Microbially Influenced Corrosion of Stainless Steel by *Acidithiobacillus ferrooxidans* Supplemented with Pyrite: Importance of Thiosulfate

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ABSTRACT Microbially influenced corrosion (MIC) results in significant damage to metallic materials in many industries. Anaerobic sulfate-reducing bacteria (SRB) have been well studied for their involvement in these processes. Highly corrosive environments are also found in pulp and paper processing, where chloride and thiosulfate lead to the corrosion of stainless steels. *Acidithiobacillus ferrooxidans* is a critically important chemolithotrophic acidophile exploited in metal biomining operations, and there is interest in using *A. ferrooxidans* cells for emerging processes such as electronic waste recycling. We explored conditions under which *A. ferrooxidans* could enable the corrosion of stainless steel. Acidic medium with iron, chloride, low sulfate, and pyrite supplementation created an environment where unstable thiosulfate was continuously generated. When combined with the chloride, acid, and iron, the thiosulfate enabled substantial corrosion of stainless steel (SS304) coupons (mass loss, 5.4 ± 1.1 mg/cm² over 13 days), which is an order of magnitude higher than what has been reported for SRB. These results were verified in an abiotic flow reactor, and the importance of mixing was also demonstrated. Overall, these results indicate that *A. ferrooxidans* and related pyrite-oxidizing bacteria could produce aggressive MIC conditions in certain environmental milieus.

IMPORTANCE MIC of industrial equipment, gas pipelines, and military material leads to billions of dollars in damage annually. Thus, there is a clear need to better understand MIC processes and chemistries as efforts are made to ameliorate these effects. Additionally, *A. ferrooxidans* is a valuable acidophile with high metal tolerance which can continuously generate ferric iron, making it critical to copper and other biomining operations as well as a potential biocatalyst for electronic waste recycling. New MIC mechanisms may expand the utility of these cells in future metal resource recovery operations.

KEYWORDS *Acidithiobacillus ferrooxidans*, biocorrosion, biomining, iron oxidizers, sulfur

Corrosion affects a variety of industries with severe economic consequences, and microbially influenced corrosion (MIC) is estimated to be responsible for ~20% of total corrosive damage (1). MIC refers to corrosion influenced by electrochemical processes associated with microorganisms. With the many reactions in which microorganisms can play a role, MIC mechanisms can either enhance corrosion, or they can inhibit corrosion through the formation of biofilms or chemical inhibitors (2). MIC is known to significantly affect the sewage, oil, and shipping industries, and it is estimated to cause several billions of dollars in damage in gas pipelines (3).

As MIC has been studied, biofilm formation by microorganisms has been shown to influence corrosion by the formation of concentration gradients and local conditions which result in the formation of anodic and cathodic sites on metal surfaces (4).

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Although there is a broad range of bacteria that can be involved in MIC, sulfate-reducing bacteria (SRB) have been most frequently studied because anoxic, sulfate-rich waters are widespread, and laboratory studies of corrosion have matched the severe rates of corrosion found for metals in the environment (5). SRB have been known to cause the corrosion of stainless steels, which are typically more corrosion resistant than are mild carbon steels. The formation of sulfur species, such as sulfide, results in conditions where 304L stainless steels (SS304L) and 316 stainless steels (SS316) are not immune to MIC attack (6). Previous studies have offered the cathodic depolarization theory as one explanation of the involvement of SRB in corrosion; however, work remains to elucidate the true involvement of SRB in biofilms on steel surfaces. Compared to pure cultures of SRB that are used for laboratory studies of MIC, mixed cultures of SRB are found in marine sediments where synergistic interactions between species in a consortium of bacteria causes severe MIC, which has been difficult to investigate and mitigate (7).

While SRB are the most studied anaerobic bacterial species causing MIC with the ability to cause corrosion of SS316 stainless steel even in seawater, other aerobic bacterial species that oxidize inorganic compounds such as sulfur, iron, or manganese are thought to be involved with MIC of stainless steels (8–11). However, the extent of MIC reported using aerobic bacteria appears to be widely varied and much more limited in its extent. Microbial colonization and biofilm formation occur on the surface of stainless steel, but the localized corrosion is primarily seen as pitting on the micrometer-length scale and is not as dramatic as is observed with anaerobic MIC (12). Despite the limited corrosion that aerobic microorganisms appear to cause on the surface of metals, it is known that there are many reaction mechanisms that bacteria can modulate to affect the corrosion process (13).

Acidithiobacillus ferrooxidans is an acidophilic bacterium that oxidizes ferrous iron and reduced inorganic sulfur compounds under aerobic conditions and is known to play a key role in the industrial biomining of copper and other sulfide-rich ores (14, 15). Although *A. ferrooxidans* is an obligate anaerobe and can grow on reduced inorganic sulfur compounds (RISCs) under anaerobic environments, it is commonly grown aerobically for the leaching of sulfide minerals in mining operations (16, 17). There has been recent interest in using *A. ferrooxidans* and related bacteria in the recycling of electronic waste, as these organisms have the potential to leach metals, including copper, silver, aluminum, nickel, cobalt, and cadmium, from waste printed circuit boards, spent batteries, and industrial sewage sludges (18–22). Despite the association of *A. ferrooxidans* with the dissolution of metals and its high tolerance of soluble metals, the role of this bacterium in MIC is poorly characterized (23). A recent study measuring the corrosion rate of C1010 steel with and without the inoculation of *A. ferrooxidans* found that the iron oxidation activity of the cells increased corrosion by 3 to 6 times over that of an abiotic acidic medium (24). These results showed that neither biofilm formation on the surface of the steel nor direct contact of the cells was needed, as the ferric iron species continuously produced by the cells increased the MIC process. Furthermore, while the study tested MIC from *A. ferrooxidans* with 304 stainless steel (SS304) coupons, no weight loss or change in surface roughness was reported, indicating that the conditions used were insufficient for the breaching of the stainless steel passivation layer.

Thiosulfate ions are known to cause corrosion in stainless steel, resulting in pitting, stress corrosion cracking (SCC), and crevice formation in equipment found in the pulp and paper industry, refineries, and nuclear material storage facilities which involve pH conditions higher than what is necessary for the growth of *A. ferrooxidans* (25). Under specific conditions, thiosulfate alone can cause pitting corrosion. However, thiosulfate and chloride together can act synergistically, as the thiosulfate helps to weaken the passivation layer for the chloride to form pits and accelerate corrosion (26). Prior research has shown that there is an optimal redox potential and proportions of thiosulfate and chloride to maximize pitting (27). Pitting of SS316 was observed at

FIG 1 Ferric iron caused the corrosion of several metals and alloys. However, no corrosion was observed for stainless steel. Abiotic immersion tests were conducted for 312 h using 50 mg of metal in 10 ml of AFM3 Fe(III) medium, and the mass loss of the 50-mg coupons was measured in triplicate; error bars indicate the standard deviation. SS304, 304 grade stainless steel.

thiosulfate-to-chloride ratios similar to those observed with SS304, but the more-corrosion-resistant SS316 required a higher ionic strength (28).

Here, we investigate the role *A. ferrooxidans* plays in the corrosion of SS304 stainless steel and the conditions under which enhanced MIC can occur with these bacteria. A range of conditions similar to ones that had been used for the cultivation of the bacterium were tested to determine MIC mechanisms that were compatible with its metabolic processes. As thiosulfate is involved in the sulfur oxidation pathways used by *A. ferrooxidans*, the oxidation of sulfur or sulfide minerals such as pyrite was explored (29, 30). We uncovered new conditions under which *A. ferrooxidans* could enable aggressive MIC of stainless steel in aerobic, acidic environments where thiosulfate can be continuously generated during pyrite oxidation.

RESULTS

Corrosion by ferric iron in acidic medium. Under acidic conditions, ferric iron and other metal ions are soluble, leading to the rapid corrosion of metals and many alloys, with the ferric iron acting as an oxidant, as shown in Fig. 1. Furthermore, the acidic medium provides protons as another oxidative species, leading to more metal dissolution than predicted by the stoichiometric reaction of ferric iron with the metals. With the reaction of both ferric iron and protons with the metals during the corrosion of the metal coupons, ferric salts were seen as an orange precipitate at the bottom of the samples. In AFM3 Fe(III) medium, the large amounts of sulfate associated with ferric sulfate salts do not prevent the redox reactions from occurring. However, the SS304 grade stainless steel did not experience mass loss over the 312-h incubation period. SS304 was found to be corrosion resistant to diluted concentrations of sulfuric acid typically involved with the growth medium used for *A. ferrooxidans*, and the passivation layer formed by the addition of chromium into the alloy prevented ferric iron from attacking the coupon (24). The elemental composition of the SS304 metal used in this study is displayed in Table S1 in the supplemental material.

Corrosion of SS304 caused by *A. ferrooxidans* in the presence of chloride. While *A. ferrooxidans* cells are able to oxidize ferrous iron to ferric iron for cell growth, the presence of 45 mM chloride in AFM3 medium does not cause much visible corrosion in SS304 stainless steel. The iron oxidation activity by *A. ferrooxidans*, which changes the medium color from a pale yellow to a darker orange as the ferrous iron is converted to ferric iron, was slowed by the inhibition from the chloride, which is consistent with the decrease in activity observed in previous studies (31, 32). The bulk changes in the metal coupons observed visually before and after the 312-h immersion test were minor (Fig. 2), with only some rounding of the edges and precipitation of ferric salts onto the surface. Under these conditions, 3.4 ± 0.5 mg of coupon mass loss was measured.

FIG 2 Corrosion of SS304 by *A. ferrooxidans* cells in AFM3 medium with chloride supplementation. Ferrous iron oxidized by *A. ferrooxidans* in the presence of 45 mM chloride caused little visible changes during a 312-h immersion test. On the left are the coupons before immersion, and on the right are the coupons after the immersion.

Although ferric chloride is known to be a corrosive agent to SS304, the presence of 100 mM sulfate in AFM3 medium was greater than the added chloride concentration which inhibited the corrosive attack. This is consistent with previous research which has shown that increasing the sulfate concentration above the chloride concentration prevented crevice formation for SS304 (26).

Development of chloride-containing, low-sulfate medium for SS304 corrosion.

A new medium formulation (CM5) was created with a lower sulfate concentration with the goal of promoting more pitting corrosion from the chloride. The CM5 medium contains 14 mM total sulfate, which is lower than the 45 mM concentration of chloride used. Over the 312-h immersion test in the CM5 medium, SS304 coupons lost 5.1 ± 0.8 mg of mass by corrosion (not shown). Under these conditions, the ferric and chloride ions resulted in increased corrosive attack. This medium formulation was used for the remainder of the experiments and explored further under various conditions compatible with the unique physiology of *A. ferrooxidans* to understand the mechanisms that could further promote MIC by these cells.

Addition of pyrite causes additional corrosion in CM5 medium. Previous experiments investigating the oxidation of pyrite indicated that thiosulfate was the first detectable sulfur compound released when pyrite was leached with ferric iron (30). We hypothesized that pyrite could serve as a source of thiosulfate that could be produced via leaching by ferric iron that can be continuously produced by the cells. Increased corrosion of SS304 coupons occurred when the coupons were immersed in sterile CM5 medium with the addition of 10 g/liter pyrite aerobically. A total of 9.3 ± 0.5 mg of mass was lost from the coupons over the 312-h incubation period with 200-mesh pyrite, and 11.5 ± 2.4 mg of mass loss from SS304 occurred over the 336-h incubation period with 325-mesh pyrite (Fig. 3).

To better understand the mechanism leading to increased corrosion of the coupons with the addition of pyrite into CM5 medium in the absence of cells, the redox potentials (ORPs), pH, ferrous iron and thiosulfate concentrations, and total iron concentrations were measured with the 325-mesh pyrite and 200-mesh pyrite included in immersion tests, as shown in Fig. 4 and S1. As the initial ferric iron included in the medium was reduced to ferrous iron by reacting with the pyrite, ORP values decreased from 749 ± 1 mV to 571 ± 1 mV over the first few days. Once the initial consumption of ferric iron was complete, the oxidation of pyrite by molecular oxygen or the corrosion products of SS304 coupons did not lead to further changes in the ORP. The changes in pH, ferrous iron and thiosulfate concentrations, and total iron concentrations for both pyrite particle sizes were similar. The decrease in pH from an initial value of 2.65 to 1.90 ± 0.06 for the 200-mesh pyrite was coupled with a steady increase in concentration of the species measured in this study because the oxidation of pyrite under low ferric iron released ferrous iron, thiosulfate, and protons, as shown in equation 1:

FIG 3 The addition of pyrite increased the corrosion of stainless steel in CM5 medium. Abiotic immersion tests were conducted for 312 h using 100 mg of stainless steel and 1 g of each pyrite in 100 ml of CM5 medium. The mass loss of the coupons was measured in triplicate, and error bars indicate the standard deviation. Statistical significance was calculated using an unpaired *t* test (*, $P < 0.05$; **, $P < 0.005$; n.s., not significant).

while the dissolution of the metal coupon mostly contributed additional iron in the ferrous state to the solution. This partial oxidation of pyrite contrasts with the full oxidation of pyrite where the sulfur species are converted to sulfate, as shown in equation 2:

Ferric iron readily oxidizes thiosulfate to tetrathionate, as shown in equation 3:

which begins the series of complex decomposition reactions to sulfate. As the difference between the ferrous iron and thiosulfate concentrations and the total iron concentration was minimal (<1 mM) throughout the test, thiosulfate ions did not accumulate appreciably in the medium. As there were no significant differences obtained in the mass loss of the metal coupon with 325-mesh and 200-mesh pyrite, the 200-mesh pyrite was used for the rest of this study.

Pyrite oxidation by *A. ferrooxidans* enhances MIC in the CM5 medium. The inoculation of *A. ferrooxidans* cell to the CM5 medium containing pyrite substantially increased the corrosive environment for the rate of SS304 coupons. *A. ferrooxidans* grown on either ferrous iron or pyrite prior to inoculation into CM5 medium was tested,

FIG 4 Corrosion of stainless steel in abiotic, aerobic, acidic medium with high chloride and low sulfate concentrations supplemented with pyrite. The CM5 medium with the addition of 325-mesh pyrite was sampled over a 336-h immersion for pH, redox potential (ORP), ferrous iron and thiosulfate concentrations, and total iron concentration in triplicate; error bars indicate the standard deviation.

FIG 5 The inoculation of *A. ferrooxidans* into CM5 medium containing pyrite was measured over the 312-h immersion period. (A) Cultures inoculated with pyrite-grown cells. (B) Cultures inoculated with iron-grown cells. (C) Crevice formation in one of the coupons, circled in white. Both sets of cultures were run in triplicate, and error bars indicate the standard deviation.

as the bacterium is known to secrete extracellular polymeric substances (EPS) depending on the substrate used for their growth (33). The cells grown on ferrous iron were expected to mediate pyrite dissolution through the generation of ferric iron planktonically, while the cells grown on pyrite were expected to reattach to the mineral surface and accelerate pyrite oxidation through the complexation of ferric iron in the EPS near the mineral surface (34). As shown in Fig. 5A and B, *A. ferrooxidans* cells caused additional oxidation of pyrite, as evidenced by the steady decline in pH and increases in sulfate concentrations during the incubation period compared to the abiotic experiments (Fig. 4). At the end of the 312 h, the pH in the culture with the pyrite-grown cells reached 1.74 ± 0.03 , while the pH in the cultures with the iron-grown cells reached 1.58 ± 0.09 (Fig. 5B). The increase in sulfate concentration was approximately double in samples containing iron-grown cells with 42.0 ± 16.1 mM sulfate compared to the 22.7 ± 1.0 mM sulfate measured in the samples with pyrite-grown cells. Both of these indicators suggest that more pyrite oxidation occurred when iron-grown cells were

FIG 6 Greater coupon mass loss is observed under low-ferric-iron and -sulfate conditions with the addition of chloride and pyrite when *A. ferrooxidans* is inoculated into the culture. All immersion tests were run for 312 h using 100-mg SS304 coupons. Statistical significance was calculated using an unpaired *t* test (*, $P < 0.05$; **, $P < 0.005$).

added to the medium. *A. ferrooxidans* mediates the oxidation of ferrous iron to ferric iron, as shown in equation 4:

The comparison of the ferrous iron and thiosulfate concentrations to the total iron concentration demonstrates that soluble iron largely remains in the reduced form despite the presence of the cells. However, different from the abiotic immersion tests (Fig. 4), a transient accumulation of thiosulfate is seen in the first half of the immersion test as the ferrous iron and thiosulfate concentrations exceed the measured total iron concentration. As a result of increased pyrite oxidation, 15.9 ± 3.0 mg and 17.7 ± 3.7 mg were lost from the SS304 coupons using pyrite-grown and iron-grown cells, respectively, which is a significant increase in corrosion compared to the abiotic tests. However, no significant differences were noted in the final extent of corrosion that occurred from the iron-grown cells compared to the pyrite-grown cells. The aggressive nature of MIC was visually noted by the formation of small crevices in most (2/3) of the coupons (Fig. 5C).

Figure 6 shows a comparison of the results obtained. The reduction of sulfate and addition of chloride lead to a sterile medium formulation (CM5) that is more corrosive to stainless steel than is observed with cells growing on an iron- and sulfate-rich medium (AFM3). The addition of pyrite to the sterile CM5 medium enhanced the corrosion of stainless steel, as the available ferric iron likely produced thiosulfate from the pyrite. The most corrosive environment for stainless steel occurred with pyrite and *A. ferrooxidans* in the CM5 medium as the cells are able continuously produce ferric iron, leading to a continuous production of corrosive thiosulfate from the pyrite.

Acid, chloride, and thiosulfate cause stainless steel corrosion. To further verify the mechanism for pyrite-mediated MIC by *A. ferrooxidans*, a plug flow reactor (PFR) was constructed to test the various compounds involved in this MIC environment. The presence of sufficient acid, chloride, and thiosulfate was hypothesized to be necessary to induce the corrosion of SS304 stainless steel. Therefore, a solution of 45 mM HCl and 1 mM sodium thiosulfate was flowed through the PFR containing a 100-mg SS304 coupon to mimic the conditions generated by the addition of pyrite to CM5 medium. Under these conditions, no corrosion of the coupon was observed, and a white precipitate was formed over time in the solution, likely from the acid decomposition of thiosulfate. To minimize the decomposition of thiosulfate and cause corrosion of the stainless steel, 90 mM HCl and 10 mM sodium thiosulfate solutions were prepared separately and mixed at the point of entrance to the PFR at equal flow rates. With an

FIG 7 XPS analysis of coupons immersed in the PFR. The $2p_{3/2}$ binding energy of sulfur and iron indicates a depletion of iron in the coupon in the 45 mM HCl plus 5 mM $\text{Na}_2\text{S}_2\text{O}_3$ solution where the black compound formed at the surface.

effective initial solution composition of 45 mM HCl and 5 mM sodium thiosulfate within the PFR, a black compound formed at the surface of the coupon within 15 min of starting flow through the PFR, and degradation of the coupon was readily visible. Over the 168 h for which the PFR was operated, the surface of the coupon remained covered with the black compound, and 79.5 mg of the 100-mg coupon was lost. A duplicate run of the PFR resulted in a 39.5-mg loss from the coupon.

The coupons from the PFR were analyzed by X-ray photoelectron spectroscopy (XPS) to characterize the black surface that had formed when flowing the 5 mM sodium thiosulfate solution. The coupons that had been exposed to the two concentrations of sodium thiosulfate were compared against a pristine coupon that had not been immersed. As shown in Fig. 7, all coupons had a sulfur peak indicating the presence of sulfur in the surface layer, as SS304 contains trace amounts of sulfur. The sulfur and iron peaks from the coupon immersed in the 1 mM sodium thiosulfate and pristine SS304 coupon are similar in shape and intensity. However, the coupon immersed in the 5 mM sodium thiosulfate, which reacted to form the black surface layer, displayed a much lower intensity in iron and more prominent sulfur $2p_{1/2}$ and $2p_{3/2}$ peaks. This supports the notion that the black compound is a result of thiosulfate adsorption onto the surface, as shown in equation 5:

where O_{ads} and S_{ads} represent adsorbed oxygen and sulfur, respectively. Furthermore, the formation of black deposits by thiosulfate and chloride at higher pH and the depletion of iron at the surface of the coupon by energy-dispersive X-ray spectroscopy (EDS) analysis have been reported by previous studies in abiotic solutions (26). Since no major differences were seen for the sulfur peak for the coupon in the 1 mM sodium thiosulfate where no visible changes to the coupon was observed and no mass loss was measured, sufficiently high concentrations of thiosulfate appear to be necessary to cause corrosion of stainless steel. As the black surface layer was rapidly formed for all the PFR tests, the differences in the mass losses between the two 168-h PFR runs indicate that the corrosion of the bulk metal from the coupon is controlled by the transport of ions through the layer of sulfur formed on the surface.

Based on these results and the observations made in CM5 medium with the addition of pyrite, we propose a mechanism for pyrite-mediated MIC by *A. ferrooxidans* in Fig. 8. The stainless steel is corroded in an environment where there is sufficient chloride, thiosulfate, ferric iron, and protons. This environment can be produced when *A. ferrooxidans* cells are able to produce ferric iron, which oxidizes pyrite to produce thiosulfate. The freshly generated thiosulfate can adhere to and participate in the

FIG 8 Scheme showing the effect of pyrite addition with *A. ferrooxidans* (*A. f.*) leading to enhanced MIC. 1, pyrite is oxidized by ferric iron to form ferrous iron, protons, and thiosulfate. 2, chloride initiates pitting in the passivation layer on the surface of stainless steel. 3, if the thiosulfate species is not degraded by reaction with protons, ferric iron, or pyrite itself, thiosulfate can be reduced to form adsorbed sulfur in the pits to prevent repassivation of the surface. 4, the pits stabilized by adsorbed sulfur are susceptible to additional acid dissolution. 5, *A. ferrooxidans* oxidizes ferrous iron to ferric iron for further pyrite oxidation.

corrosion of the stainless steel along with protons in chloride-induced pits before it decomposes through reaction with ferric iron, pyrite, or acid.

Role of agitation in detection of MIC. Agitation of the medium is critical for increasing the lifetime of the thiosulfate to allow it to enhance the corrosion of stainless steel. SS304 coupons were immersed into CM5 medium with pyrite either under abiotic conditions or with the inoculation of iron-grown *A. ferrooxidans* to test the extent of corrosion without shaking. After incubation for 312 h, the abiotic tests reached a final pH of 1.91 ± 0.07 and had a negligible mass loss of 0.0 ± 0.5 mg, and the tests with *A. ferrooxidans* reached a final pH of 1.55 ± 0.06 and also had no detectable mass loss (0.0 ± 0.4 mg). As the decreases in pH for both abiotic and biotic tests for these tests were similar to those obtained in the agitated samples, pyrite oxidation by ferric iron is not affected. Furthermore, without agitation of the medium, the pyrite settles to the bottom so that the metal coupon is covered by the pyrite. These results indicate that direct contact of the pyrite to the stainless steel does not contribute to MIC through galvanic interactions, and the thiosulfate must be transported away from the pyrite surface to interact with the stainless steel to induce corrosion.

DISCUSSION

A. ferrooxidans is an important microorganism in bioleaching operations due to its ability to interact directly with sulfidic ores through ferric iron (15, 35). There have been some efforts to develop these cells as a platform for biofuel production (36–40). However, its potential role in MIC processes has received less attention. Here, we have uncovered an aggressive MIC mechanism for stainless steel that results from the microbial oxidation of pyrite in a liquid medium with chloride and low sulfate. The critical components of the mechanism involving the combination of acid, chloride, and thiosulfate were verified using a continuous-flow PFR. This aerobic MIC mechanism involving acidophilic bacteria may have important implications in mining, electronic waste recycling, and the protection of military and industrial material.

The oxidation of pyrite proceeds in abiotic systems with ferric iron causing some accelerated corrosion of stainless steel. Although *A. ferrooxidans* has genes for pathways involved in the oxidation of ferrous iron and RISCs (including thiosulfate and tetrathionate), the inoculation of these compounds in immersion tests with the bacteria

further increased the corrosion of SS304, suggesting that *A. ferrooxidans* contributed to the additional production of thiosulfate through the generation of ferric iron rather than inhibiting corrosion by consuming the thiosulfate in solution (41). Despite the inherent instability of the thiosulfate ion which can react with many components of this system, including protons, ferric iron, and/or pyrite, compared to the alkaline pH involved in the pulping stage of paper processing where the thiosulfate species are more stable, MIC still occurred (42). The use of the CM5 medium creates an environment where the iron is kept in a reduced state, and *A. ferrooxidans* cells are able to enhance the oxidation of pyrite by locally generating thiosulfate near the surface of the metal to accelerate the corrosion of SS304 (30, 43).

Compared to other studies investigating the MIC of SS304, the mass loss of the coupon from the activity of *A. ferrooxidans* found here is more than an order of magnitude larger than the mass loss reported with SRB. With the inoculation of iron-grown cells into CM5 medium with pyrite, our reported values are equivalent to a loss of 5.4 ± 1.1 mg/cm² over 13 days using an average exposed surface area of 3.31 cm², whereas 0.18 ± 0.10 mg/cm² specific mass loss was reported with *Desulfovibrio vulgaris* in an anaerobic test over 7 days (44). Although SRB have been implicated as the major bacterial group involved in MIC, higher rates of corrosion have been found with the aerobic *Pseudomonas* bacteria in simulated seawater, where biotic samples exposed for 14 days had a corrosion rate of 0.0335 mm/year; this approximately corresponds to a specific mass loss of 1.0 mg/cm² (45). As strains of *A. ferrooxidans* have been abundantly found in natural environments involving metals and ores, this microorganism has the potential to severely impact the MIC of various materials.

Despite the short incubation times of these corrosion tests, the differences in EPS formation and metabolism between the iron-grown and pyrite-grown cells had a noticeable impact on the measured culture conditions. Although the composition of the EPS is similar for *A. ferrooxidans* grown on the two substrates, pyrite-grown cells are known to produce roughly 10 times more EPS to mediate attachment to the sulfide mineral (33). Additionally, as *A. ferrooxidans* adapts to grow on pyrite, many proteins are differentially expressed to allow for biofilm formation and greater resistance to environmental stresses (46–48). In the cultures with iron-grown cells, more pyrite oxidation indicated by higher sulfate accumulation suggests that the regeneration of ferric iron by cells in this metabolic state helped to release more thiosulfate to cause MIC, whereas in the cultures with pyrite-grown cells, the slower increase in total iron concentration suggests that the production of more EPS may have complexed more ferric iron to better stabilize the thiosulfate produced to similarly enhance MIC. While both of these processes increase the rate of corrosion on stainless steel, as sulfate accumulation becomes inhibitory to chloride pitting at higher concentrations, the pyrite-grown cells would likely cause greater corrosion over longer incubation periods.

There are similarities to the aerobic MIC mechanism reported here, as sulfur species and adsorbed sulfur play an important role in promoting the degradation of the stainless steel. When SRB produce the metabolite, H₂S, adsorption of the sulfide species hinders the reformation of the oxide film passivating the surface of the stainless steel (49). Likewise, when *A. ferrooxidans* generates thiosulfate from the oxidation of pyrite, the adsorption and reduction of the thiosulfate lead to an enrichment of sulfur at the surface of the metal, weakening the passivation layer, which allows MIC to proceed through acid dissolution of the bulk metal. However, as no black deposit was visible on the metal coupons immersed in CM5 medium with pyrite and *A. ferrooxidans*, the extent of the MIC demonstrated here appears to be limited not only by the stability of the produced thiosulfate but also by the adsorbed sulfur that forms on the surface of the stainless steel.

As the role of *A. ferrooxidans* and its activity in bioleaching and biomining are better understood, bioleaching technologies can be developed to exploit discarded materials, such as e-waste and military metals, to recover economically valuable metals (50). The novel corrosion mechanism introduced here could be utilized as an alternative to the ferric iron dominated metal dissolution that is commonly associated with these bacteria

for materials that are difficult to leach. Should the genetic tools that are being developed to modify the bacterium become implemented to improve various leaching processes to be more efficient, engineering the genes related to sulfur oxidation, metal tolerances, and salt tolerance of these acidophiles will allow for more development of *A. ferrooxidans* for new applications (23, 36, 37, 51, 52).

MATERIALS AND METHODS

Chemicals, reagents, media, and culturing of *Acidithiobacillus ferrooxidans*. All chemicals were sourced from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Two hundred-mesh pyrite was provided as a kind gift from Freeport-McMoRan (Phoenix, AZ). *Acidithiobacillus ferrooxidans* ATCC 23270 was obtained as described previously (37). All *A. ferrooxidans* cultures were initiated with a starting optical density at 600 nm (OD_{600}) of 0.001, corresponding to a cell density of 8.3×10^6 cells/ml (53). *A. ferrooxidans* was maintained for use in experiments by weekly subculture into 100 ml of AFM3 medium for iron-grown cells or 100 ml of AFM3 Fe(III) medium for pyrite-grown cells which contained either 100 mM Fe(II) using ferrous sulfate heptahydrate or 100 mM Fe(III) using ferric sulfate hydrate, respectively, and 0.80 g/liter $(NH_4)_2SO_4$, 2.0 g/liter $MgSO_4 \cdot 7H_2O$, 0.1 g/liter K_2HPO_4 , and 5 ml/liter trace mineral supplement (ATCC MD-TMS) adjusted to pH 1.8 using concentrated sulfuric acid. Two hundred-mesh pyrite (10 g/liter) was added to AFM3 Fe(III) aseptically after filtration for pyrite-grown cells. All cultures were incubated at 30°C and shaken at 140 rpm. Cells were harvested by centrifugation at $5,000 \times g$ for 7 min. Harvested cells were kept in 10 ml of AFM3 medium and maintained viability for 1 to 2 weeks stored at 4°C.

Immersion corrosion experiments were conducted in 10 ml of AFM3 Fe(III) medium, 100 ml AFM3 medium, or 100 ml of CM5 medium which contained 0.80 g/liter $(NH_4)_2SO_4$, 2.0 g/liter $MgSO_4 \cdot 7H_2O$, 0.02 g/liter K_2HPO_4 , 0.81 g/liter $FeCl_3$, 5 ml/liter trace mineral supplement (ATCC MD-TMS), and 2.24 g/liter KCl. Media were sterilized with a 0.2- μ m filter (Thermo Fisher Scientific, Waltham, MA). Pyrite, at a concentration of 10 g/liter, was added to CM5 medium after filtration using aseptic techniques.

Preparation and analysis of metal coupons. For testing the effects of ferric ion-mediated corrosion, 0.003-inch-thick brass, low-carbon steel, aluminum, and SS304 stainless steel shims and a 0.005-inch-thick bronze shim were obtained from McMaster-Carr (Robbinsville, NJ). Each metal was cut into 50-mg coupons and weighed using an analytical balance (Accu-124; Fisher Scientific, Hampton, NH). For the immersion corrosion tests using either AFM3 or CM5 medium, the stainless steel shim was cut into 100-mg coupons and weighed using an analytical balance. All coupons retrieved after immersion were rinsed with distilled water, air dried, and weighed using an analytical balance.

Analysis of immersion medium. The pH values of the medium samples were measured using the pH 700 benchtop meter (Oakton, Vernon Hills, IL). The redox potentials of the media were measured using a Mettler Toledo InPro3253SG pH probe. The redox potentials were calibrated against an ORP standard at $E_H = +420$ mV against a standard hydrogen electrode. The combined ferrous iron and thiosulfate concentrations were measured by titrating 1 ml of the sampled medium mixed with 10 μ l of ferroin indicator using a 0.1 M cerium sulfate solution and noting the color change of the solution from a red to a cyan color, which indicated that the reduced species had been oxidized. The combined concentrations are reported in the equivalent concentration of ferrous iron. Total iron concentrations were measured using the iCE 3300 atomic absorption spectrometer (AAS; Thermo Fisher Scientific, Waltham, MA). A standard curve was obtained from 1-ppm, 2-ppm, 3-ppm, and 4-ppm Fe standards in distilled water, prepared from a 1,000 mg/liter AAS iron standard. Culture samples were diluted using distilled water to be within the range of the standard curve for the measurement of total iron.

Sulfate concentrations were measured using a barium sulfate turbidimetric method. The Bapolyethylene glycol (Ba-PEG) reagent was prepared using 9.77 g/liter $BaCl_2 \cdot 2H_2O$ and 150 g/liter of polyethylene glycol with an average molecular weight of 8,000. Fifty milliliters of this reagent was transferred to a beaker, and 100 μ l of 50 mM Na_2SO_4 solution was added dropwise while stirring magnetically to produce the precipitant solution. A standard curve for sulfate was obtained from 0.5, 1, 1.5, and 2 mM sulfate standards in distilled water, prepared from a 1,000 mg/liter ion chromatography (IC) sulfate standard. Half a milliliter of the standard was mixed with 0.5 ml of the precipitant solution and vortexed for 1 min. The mixture was allowed to stand for 5 min, and the absorbance of this mixture at 600 nm was measured. Culture samples were diluted using distilled water to be within the range of the standard curve and mixed with the precipitant solution similarly for the measurement of sulfate. A standard curve can be found in Fig. S2.

Operation and analysis of corrosion tests in plug flow reactor. A Masterflex L/S pump was connected to a plug flow reactor (PFR) containing the stainless steel coupon. When a single solution was flowed into the PFR, a flow rate of 1 liter/day was used. When two solutions were used, a flow rate of 0.5 liter/day was used to have a total inlet flow rate of 1 liter/day. The solutions were allowed to fill the PFR, submerge the coupon, and exit from the other end.

Coupons retrieved from the PFR after immersion were rinsed with distilled water, air dried, and weighed using an analytical balance. An X-ray photoelectron spectroscopy (XPS) system was used for surface analysis of the stainless steel coupons before and after immersion in the PFR. A PerkinElmer 04-303A Ar ion gun was used to etch the top 0.5 nm of the samples for precleaning. A *Phi 5500* X-ray photoelectron spectrophotometer equipped with a monochromatic Al K_{α} source (photon energy = 1,486 eV, work function = 3.41, scale factor = 20.0119) was used to scan the samples for the presence of iron and sulfur. The scan rate was employed with 0.05 eV per step, and multiple scans were conducted for each sample to improve the spectral clarity.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01381-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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