Effects of Iron-Reducing Bacteria on Carbon Steel Corrosion Induced by Thermophilic Sulfate-Reducing Consortia

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Introduction

Microbe-influenced corrosion (MIC) is due to the presence and activity of microorganisms, including bacteria and fungi, and affects a wide range of industries, resulting in severe economic losses [19]. Carbon steel, although susceptible to corrosion, is frequently employed in industries and infrastructure because of its strength, availability, relatively low cost, and fire resistance [19]. Infrastructure affected by MIC (e.g., buried pipelines) is often operated under anaerobic or microaerobic conditions [20]. Most studies on anaerobic-microaerobic MIC use sulfate-reducing bacteria (SRBs) because they are sulfide producers and promoters of the cathodic depolarization process in steel [14, 38]. According to von Wolzogen and Van der Vlugt [36], under anaerobic conditions, electrons from the metal surface (cathode) reduce protons to form hydrogen, which forms a film that prevents further proton reduction, thus producing electrostatic isolation (passivation). Different bacteria that colonize the metal surface can consume the hydrogen film, resulting in Fe2+ release from the metal surface. Sulfide produced by SRBs combines with Fe2+ to form ferrous sulfide and generate an adhesive film. This mineral film acts as a cathode for hydrogen evolution, thus increasing the corrosion rate [16], but can also have a protective passivation effect depending on the crystalline mineral composition [9, 11, 22]. The differences in SRB-related corrosive effects are also caused, at least in part, by the SRB species themselves [26, 34], in addition to ambient factors.

Under microaerobic conditions, a protective mineral film may be formed with ferric oxides. The dissolution of this protective film by iron-reducing bacteria (IRBs) may result in increased corrosion rates [10, 25]. SRBs and IRBs frequently coexist in association with buried metallic structures [1, 20]. The corrosive effect of microbial cocultures (consortia) composed of SRBs and IRBs is controversial [12]. It has been suggested that ferrous ions derived from bacterial ferric reduction may prevent formation of the protective sulfide film [25], thus increasing corrosion rates, and that bacterial consortia containing both bacterial types produce higher corrosion rates than axenic cultures [33, 35]; however, other studies have shown that IRBs inhibit steel corrosion.
corrosion in a dual-species biofilm with SRBs [8, 21]. SRBs and IRBs coexist in heterogeneous communities with different bacterial populations that modify the metabolism of the entire community [13, 39], thus likely modifying their corrosive effect.

In natural environments, the reactions leading to steel corrosion intensify with temperature [4], and high-temperature environments are characterized by diverse bacterial communities [32], many of which may have corrosive effects [2, 29]. In a previous study with a microcosm approach [33], we reconstructed different thermophilic bacterial consortia and found that when IRBs and SRBs were co-integrated in a consortium with Bacillus spp., they produced steel corrosion rates 4.8 and 5.0 times higher than those of sterile controls. Here, we provide evidence that, in bacterial consortia containing IRBs-SRBs, IRBs produce high ferrous ion concentrations that destabilize the mineral protective film formed on the steel surface, thus increasing the corrosion rate.

Materials and Methods

Bacteria Employed and Culture Conditions

We used the bacterial strains Bacillus sp. G2 (facultative anaerobic, IRB) reclassified as Geobacillus sp. G2 by Nazina et al. [23], Bacillus sp. G9a (facultative anaerobic, fermentative, slime forming), Bacillus sp. G11 (strictly aerobic, growth-factor requiring), and Desulfotomaculum sp. SRB-M (strictly anaerobic, hydrogen consuming, SRB) that were isolated from two hot springs in central Mexico and characterized as previously described [33]. The bacterial strains were cultured in medium D2 (g/l): MgSO\(_4\)·7H\(_2\)O, 1.50; Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\), 0.10; glucose, 5.00; casein peptone, 5.00; meat extract, 3.00; yeast extract, 0.20; pH 8 [3]; they were maintained by subculture in D2 medium, in SIRC cultures, or in SRC cultures. After 3 days of incubation (short period), the steel coupons incubated in medium, in SIRC or SRC, or in sterile D2 medium, for 3 or 25 days. After the incubations, the coupons were removed and dehydrated in absorbent paper under a N\(_2\) stream, and stored under ultraviolet light in a stream of warm, sterile air as a slight modification of the method of Obuekwe et al. [24]; and then placed in culture tubes (volume, 70 ml; 2.2 cm diameter × 20 cm length) containing 35 ml of culture medium D2 and 35 ml of air. At 55°C, the oxygen in the culture medium was of 28 µmol/l at 1 cm from the aerobic interface and 15 µmol/l at the bottom of the tube (10 cm from the aerobic interface). The tubes were inoculated with a sulfate- and iron-reducing consortium (SIRC) integrated by overnight culture of 0.5 ml of Geobacillus sp. G2, Bacillus sp. G9a, Bacillus sp. G11, and Desulfotomaculum sp. SRB-M, or they were inoculated with a sulfate-reducing consortium (SRC) integrated with the same bacteria except for the IRB Geobacillus sp. G2. In all the experiments, non-inoculated tubes were included as sterile controls. Tubes were incubated at 55°C for 3 or 25 days in a microoxic environment. After incubation, the coupons were cleaned using a slight modification of the method of Bryant et al. [5] in which the coupons were sonicated in citric acid (5% (w/v)) for 5 min for remove the bio- and mineral film, and then rinsed in distilled and deaerated water for 1 min. The coupons were flame and weighed. Corrosion was measured as weight loss divided by the coupon area [33].

Analytical Techniques

The Fe\(^{2+}\) concentration in the cultures was determined by the ferrozine assay [31]. The dissolved sulfide content was measured in the culture media by the methylene blue method [6] with a simple modification: to eliminate bacterial growth-related turbidity interference, the samples were centrifuged and the supernatant was assayed. The pH of the solution was measured using a potentiometer. Oxygen in the culture medium was measured with a dissolved oxygen meter.

Steel Corrosion by Bacterial Consortia

Carbon steel 1018 (AISI-SAE) coupons with an average area of 12.9 cm\(^2\) were used. The individual areas of the coupons were estimated according to their density, weight, and geometry. Each coupon was initially sterilized by immersion in 70% ethanol for 10 min; degreased in 100% ethanol for 15 min; and quickly dried under ultraviolet light in a stream of warm, sterile air as a slight modification of the method of Obuekwe et al. [24]; and then placed in culture tubes (volume, 70 ml; 2.2 cm diameter × 20 cm length) containing 35 ml of culture medium D2 and 35 ml of air. At 55°C, the oxygen in the culture medium was of 28 µmol/l at 1 cm from the aerobic interface and 15 µmol/l at the bottom of the tube (10 cm from the aerobic interface). The tubes were inoculated with a sulfate- and iron-reducing consortium (SIRC) integrated by overnight culture of 0.5 ml of Geobacillus sp. G2, Bacillus sp. G9a, Bacillus sp. G11, and Desulfotomaculum sp. SRB-M, or they were inoculated with a sulfate-reducing consortium (SRC) integrated with the same bacteria except for the IRB Geobacillus sp. G2. In all the experiments, non-inoculated tubes were included as sterile controls. Tubes were incubated at 55°C for 3 or 25 days in a microoxic environment. After incubation, the coupons were cleaned using a slight modification of the method of Bryant et al. [5] in which the coupons were sonicated in citric acid (5% (w/v)) for 5 min for remove the bio- and mineral film, and then rinsed in distilled and deaerated water for 1 min. The coupons were flame and weighed. Corrosion was measured as weight loss divided by the coupon area [33].

Scanning Electronic Microscopy

Carbon steel 1018 coupons with an average area of 2.4 cm\(^2\) were degreased and experimentally oxidized in Petri dishes with distilled water (depth, 2 mm), air drilled, ethanol-sterilized, and stored in vacuum in hermetic vials. The other coupons were incubated for 72 h in tubes with 35 ml of D2 medium inoculated with Desulfotomaculum sp. SRB-M to produce a biogenic ferrous sulfide film. Then, in an anaerobic chamber, the coupons were removed from the tubes, submerged in 70% deaerated ethanol for 10 min, drilled in absorbent paper under a N\(_2\) stream, and stored under vacuum conditions. A third coupon group was not pretreated and was only sterilized in 70% ethanol.

Coupons for the three treatments were incubated with cultures of the SIRC or SRC, or in sterile D2 medium, for 3 or 25 days. After the incubations, the coupons were removed and dehydrated in increasing concentrations of 20%, 40%, 60%, 80%, and 100% ethanol (in water (v/v)) for 5 min each. The coupons were stored in vacuum for metalization with copper and were examined using a scanning electronic microscope (JEOL JSM-6400) at 10 kV.

Results

Corrosion Experiments with Reconstructed Thermophilic Bacterial Consortia

Carbon steel 1018 coupons were incubated in a sterile medium, in SIRC cultures, or in SRC cultures. After 3 days of incubation (short period), the steel coupons incubated in sterile media showed corrosion of nearly 2.7 g/m\(^2\) (abiotic corrosion), whereas the SIRC and SRC culture media showed a slightly alkaline pH, with a ferrous ion concentration...
Corrosive Effect of Iron-Reducing Bacteria

From steel dissolution (close to 1 mmol/l) and a basal sulfide concentration provided by the culture medium (Table 1). Coupons incubated for 3 days in SRC cultures did not show corrosion, as measured by weight loss, but showed a slight increment in weight (0.05 g/m²), probably because of mineral deposits on the metal surface. The culture medium had a moderate acidic pH, the ferrous ion levels were much lower (36 µmol/l) than those in the abiotic controls, and the sulfide concentration was the highest among all conditions in the entire experiment (110 µmol/l). In contrast, coupons incubated in the SIRC cultures showed the highest corrosion, almost two times higher than that of the coupons incubated in sterile medium. The culture medium of the SIRC had a pH nearly identical to that of the SRC medium, but the ferrous ion concentration was 10 times higher and the sulfide concentration was 3.5 times lower (Table 1).

When the steel coupons were incubated for 25 days (long period), similar results were obtained. Coupons incubated in sterile media showed abiotic corrosion of 4.9 g/m² with a culture medium that had a slightly alkaline pH, but a relatively low ferrous ion concentration (close to 1 mmol/l) and a basal sulfide concentration. Coupons incubated with the thermophilic consortia were also affected by the presence or absence of the IRB Geobacillus sp. G2. The pH in both consortia was close to neutral, but in the SRC culture medium, the ferrous ion concentration was much lower and the sulfide concentration was two times higher than that in the SIRC medium. The formation of floccules of a dark precipitate was very clearly seen at the bottom of the culture tubes with SIRC. The difference in steel corrosion was dramatic; the corrosion was 10-fold higher in coupons incubated in SIRC relative to coupons incubated in SRC (Table 1).

**Examination of Carbon Steel Coupons by Scanning Electron Microscopy**

Carbon steel coupons were incubated for 3 or 25 days under sterile conditions, in SIRC cultures, or in SRC cultures. Some coupons were previously covered with an oxide or biological sulfide film. After 3 days of incubation, steel coupons incubated in sterile medium showed shallow pits with limited extension and were essentially intact without any mineral film (Fig. 1A). Coupons previously covered with an oxide film and then incubated in sterile medium showed an irregular dense mineral layer with numerous edges (Fig. 1C) characteristic of iron oxides [24], whereas coupons previously treated with biogenic sulfide and then incubated in sterile medium showed a dense homogeneous mineral layer with granular inclusions but without evident cracks (Fig. 1F). A similar mineral layer was found in coupons incubated with SIRC, regardless of whether they were coated with a mineral layer (Fig. 1B) or had been previously coated with an oxide (Fig. 1E) or sulfide layer (Fig. 1H), although it is notable that cracks were apparent with these three treatments. The mineral films were colonized by bacteria. In contrast, coupons incubated in SRC cultures showed dense mineral layers, without evident cracks (Figs. 1D and 1G), and were colonized by fewer bacteria than coupons exposed to SIRC. When the steel coupons were incubated for 25 days, the trend was the same as that at 3 days. However, all coupons incubated in sterile media showed a similarly homogeneous dense mineral film, regardless of whether they were pre-

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**Table 1. Comparison of parameters linked to corrosion of carbon steel incubated with SIRC or SRC.**

<table>
<thead>
<tr>
<th>Incubation Period</th>
<th>Steel corrosion (g/m²)</th>
<th>Culture medium (µmol/l)</th>
<th>Culture medium (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>Fe⁺ concentration</td>
</tr>
<tr>
<td>Sterile control</td>
<td>2.69 ± 0.24</td>
<td>7.4 ± 0.0</td>
<td>874 ± 99</td>
</tr>
<tr>
<td>SRC</td>
<td>-0.05 ± 0.11</td>
<td>5.8 ± 0.1</td>
<td>36 ± 14</td>
</tr>
<tr>
<td>SIRC</td>
<td>4.80 ± 0.82</td>
<td>5.7 ± 0.1</td>
<td>654 ± 258</td>
</tr>
<tr>
<td>After 25 days of incubation</td>
<td>4.92 ± 4.9</td>
<td>7.4 ± 0.0</td>
<td>1,207 ± 85</td>
</tr>
<tr>
<td>SRC</td>
<td>1.24 ± 8.5</td>
<td>6.3 ± 0.1</td>
<td>66 ± 12</td>
</tr>
<tr>
<td>SIRC</td>
<td>13.17 ± 10.5</td>
<td>6.9 ± 0.3</td>
<td>4,028 ± 690</td>
</tr>
</tbody>
</table>

Values shown represent the average of four replicates ± SE; letters are used to indicate that means on the same incubation day differ significantly by Duncan’s multiple range test (p < 0.05).
coated with a mineral layer (Fig. 2A) or were pre-coated with an oxide film (Fig. 2D) or a sulfide film (Fig. 2G). Fig. 2G also shows the bacteria responsible for the production of the biogenic sulfide film embedded in the mineral layer; these bacteria were killed by flaming before incubation in the sterile medium. The exposure of these bacteria indicated weathering in the biogenic ferrous sulfide film.

After 25 days of incubation with the thermophilic bacterial consortia, a dense mineral film was found, and when the coupons were incubated with SRC, the mineral film did not show evident cracks (Figs. 2B and 2E), but when the coupons were incubated with SIRC, the mineral film cracked (Figs. 2C, 2F, and 2H). The width of the fractures increased by 4-fold from 3 to 25 days, and the cracks were colonized by bacteria (Figs. 2F and 2H).

Discussion

MIC of steel is a complex phenomenon that involves diverse factors such as oxygen concentration (aerobic, microaerobic, or anaerobic conditions), the environment (temperature, pH, and nutrients), the groups of microorganisms (species, physiological factors, and ecological factors) [26, 34], and the particularities of steel itself. Because of this complexity, generalizing the data available in the literature has been difficult. Rodin et al. [28] previously showed that diverse bacterial consortia that include SRBs have a corrosive or protective effect on steel according to the culture conditions. Various studies have shown that IRBs—in combination with SRBs—have a protective effect on steel [8, 21], but other studies have shown that IRBs enhance the corrosive effect of SRBs [33, 35]. In the present study, we found that addition of the IRB Geobacillus sp. G2 reversed the protective effect of an SRC against steel corrosion.
The protective effect of the SRC was observed at both short (3 days) and long (25 days) incubation times, but at both time points, the pH was not the most important factor whenever both consortia contained the fermenting bacterium G9a, which produced the same pH in the respective media. Fe$^{2+}$ levels were much higher in SIRC cultures than in SRC cultures. At the short incubation time, the sterile controls had an Fe$^{2+}$ concentration similar to that observed for SIRC, but in our microaerobic system, it is expected that the Fe$^{2+}$ can be re-oxidized; therefore, at the long incubation time, the Fe$^{2+}$ level in SIRC cultures was 3-fold higher than that in sterile controls and 60-fold higher than that in SRC cultures (Table 1), probably because of the iron-reducing activity of *Geobacillus* sp. G2. Additionally, the higher Fe$^{2+}$ concentration in SIRC cultures probably caused two other observed effects: (i) lower sulfide concentration in SIRC than in SRC cultures resulting from the formation of ferrous sulfide floccules and (ii) increased steel corrosion by SIRC compared with SRC (Fig. 3).

Sulfide is corrosive [18], but at low concentrations, it reacts with the steel surface and produces a protective film of ferrous sulfide crystallized as mackinawite [30]; in our system, the maximum concentration of sulfide was 110 μmol/l, which was lower than the level in other systems [18, 21] and may thus have caused the protective effect in SRC [9, 33, 37]. King et al. [15, 17] previously showed that in SRB cultures with a high ferrous concentration, the breakdown of the mackinawite protective film by transformation into other less-adhesive sulfide conferred less protection to the underlying metal. It is therefore not surprising that SIRC showed the highest steel corrosion in the present study.

To evaluate the mineral film in different cultures, steel coupons covered with an oxide film, a biogenic sulfide film, or not covered were included in the two consortium cultures (Figs. 1 and 2). Steel coupons in these cultures presented a dense mineral film. Given that coupons pre-covered with oxide appeared similar to coupons biogenically covered with a sulfide film, it is possible that a secondary mineral film of ferrous sulfide covered the surface. Steel coupons incubated in SIRC cultures were extensively fractured, regardless of whether the steel surfaces were pre-coated, whereas SRC cultures did not show such fractures. Over time, the fractures became wider and reached a thickness of nearly 1 μm. Thus, the integrity and fracture condition of the mineral film correlated with the protective and corrosive effects of the SRC and SIRC, respectively. This finding suggests that, in SIRC cultures, the mineral film was transformed to a poorly adhesive sulfide material with poor integrity and without protective characteristics. A fractured mineral film permits diffusion of ferrous ions from the corroded steel surface to the

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**Fig. 3.** Schematic diagram of the proposed reaction mechanism for acceleration of steel corrosion by IRBs in the presence of SRBs.

The diagram shows a steel surface submerged in a medium in the presence of SRBs (A) or in the presence of SRBs and IRBs (B), under microaerobic conditions. Anaerobe facultative bacteria (not schematized) that consume oxygen from an aerobic interface maintain microaerobic conditions. Ferrous ions obtained from steel dissolution are oxidized to ferric ions at the aerobic interface, which are precipitated or produce ferric hydroxides on the metal surface. SRBs oxidize H$_2$ from the steel or from the glucose in the medium and reduce sulfate to produce sulfide. At low ferrous ion concentrations (A), sulfide reacts with the steel surface and produces a protective film of ferrous sulfide crystallized as mackinawite. The protective sulfide film isolates the steel surface from the protons present in the medium and limits ferrous ion diffusion, thereby inhibiting steel corrosion. In the presence of IRBs (B), ferric ions are reduced by IRBs, leading to the accumulation of ferrous ions in the medium; sulfide reacts with these ferrous ions from the medium to produce poorly adhesive ferrous sulfide floccules. These floccules form a film that is easily fractured and allows the interaction of protons from the medium with the steel surface; new H$_2$ evolution; and ferrous ion diffusion, all of which enhance steel corrosion.
medium and facilitates the diffusion of aggressive species such as sulfides to the steel surface for new corrosion cycles. Dong et al. [7] proposed that transformed ferrous sulfide minerals are conductive and undergo cathodic depolarization; therefore, a porous layer of these minerals also results in a large specific surface area composed of porous films. A similar effect can be expected on fractured sulfide film.

IRBs have been found to be protective against corrosion when added to SRB-containing environments, probably by depleting oxygen and biocompetitively excluding SRBs [27]. The present study showed that (i) under microaerobic conditions where ferrous ions can potentially accumulate, with a low sulfide ion concentration, IRB addition to a consortium of SRBs and fermentative bacteria increases steel corrosion, possibly through destabilization of the protective sulfide film, and (ii) that IRBs must be taken into account in MIC diagnosis.

Acknowledgments

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