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Isolation of a sulfide-producing bacterial consortium from cooling-tower water: Evaluation of corrosive effects on galvanized steel

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ABSTRACT

Sulfidogenic *Clostridia* and sulfate reducing bacteria (SRB) often cohabit in nature. The presence of these microorganisms can cause microbially influenced corrosion (MIC) of materials in different ways. To investigate this aspect, bacteria were isolated from cooling tower water and used in corrosion tests of galvanized steel. The identity of the isolates was determined by comparative sequence analysis of PCR-amplified 16S rDNA gene fragments, separated by denaturing gradient gel electrophoresis (DGGE). This analysis showed that, in spite of the isolation process, colonies were not pure and consisted of a mixture of bacteria affiliated with *Desulfosporosinus meridiei* and *Clostridium* sp. To evaluate the corrosive effect, galvanized steel coupons were incubated with a mixed culture for 4, 8, 24, 72, 96, 168, 360 and 744 h, along with a control set in sterile culture medium only. The corrosion rate was determined by weight loss, and biofilm formation and corroded surfaces were observed by scanning electron microscopy (SEM). Although the sulfide-producing bacterial consortium led to a slight increase in the corrosion of galvanized steel coupons, when compared to the previous studies it can be said that *Clostridium* sp. can reduce the corrosive effect of the *Desulfosporosinus* sp. strain.

1. Introduction

Cooling towers are used for removing waste heat generated by industrial processes, such as in the petrochemical industry. They are also used, on a smaller scale, to remove excess heat from air conditioning systems in public buildings. These cooling towers, especially those of the open type, have conditions favorable for microbial growth and biofilm formation [1]. Biofilms often consist of both aerobic and anaerobic bacteria, including sulfate reducing bacteria (SRB) that are well-known as biocorrosion agents [2–4]. *Desulfovibrio* sp. and *Desulfosporosinus* sp. are the most abundant and dominant SRB in cooling tower water [5].

Microbiologically influenced corrosion (MIC) or biocorrosion is initiated or accelerated by the activities of microorganisms, which are often present as biofilms [6]. In aerated biofilms, O_2 is removed by both the cathodic reactions during the corrosion process and the microbial activity of aerobic bacteria [7,8]. In a developing biofilm, these processes cause the formation of different oxygen gradients within the biofilm, promoting the growth of anaerobic SRB in a fully aerated system, such as a cooling tower [9]. Although several anaerobes may be associated with corrosion, SRB are considered the main cause of MIC [8,10,11]. Several mechanisms explain anaerobic MIC from a single SRB strain. These include cathodic depolarization [7], iron sulfide precipitation that can induce cathodic depolarization [12,13], anodic depolarization by local acidification of the anode [14], chelation of metal ions owing to extracellular polymeric substances (EPS) [14], and galvanic coupling with EPS [15]. Additionally, a hydrogenase enzyme was shown to induce a cathodic reaction on steel surfaces and so causing MIC [16].

Each SRB strain is known to show different corrosive effects via distinctive corrosion mechanisms [16–18]. Additionally, each SRB strain is known to form a different biofilm structure, which affects its corrosion behavior in different ways [19]. To understand the MIC

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mechanism, laboratory studies with pure cultures isolated in nature need to be performed; however, in nature, microorganisms rarely exist in a pure form. Herro and Port [20] reported on results of a microbiological analysis of carbon-steel service water. They found out that sulfidogenic *Clostridia* are commonly detected in locations featuring possibly corrosive sulfate reducers and that their metabolic products are utilized as substrates by SRB.

Some *Clostridium* species are known to produce hydrogen sulfide and organic acids [21] and therefore enhance the corrosion of metals [22,23]. Furthermore, the activity of the hydrogenase enzyme produced by *C. pasteurianum* has been shown to catalyze the removal of cathodically produced hydrogen from mild steel when suitable electron acceptors are available [24]. These findings highlight the importance of assessing the influence of SRB and *Clostridia* on corrosion processes.

Cooling towers are often made of galvanized steel owing to its high resistance to corrosion, mechanical workability, and ability to resist biofouling. However, these systems are still hampered by biofilm formation and biocorrosion, which influences the cooling efficiency and increases maintenance costs [4]. Ilhan-Sungur et al. [19,25] reported that *Desulfovibrio* sp. and *Desulfosporosinus* sp. can colonize on galvanized steel coupons and corrode them under laboratory conditions.

Because *Clostridium* sp. provides hydrogen as an electron donor, it is of great importance to SRB [20]. However, no studies have reported on the MIC of galvanized steel by *Clostridia* with or without SRB in cooling towers. The main aim of this study is to evaluate the corrosive effect of a sulfide-producing bacterial consortium consisting of *Desulfosporosinus meridiei* and *Clostridium* sp. on galvanized steel. For this purpose, this study was conducted in two stages: (i) a sulfide-producing bacterial consortium of *Desulfosporosinus meridiei* and *Clostridium* sp. was isolated from cooling water, and (ii) the corrosivity of this consortium on galvanized steel was determined under laboratory conditions over 744 h. Our results may have broad implications, because similar microorganisms are found in industrial systems such as sewage systems, heat exchangers, and those used in the petrochemical industry.

2. Materials and methods

2.1. Isolation and identification of a sulfide-producing bacterial consortium

2.1.1. Isolation of anaerobic bacteria

Water samples were collected from five different cooling towers belonging to different hotels in Istanbul. Dark-colored sterile glass bottles were completely filled with water samples and sealed tightly to maintain anoxic conditions. All samples were brought directly to the laboratory and processed on the same day. Water samples were inoculated into sterile Postgate's medium B (PB) to enrich the sulfide-producing bacteria [26]. The tubes were incubated in darkness for 3 months at 30 °C. Growth was revealed by the turbidity and black FeS precipitate formation [26]. Inoculations from the growth-positive tubes were conducted into the semi-solid Postgate's medium C (PC) [26]. The plates were placed in anaerobic jars with AnaeroGen sachets (Oxoid, Thermo Fisher Scientific) in less than 1% O_2 and 9–13% CO_2 atmosphere, and the jars were then incubated in darkness at 30 °C for 10–21 days. A number of blackish-brown colonies with similar macroscopic characteristics were selected aseptically by using sterile Pasteur pipettes with a stereo microscope; these were then inoculated separately in a sterile liquid PB medium, and the procedure was repeated in a solid PC medium to realize the formation of colonies of single bacterial strains [27,28]. The obtained sulfide-producing bacterial cultures were identified using molecular assays.

2.1.2. DNA extraction and PCR amplification of 16S rRNA gene

Two ml of the selected cultures was centrifuged at 10,000 g for 10 min, and the genomic DNA of the cell pellet was extracted using the UltraClean® Microbial DNA Isolation Kit (MOBIO Laboratories, Inc., CA) as described by the manufacturer. The genomic DNA was examined through 0.8% (w/v) agarose gel electrophoresis and stored at −20 °C until use. 16S rRNA gene fragments were amplified from the extracted DNA using universal primer 341F with a GC clamp (5′-CCTACGGGAGGCAGCAG-3′) and primer 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) [29]. The 25 μl reaction mixture contained 1.25 μl of genomic DNA (undiluted or diluted), 0.19 μl of each primer, 10.88 μl of RNAse-free water (Qiagen, Germany), and 12.5 μl of Taq master mix (Qiagen, Germany). Amplification was conducted in a Thermocycler (Westburg, The Netherlands) by the following steps: initial denaturation (95 °C; 5 min), 32 cycles of denaturation (95 °C; 30 s), annealing (57 °C; 40 s), elongation (72 °C; 40 s), and final elongation (72 °C; 30 min). The PCR products were then cooled to 4 °C.

2.1.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was conducted using the D-Code System (Bio-Rad Laboratories, USA). Twenty-five μl of the PCR product (200–300 ng) was mixed with 5 μl of loading dye and then applied directly onto a 1-mm-thick 6% acrylamide gel containing a 20%–70% urea-formamide gradient. Electrophoresis was then performed for 16 h at 100 V in a 1xTAE solution heated to 60 °C. After electrophoresis, the gels were stained with SYBR Gold (Invitrogen, USA) in a 1 x TAE solution for 20 min and photographed. The bands were excised under UV illumination, placed in 40 μl of 1xTRIS buffer (pH 8), and kept at 4 °C for 2 days. The eluted DNA was re-amplified using the same primer pair by the following PCR protocol: 25 μl of reaction mixture containing 0.5 μl of template DNA, 0.125 μl of each primer, 12.5 μl of Taq master mix, and 11.75 of nuclease-free water. The reaction was performed with 25 cycles [30]. Subsequently, 2.5 μl of PCR products was run on 1.5% (w/v) agarose gel for quantification.

2.1.4. Sequencing of PCR products

By using a Qiaquick PCR purification kit (QIAGEN GmbH, Hilden, Germany), the PCR products were purified, sequenced using a Macrogen (Seoul, Korea), and finally placed in a sterile 96-well plate. The reverse primer 907R was used to sequence the 16S rRNA gene fragments.

2.1.5. Comparative sequence analysis

The DNA sequences were aligned using CodonCode Aligner software and checked manually for gaps and undesignated bases. Then, they were compared with the sequences stored in the GenBank nucleotide database (NCBI) by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [31], and the closest affiliations were selected.

The 16S rRNA gene sequences are now located in the GenBank database with the accession numbers KR132557.1, KR132558.1, and KR132559.1.

2.2. Corrosion experiments

2.2.1. Test material

The test material was galvanized steel of the type used in constructing cooling towers. The material had a 5-μm-thick Zn coating. The coupons, measuring 50 × 25 × 0.5 mm in size, were arranged according to the guidelines of the American Society for Testing and Materials (ASTM) G1–72 [32]. The coupons were weighed, and each coupon's total surface area was calculated. Epoxy Zn phosphate primer (Moravia, Turkey) (gray) was used to coat the cut edges of