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Microbial Corrosion of C1018 Mild Steel by A Halotolerant Consortium of Sulfate Reducing Bacteria Isolated from an Egyptian Oil Field

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IN THIS study, a consortium of halotolerant sulfate reducing bacteria (SRB) was obtained from a water sample collected from an Egyptian oil-field. North Bahreyia Petroleum Company (NORPETCO) is one of the petroleum companies that suffer from severe corrosion. The present study aim to investigate the microbial structures of this consortium and their potential contributed to microbial corrosion. Dissimilatory sulfite reductase *dsrAB* gene sequences analysis indicated that the mixed bacterial consortium contained two main phylotypes: members of the *Proteobacteria* (*Desulfomicrobium* sp., *Desulforhopalus* sp., and *Desulfobulbus* sp.) and *Firmicutes* (*Desulfotomaculum* sp.). Mild steel C1018 coupons were incubated in the presence of SRB consortium for a period of 35 days, the evolution of corrosion was studied using weight loss and dissolved sulfide production of SRB consortium. Results indicated that, the corrosion rate in the presences of SRB was approximately 15 times of that for the control. Furthermore, sessile cells (biofilm) and subsequent corrosion products that developed were characterized by scanning electron microscope coupled with energy dispersive X-ray spectroscopy (EDX).

Keywords: Microbial corrosion, Sulfate-reducing bacteria, Dissimilatory sulfite reductase; *dsrAB,* dissolved sulfide.

Introduction

Metal corrosion is considered as a major problem affecting oil and gas industry that leading for serious economic and environmental problems. It has been estimated that total annual corrosion cost was exceeded $90 billion per year [1, 2]. The corrosion problems were mainly attributed to chemical and microbiological activities. Microbiological influenced corrosion (MIC) activities were responsible for 20 – 30 % of these problems. Sulfate-reducing bacteria (SRB) are considered as the main actor implicated in MIC [2]. SRB are anaerobic bacteria that reduce sulfate to sulfide *via* a process of dissimilatory anaerobic respiration. A key enzyme in these species is dissimilatory sulfite reductase (Dsr), which catalyzes the last step in sulfate reduction, the reduction of sulfite to sulfide. In sulfate-reducing bacteria (SRB), the genes encoding Dsr are commonly organized in a *dsrAB* operon, where *dsrA* and *dsrB* encode the α and β -subunits of Dsr, respectively [3]. Sequence analysis of *dsr*-gene was considered a useful tool in the identification of SRB especially within a complex microbial population [4]. The SRB microbial consortium was recorded as big problem in oil and gas industry where they cause pitting corrosion of tanks and pipelines [5]. The corrosiveness of these microorganisms is due to metabolites produced such as hydrogen sulfide (H_2S), the supposed electrochemical effect termed "cathodic depolarization", and microbial colonization on the metal surface [6]. The undesirable production of H_2S in oil reservoirs (souring) often occurs during secondary oil recovery when seawater is injected to produce the remaining oil. Consequently, oil reservoirs receive high sulfate concentration,

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about 330 mM [7]. Under anaerobic conditions, dissimilatory sulfate-reducing bacteria utilize sulfate as a terminal electron acceptor in the degradation of organic matter and produce the toxic and the corrosive H_2S [3]. This work was directed to (i) collection of water sample with high salinity from an oil field water tank with previous problem and use this sample as a case study, (ii) estimation the microbial composition of the enriched SRB consortium using *dsrAB* genes analysis, (iii) evaluation of the corrosion activity of the enriched SRB consortium by measuring the total dissolved sulfide in the bulk phase and calculating the metal corrosion rate.

Materials and Methods

Sample collection and SRB enrichment.

One water sample with high salinity content (110,000 ppm NaCl) was collected from North Bahreyia Petroleum Company (NORPETCO), Egypt. The water sample was delivered to the Egyptian-petroleum research institute`s laboratory under a cooling and an anaerobic condition for further analyses. On site, the water sample was inoculated in an anaerobic Postgate´s-B medium as reported by Postgate [8]. The Postgate´s medium was prepared by applying the original water salinity (110,000 ppm NaCl) and pH during preparation. The medium was prepared, sparged with nitrogen gas. The culture medium was inoculated with 10 % (*v/v*) water sample and incubated at 37 °C in darkness for 21 days. Medium preparation and cultivation were achieved according to the modified Hungate's technique for anaerobes [9]. The medium is useful since the abundant precipitate actually provides a favorable microenvironment for the preservation of viable cells [10]. The black precipitation (Ferrous sulfide) was used as a marker for sulfate-reduction bacterial activity.

Sulfate-reducing bacterial diversity

DNA extraction and PCR amplification of dsrAB genes (marker gene):

Two ml of the cultivated SRB was centrifuged at 10,000 g for 10 min and the genomic DNA of the cell pellet was extracted using E.Z.N.A® Bacterial DNA kit (3350-01, OMEGA biotech, USA) as described by the manufacturer. The extracted DNA was examined through 0.9% (w/v) Agarose gel electrophoresis and stored at -20°C until use. An approx. 1.9 kb *dsrAB* segment was PCR amplified by using an equimolar mix of forward primer DSR1F (5′-AC[C/G] CACTGGAAGCACG-3′) and reverse primer DSR4R (5′-GTGTAGCAGTTACCGCA-3′) as described by Wagner et al. [11]. Specific DNA was amplified through Gene Amp Polymerase Chain Reaction (Creacon, Thermo cycler, Holand) system cycler 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) [12].

The successful PCR amplification of the *dsrAB* fragment was checked by running amplificates on horizontal agarose gel electrophoresis (1%). Specific DNA fragments of *dsrAB* were eluted from agarose gel. Resultant PCR products were purified with Microspin filters (E.Z.N.A.®Gel Extraction Kit, D2500-01, OMEGA bio-tek, USA) and quantitied spectrophotometrically for preparing to cloning procedure.

Molecular cloning of dsrAB genes

TOP10 competent Escherichia coli cells (TA/TOPO TA Cloning Kits, Invitrogen) hosting plasmids (pCR2.1-TOPO or pCR-XL-TOPO vectors, Invitrogen) were applied for ligation, transformation and cultivation of clones according to the manufacturer instructions. Recombinant clones were cultured overnight in LB media in the presence of the respective antibiotic (Ampicillin, kanamycin) to prevent cells from losing the plasmids. 4 ml overnight cultures were harvested by centrifugation. Pellet was used for plasmid extraction by E.Z.N.A.®Plasmid DNA Midi Kit (D6904-03 , OMEGA bio-tek, USA). The PCR amplicons from the individual clones were subjected to restriction enzyme Hin PII (Hin61) and HaeIII (BsuR1) (Fermentas) digestion. Clones containing inserts that produced identical restriction patterns were grouped and then one of the different groups were purified and sequenced.

Sequencing of PCR products

Corrected size of inserted *dsrAB* gene fragment was sequenced with Long-Read DNA Sequencing on the Applied Biosystems 3130xl Genetic Analyzers according to the manufacturer instructions. Then, they were compared with the sequences stored in the Gen- Bank nucleotide database (NCBI) by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the closest affiliations were selected.

Sulfate-reducing bacterial corrosion activity

Mild steel C1018 coupons purchased from (Rohrback Cosasco Systems, UK) with a chemical

composition as shown in Table 1, and dimensions of (73 mm x 22 mm x 3 mm) were used as a test material. The coupons were polished with a progressively finer sand grinding paper until a final grit size of 1000 µm was obtained. After polishing, the coupons were rinsed with distilled water, degreased in acetone and sterilized by exposing to pure ethanol for 24 h [13]. Corrosion experiments were initiated by hanging polished coupons on a nylon string in l L-glass bottles then submerged in 800 ml Postgate medium C [8], sealed with butyl rubber septa, purged with a N_2 flux and autoclaved for 20 minutes at 120ºC. The size of the inoculum was equal to 0.5 Mcfalard of a 3-day-old bacterial culture. A control system (without inoculation) was run at the same time with the test one. The inoculated bottles incubated at 37°C in the dark for 35 days.

Planktonic bacterial numbers were established using the most probable number (MPN) technique in Postgate's B medium as described by NACE standard (TM0194- 2004) [14]. Furthermore, the total dissolved sulfide in the bulk medium was determined every 7 days along 35 days incubation period. The dissolved sulfide was measured by the turbidimetric method as reported by Cord-Ruwisch [15].

At the end of the incubation period, 35 days, the coupons were removed, immersed in the Clarke solution (1 L 36 % HCl, 20 g Sb_2O_3 and 50 g $SnCl_2$) for 10-15 sec, washed with deionized water, ethanol and then dried in the desiccator. Subsequently, the dried coupons were weighed and the weight loss was determined by difference the weight of the coupons after and before cultivation. The equation proposed by the ASTM standard G 1-72 (see below) and represented as mdd (mg dm^{-2} day^{-1}) was used to determine the corrosion rate [16]. Furthermore, the metal surface with the cultivated sample in this study was examined using Scanning Electron Microscopy (SEM) model (Quanta FEG 250, Thermo Fisher Scientific) coupled with energy dispersive X-ray spectroscopy (EDX) to evaluate the morphology and chemical composition of the biofilm and corrosion products. The grown biofilm (attached cells), on the surface of mild steel coupons, fixed with 4 % glutaraldehyde in phosphate buffer solution, pH 7.3–7.4 for 4 h, dehydrated with 4 ethanol solutions (15 minutes each) of 25, 50, 75 and 100% successively, air dried and then gold sputtered [13].

Eq (1): Corrosion Rate = (K *W) (A* T* D)[16]

***Where: K** = a constant, **W**= mass loss in grams, **A**= area in cm^2,*

***T**= Time of exposure in hours, and **D**= Density in g/cm^3*

Results and Discussion

Sulfate-reducing bacterial characterization

The 1.9 kb DNA fragments, encoding most of α and β subunits of dissimilatory sulfite reductase, was amplified by PCR to assess the community composition of SRB microbial consortium [17]. The amplified *dsrAB* gene fragments are present in all sulfate reducers examined so far, so that the *dsr* is a good phylogenetic marker molecule for dissimilatory sulfate reducing microorganisms [12]. The successful PCR amplification of the *dsrAB* fragments were checked by agarose gel electrophoresis. The clones containing inserts that produced identical restriction patterns were grouped and then one of the different groups were purified and sequenced. The restriction Fragment Length Polymorphism (RFLP) pattern has displayed four different groups (C1, C2, C3 and C4) that were successfully sequenced. From results shown in Table 2, the dominate bacterial phylotype was *Proteobacteria* and include members of *Desulfovibrionales* and *Desulfobacterales.* The order *Desulfovibrionales* were represented by one genus *Desulfomicrobium*. While the order *Desulfobacterales* were appeared by two genera, *Desulforhopalus* and *Desulfobulbus*. Furthermore, the second phylotype present in the bacterial consortium was *Firmicutes* and represented by only one genus *Desulfotomaculum*.

Finally, Bioinformatics analysis and Aligned sequences using BLAST showed that, the four bacterial isolates (C1, C2, C3 and C4) were identified as *Desulfomicrbium apsheronum* C1

TABLE 1. The chemical composition of mild steel C1018 coupons (weight %)

Element	C	Al	Cr	Cu	Mn	Mo	N	Ni	Pb
(wt%)	0.16	0.003	0.064	0.216	0.73	0.014	0.008	0.085	0.001
Element	P	Pb	S	Si	Sn	Ti	V	P	Fe
(wt%)	0.008	0.001	0.011	0.19	0.013	0.001	0.004	0.008	Balance

with 100% sequence identity, *Desulfotomaculum acetoxidans* C2 with sequence identity 98%, *Desulforhopalus singaporensis* C3(99%) and *Desulfobulbus rhabdoformis* C4 with sequence identity 99%.

The type strain *Desulfomicrbium apsheronum* (C1) was isolated previously from stratal waters of oil-bearing deposits in the Apsheron peninsula, tolerate up to 8% NaCl for growth and oxidizes incompletely lactate to acetate [18]. Moreover, they have been isolated from different oil fields in North Sea [19] and in 5 of 6 different Alberta oil fields in Canada as observed by Voordouw and colleages [20].

One notable finding of this work is the presence of *Desulfotomaculum* genus in the cultivated sample. The halotolerant character (tolerate up to 11% NaCl) of the isolate *Desulfotomaculum acetoxidans* (C2) has not been observed in the genus *Desulfotomaculum*. Only two strains, one of them represented as a subspecies, *D. nigrijicans* subsp. *salinus*, has been described as a slightly halophilic, it was able to tolerate up to 4 % NaCl [21]. The other strain described by Tardy-Jacquenod et al. [22] was sufficiently different from other members of the genus *Desulfotomaculum* and representative as a new species *Desulfotomaculum halophilum* sp., tolerate up to 14% NaCl.

Desulforhopalus singaporensis (C3) as reported by Lie et al. [23] has been isolated from marine sulfidogenic mud from a saltwater marsh in Singapore, and grown in cultures containing lactate (4.8 mM) plus sulfate (10 mM) to produced sulfide (3.6 mM) and acetate (3.5 mM) as end products.

The strain type *Desulfobulbus rhabdoformis* (C4) was isolated from the water-oil separation system on field platform in the Norwegian sector of the North Sea, the isolate considered as a slightly halotolerant with maximum growth up to 2% NaCl as reported by Lien et al. [24]. In fact, the output of this part of results need further research to increase information about the ability of some identified microbes to tolerate high concentration of salinity, for example; *Desulfotomaculum acetoxidans* C2.

TABLE 2ز Sulfate-reducing bacterial diversity using molecular cloning of dsrAB genes

Identified species	Relative Microorganism	Nucleotides sequences (bp)	Query coverage	Identity	GenBank code:
Bacteria; Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfomicrobiaceae; Desulfomicrobium.	*Desulfomicrobium apsheronum* strain (DSM 5918)	1838	89 %	100%	AF482459.1 C1
Bacteria; Firmicutes; Clostridia; Clostridiales; Peptococcaceae; Desulfotomaculum	*Desulfotomaculum acetoxidans* (DSM 771)	1881	100 %	98%	CP001720.1 C2
Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulforhopalus.	*Desulforhopalus singaporensis* strain (DSM 12130)	1886	85 %	99%	AF418196.1 C3
Bacteria; Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfobulbus.	*Desulfobulbus rhabdoformis*	1883	89%	99 %	AJ250473.1 C4

Sulfate-reducing bacterial corrosion activity

Bacterial growth and dissolved sulfide production

The growth process of planktonic SRB consortium associated with the sulfide production in the enriched growth medium were shown in Fig.1. The results indicate that the growth process can be divided into three main stages. The first stage, one to seven days, is called the exponential or lag phase. During this stage, the number of viable SRB species increased quickly to approximately the maximum value of $1.6x10^9$cells/ml. It has been shown that during the lag phase, the concentration of hydrogen sulfide (H_2S) increases correspondingly with increasing number of cells [25]. After the 7th day, the growth process reaches the 2nd stage, the stationary phase, which takes place during the period from 7 to 21 days. During this stage the level of dissolved sulfide increased drastically for the first 14 days (14.86 mmol/l) then it sharply decreased where there was no increase in the number of cells and the growth was limited by deficient nutrient and by product accumulations. The last phase of bacterial growth (3 rd stage) is the declining or death phase that starts after 21 days. The accumulation of high concentrations of hydrogen sulfide, produced during the exponent phase, can badly impact and inhibit the reinitiating of cell division. It was reported that at hydrogen sulfide concentrations of about 547 mg/L (16.1 mM), *Desulfovibrio* sp., culture growth is completely inhibited [26]. The production of hydrogen sulfide and the oxidation of iron (anodic reaction), promotes the formation of iron sulfide as follows [27].

$$Fe \rightarrow Fe^{+2} + 2e$$

$$Fe^{+2} + H_2S \rightarrow FeS + 2H^+$$

Weight loss measurement

At the end of incubation period, the weight loss of the coupon was measured and metal corrosion rate was calculated (Table 3) as mdd (mg dm^{-2} day^{-1}). In the present study, the test salinity was 110,000 ppm and the corrosion rate in culture medium inoculated with SRB reached to 15 mdd while with no SRB was <1. Medium salinity is considered as one of important factors increasing the metal corrosion where chloride anion increases the solubility of the corrosion products and rises significantly the conductivity of the electrolyte layer over the metal surface. Thus it destroys the passive film existing on the metal surface and produce pits and crevices corrosion [28], [29].

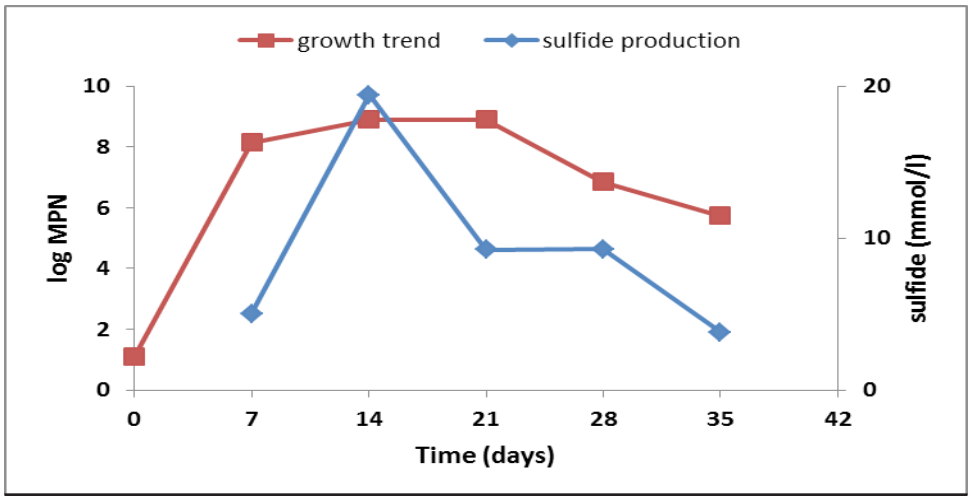

Fig. 1. Growth trend and dissolved sulfide production of planktonic bacteria in Postgate's C medium over 35 days of incubation.

TABLE 3. Average weight loss and corrosion rate of test and control medium after 35 days

Medium	Average weight loss (g)	Corrosion rate (mdd; mg dm^{-2} day^{-1})
Culture medium (control)	0.01	0.85
Culture medium + SRB (test)	0.184	15.67

Also as reported by Zhao et al. [30] the corrosion rate of Q235 steel varied with different additive amount of chloride ions. SRB and chloride ions at certain concentration had a synergistic effect on the corrosion of Q235 steel.

Surface Analysis of Coupons Exposed to SRB

The characteristics of the layer that developed in the presence of the sulfate-reducing microbial consortium over 35 days were shown in Fig.2 a and b. The morphology of most SRB cells was rod- and oval shaped, with different length and sizes. The cells were of 1.5-2 µm in length and they link to form an elongated thread-like structure, *Desulfomicrobium* sp. may have rod or oval morphology [24]. Most commonly, the exopolymeric substances (EPS) and corrosion products occupied 75-95 % of produced biofilm volume, while 5-25% is occupied by the cells [31]. Bacterial EPS consists mostly of polysaccharides, proteins, nucleic acids and phospholipids. EPS helps the bacteria attach to the metal surface and play a significant role in the corrosion process as it has the capacity to bind metal ions to form concentration cells and, in turn, result in galvanic coupling [32].

The results of EDX analysis of corrosion products of mild steel C1018 immersed in the biotic system were shown in Fig 3. The quantitative analysis refers to the presence of higher amounts of carbon, oxygen, sulfur, iron and sodium salts. The results support the formation of siderite ($FeCO_3$). Furthermore, Similar features have been reported by Venzlaff et al. [6] for steel surfaces exposed to the SRB species *Desulfopila corrodens*.

The capacity of *Desulfomicrobium* to convert the carbon source (lactate) through pyruvate to acetate with production of carbonate supports the formation of siderite [6], [26]. In addition, the presence of sodium chloride in the growth media might lead to the precipitation of sodium chloride on the surface

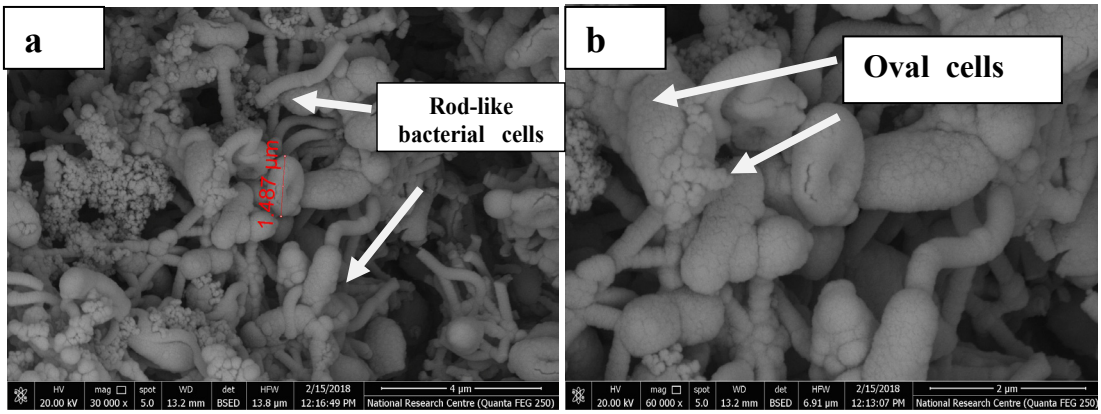

Fig. 2. SEM image for the biofilm (sessile cells) developed on C1810 mild steel exposed to SRB consortium at different magnification a) 30000X b) 60000 X.

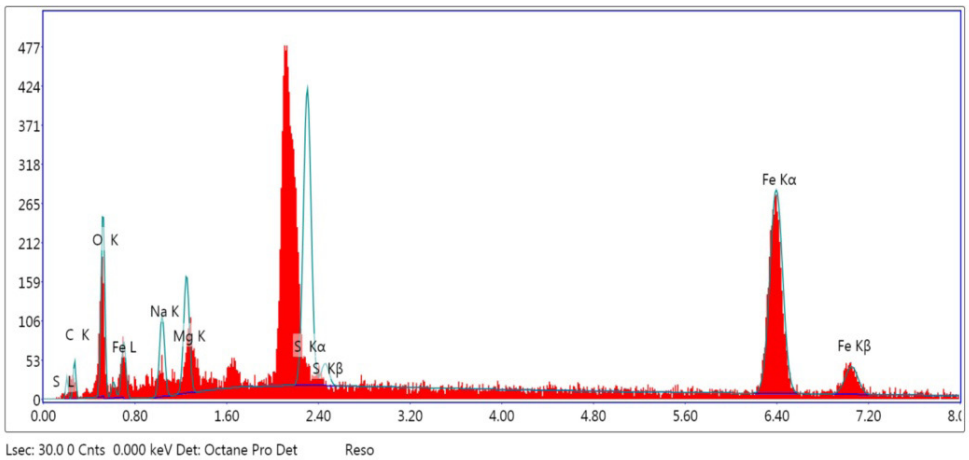

Fig 3. EDX spectrum from the mild steel C1018 surface after 35 days of exposure in the presence of SRB.

Conclusion:

The planktonic consortium of halotolerant SRB consisted of two main phylotype: *Proteobacteria* and *Firmicutes*. The corrosion experiments with this consortium on mild steel C1018 showed increased corrosion of the steel. Moreover, the production of the corrosive sulfide reached the maximum at 14 days of incubation during the cellular exponential growth phase. From weight loss results, steel corrosion in the presence of SRB was approximately 15 time that for the control. In this study, SRB and chloride ions had a potent effect on C1018 steel corrosion.

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