concentrations of methane and the lowest concentration of sulfate in sediments. In contrast, sites LSI-H7 and LSI-H8 had the lowest concentrations of methane and the highest concentration of sulfate (Table 2). These results are consistent with the hypothesis that methanogenesis is attenuated by sulfate-reducing bacteria at non-limiting sulfate concentrations [27]. Considering that methanogenic archaea are abundant only in habitats where electron acceptors such as SO_4^{2-} are limiting, the cell densities of methanogens in the samples analyzed may have been low, since the concentrations of sulfate in sediments ranged from 4.12 to 8.36 g/kg. Nevertheless, methanogenesis is also constrained by ecological interactions (both stimulatory and competitive) and/or physicochemical environmental factors that act at biochemical or bioenergetic levels. In addition to physicochemical "extremes" (mainly temperature, salinity, and pH), other factors affect the environmental distribution of methanogens, which is constrained to a great extent by energy availability, the environmental distributions of oxygen (biochemical inhibition), and the seawater anion sulfate content (competitive effects that act at a bioenergetic level) [27]. More exhaustive sampling will be needed to establish which physicochemical factors (salinity, temperature, pH, moisture, water activity and nutrients) have the greatest influence on microorganism distributions in different natural environments [9].

An alternative explanation for the low concentrations of methane, which will not be considered further here, is the aerobic and anaerobic oxidation of methane, which has been reported in microbial mats in salt marshes [4].

Production of methane from samples incubated with substrates. To further explore the metabolic processes giving rise to the methane detected at our field sites, microcosm incubations were conducted. Determination of the use of substrates in methanogenesis is of great relevance for explaining trophic relationships and the level of activity in nature. As an example, only members of the Order Methanosarcinales use methylamines as catabolic substrates. These organisms also use H_2 + CO_2, while the other four Orders only contain hydrogenotrophic or acetoclastic members.

Although the production of CH_4 within the upper layer (0–20 mm) of hypersaline microbial mats has been previously correlated with the cyanobacterial production of H_2 [13], microcosm experiments with different substrates showed that methanogenesis was not stimulated by the addition of hydrogen; rather, only TMA stimulated methane production in samples from ESSA-A1 and H7 and H8 of LSI (Fig. 1). These results are congruent with previous studies that have recorded methane production in media designed to enrich methylotrophic methanogens on TMA using sulfate-rich samples from hypersaline microbial mats in the Napoli mud volcano [19]. Methanol additions also resulted in an increase in methane production in samples from LSI.

Substrates utilized by hydrogenotrophic and acetoclastic methanogens (H_2/CO_2, formate and acetate) did not increase

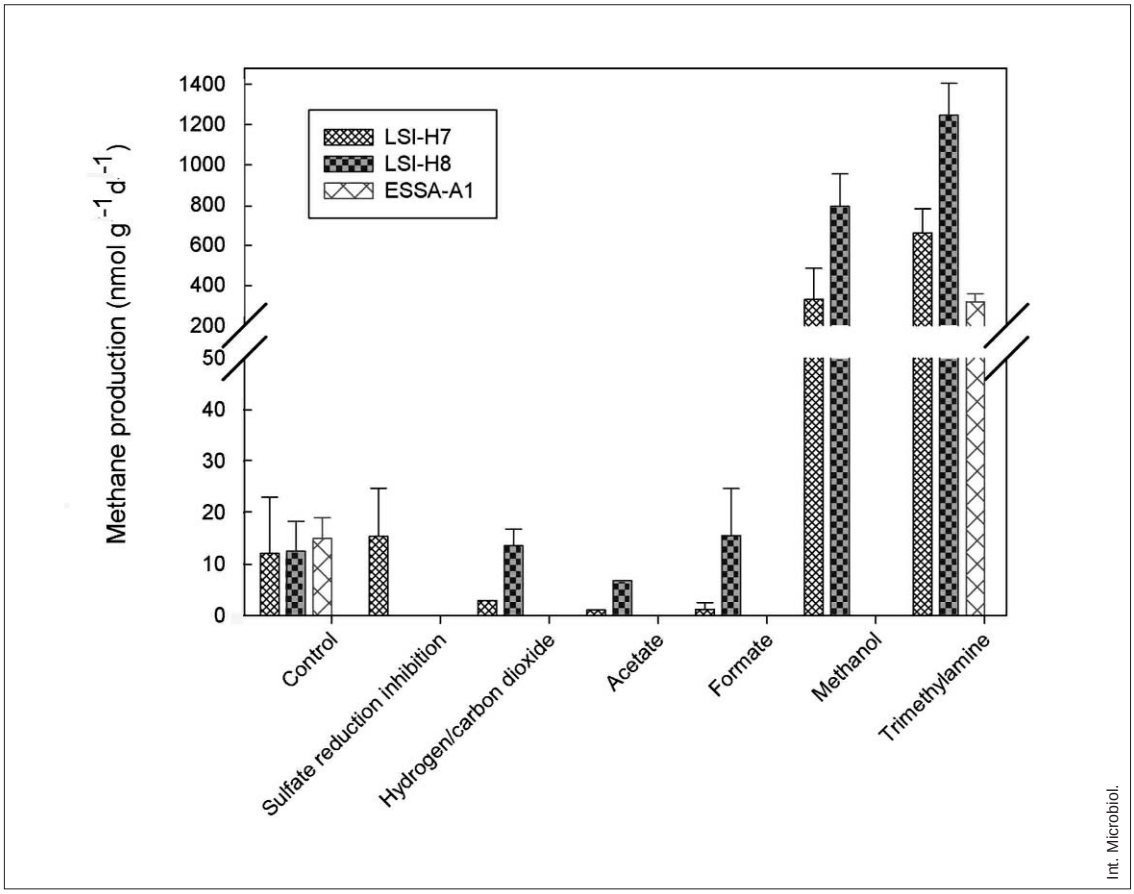

Fig. 1. Methane production rates from microbial mat samples from Laguna San Ignacio (sites H7 and H8) and the site ESSA-A1 in Guerrero Negro amended with site field water and non-competitive and competitive substrates under anoxic conditions. Samples of ESSA-A1 were incubated at room temperature (ca. 25 °C) for 48 h, while LSI samples were incubated at 30 °C for 33 days. Error bars indicate one standard deviation about the mean of two replicate samples.

methane production in the LSI samples (Fig. 1), confirming previous studies in which acetate, hydrogen, or methionine additions did not stimulate methanogenesis in freshly collected marsh sediments from intertidal sediments [43]. Acetoclastic and hydrogenotrophic methanogens, with their lower energetic yields, are therefore more susceptible than methylotrophic methanogenesis, which further explains the predominance of methylotrophic methanogens in hypersaline environments [30].

Our understanding of how methanogenesis is coupled to energy conservation has proceeded more slowly. As for all respirers, energy conservation is fundamentally chemiosmotic. A methyl transfer step plays a central role in most methanogenic pathways and directly drives the export of sodium ions. Other components of the energy conservation apparatus appear to differ in the methylotrophic and hydrogenotrophic methanogens. Methylotrophic methanogens have cytochromes and a proton-translocating electron transport chain, which they use to conserve energy in the last, exergonic step in methanogenesis. Hydrogenotrophic methanogens, however, lack these components, and it is not clear how these organisms achieve a net positive gain in energy conservation, because the first step in methanogenesis from CO_2 is endergonic [21]. A proposed mechanism involving electron bifurcation, in which exergonic electron flow directly drives endergonic electron flow, could resolve this conundrum [42].

To assess whether the activity of sulfate-reducing bacteria attenuated hydrogenotrophic methanogenesis at non-limiting sulfate concentrations, sulfate reduction was inhibited in microcosm experiments. Thus, the addition of sodium molybdate resulted in a 76–78 % decrease in hydrogen sulfide production in the microcosm (data not shown), indicating a sharp decrease in sulfate reduction rates. This decrease

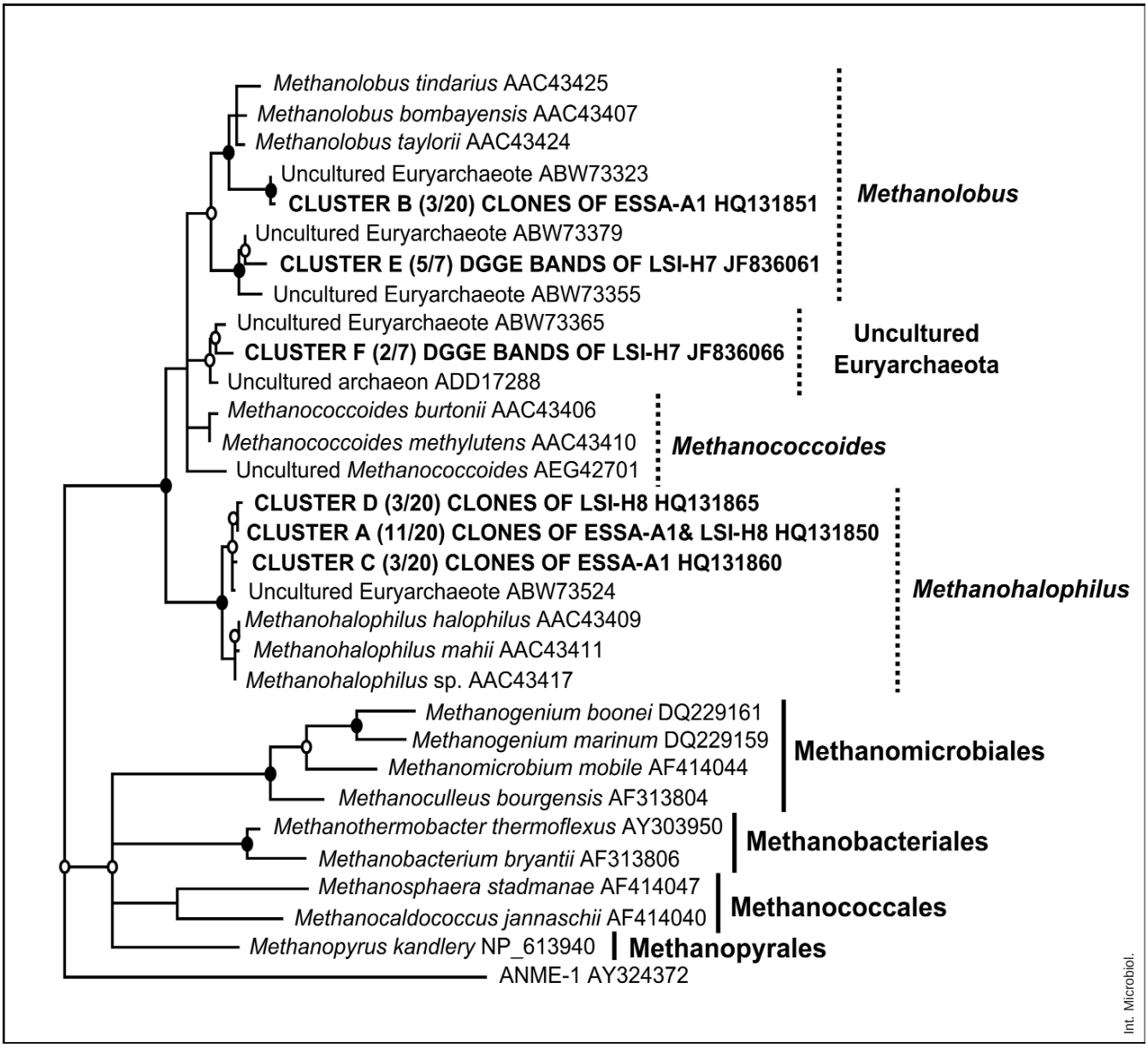

Fig. 2. Phylogenetic tree based on comparison of inferred amino acid sequences of *mcrA* gene from methanogenic euryarchaeota from natural (clusters A-D) and manipulated (clusters E, F) samples of microbial mats from hypersaline environments. Branch nodes supported by phylogenetic analysis (bootstrap values >95 % by both maximum parsimony; MP and neighbor-joining; NJ analyses) are indicated by filled circles. Open circles indicate >75 % bootstrap support by either MP or NJ analysis, while branch nodes without circles were not resolved (bootstrap value <75 %). Bootstraped trees were generated with 1000 resamplings. The tree is rooted using an environmental sequence related to anaerobic methanogenic-oxidizing archaea group 1 (ANME-1) as the outgroup. Abundance of each phylotype cluster (97 % identity) detected is stated in parentheses. The bar represents 0.2 changes per amino acid.

did not, however, increase methane production (Fig. 1), which might be explained by the low abundances of methanogens capable of using H_2/CO_2 or acetate as substrate in those samples. Methane production in the unamended samples best represented natural conditions, which are probably suboptimal for methylotrophic methanogens due to the low concentrations of methylamines (Kelley et al., personal communication).

Phylogenetic diversity of methanogens. Methanogens are frequently studied without cultivation, owing to a generally good correspondence between phylogeny and phenotype, which is less typical in other groups [30]. The phylogenetic diversity of methanogens was different for all the studied sites. Clone libraries of *mcrA* from natural ESSA-A1 samples consisted exclusively of sequences related to methy-

lotrophs of the Order Methanosarcinales, including moderately halophilic and marine members of the genera *Methanohalophilus* (8 sequences from cluster A and 3 sequences from cluster C) and *Methanolobus* (3 sequences from cluster B). Sequences closely related to members of the genera *Methanohalophilus* were also retrieved from natural samples from LSI-H8 (3 sequences from cluster A and 3 sequences from cluster D) (Fig. 2). This confirms earlier reports that the methanogenic community in hypersaline environments is dominated by methylotrophic methanogens [44]. The *mcrA* sequences retrieved from DGGE bands from LSI-H7 samples incubated with methanol and TMA were similar to sequences of uncultured Euryarchaeota that phylogenetic analyses showed to be distantly related to slightly halophilic and marine organisms of the genera *Methanococcoides* (2 sequences from cluster F) and *Methanolobus* (5 sequences from cluster E) (Fig. 2). However, we were not able to detect hydrogenotrophic or acetoclastic methanogens using the described molecular approaches, presumably due to the low abundances of methanogens that utilize the pathway involving CO_2 reduction and acetoclastic reaction. Members of the genus *Methanococcoides* have been frequently found in anoxic marine sediments [17], but have also been reported for incubations of surface layers of microbial mats from hypersaline environments [33]. Enrichment cultures have also shown the presence of viable methylotrophic *Methanococcoides* in shallow sediment layers from hypersaline microbial mats in the Napoli mud volcano [19], suggesting that this genus is common in hypersaline environments.

Enrichments made it possible to assess the differential effects of environmental factors imposed on mixed microbial populations, as well as to select organisms capable of attacking or degrading particular substrates or of thriving under unusual conditions [18]. The retrieval, from enriched samples, of sequences that were not found in natural samples showed the importance of applying different approaches to the characterization of methanogenic community composition.

There is uncertainty about the phenotype of uncultivated organisms giving rise to sequences that cluster within the Euryarchaeota but outside of known methanogens [30]. Recent studies, however, have shown the importance of studying uncultured microorganisms, because novel microbes can be detected with molecular data [12]. Examples of this are the recently described novel major lineage of Nanohaloarchaea, from hypersaline microbial communities [31], and the report of a new candidate division, MSBL1, which branchs deeply within the Euryarchaeota, from extremely halophilic microbial communities in anaerobic sediments from a solar saltern [25].

Although *mcrA* sequences of the same genera of methylotrophic methanogens found in our samples have been reported from other hypersaline environments [33,37,40], the relative abundances of retrieved sequences were different at the field sites reported here. Previous studies at the site ESSA-A4 reported that *Methanolobus* and *Methanococcoides* sequences were the most abundant while *Methanohalophilus*-like sequences were retrieved in lower abundances [33,37]. In our study, > 60% of the sequences (17 sequences) were related to *Methanohalophilus*. These results show the importance of studying the microbial community composition from different hypersaline environments even though many identical phylogenetic groups are detected in sediments independent of their geographic location [17]. In contrast to the hypothesis that microbial diversity in hypersaline environments is essentially the same at different geographical locations, there can apparently be great heterogeneity in phylogenetic diversity between sites that share physiographic setting and climate.

We can conclude that geochemical and molecular evidence confirm the presence of methanogenesis in these hypersaline environments. The detection of methane suggests that physicochemical extreme conditions in hypersaline environments should not prevent methanogenesis. The retrieval of *mcrA* sequences showed that the methanogen community was dominated by moderately halophilic organisms of the genus *Methanohalophilus* (more than 60 of *mcrA* sequences retrieved). Nevertheless, slightly halophilic and marine organisms of the genera *Methanococcoides* and *Methanolobus*, respectively, were identified at lower abundances. These results suggest that the community composition of methanogens differs even in similar ecosystems. All sequences were related exclusively to methylotrophic members of the Family Methanosarcinaceae.

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