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SHORT CONTRIBUTION

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Phylogenetic diversity of a SRB-rich marine biofilm

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Abstract This study was conducted to characterize the phylogenetic diversity of a corrosive marine biofilm based on 16S rDNA. Results of phylogenetic analysis indicated that, out of the 112 clones developed, 52 clones (46.4%) were affiliated with two families of sulfate-reducing bacteria: *Desulfovibrionaceae* and *Desulfobacteriaceae*. Another 44 clones (39.3%) were affiliated with the *Clostridiaceae* family of low G+C, Gram-positive bacteria. Three clones (2.7%) were closely related to *Chlorobium vibrioforme*, a green sulfur bacterium.

Introduction

Microbial-influenced corrosion (MIC) has been studied for decades since the electrochemical process caused by sulfate-reducing bacteria (SRB) was identified. Although most biofilms contain mixed populations, MIC studies conducted to date have focused on pure cultures (Jayaraman et al. 1999), or artificial consortia comprising two or three known species (Angell et al. 1997). As a consequence, the influence of microbial interactions on MIC has often been overlooked.

This study was conducted to characterize the phylogenetic diversity of microbial populations present in a corrosive marine biofilm (Xu et al. 1999) using molecular techniques including denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) and cloning and sequencing of selected 16S rDNA fragments (Sekiguchi et al. 1998). This approach provided a better understanding of the bacterial populations associated with corrosion in the marine environment.

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Materials and methods

DNA extraction and amplification

A SRB-rich marine biofilm was developed for the study of biocorrosion of mild steel as described previously (Xu et al. 1999). The genomic DNA in the biofilm after 90 days was extracted (Liu et al. 1997). For DGGE screening, the extracted DNA was amplified by polymerase chain reaction (PCR) as follows: an initial denaturation at 94°C for 7 min; 35 cycles of denaturation (1 min at 92°C), annealing (1 min at 54°C) and extension (1 min at 72°C); and a final extension at 72°C for 10 min. The same procedures were also applied for DNA cloning, except with only 25 cycles of denaturation, annealing and extension. All PCR products were stored at 4°C.

DGGE screening

Before cloning of extracted rDNA, sequence variations were assessed by DGGE. The DNA extracts were PCR-amplified using the primer set Eub968F (5′-AACGCGAAGAACCTTAC) plus GC-clamp (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG) and Univ1392R (5′-ACGGGCGGTGTGTRC). The DGGE profile of the PCR-amplified DNA was obtained following the method of Muyzer et al. (1993). Each stained band in the DGGE profile represented a unique DNA fragment.

Cloning

The extracted DNA was PCR-amplified using the primer set GM3F (5′-AGAGTTTGATCMTGGCTCAG) and GM4R (5′-GGTTACCTTGTTACGACTT). Cloning of these fragments was conducted using the TA cloning kit (Invitrogen, Carlsbad, Calif.). A total of 112 colonies were selected for inoculation of 1.0 ml LB medium containing 50 mg l^{-1} kanamycin. After 18 h incubation at 37°C, the plasmids were recovered. The primer set of M13F (5′-GTTTGATCCTGGCTCAG) and M13R (5′-CAGGAAACAGCTATGAC) was used to amplify the inserted rDNA fragments carried on the plasmid.

DNA sequencing and phylogenetic analysis

Using the DGGE profiles of the PCR products, a total of 112 clones were selected. They were composed of 24 unique DNA sequences, commonly referred as operational taxonomy units (OTUs). Eleven major OTUs were shared by three or more clones. The DNA of these OTUs was then sequenced using an auto se-

quencer (ABI model 377A, Perkin-Elmer, Foster City, Calif.) and dRhodamine terminator cycle sequencing FS ready reaction kit (Perkin-Elmer). All sequences obtained were aligned with the closest match 16S rDNA sequences found in the GenBank by BLAST search (Altschul et al. 1990) using Clustal W1.8 (Thompson et al. 1994), and phylogenetic trees were constructed using the neighbor-joining method with MEGA 2.1 (Kumar et al. 1993).

Accession numbers

The nucleotide sequence data reported in this paper have been submitted to the GenBank, EMBL and DDBJ databases, and assigned the following accession numbers: AF295107 (90dCC9), AF295108 (90d11), AF295109 (90d4), AF295110 (90dCB10), AF295111 (90dCB13), AF295112 (90dCB36), AF295113 (90dCB40), AF295114 (90dCC18), AF295115 (90dCC26), AF295116 (90dCC28), and AF295117 (90d10).

Results

Table 1 shows that a total of 52 clones (46.4%) comprising five OTUs were affiliated with the two SRB families *Desulfovibrionaceae* and *Desulfobacteriaceae*. Another 44 clones (39.3%) comprising five different OTUs were affiliated with members of the *Clostridiaceae* family of low G+C, Gram-positive bacteria. Three clones (2.7%) comprising one OTU were closely related to *Chlorobium vibrioforme*, a green sulfur bacterium (GSB).

Among the SRB-related clones, 37 clones (33.0%) comprising three OTUs were related to the family *Desulfovibrionaceae*, and 15 (13.4%), comprising two OTUs, to the family *Desulfobacteriaceae*. A total of 28 clones (25%) that fell within the OTU 90d4 were most closely related (92% similarity) to a single SRB species,

Table 1 Phylogenetic affiliation of OTUs

Operational taxonomy unit (OTU)	Sequence length	Phylogenetic relationship			No. of clones	Abundance (%)
		Family	Closest species in GenBank	Similarity (%)		
90d4	613	*Desulfovibrionaceae*	*Desulfovibrio sp.* BG50	92	28	25.0
90dCB13	282	*Desulfovibrionaceae*	*Desulfovibrio caledoniensis*	93	5	4.5
90dCC9	294	*Desulfovibrionaceae*	*Desulfomicrobium baculatum*	86	4	3.6
90dCB36	379	*Desulfobacteriaceae*	*Desulfobacterium sp.* BG33	92	12	10.7
90dCC28	269	*Desulfobacteriaceae*	*Desulfobulbus sp.* BG25	96	3	2.7
90d10	714	*Clostridiaceae*	*Clostridium litorale*	96	17	15.2
90dCB40	349	*Clostridiaceae*	*Clostridium litorale*	96	5	4.5
90dCC18	397	*Clostridiaceae*	*Clostridium litorale*	95	2	1.8
90d11	452	*Clostridiaceae*	*Ruminococcus hydrogenotrophicus*	88	13	11.6
90dCB10	370	*Clostridiaceae*	*Ruminococcus hydrogenotrophicus*	90	7	6.3
90dCC26	410	*Chlorobiaceae*	*Chlorobium vibrioforme*	92	3	2.7
Others[a]					13	11.6
				Total	112	100.0

[a] One clone each

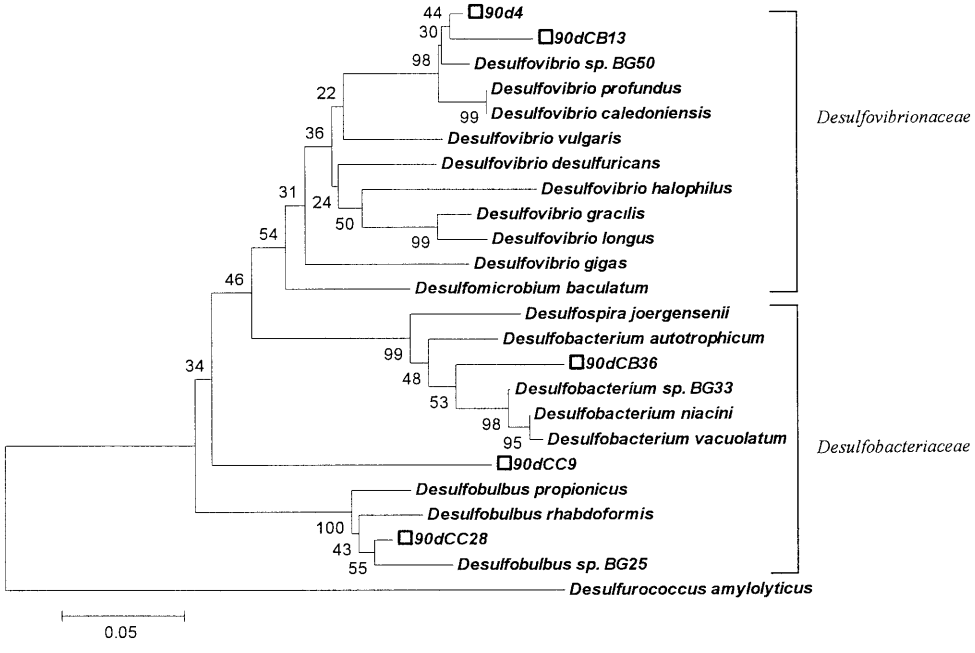

Fig. 1 Phylogenetic tree of five operational taxonomy units (OTUs) and their close relatives in the order *Desulfovibrionales* based on 260 nucleotides in 16S rDNA sequence. The tree, based on Jukes-Cantor distance, was constructed using the neighbor-joining algorithm with 500 bootstrappings. *Desulfurococcus amylolyticus* was selected as the outgroup species. The scale bar represents 0.05 substitutions per nucleotide position. Numbers at the nodes are the bootstrap values

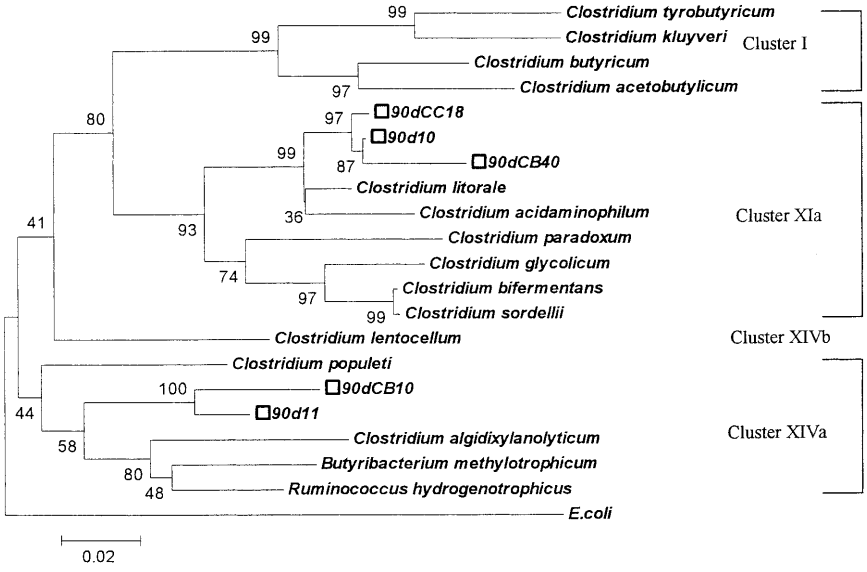

Fig. 2 Phylogenetic tree of five OTUs and their close relatives in the *Clostridiaceae* family based on 350 nucleotides in 16S rDNA sequence. The clusters I, XIa, XIVa and XIVb are the rDNA clusters in the genus *Clostridium*. The tree, based on Jukes-Cantor distance, was constructed using the neighbor-joining algorithm with 500 bootstrappings. *Escherichia coli* was selected as the outgroup species. The scale bar represents 0.02 substitutions per nucleotide position. Numbers at the nodes are the bootstrap values

Desulfovibrio sp. BG50 in the family *Desulfovibrionaceae*. Nine additional clones (8.1%) comprising two OTUs, 90dCB13 and 90dCC9, were most closely related to two other species in the same family, *Desulfovibrio caledoniensis* and *Desulfomicrobium baculatum*, respectively. On the other hand, 15 clones (13.4%) comprising two OTUs, 90dCB36 and 90dCC28, were most closely related to *Desulfobacterium sp.* BG33 and *Desulfobulbus sp*. BG25, respectively, of family *Desulfobacteriaceae*.

Among those related to *Clostridiaceae*, 24 clones (21.4%) comprising three OTUs, 90d10, 90dCB40 and 90dCC18, were most closely related to the species *Clostridium litorale*, with 95–96% similarity. *C. litorale* is a mesophilic species, with an optimum growth temperature of 30°C, found in anoxic marine environments similar to the marine sediment used as the seed sludge of this study. Another 20 clones (17.9%) comprising two OTUs, 90d11 and 90dCB10, were loosely related, with 88–90% similarity, to the species *Ruminococcus hydrogenotrophicus.*

Figure 1 illustrates the phylogenetic tree for five OTUs closely related to the families *Desulfovibrionaceae* and *Desulfobacteriaceae*, whereas Figure 2 illustrates that of five OTUs closely related to *Clostridiaceae*.

Discussion

Figure 1 illustrates that OTUs 90d4 and 90dCB13 are closely related to each other, and form a cluster with *Desulfovibrio caledoniensis*, *Desulfovibrio profundus* and *Desulfovibrio* BG50 with a 98% bootstrap value. Thus, these two OTUs might be assigned to the genus *Desulfovibrio*, which is capable of reducing sulfate, sulfite and thiosulfate, using hydrogen and lactate as electron donors to produce acetate (Postgate 1984). The OTU 90dCC9 appears to belong to the order *Desulfovibrionales*, but is not closely related to any known species in that order. Figure 1 also illustrates that OTU 90dCB36 might be assigned to the genus *Desulfobacterium*, abundant in brackish and marine environments, and capable of reducing sulfate, sulfite and thiosulfate to hydrogen sulfide (Holt et al. 1994). Figure 1 further illustrates that OTU 90dCC28 was affiliated with the genus *Desulfobulbus*, a group of SRB that has not been associated with MIC. Studies of MIC have focused primarily on the SRB. SRB-related MIC has been attributed to either cathodic hydrogen removal (Cord-Ruwisch et al. 1987) or production of hydrogen sulfide and fatty acids (Boopathy and Daniels 1991). Of the SRB, the genus *Desulfovibrio* has been most widely linked to MIC (Jayaraman et al. 1999). In this study, 33 clones (29.5%) comprising two OTUs were closely related to *Desulfovibrio*.

Figure 2 illustrates that OTUs 90d10, 90dCB40 and 90dCC18 by themselves form a new group, which clusters with *C. litorale* and *Clostridium acidaminophilum* with a 99% bootstrap value. They are also closely related to other species of *Clostridium* in the cluster XIa. Thus these three OTUs might be assigned to the cluster XIa of genus *Clostridium* (Stackebrandt and Rainey 1997). Some *Clostridium* species are capable of producing hydrogen sulfide and organic acids (Holt et al. 1994), and have been associated with corroding metals. Other *Clostridium* species, such as *C. acetobutylicum*, may produce hydrogen for SRB (Nandi and Sengupta 1998), lowering the hydrogen demand of SRB at the cathode and thus resulting in slower corrosion rates. Most hydrogen-producing *Clostridium* species characterized to date are freshwater species. Information is scarce on marine species such as *C. litorale*. Most hydrogen-producing bacteria in the genus *Clostridium* are in cluster I (Ia, Ii, Ig and Ih) with a few exceptions in clusters III and XIb. In this study, OTUs 90d10, 90dCB40 and 90dCC18 were affiliated with cluster XIa. The hydrogen-producing capabili-

ty of strains of *Clostridium* affiliated with this cluster has not been elucidated.

Hydrogenotrophic anaerobes other than SRB might also contribute to MIC (Boopathy and Daniels 1991). *R. hydrogenotrophicus* is the closest known species to the OTUs 90d11 and 90dCB10, but only with 88–90% similarity. Figure 2 also shows that OTUs 90dCB10 and 90d11 form a new group which shares little relationship with any known species, although they might be assigned to Cluster XIVa of the *Clostridium* genus (Stackebrandt and Rainey 1997).

Table 1 shows that OTU 90dCC26 is related (92% similarity) to *Chlorobium vibrioforme*, a photosynthetic GSB, but it cannot be assigned to any genus. According to 16S rRNA analysis, most phenotypic traits of GSB are of little phylogenetic significance (Overmann and Tuschak 1997). GSB may be syntrophically associated with SRB by oxidizing sulfide to sulfate; this would result in an increase of corrosion rate. On the other hand, GSB may also compete with SRB for hydrogen (Holt et al. 1994), resulting in a decrease of corrosion rate. The net effect is still not clear.

A recent study showed that a mixed-culture SRB-rich biofilm, similar to the biofilm of this study, was ten times less corrosive than biofilms of pure SRB cultures (Xu et al. 1999). The results of this study suggest that *Clostridium* and GSB in the mixed culture might be the reason for lower corrosion; the former reduces the cathodic hydrogen utilization by SRB, while the latter removes hydrogen sulfide at the anode.

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