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Characterization of bacterial community associated to biofilms of corroded oil pipelines from the southeast of Mexico

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Abstract

Microbial communities associated to biofilms promote corrosion of oil pipelines. The community structure of bacteria in the biofilm formed in oil pipelines is the basic knowledge to understand the complexity and mechanisms of metal corrosion. To assess bacterial diversity, biofilm samples were obtained from X52 steel coupons corroded after 40 days of exposure to normal operation and flow conditions. The biofilm samples were directly used to extract metagenomic DNA, which was used as template to amplify 16S ribosomal gene by PCR. The PCR products of 16S ribosomal gene were also employed as template for sulfate-reducing bacteria (SRB) specific nested-PCR and both PCR products were utilized for the construction of gene libraries. The V3 region of the 16S rRNA gene was also amplified to analyse the bacterial diversity by analysis of denaturing gradient gel electrophoresis (DGGE). Ribosomal library and DGGE profiles exhibited limited bacterial diversity, basically including *Citrobacter* spp., *Enterobacter* spp. and *Halanaerobium* spp. while *Desulfovibrio alaskensis* and a novel clade within the genus *Desulfonatronovibrio* were detected from the nested PCR library. The biofilm samples were also taken for the isolation of SRB. *Desulfovibrio alaskensis* and *Desulfovibrio capillatus*, as well as some strains related to *Citrobacter* were isolated. SRB consists in a very small proportion of the community and *Desulfovibrio* spp. were the relatively abundant groups among the SRB. This is the first study directly exploring bacterial diversity in corrosive biofilms associated to steel pipelines subjected to normal operation conditions.

Keywords: Biofilm; Corrosion; Oil pipeline; Bacterial community; *Citrobacter*

1. Introduction

Metal corrosion is one of the main damages causing great economic losses in pipeline systems of the petroleum industry. Around 40% of the internal pipeline corrosion in the gas industry has been attributed to microbiologically influenced corrosion (MIC) [1,2]. However, the mechanistic basis of MIC is remained unclear.

A theory about the MIC holds that biofilms promote corrosion by inducing the formation of corrosion cells. This is thought to occur as a consequence of aerobic respiratory activity within biofilms that leads to the establishment of local cathodic and anodic regions on the steel surface, which promotes electron flow [3]. Other explanations for MIC include corrosion promoted by anaerobes such as sulfate-reducing and iron-reducing bacteria. In the biofilm, the sulfate reducers promote corrosion by consuming hydrogen and inducing the formation of ferrous sulfide and the iron reducers promote corrosion by reductively dissolving the protective ferric oxide coat that forms on the steel surface [3,4]. The

Abbreviations: MIC; Microbiologically influenced corrosion; SRB; Sulfate-reducing bacteria; AFB; Anaerobic fermentative bacteria; DGGE; Denaturing gradient gel electrophoresis; SEM; Scanning electron microscopy

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bacterial communities in biofilms developed on the surface of materials in natural environments are heterogeneous, and therefore there is significant uncertainty concerning how these communities affect corrosion in a given environment. The knowledge of bacterial diversity in the biofilms is helpful to understand the interactions between corrosive bacteria and metal surface, as well as with other micro-organisms, and provides the basis for the development of new and better means for the detection and prevention of corrosion.

Few studies, based on culture and culture-independent techniques, have been performed to investigate the composition of microbial communities in corrosive biofilms formed under laboratory conditions using samples collected from gas pipelines affected by corrosion and marine sediments [5–7]. However, the isolation of bacterial species presented in pipeline transportation systems and storage tanks used in petroleum exploitation do not reflect the complexity of the bacterial community [8–11]. Some researches on bacterial communities in the oil-contaminated sites or oil-storage cavity were carried out [12,13], but there is no such report about biofilms associated to corrosion in oil pipelines.

The aim of this study was to analyse through molecular and culture methods the diversity of bacterial communities in biofilm samples developed in oil pipelines under normal operation conditions with evident corrosion signals from Mexican oil fields. This topic is relevant for the recognition of bacterial species found in biofilms attached to metal surfaces of pipeline. The data may contribute to elucidate which bacterial species contribute in the MIC process and to gain a better understanding of the microbial community composition of biofilms.

2. Materials and methods

2.1. Sampling of biofilms

To obtain the biofilm samples, two API X52 steel coupons (surface area with 4 cm^2) were placed at inner surface (Fig. 7A two different pipelines made of the same material and exposed for 40 days to the flow of petroleum. The involved pipelines were located in the Mexican Southeast region, Tabasco, where Maya crude is extracted. In general, Maya crude oil has a high viscosity, high contents of asphaltene, high concentration of heavy metals and sulfur (3.3 wt%), it contains in average 6% of water with 60 000–80 000 ppm of NaCl and 350–400 ppm of sulfate. The coupons were detached from the inner region of oil pipelines and the biofilms formed on the surfaces of each coupon were removed with sterile brushes and collected into sterile bottles with 50 mL phosphate-buffered saline, pH 7 [14]. The biofilm samples were named as M1 and M2, corresponding to each oil pipeline.

2.2. DNA extraction from biofilm samples and PCR amplification

The metagenomic DNA from biofilm samples was extracted using a previously reported method [15]. The DNA extracts required five additional 70% ethanol washes to eliminate salts and to obtain an amplifiable DNA. DNA concentrations and A_{260}/A_{280} ratio were determined with a spectrophotometer (Lambda 1A; Perkin-Elmer). An A_{260}/A_{280} ratio of 1.8–2.1 was considered acceptable for PCR-based procedures. The extracted DNA of biofilms was used as template to amplify the 16S rRNA genes by PCR with the universal forward primer 8FPL (5′-GCGGATCCGCGGCCGCTGCAGAGTTTG-3′) and reverse primer 1492RPL (5′-GGCTCGAGCGGCCGCCCGGGTTACCTT-3′) [16]. The reaction mixture (25 μL) contained 100 ng of DNA; the appropriate primer at 0.8 pM; dATP, dCTP, dCTP, and dTTP, each at a concentration of 800 μM; 2.5 mM $MgCl_2$; and 1 U of *Taq* DNA polymerase in the PCR buffer provided by the manufacturer (Invitrogen Life Technologies, Sao Paulo, Brazil). Amplification conditions included a denaturation step for 5 min at 92 °C followed by 21 cycles consisting of 1 min at 92 °C, 1 min at 53 °C and 2.5 min at 72 °C, and a final extension step for 10 min at 72 °C. The amplification was done with a Gene Amp® PCR System 9700 (Applied Biosystems, Foster City, CA). The expected size of the fragment amplified from the 16S rRNA gene was approximately 1500 bp. PCR products were purified by means of the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA).

2.3. Construction of 16S rRNA gene libraries and 16S rRNA gene sequencing

The purified PCR products of 16S rRNA genes from the biofilm samples were cloned into the vector pCR® 2.1-Topo® by using the TOPO TA Cloning Kit (Invitrogen Life Technologies Carlsbad, CA). The positive clones were detected by the appearance of white colonies in solid LB medium containing 80 μg/mL X-gal, 160 μg/mL IPTG, and 100 mg/mL ampicillin. Recombinant plasmids were isolated from overnight cultures by alkaline lysis [14], and a restriction analysis with *Eco*RI to detect the insertion was performed. The restriction endonucleases, *Msp*I and *Hha*I, were used to digest the recombinant plasmids. The RFLP patterns were revealed by electrophoresis in 3% high-performance Agarose 1000 (GIBCO Laboratories, Grand Island, NY). The clones were grouped according to their RFLP patterns and one clone for each RFLP pattern was selected for sequencing with the ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA) using M13 primers. The relative abundance was estimated considering the proportion of clones of each pair of theorical RFLP phylotypes per species.