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Bisulfite reductase and nitrogenase genes retrieved from biocorrosive bacteria in saline produced waters of offshore oil recovery facilities

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ABSTRACT

Water-flooding is a common strategy to enhance oil recovery in reservoirs. Maintaining quality and standards of produced water avoids oil biodegradation, biogenic souring and biocorrosion during operations, which are influenced by sulfate-reducing (SRB) and Fe (III) reducing bacteria. The aim of this work was to increase our knowledge of corrosive bacterial communities inhabiting saline produced waters of offshore oil exploitation facilities through retrieving sequences of functional genes, for instance, *dsr*AB and *nif*D of Desulfovibrionales, Desulfobacterales and Desulfuromonadales taxonomical orders. Five clone libraries were generated with retrieved sequences acquired from different saline produced waters, with and without biocide dosing. The *dsr*AB phylogenetic analyses showed *Desulfomicrobium*, *Desulfovibrio*, and *Desulfohalobium* as well as *Desulfococcus*, *Desulfosarcina*, *Desulfobacter*, *Desulfobacterium* and *Desulfobulbus*. The retrieved *nif*D genes displayed the Fe (III) reducing bacteria (Desulfuromonadales) such as *Desulfuromusa*, *Pelobacter*, *Malonomonas*, and *Desulfuromonas*. The relative abundance in all waters was: the Desulfovibrionales were represented by 55.28% of analyzed clones; the Desulfobacterales by 26.83% and 17.89% for the Desulfuromonadales. Diversity measures were calculated by the Shannon index (H'), which showed that there was a high degree of diversity between all produced waters; however, dominance in produced water with biocide was detected by a *Desulfovibrio* taxon.

1. Introduction

The sulfate-reducing bacteria (SRB) are widespread in nature and have been found in virtually every anaerobic environment that has been investigated. They play an important role in the global sulfur cycle, and in marine sediments, they are involved in up to 50% of the total carbon mineralization process (Birkeland, 2005).

The SRB produce metabolites, like hydrogen sulfide, which causes several problems during oil production, such as corrosion of iron and steel alloys in the oil wells, the reduction of the oil quality by souring oil and gas, and the reduction of the permeability of the oil formation in the reservoir (Cord-Ruwisch et al., 1987; Muyzer and Stams, 2008; Barton and Fauque, 2009). However, other bacterial species ecologically involved with SRB contribute to corrosion development (Zhu et al., 2003).

The dissimilatory Fe (III) reducers can completely oxidize acetate and other short-chain fatty acids to carbon dioxide, with Fe (III) being the sole electron acceptor (Lovley, 1997). These bacteria like *Geobacter*, *Desulfuromonas*, *Pelobacter* and *Desulfuromusa*, conform a monophyletic group inside the Deltaproteobacteria class as well as share the Fe (III) and / or S° reducing ability (Lonergan et al., 1996). The Fe (III) reducers have been isolated from corroded steel pipelines and they accelerate corrosion by removing Fe (III) oxide over metal surfaces acting as an electron acceptor (Obuekwe et al., 1981). Currently, it is well-known that *Geobacter sulfurreducens* is implicated in the corrosion of mild steel, ferritic and austenitic stainless steels, by extracting the electron directly from metal and creating a cathodic reaction on material surfaces (Mehanna et al., 2009a; Mehanna et al., 2009b).

In the oil and gas industry, high salinity waters are routinely injected into formations for pressure maintenance by water flooding. The enhanced oil recovery (EOR) processes, like steam drive, *in situ* combustion and carbon dioxide, micellar-polymer,

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polymer and alkaline flooding, require large quantities of water available from sea and aquifers (Royce et al., 1984). In order to avoid supply problems, produced water from reservoirs is re-injected after physical and chemical treatment processes, like filtration, chlorination and biocide addition. High-quality water is needed to achieve EOR operations and to keep from being damaged the geological-formation of exploited reservoirs as well as to avoid ineffective production operations. Although oil reservoirs have some similarities, each one is unique and should be evaluated individually to apply EOR technologies, including the microbiological quality of the water (Royce et al., 1984; Bao et al., 2009). The SRB, the Fe (III) reducing and total bacteria count values are mainly suggested before and after injection of water operations with the purpose of preventing souring and corrosion processes (Royce et al., 1984; Collins and Wright, 1985; Bao et al., 2009).

Several studies have been conducted in order to know the diversity of the microbiota in oil reservoirs, and their exploitation facilities by using 16S rRNA genes (Voordouw et al., 1996; Orphan et al., 2000; Zhu et al., 2003; López et al., 2006; Neria-Gonzalez et al., 2006; Korenblum et al., 2010). At the present time, the functional genes that are implicated in metabolic processes are being incorporated into ecological studies (Agrawal and Lal, 2009; Duncan et al., 2009; Gittel et al., 2009). The SRB have dissimilatory sulfite reductase (DSR), a key enzyme in the anaerobic sulfate respiration pathway. The *dsr*AB gene encodes alpha and beta subunits of DSR mature enzyme and it has been sequenced in several SRB lineages as well as used to infer the evolutionary history of DS reductases through sulfate reducing prokaryotes (Wagner et al., 1998; Zverlov et al., 2005). Another potential target for the detection of bacteria associated with corrosion is the *nif*D gene that encodes the α subunit of the dinitrogenase enzyme (NIFD). It catalyzes N_2 fixation, a process coupled with Fe (III) reduction in *Geobacteraceae* and in some species of the Desulfuromonadales order; however, the gene has not been detected in other metal reducing bacteria (Holmes et al., 2004a; Holmes et al., 2004b). Since it has been demonstrated that *Geobacteraceae* plays a significant function in corrosion (Mehanna et al., 2009a; Mehanna et al., 2009b), detection methods for other Fe (III) respiring microorganisms, that may also be involved in corrosion process, are a subject for further study in oil fields. The aim of this work was to increase our knowledge of corrosive bacterial communities inhabiting saline produced waters, with and without biocide, of offshore oil exploitation facilities, through the *dsr*AB and *nif*D functional genes.

2. Materials and methods

2.1. Sample collection

Six saline produced water samples (PW) were aseptically obtained from an offshore platform of water systems for EOR program in Mexican oil field. PW1, PW2, PW3 and PW4 samples were taken from different points of EOR facilities after commercial biocide dosing with tetrakis (hydroxymethyl) phosphonium sulfate (THPS). PW5 and PW6 were acquired from two different sites without biocide treatment. In this oil reservoir there is no nitrate treatment in order to stimulate nitrate-reducing bacteria that outcompete SRB for electron donors, which promote the inhibition of SRB by the production of nitrite and nitrous oxides.

All samples were stored in sterilized bottles sealed with rubber stoppers at 4 °C. The physicochemical values were determined for all water samples. The conductivity/salinity was measured by EPA Method 120.1. The sulfate was determined according to ASTM D516-02 method; for the iron analysis the Hach Method 8008 was followed; the pH and temperature were measured by the SM 4500+H B and SM 2550 methods, respectively.

2.2. Reference strains

Anaerobic reference strains were acquired from the DSMZ bank (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). *Desulfobacter postgatei* DSM 2034; *Desulfobulbus rhabdoformis* DSM 8777, and *Desulfovibrio desulfuricans* subsp. *desulfuricans* DSM 642 were used to test PCR of the *dsr*AB gene. *G. sulfurreducens* DSM 12127, *G. metallirreducens* DSM 7210 and *Desulfuromonas palmitatis* DSM 12931 were used to test PCR of the *nif*D gene, and *Klebsiella variicola* F2R9 (=ATCC BAA-830T) was the positive quality assay control. All DSM strains were cultivated in liquid media under specific conditions described by DSMZ culture protocols (www.dsmz.de). Additionally, in all PCRs to test *dsr*AB gene, the template DNA used as negative controls was obtained from *K. variicola*, *Escherichia coli* and *Bacillus megaterium*; they were grown on nutrient broth and were incubated at 35 °C.

2.3. DNA extraction

The DNA from strains was extracted from 1 ml of culture medium by using UltraClean Microbial DNA Isolation Kit (MoBio Laboratories). The produced water samples (100–150 ml for each one) were centrifugated and harvested by filtration in a sterile 0.22 μm cellulose acetate membrane (Millipore). The collected biomass was washed with 50 ml of sterile buffer solution (3 g l^{-1} Na_2HPO_4; 2 g l^{-1} KH_2PO_4; 5 g l^{-1} NaCl; 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$ diluted in DNAse and RNAse-free water; pH 6.5). Metagenomic DNA was extracted using the UltraClean soil DNA extraction kit (MoBio Laboratories), whilst following the manufacturer's instructions. The DNA quality was estimated by electrophoresis in 1.2% agarose gels in 1X TBE buffer (0.089 M Tris; pH 8.3; 0.089 M boric acid; 0.002 M EDTA); it was stained in a 0.5 mg ml^{-1} ethidium bromide solution. The DNA concentrations were determined by spectrophotometry and an A260/A280 ratio of 1.8:2.1 was considered to be acceptable.

2.4. Oligonucleotide primers

A total of 85 *dsr*A genes of Desulfobacterales, 101 *dsr*B genes of Desulfovibrionales and 31 *nif*D genes of Desulfuromonadales species were obtained from GeneBank in the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and were analyzed by the BLAST program (Altschul et al., 1990). Multiple alignment analyses were performed with CLUSTAL X (Thompson et al., 1997), and highly conserved gene regions were considered for the design of PCR primer oligonucleotides. Melting temperatures (Tm), self and two primer-complementary configurations, and mispriming analyses were estimated by DNAMan software. All primers were synthesized by Invitrogen Life Technologies. Primers were probed with template DNA extracted from reference strains as positive and negative controls. Additionally, the amplicons obtained of the designed primers were compared against the public database by the BLAST program in order to ensure that the correct target was being amplified. The set of designed sequences to amplify *dsr*A, *dsr*B and *nif*D genes and the expected size of PCR products are presented in Table 1. It means that five clone libraries were generated, one clone library per set of primers. Their target-taxa include three orders and seven families of bacteria related to biocorrosion processes.

2.5. PCR procedures

A nested PCR with primers DSR1F and DSR4R (Wagner et al., 1998) was conducted to amplify the *dsr*AB gene. The PCR conditions were an initial activation step of 15 min at 95 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; the