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Methanogen population in a marine biofilm corrosive to mild steel

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Abstract This study was conducted to analyze the methanogen population in a corrosive marine biofilm based on 16S rDNA analysis, using a PCR-cloning-sequencing approach. There were 80 methanogen clones developed from the PCR-amplified DNA extracted from the biofilm on the mild steel surface. All clones were categorized into one of five operational taxonomy units (OTUs). Two OTUs (comprising 57 clones) were affiliated with the acetotrophic *Methanosaeta* genus; the remaining three OTUs (23 clones) were affiliated with the hydrogenotrophic genera of *Methanogenium*, *Methanoplanus* and *Methanocalculus*. The hydrogenotrophic methanogens could directly cause metal corrosion through cathodic depolarization, whereas the acetotrophic methanogens grew syntrophically with corrosion-causing sulfate-reducing bacteria, as observed by fluorescent in situ hybridization, and thus contribute indirectly to metal corrosion.

Introduction

Sulfate-reducing bacteria (SRB) and methanogens coexist in natural habitats, such as soil (Daniel and Stephan 2001) and sediments (Ward and Winfrey 1985), as well as in artificial ecosystems, such as granular sludge (Li et al. 1996) and biofilms in wastewater treatment (Raskin et al. 1996). Some SRB species may compete with methanogens for carbon and electron sources; yet others may grow syntrophically with methanogens, in which case the metabolites of SRB become the substrates of methanogens.

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Species of both SRB and methanogens may be hydrogen scavengers, which could cause metal corrosion by removing electrons from the metal surface. Although corrosion induced by SRB has been extensively studied (von Wolzogen Kuhr and van der Vlugt 1934; Wilson et al. 1997; Xu et al. 1999), corrosion induced by methanogens has only been reported for some pure methanogen cultures (Daniels et al. 1987; Boopathy and Daniels 1991). Very limited information is available on corrosion induced by methanogens in a mixed culture community, especially in the presence of SRB.

In a previous study of marine biofilm corrosion (Zhang and Fang 2001), this research group analyzed the *Eubacteria* population in the biofilm using a 16S-rRNA-based molecular method. Four SRB and two *Clostridium* species were identified as a result. This study was conducted to further analyze the methanogen population in the same biofilm, and to investigate SRB-methanogen interactions in the metal corrosion process.

Materials and methods

Biofilm incubation

The biofilm was developed on the surface of a mild steel coupon (40×15×1.5 mm) in modified Postgate's medium C (Videla 1996) prepared from seawater at 20±2°C. The medium was seeded with a mixed culture at an initial concentration of about 1×10^6 cells/ml enriched from the sediment of the Victoria Harbour in Hong Kong. The system was kept under anaerobic conditions by purging with nitrogen. Half of the mixed liquor was replaced every 10 days by an equal volume of fresh medium. Biofilm on the steel coupon surface became visible after 10 days. Microbial analyses were conducted for the biofilm after 90 days. Details of the biofilm incubation process have been reported previously (Zhang and Fang 2000, 2001).

DNA extraction

The biofilm was removed from the coupon and resuspended in 2 ml lysis buffer (0.75 M sucrose, 100 mM EDTA, 100 mM Tris, pH 8.0). After repeating a freezing (–80°C) and thawing (37°C) cycle four times, the sample was homogenized in a Mini-

Beadbeater (Biospec Products, Bartlesville, Okla.) with 0.2 g 0.1 mm glass beads at 5,000 rpm for 10 s. The process was repeated three times. The homogenized sample was then combined with 3 ml of lysis buffer, 100 μl each of lysozyme solution (50 mg/ml) and achromopeptidase solution (10 mg/ml), and incubated at 37°C for 30 min, followed by the addition of 0.5 ml 10% lauryl sulfate and 50 μl proteinase K solution (20 mg/l). The mixture was further incubated at 37°C for 2 h, before adding 0.8 ml 5 M NaCl solution and 0.6 ml of a mixed solution containing 10% cetyltrimethylammonium bromide and 4.1% NaCl, followed by another 20 min incubation at 65°C. The solution was then mixed with 6.6 ml of phenol:$CHCl_3$:isoamyl-alcohol (25:24:1) solution, and centrifuged at 4,000 rpm for 20 min. The supernatant (5.0 ml) was mixed with an equal volume of $CHCl_3$:isoamyl-alcohol (24:1) solution, and centrifuged again. The supernatant (4.0 ml) was mixed with 2.4 ml isopropanol, and kept at 4°C overnight. The DNA precipitate was centrifuged the following day at 12,000 rpm for 10 min, and rinsed with 0.8 ml 70% ethanol. After another centrifugation, the DNA precipitate was air-dried for 5 min, before dissolving in 100 μl water.

Polymerase chain reaction amplification

The extracted biofilm DNA was amplified by polymerase chain reaction (PCR) using the primer set ARCH622 (TGA AAT CYY RTA ATC CC) (Liu et al. 2002) plus GC-clamp and ARCH934 (GTG CTC CCC CGC CAA TTC CT) (Giovannoni et al. 1990). All PCR amplifications were conducted in 30 μl of a pH 8.3 buffer (Pharmacia, Piscataway, N.J.) containing 200 μM each of the four deoxynucleotide triphosphates, 15 mM $MgCl_2$, 0.1 μM each primer and 1 U *Taq* polymerase (Pharmacia). An automated thermal cycler (GeneAmp PCR 9700, Perkin-Elmer, Foster City, Calif.) was used for PCR amplification using the following program for denaturing gradient gel electrophoresis (DGGE): an initial denaturation at 94°C for 7 min; 35 cycles of denaturation (1 min at 92°C), annealing (1 min at 56°C) and extension (1 min at 72°C); and a final extension at 72°C for 10 min before storing at 4°C.

Denaturing gradient gel electrophoresis

DGGE was performed with a DCode Universal Mutation Detection System (Bio-Rad, Hercules, Calif.) (Zhang and Fang 2000). A denaturant gradient maker was used to make 8% (w/v) polyacrylamide gels with denaturing gradient from 50% to 70% [100% denaturant corresponds to 7 M urea and 40% (v/v) formamide deionized with AG501-X 8 mixed-bed resin (Bio-Rad)], using acrylamide-bisacrylamide gel stock solution (37.5:1; Fluka, Buchs, Switzerland). Before gel loading, a GelBond PAC film (BMA, Rockland, Me.), which has a hydrophobic and a hydrophilic side, was placed on a glass plate of the gel maker to support the gel during the silver-staining process. The hydrophilic side of the film was facing the gel. After loading, gels were allowed to polymerize for 2 h. Electrophoresis was conducted in 1× TAE buffer (20 mM Tris, 10 mM sodium acetate, 0.5 mM Na_2 EDTA, pH 7.4) for 4 h at 200 V and 60°C.

For staining, the gel was immersed in fixation solution (10% ethanol, 0.5% acetic acid) overnight. The next day, the fixation solution was replaced by a silver staining solution. After 30 min, the staining solution was removed and the gel was briefly rinsed with distilled water. The gel was then immersed in development solution, and the gel image was capture by CCD camera (Leica, Germany)

Cloning and sequencing

PCR-amplified DNA fragments for cloning followed the same PCR procedures as for DGGE, except that only 25 cycles (instead of 35 cycles for DGGE) of denaturation, annealing and extension were performed. The PCR-amplified products were cloned using a TA cloning kit (Invitrogen, Carlsbad, Calif.) (Zhang and Fang 2001). A total of 80 colonies were selected for inoculation in 1.0 ml Luria-Bertani medium containing 50 mg/l kanamycin. After 18 h of incubation of the inoculated clones at 37°C, the plasmids were recovered (Sambrook et al. 1989), and dissolved in 50 μl pure water. The insert in the plasmid of each individual clone was then sequenced with the primer M13R (5′-CAGGAAACAGCTATGAC-3′) using an auto sequencer (ABI model 377A, Perkin-Elmer) and a dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer).

Phylogenetic analysis

Each DNA sequence was analyzed using the CHECK-CHIMERA program (Maidak et al. 1997) to detect possible chimeric artifacts, which, if found, were excluded from further analysis. All sequences were compared with those of reference microorganisms available in the GenBank by BLAST search (Altschul et al. 1990). The closest 16S rDNA sequences were retrieved from GenBank, and aligned using BioEdit (Hall 1999). A phylogenetic tree was then constructed by the neighbor-joining method using MEGA 2.1 (Kumar et al. 1993). Bootstrap re-sampling analysis (Felsenstein 1985) for 1,000 replicates was performed to estimate the confidence of tree topologies.

Fluorescent in-situ hybridization

The morphology and distribution of microbial species in the biofilm were examined by FISH. Table 1 lists the major characteristics of the four oligonucleotide probes, i.e., MX825, ARCH915, SRB385 and EUB338, used in this study. The probes were labeled at the 5′-end with either Cy3 or 6-carboxy-fluorescein (Integrated DNA Technologies, Coralville, Iowa). Biofilm samples were first fixed with an aqueous solution containing 4% (v/v) paraformaldehyde, air-dried on a glass slide, and then dehydrated sequentially by ethanol solutions of 50, 80 and 96% for 3 min each. The dehydrated sample was air-dried again, and hybridized with 1 μl each of the

Table 1 16S rRNA oligonucleotide probes used in fluorescent in-situ hybridization

Probe	Oligo probe database	Target microbe	Sequence (5′–3′)	Fluorescence label	Formamide (%)	Reference
EUB338	S-D-Bact-0338-a-A-18	Eubacteria	GCT GCC TCC CGT AGG AGT	Cy3	0–80	Amann et al. 1995
ARCH915	S-D-Arch0915-a-A-20	Archaea	GTG CTC CCC CGC CAA TTC CT	6-Carboxy-fluorescein	20	Amann et al. 1990
MX825	S-F-Msae-0825-a-A-23	Methanosaeta	TCG CAC CGT GGC CGA CAC CTA GC	6-Carboxy-fluorescein	20	Raskin et al. 1994
SRB385	S-Sc-dProt-0402-a-A-18	Delta-Proteobacteria	CGG CGT CGC TGC GTC AGG	6-Carboxy-fluorescein	35	Amann et al. 1990

oligonucleotide probe solutions (100 ng/μl) and 10 μl of a formamide-containing hybridization buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.2), the concentrations of which are also shown in Table 1. Hybridization took place at 46°C in a sealed moisture chamber for 90 min, followed by incubation in washing buffer (0.07 M NaCl, 0.01% SDS, 20 mM Tris/HCl, 5 mM EDTA, pH 7.2) at 48°C for 30 min. The glass slide was then rinsed with distilled water and air-dried before examination under a confocal laser scanning microscope (LSM 5 Pascal, Zeiss, Jena, Germany). The relative abundance of each microbe was calculated from the fluorescent area hybridized with the microbe-specific probe using the image analysis software MetaView (Universal Imaging, Downingtown, Pa.).

Membrane hybridization

Methanogen and SRB populations in five biofilms sampled on days 10, 20, 40, 60 and 90 were quantified using the membrane hybridization technique (Liu et al. 2002). The rRNA in the biofilm was extracted using a commercially available kit (Tri-Reagent, Molecular Research Center, Cincinnati, Ohio), denatured, and then blotted onto a magna charged nylon membrane. The membrane was then hybridized using the three following radioactively labelled probes: UNIV1392 (ACG GGC GGT GTG TRC) targeting all the microbes; ARCH915 (GTG CTC CCC CGC CAA TTC CT) targeting *Archaea*, and SRB385 (CGG CGT CGC TGC GTC AGG) targeting SRB in the delta sub-division of *Proteobacteria*. The washing temperatures were 42°C for probe UNIV1392 (Stahl et al.1988), 59°C for probe SRB385 (Sandaa et al. 2001), and 56°C for probe ARCH915 (Amann et al. 1990). The abundance of each group was determined by comparing the radioactivity of the group-specific probe measured by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, Calif.) relative to the radioactivity of the universal probe UNIV1392. Reference rRNAs were obtained from pure cultures of *Desulfovibrio fluorescens* (DSM 8712) and *Methanosaeta soehngenii* (DSM 2139).

Accession numbers

The16S rDNA sequences obtained in this study are available in the GenBank, EMBL and DDBJ databases under the following accession numbers: AY253745 (90D-A-61), AY253746 (90D-A-53), AY253747 (90D-A-55), AY253748 (90D-A-60), AY253749 (90D-A-31).

Results

Phylogenetic analysis

The DGGE image of the PCR-amplified DNA in Fig. 1 shows that there were only a few detectable bands, indicating that the methanogen population in the biofilm was not diverse. A total of 80 clones were obtained. Based on the DNA sequences, these clones could be classified into five operational taxonomy units (OTUs). Of these, two OTUs, comprising 57 clones, were affiliated with the *Methanosaeta* genus in the family *Methanosaetaceae* of the order *Methanosarcinales*, two OTUs (19 clones) were affiliated with the genera of *Methanogenium* and *Methanoplanus* in the family *Methanomicrobiaceae* in the order *Methanomicrobiales*, and the remaining OTU (4 clones) with the *Methanocalculus* genus in the order *Methanomicrobiales*.

Fig. 1 Denaturing gradient gel electrophoresis (DGGE) image of methanogens in the biofilm. The denaturant gradient was from 50% (left) to 70% (right)

According to substrate specificity, methanogens may be categorized into four groups. Three are very substrate specific using, respectively, acetate (e.g., the *Methanosaeta* genus), H_2/CO_2 and/or formate (e.g., the genera of *Methanogenium* and *Methanospirillum*) and methyl compounds (e.g., the genera of *Methanococcoides* and *Methanolobus*) as substrates. The fourth group is less substrate specific, an example being *Methanosarcina*, which may use either acetate, H_2/CO_2, or methyl compounds as substrate. Based on the phylogenetic tree in Fig. 2, the five OTUs found in this study belong to the acetotrophic group (*Methanosaeta*) and the group using H_2/CO_2 and/or formate as substrate (*Methanogenium*, *Methanoplanus* and *Methanocalculus*).

Figure 2 illustrates that OTU 90D-A-55 (comprising 49 clones) and OTU 90D-A-31 (8 clones) may be assigned to the acetotrophic *Methanosaeta*. OTU 90D-A-55 is closely related to some uncultured *Methanosaeta* clones, which include the uncultured *Methanosaeta* sp. clone A1 (99% similarity) retrieved from a methane-producing community using long-chain alkanes (Zengler et al. 1999), an uncultured archaeon Arc No.5 (accession number: AF395423) from acetate-enriched culture (99%), an uncultured archaeon clone clH28arc (accession number: AF364943) from high-temperature continental oil reservoirs (98%), and an uncultured archaeon AT425_ArC3 associated with Gulf of Mexico gas hydrates (97%) (Lanoil et al. 2001). It also shares 95% similarity with *Methanosaeta thermophila*, which is also known by several other names, including *Methanothrix thermoacetophila*, *Methanosaeta thermoacetophila*, *Methanosaeta thermacetiphila*, *Methanosaeta thermoacetophila* and *Methanothrix thermophila*. OTU 90D-A-31 likewise has similarities with these species or clones.

The remaining three OTUs, on the other hand, belong to the order of *Methanomicrobiales*, which use H_2/CO_2 and/or formate as substrate. Among them, OTU 90D-A-61 (10 clones) is most closely related to *Methanogenium organophilum* (98%), followed by *Methanogenium cariaci* (97%), an uncultured archaeon 2MT120 (accession number: AF015983) from a coastal saltmarsh (97%), and an uncultured archaeon CS_R002 from hydrothermal sediments in the Guaymas Basin (95%) (Teske et al. 2002). OTU 90D-A-60 (9 clones) is most closely related to *Methanoplanus petrolearius* (97%), followed by an uncultured archaeon O23C7 in high-temperature petroleum reservoirs (96%) (Orphan et al. 2000). Lastly, OTU 90D-A-53 (4 clones) is most closely related to three known species (98% each), i.e., *Methanocalculus taiwanense*, *Methanocalculus pumilus* and *Methanocalculus halotolerans*, followed by *Methanocalculus* sp. O1F9702c

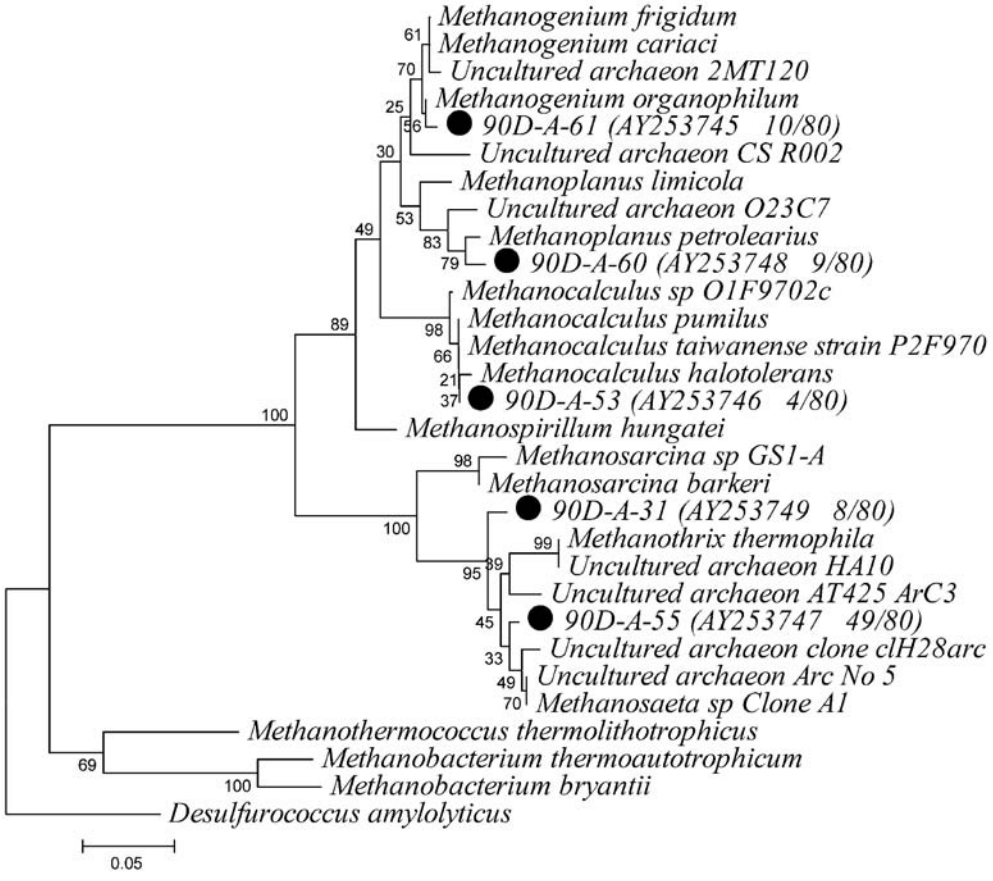

Fig. 2 Phylogenetic tree of the methanogens in a corrosive biofilm based on partial 16S rDNA sequence. The tree was based on Jukes-Cantor distance and constructed using the neighbor-joining algorithm with 1,000 bootstrappings. *Desulfurococcus amylolyticus* was selected as the out-group species. Numbers at the nodes are the bootstrap values. Numbers in parenthesis indicate the accession number and clone number of that operational taxonomy unit (OTU) in the library. *Scale bar* represents 0.05 substitution per nucleotide position

(accession number: AY026256) isolated from estuarine water (97%).

Populations estimated from membrane hybridization results

Based on the radioactivity measurements of group-specific probes used in membrane hybridization, the abundances of SRB and methanogens were estimated for five biofilms sampled from day 10 to day 90. Results in Fig. 3 illustrate that both SRB and methanogen populations increased over time. The relative abundance of SRB increased from 18% on day 10 to 59% on day 90, whereas the corresponding values for methanogens were 2% and 39%.

Fluorescent in situ hybridization

The SRB and methanogen populations in the biofilm were also determined based on FISH images using various probe combinations. Figure 4a illustrates the FISH image using the *Methanosaeta*-specific probe MX825 and the *Eubacteria*-specific probe EUB338, whereas Fig. 4b shows the *Archaea*-specific probe ARCH915 and EUB338, and Fig. 4c the SRB-specific probe SRB385 and EUB338.

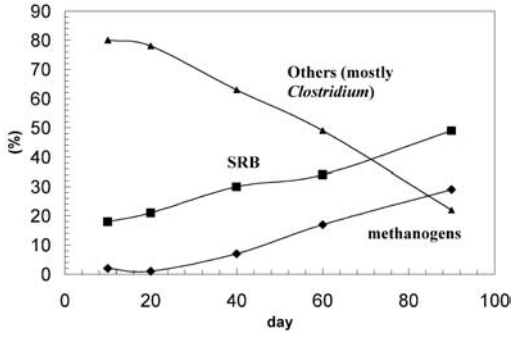

Fig. 3 The relative abundance of 16S rRNA of sulfate-reducing bacteria (*SRB*), methanogens and others (mostly *Clostridium*) in the biofilm community over time

Discussion

Corrosion caused by methanogens

Methanogen corrosion of metals is not as well known as SRB corrosion. In the corrosion process, both SRB and methanogens use the cathodic hydrogen released from metal as the electron donor. In the process, SRB reduce sulfate to hydrogen sulfide, whereas methanogens reduce carbon dioxide to methane. The corrosion rates by both microbes are comparable. Boopathy and Daniels (1991)

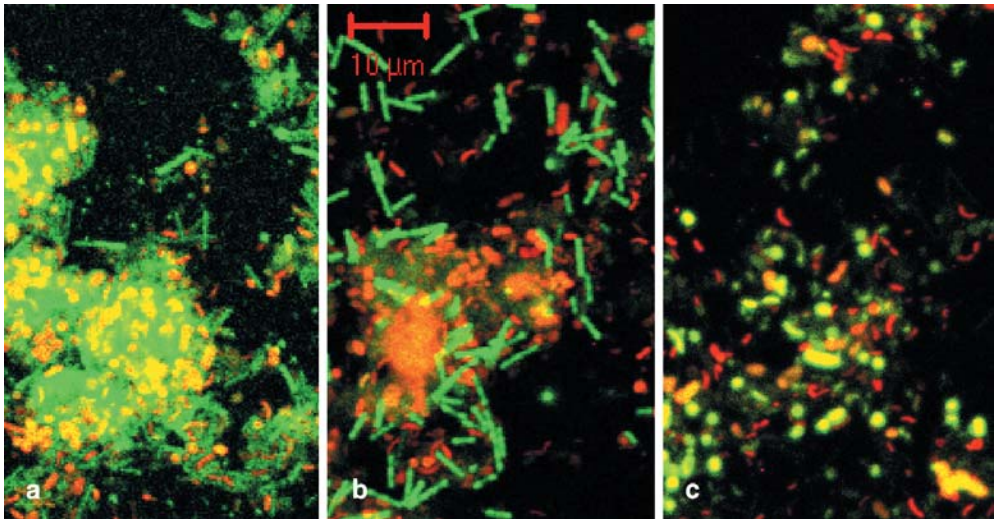

Fig. 4a–c Fluorescent in situ hybridization (FISH) images of the biofilm using various probe combinations. **a** ARCH915-FITC/EUB338-Cy3 (*Archaea* in green, *Eubacteria* in red; yellow represents overlap of *Archaea* and *Eubacteria*); **b** MX825-FITC/EUB338-Cy3 (*Methanosaeta*-like species in green and *Eubacteria* in red; **c** SRB385-FITC/EUB338-Cy3 (SRB in yellow and green, other *Eubacteria* in red). *Bar* 10 µm

found that the rate of methanogen corrosion was 10–14 mg day^{-1} dm^{-2}, as compared to the rates of 1.3–21 mg day^{-1} dm^{-2} by several pure *Desulfovibrio* cultures.

Daniels et al. (1987) studied the corrosion of pure methanogen cultures, including *Methanosarcina barkeri*, *Methanococcus thermolithotrophicus*, *Methanobacterium thermoautotrophicum*, *Methanobacterium bryantii* and *Methanospirillum hungatei*. They found that the increase in methanogen population correlated well with the increase in the degree of corrosion, as evidenced by the increased concentration of the corrosion product, i.e., ferrous ion.

Methanogens in the marine biofilm

The two most predominant methanogen groups found in this study were OTUs 90D-A-55 and -31, both of which are closely related to the acetotrophic *Methanosaeta*. Since *Methanosaeta* are not hydrogenotrophic, OTUs 90D-A-55 and -31 probably did not cause metal corrosion directly. Instead, they could cause metal corrosion only indirectly via syntrophic interactions with the corrosion-causing acetogenic SRB.

The remaining OTUs 90D-A-53, -60 and -61 are, on the other hand, closely related to hydrogenotrophic methanogens. Therefore, they probably interacted syntrophically with lactate-degrading SRB, using the hydrogen produced by the SRB as the electron donor. Since they are hydrogen scavengers, these three OTUs are likely responsible for metal corrosion through the cathodic depolarization mechanism.

In this study, the presence of *Methanosarcina* species was not detected. *Methanosarcina* may compete with *Methanosaeta* for acetate, but competition is unfavorable at acetate concentrations below 200 µM (12 mg/l) (McHugh et al. 2003). The absence of *Methanosarcina* indicates that the acetate was effectively consumed in the biofilm, most likely by *Methanosaeta*.

Interactions between methanogens and SRB

It was found in a previous study (Zhang and Fang 2001) that the same biofilm was composed of SRB and *Clostridium*. The SRB found in the biofilm, including *Desulfobacterium*, *Desulfovibrio*, *Desulfomicrobium* and *Desulfobulbus*, were those capable of using lactate as substrate to produce acetate and hydrogen. None of these SBR species are capable of further degrading acetate (Holt et al. 1994). On the other hand, methanogens found in this study in the same biofilm were those capable of utilizing acetate (*Methanosaeta*) and H_2 (*Methanogenium*, *Methanoplanus* and *Methanocalculus*) even though neither acetate nor hydrogen was present in the culture medium. Thus, SRB and methanogens appeared to grow syntrophically in the biofilm, based on their physiological characteristics. SRB converted lactate into acetate and hydrogen, both of which were subsequently utilized by methanogens for the production of methane. Such a syntrophic relationship is also evidenced in Fig. 3, which clearly illustrates the synchronous growth of SRB and methanogen populations, as measured by the membrane hybridization technique.

Although the *Clostridium* population was not measured in membrane hybridization, the increase in both SRB and methanogen populations over time, as illustrated in Fig. 3, indicates a decrease in the *Clostridium* population. This may be due to *Clostridium's* inability to use lactate and acetate in the biofilm as substrate. Although many *Clostridium* can degrade yeast extract, the decrease in the *Clostridium* population is likely due to the reduced availability of yeast extract from the bulk solution, as a result of increased biofilm thickness.

Conclusion

Based on 16S rDNA analysis using a PCR-cloning-sequencing approach, the two most predominant groups of methanogen in the corrosive marine biofilm in this

study were closely related to the acetotrophic *Methanosaeta* and the hydrogenotrophic *Methanogenium*, *Methanoplanus* and *Methanocalculus*. The hydrogenotrophic group might corrode metals directly through cathodic depolarization. The acetotrophic group might, on the other hand, corrode metals indirectly via syntrophic growth with corrosion-causing SRB.

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