Proteins identified through predictive metagenomics as potential biomarkers for the detection of microbiologically influenced corrosion

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Abstract: The unpredictability of microbial growth and subsequent localized corrosion of steel can cause significant cost for the oil and gas industry, due to production downtime, repair, and replacement. Despite a long tradition of academic research and industrial experience, microbial corrosion is not yet fully understood and thus not effectively controlled. In particular, biomarkers suitable for diagnosing microbial corrosion which abstain from the detection of the classic signatures of sulfate-reducing bacteria are urgently required. In this study, a natural microbial community was enriched anaerobically with carbon steel coupons and in the presence of a variety of physical and chemical conditions. With the characterization of the microbiome and of its functional properties inferred through predictive metagenomics, a series of proteins were identified as biomarkers in the water phase that could be correlated directly to corrosion. This study provides an opportunity for the further development of a protein-based biomarker approach for effective and reliable microbial corrosion detection and monitoring in the field.

Keywords: microbial corrosion, predictive metagenomics, biomarkers, sulfate-reducing bacteria, pipeline’s microbiome

Introduction

Microbiologically influenced corrosion (MIC) can be involved in the deterioration of steel and other structural materials in a wide range of industries, and its economic impacts may be highly significant (Bennett, 1978). In particular, within the oxygen-free engineered environments of oil and gas operations, MIC often becomes the dominant process. Microbes responsible for MIC in production pipelines of the petroleum industry are typically affiliated to many different Bacteria and Archaea, organized in microbial communities with different levels of interactions. The composition of such pipelines’ microbiome will ultimately govern the occurrence of specific microbial metabolisms, and thus the extent of microbial corrosion. Specifically for pipelines, the local conditions can vary quickly. For example, temperatures closer to the wellhead are usually several degrees higher than in the center of the pipeline and, especially for offshore deep pipelines, seasonal temperature variability can be observed even for buried pipelines (Sund et al., 2015). Similarly, pH can vary dramatically in relation to mixing with water from various sources and because of abiotic and biotic generation of H₂S (Hutcheon, 1998) and CO₂ (Bai et al., 2018). Shear stress deriving from pipeline flow can also hinder especially planktonic microbes (Rochez et al., 2008). To partially alleviate these drawbacks, microbial communities inside pipelines tend to attach to the inner wall, preferentially at the bottom, or “6 o’clock position,” where the water and oil phases separate and where the flow velocity and shear stress are lower (Nakayama, 2018). Additionally, the 6 o’clock position in pipelines, can accumulate particles, especially in the presence of bends (Leung et al., 2018), providing a potential source of macronutrients, such as N and P, as well as micronutrients, such as Cu, Fe, Mn, and S. In the presence of a surface for attachment, water and nutrients, the pipeline’s microbiome can establish by forming a biofilm, which is likely to be a prerequisite for the occurrence of MIC (Eckert, 2015). The central role of sulfate-reducing bacteria (SRB) as main culprit of MIC has been extensively reported (Beech et al., 1994, Booth, 1964, Hamilton, 1985; Lee et al., 1995; Paisse et al., 2013). SRB in pure cultures can cause corrosion via organotrophic or lithotrophic growth (Enning & Garrels, 2014). In the first case, an organic substrate is normally used as electron donor for sulfate reduction (Muyzer & Stams, 2008), while in the second scenario, the iron from steel serves directly as electron donor (Enning et al., 2012). Under both organotrophic and lithotrophic conditions, hydrogen, generated either biologically or abiotically, might also constitute a suitable electron donor for SRB. Under every electron donor regime, moreover, release of H₂S as main byproduct of sulfate reduction facilitates the corrosion of steel (Wikjord et al., 1980). Furthermore, other microbial electron-accepting metabolisms, with either organic carbon, H₂ or Fe from steel as electron donor, have been described as responsible for MIC. These include nitrate-reduction (Hubert et al., 2005), thiosulfate-reduction (Magot et al., 1997), methanogenesis (Mori et al., 2010), and acetogenesis (Dowling et al., 1992; Mand et al., 2014). Even though a vast amount of knowledge on MIC exists, an effective and economic approach to detect and monitor MIC is still lacking (Little et al., 2020). Here, we hypothesize that a diverse soil microbiome can guarantee the establishment of corrosive microbial consortia under different, including nonmesophilic, conditions. In this study, we enriched anaerobically a natural microbial community from marine soil under various physiochemical conditions and in the presence of carbon steel. These conditions were chosen to mimic several parameters, such as variability in temperature, pH, and CO₂ concentrations, often detected in oil and gas production pipelines. Microbiologically mediated changes in...
Materials and Methods
Sample Collections and Setup of Lab Enrichments
Coastal marine sediment was collected in sterile glass bottles on the northeastern tip of Galveston Island, Texas. The silt, gray-black sediment was taken from approximately 3 to 15 cm depth and smelled of hydrogen sulfide. The collection bottle was filled up to minimize oxygen exposure, and then sent to the lab under cooling. The original sediment microbial community was analyzed for approximately 2 months.

Preparation of Enrichment Medium
The enrichment medium used in this study was prepared by adding the following components (all from Sigma-Aldrich, St. Louis, MO, USA): MilliQ water: KH2PO4 (0.5 g/l), NH4Cl (1 g/l), CaCl2 (0.41 g/l), NaCl (15.5 g/l), KCl (0.11 g/l), and MgCl2 (0.33 g/l); 20 mM (2.84 g/l) Na2SO4 was also added as electron acceptor. Afterward, the liquid phase of the medium was purged with N2 for 35 min and 2-(N-morpholino) ethanesulfonic acid (MES) added as buffer (6.5 g/l), as well as resazurin (0.2 mg/l) as oxygen indicator. Also, 80 μl/l of 70% thioglycolic acid and 1.25 g/l L-cysteine were included as reducing agents. Subsequently, the liquid was further purged with CO2:N2 (either at 15:85 or 7:93, vol/vol) for 15 min, and the bottle was equipped with butyl stoppers and crimped with aluminum rings. Then, 80 ml of medium was dispensed in 120-ml serum bottles equipped with butyl stoppers and crimped with aluminum rings. Also the liquid phase and the headspace of each serum bottle were flushed with 15% CO2 for about 5 min each. Initial inoculum of approximately 1.5 g of sediment slurry was enriched and 2 ml of such enrichment were repeatedly transferred to fresh medium bottles until virtually sediment-free cultures were obtained. From the latter, 2 ml were then transferred to fresh serum bottles for each given condition. These final bottles were equipped with a unique tailor-made coupon holder consisting of a Teflon thread rod and bolts holding in place 2 Teflon discs with slots for fitting two X-52 carbon steel coupons (5 × 20 × 1 mm) per holder. Each cleaned steel coupon, with a 600-μm sandpaper surface finish, was weighted in triplicate prior to serum bottle assembling. Each holder hung suspended from the neck of a serum bottle via a thin nylon wire piercing the butyl stopper of the bottle (Fig. S1). Assembled bottles and holders were flushed with oxygen-free nitrogen through a heated, H2-reduced, copper furnace (Supelco), and then autoclaved prior to medium addition.

Half of the bottles contained acetate (10 mM) and propionate (5 mM) as organic carbon source and electron donor, while the other half did not contain any organic carbon and both were set at different initial, buffer-controlled pH (5.6.5, and 8). The entire series of conditions was replicated for CO2 concentrations of either 7 or 15% in N2. Furthermore, an extra series of bottles containing organic carbon source, 15% CO2 but no inoculum was set as negative controls. All the bottles were incubated at selected temperatures (20, 40, and 60°C) and under moderate shaking (60 rpm) for approximately 2 months.

Time-Resolved Monitoring of Anions from Enrichments’ Water Phase
Duplicate anion quantification using high-pressure ion chromatography (HP-IC) was applied on 1 ml of subsamples collected at least weekly. An HP-IC 5000 + Reagent-Free system (ThermoFisher Scientific, Massachusetts, USA) equipped with Dionex IonPac AS 11-HC column, EluGen® III Potassium Hydroxide capillary cartridge and suppressed conductivity detector Dionex ACES 300, was used for the analyses. All the samples were centrifuged at 5,000 rpm for 2.5 min and the supernatants were filtered through 0.2-μm cellulose acetate filters to remove the particulate and biological matter and then diluted 1:100 for final analysis. The pump flow rate was 1 ml/min; injection volume of the samples was 25 μl and a column temperature of 30°C was used. An eluent gradient method was established for separation of acetate, propionate, and sulfate. The KOH gradient started with initial concentration of 1 mM for 1 min and then at increments of 0.3 mM/min from 1 to 12 min, 5 mM for 13 min, 20 mM for 25 min, and final 20 mM hold for 35 min. Calibration standards with known concentrations of acetate, propionate, and sulfate were prepared as well and used to quantify the samples.

Corrosion Product Analysis and Quantification of Corrosion Rates
Biofilms on the coupon surface were removed by vortexing (2,500 rpm, 5 min) and sonication (40 kHz, 15 min) and the resulting biomass from both the planktonic and biofilm phase was collected by centrifugation (3,500 rpm, 30 min, and 4°C). The resulting supernatants were discarded and the pellets were immediately frozen at −80°C for molecular analyses. The coupon with remaining biomass and corrosion products were further vortexed at maximum speed for additional 5 min in acetone. Subsequently,
the acetone phase was filtered using Anodisc filter membrane (47 mm in diameter with a pore size of 0.02 μm) and the corrosion products were collected and analyzed by a Rigaku Mini-Flex II X-ray diffraction (XRD) system for phase identification. The coupons, now free of biofilm and loose corrosion products, were examined with a LEO Gemini 1530 scanning electron microscope (SEM), along with energy dispersive X-ray spectroscopy (EDS) for surface residual corrosion product analysis. Coupons with corrosion product or scale left on the surface after biofilm removal were also analyzed by XRD to identify the crystal structure of the corrosion products. After the corrosion products were analyzed, the scale was removed from each coupon using a passive Clarke solution (20 g Sb₂O₃ and 50 g SnCl₂ dissolved in 1 L of 36% HCl, (G1–03, 2011)) and the weight of the descaled coupon was recorded. General weight loss dependent corrosion rate of each coupon was determined by measuring the difference between pre- and posttest coupon weights, as described in ASTM G1-03 standard (G1–03, 2011). Triplicate weights of descaled metal coupons were recorded to calculate corrosion rates in mm/year, using the formula:

$$\frac{kw}{(At)}$$

where $k$ is a constant ($k = 8.76 \times 10^4$), $w$ is the weight loss (initial—final weight) in grams, $A$ is the surface area of the metal coupons in cm² (1 cm²), $t$ is the incubation time in hours ($\sim 1,440$ hr), and $ρ$ is the density of the material in g/cm³ (7.85 g/cm³ for X-52 carbon steel). Factor analysis on covariance from corrosion data of the enrichments (excluding negative controls) was performed using the maximum likelihood method with varimax rotation as implemented in SAS JMP® Pro V 15.1.0.

DNA Extraction, Next-Generation Sequencing and Bioinformatics

Pellets from one of the two biological replicates were suspended in 2 mL of phosphate buffered saline (PBS, pH 7.4) and DNA was extracted using the E.Z.N.A.® Soil DNA Kit, following the manufacturer protocol except for the skipped column-purification step. Extracted total DNA was sequenced using MetaVx™ 16S rDNA next-generation sequencing library preparations and Illumina MiSeq sequencing at GENEWIZ, Inc. (South Plainfield, NJ, USA). Briefly, the DNA was used to generate amplicons that cover V3, V4, and V5 hypervariable regions of bacteria and archaea 16S rDNA. Indexed adapters were added to the ends of the 16S rDNA amplicons by limited cycle PCR. Sequencing libraries were validated using a DNA chip for the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit and real-time PCR (Applied Biosystems, Carlsbad, CA, USA). DNA libraries were multiplexed and loaded on an illumina MiSeq instrument according to manufacturer’s instructions (Illumina, San Diego, CA, USA). Base calling were conducted by the MiSeq Control Software (MCS) on the MiSeq instrument. Raw data were analyzed using QiIME2 as described (Bolyen et al., 2019). Briefly, raw forward and reverse paired-end reads were trimmed for quality and the Illumina adapters were removed using Dada2 (Callahan et al., 2016), as implemented in QIIME2. Subsequently, denoised data were clustered and classified to assign the appropriate taxonomy for every operational taxonomic unit (OTU). For certain OTUs of interest, that is, those affiliated with Deltaproteobacteria and Clostridia, manual classification using NCBI’s nucleotide Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov) was performed. OTU counts and sequences of each OTU were also used for predictive metagenomics using Picrust2 (Douglas et al., 2020), with the standard pipeline as described (https://github.com/picrust/picrust2/wiki/Full-pipeline-script). Predictive metagenomics output tables, as KEGG orthologs (https://www.genome.jp/kegg/) for each sample were also normalized and used for further analysis, using the Python library Pandas (https://pandas.pydata.org/). DNA sequences have been submitted to NCBI’s Short Reads Archive and are available under the Bioproject PRJNA658599, with accession numbers from SAMN15879593 to SAMN15879609.

Results

Time-Resolved Monitoring of Anions from Enrichments’ Water Phase

Fig. 1 shows the ion chromatography (IC) measurement results of sulfate from all the enrichment. Complete removal of the starting sulfate concentration of 20 mM was observed only in MIC enrichments at 20°C and pH 6.5 when organic carbon was present. More than 50% of the starting sulfate concentration was reduced under the same conditions lithotrophically. Of the other enrichments at 20°C, the organotrophic culture at pH 8 and 7% CO₂ was also removing more than 50% of the sulfate, while no more than 5 mM were reduced for any condition at pH 5. At 40°C and pH 6.5, most of the enrichments reduced ~50% of the sulfate, except for the lithotrophic culture with 7% CO₂. For the other enrichments at 40°C, at pH 5 the sulfate reduced was ~8 mM, except for the organotrophic enrichment with 7% CO₂ and, at pH8, only the organotrophic enrichment at 15% CO₂ removed ~50% of the sulfate. Little or no sulfate respiration was otherwise observed for all the other treatments as well as for the negative controls. Similarly, acetate mineralization was achieved only by microbes at 20°C and pH 6.5. Additionally, we observed acetate production in various enrichments at both 20 and 40°C under different pH (Fig. S2). At 20°C and pH 6.5, an initial acetate production that almost doubled its original concentration to ~20 mM, was followed by consumption to ~1.7 mM. Contrariwise, at 40°C, acetate values of ~25 and ~20 mM were measured at pH 5 and 6.5, respectively, until the end of the experiment. Interestingly, acetate was detected up to ~15 mM also in lithotrophic enrichments at 20 and 40°C, even though there was no acetate in the solution at the beginning of the experiments (Fig. S3). Propionate was consumed only in few of the organotrophic enrichments, especially at 40°C (Fig. S2). Similarly to the sulfate profiles, no changes in acetate or propionate were detected at 60°C and in all the negative controls (Fig. S2).

Corrosion Rate and Corrosion Product Analysis

Corrosion rates calculated from weight loss are reported in Fig. 2. A temperature gradient, with decreasing corrosion from lower to higher temperatures, can be observed for all the treatments at pH 6.5. In general, enrichments at pH 6.5 produced thick black biofilms and higher corrosion rates. In the presence of organic carbon at pH 6.5, minimal differences in corrosion rates were observed between 20 and 40°C, as well as for different CO₂ concentrations. On the other hand, for lithotrophic enrichments at pH 6.5, corrosion rates were reduced more than 35% at 40°C compared to 20°C. Corrosion rates measured at pH 5 where significantly lower than those at pH 6.5, and a generally higher background corrosion was present, due to the acidic pH. Nevertheless, several enrichments at 20 and 40°C showed visible biofilms and produced moderate corrosion rates, compared to the corresponding controls. Finally, very low corrosion rates as well as biofilm formation, barely distinguishable from the abiotic controls, were observed at pH 8. For all the conditions, 60°C apparently constituted the upper limit for microbial growth of corrosive microbes. Opposite trend of corrosion rates with increasing temperatures
was observed for negative control samples as compared to biological samples, with higher corrosion rate at higher temperature. At the end of the test, black corrosion products were observed in both the liquid phase and on the coupon surface for all the treatments, except for tests at 60°C and sterile controls. Statistical analysis of corrosion rates in relation to the different physical–chemical conditions revealed a strong correlation between corrosion rates and pH especially in the organotrophic enrichments, whereas the lithotrophic enrichments did not exhibit any particular correlation (Fig. S4). XRD and SEM/EDS analyses indicated no corrosion product formation on coupons tested at 60°C and sterile controls. For both organotrophic and lithotrophic enrichments at 20 and 40°C, XRD analysis of the entrained solids within the biofilm and the corrosion scale or residual solids adhered to the coupon surface after biofilm removal detected mackinawite (FeS) as the primary corrosion product, besides halite (NaCl), vivianite (Fe₃(PO₄)₂•8(H₂O)), and occasionally trace amounts of siderite (FeCO₃) at pH 6.5 only. This is consistent with the SEM/EDS analysis of the coupon surfaces where mackinawite was identified as the predominant corrosion product, as shown in Fig. S4. Additionally, small amount of greigite (Fe₃S₄) was also detected by XRD, along with mackinawite, for the tests at pH 5 and 20°C (data not shown).

Characterization of Microbial Communities
Selected samples, based on the IC and corrosion rate profile, were subjected to 16S rDNA amplicon sequencing, as shown in Fig. 3. More than 98% of the microbial community, consisting of 1,204 unique OTU, belonged to the Bacteria domain, while Archaea were scarcely represented. Consistently with the observed corrosion data, DNA was hardly extractable from samples growing at 60°C and from most of the samples at pH 8, due to the low biomass present in these samples. The original marine beach soil revealed a mixed microbial community characterized especially by Bacteroidetes, Cyanobacteria, Desulfobacterales, and

**Fig. 1.** Ion chromatography (IC) measurements of sulfate from enrichments growing either organotrophically (Org, blue) or lithotrophically (Lith, red). Negative control (dotted line) was prepared as organotrophic, but contained no inoculum. Shaded areas represent standard deviation from duplicate IC measurements.

**Fig. 2.** Corrosion rates calculated from weight loss measurement of duplicate coupons. Samples are grouped by temperature (x axis), pH (boxes), growth condition (organotrophic, orange and lithotrophic, purple) and CO₂ concentration (different shades).
unclassified strains of Gammaproteobacteria, along with a high number of unclassified lineages (Table S1). On the other hand, enriched consortia were represented by only 7 phyla, accounting for more than 95% of the overall diversity: Bacteroidetes, Firmicutes, Proteobacteria, Synergistetes, Tenericutes, Thermotogae, and WWE1. Additionally, Firmicutes were almost exclusively Clostridia, while Gammaproteobacteria and Deltaproteobacteria were the main representatives of Proteobacteria (Fig. 3). Given the higher relative abundance of Deltaproteobacteria and Clostridia, and their relevance in MIC studies, a breakdown of these two Classes, based on BLAST of the specific OTUs belonging from those Classes (Tables S2 and S3), is shown in Fig. 4. In terms of Deltaproteobacteria, most of the samples from pH 6.5, characterized by higher corrosion rates (above 0.2 and up to 0.5 mm/year), shared higher relative abundance of Desulfovibrio salexigens (Fig. 4A). This microbe was the single most abundant OTU in our dataset, accounting for more than 16% of all the reads sequenced. Additionally, other deltaproteobacterial SRB enriched at pH 6.5 included Desulfobacter hydrogenophilus, particularly relevant at 20°C under organotrophic conditions, Desulfocurvus thunnarius, significant mostly at 40°C. The main outlier from the enrichments at pH 6.5 was represented by the enrichment of Desulfovibrio pshycrotolerans, up to 15% in relative abundance only in the lithotrophic sample growing at 40°C with 15% CO₂. This sample was characterized by a steep decrease in sulfate at the beginning of the experiment and partial sulfate production toward the end, and the corrosion rate was also substantially lower (0.15 mm/year). The deltaproteobacterial population differed profoundly in the enrichments at pH 5, with only three main lineages, Desulfovibrio tunisiensis, Desulfobacter dechloracetivorans, and Desulfobacter hydrogenophilus, enriched with poor correlation with corrosion data. Similarly, at pH 8, D. dechloracetivorans and D. thunnarius were the main members of the community, with the addition of Desulfoarculina variabiliis enriched at 40°C only. Clostridia distribution reflects somehow what observed for Deltaproteobacteria, with increased diversity at pH 6.5 compared to pH 5 and very low abundance at pH 8 (Fig. 4B). An additional peculiarity of the enrichments at pH 6.5 was that, unlike Deltaproteobacteria, Clostridia were strongly limited by temperature, with low relative abundance at 40°C and by electron donor, with organotrophic enrichments presenting counts more than double of those from the lithotrophic ones. The main lineages enriched at pH 6.5 were related to Aminipila butyrica, De-thiosulfatibacter aminovorans, Soehngenia saccharolytica, Clostridium tepidum, and Clostridiilisalbicans paucivorans. At pH 5, no real correlation with temperature or electron donor was present and, similarly to what observed with Deltaproteobacteria, a different and less diverse community was observed, with microbes affiliated to Lacrimispora amygdalina, Hungateiclostridium clariflavum, Serpentimicella alkaliphila, and Fusibacter tunisiensis. Another important bacterial group enriched especially at pH 5, was characterized by a strong presence of the gammaproteobacterial genus Pseudomonas (Fig. 3) and by moderate corrosion rates (above 0.13 mm/year). These samples also contained smaller relative abundances of Serratia marcescens within the Gammaproteobacteria subdivision. Intriguingly, the other two samples presenting higher abundance of Gammaproteobacteria, that is, the organotrophic enrichments growing at pH 5, 20°C and 15% CO₂ and at pH 8, 60°C and 15% CO₂, both with low corrosion rates (below 0.08 mm/year), were characterized by a different gammaproteobacterial population, with members of the genus Halomonas dominating the community along with lower abundances of the genus Skewanella (data not shown). Among the
other phyla detected, the Dethiosulfovibrionaceae family within Synergistetes was enriched, up to 25%, especially at pH 8, similarly to Thermotogae and WWE1, also present mainly at pH 8. Bacteroidetes were also present in most of the samples, up to 14% in relative abundance and were largely affiliated to the Rikenellaceae family.

Predictive Metagenomics

A total of 6,522 predicted genes from the overall experiment microbiome were obtained after removal of singletons. The average Nearest Sequenced Taxon Index (NSTI) was 0.104 ± 0.015, pointing to a reasonable predictions for the metagenomes of our samples, especially considering that this mixed microbial community likely contained 16S rDNA sequences for which sequenced genomes are not readily available (Douglas et al., 2020). In order to verify that the predictions were consistent with the 16S rDNA profiling, we confirmed that genes central to sulfate reduction were representatives of the proportions of sulfate reducers present in our samples. As reported in Fig. S6, the relative abundance of the KEGG orthologs K00394 and K00395 (adenylylsulfate reductase subunits A and B, respectively) as well as K11180 and K11181 (dissimilatory sulfite reductase alpha and beta subunits, respectively) were predicted with highly similar profiles ($R^2 = 98.3\%$) to that reported for the 16S rDNA analyses of the Deltaproteobacteria (Fig. 4A) which were, in fact, largely sulfate-reducing bacteria. Among all the other predicted genes relevant across the entire dataset (Table S4), several were associated to protein responsible for chemotaxis, through the two-component system, and for transport across the membrane. Within the transport proteins, ABC transporters, polar and branched amino acid transporters, and ferrous iron uptake systems were the ones with highest relative abundance across several enrichments (Table S4). Pearson correlation coefficients calculated for the predictive metagenome against corrosion rates are shown in Table S5. The relative abundances of the four KEGG orthologs with the highest Pearson coefficients are plotted against the corrosion rates in Fig. 5 and are discussed in more detail.

Discussion

The physical and chemical testing conditions of the experimental setup did have a significant impact on MIC. Our original hypothesis was that a natural microbial community from sediment could
harbor enough diversity to grant microbial corrosion under a large variety of physicochemical parameters. Our experiments only partially validated the hypothesis, since MIC was not detectable at higher temperature and alkaline pH. Although the role of MIC at temperatures of ∼50°C has been previously reported (Alfaro-Cuevas-Villanneva et al., 2006; Lata et al., 2012), and even if the limit of microbial life is well above 60°C, MIC occurrence at higher temperatures remains unclear. Mardhiah and colleagues reported corrosion by pure cultures of Desulfovibrio vulgaris at temperatures as high as 60°C (Mardhiah et al., 2014). However, the limited amount of corrosion observed (up to 0.06 mm/year) suggests a passivation phenomenon rather than active biocorrosion. A more environmental-oriented study from Comanescu et al. found that water injection pipelines for secondary oil recovery with average temperatures 50–60°C presented deeper pits and higher bacterial counts than those with average temperatures between 60 and 120°C (Comanescu et al., 2012). Although interesting, such observations are well in line with the acetate production measured in our experiment (Figs. S2 and S3), as well as with the high amount of amino acid transport proteins computed as predicted metagenome (Table S4). In the presence of an active SRB community, biogenic H2S reacting with the steel could also produce H2 while forming FeS (mackinawite). The predominance of mackinawite, suggesting metabolic activity of SRB and an SRB-induced corrosion mechanism in our test system, is corroborated by XRD and SEM/EDS analyses (Fig. 5S) and is consistent with previous studies (Hamilton, 1985; McNeil & Little, 1990). At temperatures of 40°C, however, Clostridiales were strongly reduced but partial sulfate reduction, acetogenesis, and moderate to high corrosion were still observed. Under this scenario, amino acid fermentation would still produce acetate but, since the clostridial partner coexisting in these water-flooded wells was present, the primary cause of corrosion could have been abiotic rather than biologically induced. In our study, on the other hand, high corrosion, up to ∼0.5 mm/year, can be detected at moderate temperatures of 20 and 40°C, but MIC was always dubious at 60°C and, if it occurred, it was never to any large extent. SRB dominated the community under some of the most corrosive conditions. The most represented SRB at pH 6.5, Desulfovibrio salexigens, is a marine microbe that requires more than 0.5% NaCl for growth, which is consistent with the NaCl concentration of our medium (1.5%). Species related to D. salexigens isolated, along with other Desulfovibrionaceae, from corroded steel samples in coastal South Vietnam (Tarasov & Borzenkov, 2015), were able to utilize hydrogen, but not acetate. The conditions presenting some of the higher relative abundance of D. salexigens in our study were also correlated to higher corrosion rates, sulfate reduction, and concomitant presence of Clostridia, especially for the enrichments at 20°C. Several Clostridiales are able to consume acetate, producing CO2 and H2, to fuel a well-known cometabolism in which SRB can outcompete methanogens to remove the hydrogen (Lovley et al., 1982). At the same time, many microbes, including SRB, can also ferment amino acids, producing even more acetate (Barker, 1981). These observations are well in line with the acetate production measured in our experiment (Figs. S2 and S3), as well as with the high amount of amino acid transport proteins computed as predicted metagenome (Table S4). In the presence of an active SRB community, biogenic H2S reacting with the steel could also produce H2 while forming FeS (mackinawite). The predominance of mackinawite, suggesting metabolic activity of SRB and an SRB-induced corrosion mechanism in our test system, is corroborated by XRD and SEM/EDS analyses (Fig. 5S) and is consistent with previous studies (Hamilton, 1985; McNeil & Little, 1990). At temperatures of 40°C, however, Clostridiales were strongly reduced but partial sulfate reduction, acetogenesis, and moderate to high corrosion were still observed. Under this scenario, amino acid fermentation would still produce acetate but, since the clostridial partner converting acetate to H2 and CO2 is strongly reduced in relative abundance, accumulation of acetate is observed (Fig. S2). As a

![Fig. 5. Correlation between selected proteins from predictive metagenomics and corrosion rates. The proteins are expressed as relative abundance of the normalized functional profiling predicted with Picrust2 and are named using the corresponding KEGG ortholog ID. Shaded areas represent 95% confidence interval.](image-url)
consequence of diminished H2, also sulfate reduction is hampered in these enrichments (Fig. 1), similarly to what shown for different environments (Nanninga & Gottschal, 1985). Additional observations from our predictive metagenomics analyses (Table S4) also indicated a high potential for proteins involved in iron transport and regulation systems (K02013-K02016, K03711, and K04758). Although these proteins are mainly related to siderophores and assimilatory iron metabolisms, the role of iron regulation or chelation in microorganisms correlated to MIC remains unclear.

The conditions found at pH 5 were to some extent similar to those described for pH 6.5, with the caveat that different SRB and clostridial subpopulations were enriched. Of particular note, Desulfovibrio dechloracetivorans was isolated for its ability to grow oxidizing acetate and reducing 2-chlorophenol, but can also use sulfate as electron acceptor (Sun et al., 2000). Despite such metabolism is available to D. dechloracetivorans, the extent of sulfate reduction is limited to some of the enrichments growing at 40°C, both organotrophically and lithotrophically. In both cases, furthermore, acetate is produced, pointing to a similar fermentative metabolism of that observed at pH 6.5.

As above mentioned, some members of Gammaproteobacteria were also detected in our study, especially at pH 8. Although not correlated to the most corrosive conditions in our study, Seratia marcescens has been previously reported in corrosion studies (Rajasekar et al., 2011) and species of Shewanella and Halomonas are well known for their role in the iron cycle (Sanchez-Porro et al., 2010; Shi et al., 2007).

Some of the conditions examined in our study might represent a good approximation of the temperature and pH in pipelines subjected to MIC. Yet, most of the popular methods for assessment of MIC, such as sulfate tests or 16S rDNA detection of SRB, would not inform about the difference in corrosion. In fact, only when coupons were analyzed for corrosion rates, the true extent of MIC could be evaluated in our enrichments. However, coupons tests are not always available in the field, especially for offshore pipelines in remote location and, therefore, there is a need for biomarkers that unambiguously correlate liquid-phase samples to MIC. Our samples were collected including both planktonic and biofilm phases, since large flocks of biofilms were detaching from the coupons and sonication/vortexing of the coupons was used, adding more biofilm material to the bulk planktonic phase. This approach can be approximated to a lab-scale simulation of the removal of biofilm caused by pipelines’ flow and subsequent collection of the water fraction from a transport pipeline. Although we did not perform functional genomics or transcriptomics, we employed predictive metagenomics to explore the possibility for hypothesis testing on the microbiome functions using the cheap and widely available 16S rDNA amplicon sequencing. This study shows that dramatic changes happened to a natural mixed microbial community when shifting to a corrosive consortium. Physical and chemical conditions caused further rearrangements, with consequent differences in the extent of corrosion. Our work provides evidences of potential interactions, based on acetate cycling, between SRB and Clostridia during MIC in oil production environments and identifies proteins potentially correlated to corrosion. Our results can be further tested to develop better tools for the monitoring and mitigation of microbial corrosion in situ.

Conclusions
This study shows that dramatic changes happened to a natural mixed microbial community when shifting to a corrosive consortium. Physical and chemical conditions caused further rearrangements, with consequent differences in the extent of corrosion. Our work provides evidences of potential interactions, based on acetate cycling, between SRB and Clostridia during MIC in oil production environments and identifies proteins potentially correlated to corrosion. Our results can be further tested to develop better tools for the monitoring and mitigation of microbial corrosion in situ.

Supplementary Material
Supplementary material is available online at JIMB (wwwacademic.oup.com/jimb).

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Conflict of Interest
The authors declare no conflict of interest.

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