Enhanced microbial corrosion by *Acidithiobacillus ferrooxidans* through the manipulation of substrate oxidation and genetic engineering

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Abstract

Acidithiobacillus ferrooxidans cells can oxidize iron and sulfur and are key members of the microbial biomining communities that are exploited in the large-scale bioleaching of metal sulfide ores. Some minerals are recalcitrant to bioleaching due to presence of other inhibitory materials in the ore bodies. Additives are intentionally included in processed metals to reduce environmental and microbially influenced corrosion. We have previously reported a new aerobic corrosion mechanism where *A. ferrooxidans* cells combined with pyrite and chloride can oxidize low grade stainless steel (SS304) with a thiosulfate-mediated mechanism. Here we explore process conditions and genetic engineering of the cells to enable corrosion of a higher grade steel (SS316). The addition of elemental sulfur and an increase in the cell loading resulted in a 74% increase in the corrosion of SS316 as compared to sulfur- and cell-free control experiments. The overexpression of the endogenous *rus* gene, which is involved in the cellular iron oxidation pathway, led to further 85% increase in the corrosion of the steel. Thus, the modification of the culturing conditions and cell line, led to a more than 3-fold increase in the corrosion of SS316 stainless steel, such that 15% of the metal coupons was dissolved in just 2 weeks. This work demonstrates how the engineering of cells and the optimization of their cultivation conditions can be used to discover conditions that lead to the corrosion of a complex metal target.

Keywords: *A. ferrooxidans,* microbially influenced corrosion, rusticyanin, pyrite, sulfur oxidation

Running Title: Enhancing *A. ferrooxidans* corrosion

Introduction

Biomining technologies exploit microbial metabolisms to extract and recover metals from ores and waste materials (Rawlings, 2002). The biomining of mineral ores produces at least 15% of copper and 5% of gold globally (Johnson, 2014; Schippers et al., 2014). Furthermore, the amount of electronic waste produced annually around the world has been rapidly growing, and these waste streams contain metals of relatively high value and concentration when compared to the dilute concentrations of metals found in the earth. Chemical leaching has been studied extensively for printed circuit boards, and bioleaching has been gaining traction as it is compatible with a variety of inexpensive lixiviants (Hsu, Barmak, West, & Park, 2019). The oxidation of pure metals and minerals by microbes generally involves reactions that are well understood. However, a grand challenge in the further development of biomining and metal recycling technologies lies in the interactions that occur with other materials found in the feedstocks. For example, the bioleaching of refractory copper-containing mineral chalcopyrite is limited by inhibitory compounds that lead to passivation (Li, Kawashima, Li, Chandra, & Gerson, 2013). These inhibitory effects are exploited for the protection of metals from corrosive attack. Stainless steels are formulated with chromium and other elements to inhibit oxidation reactions.

Acidithiobacillus ferrooxidans is a well-studied acidophilic chemolithotrophic microorganism capable of oxidizing both iron and reduced inorganic sulfur species which enables the oxidation and dissolution of metal sulfides. This bacterium is one of many biomining genera involved in biomining operations, and the role of these bacteria in catalyzing redox reactions has been characterized since their identification (Gumulya et al., 2018). Recent focus on leaching low grade ores in suboptimal conditions such as in the presence of saline, reflecting the arid regions where ores are found, has spurred studies to evaluate strategies for improving traditional mining

bioprocesses. Thermophiles with their ability to operate at elevated temperatures have the advantage of higher reactions rates, but these species frequently tolerate less metal than mesophiles (Castro, Urbieta, Plaza Cazón, & Donati, 2019). Microbial consortia have been reported to outperform pure cultures in leaching due to synergistic effects from promoting and inhibiting various reactions within leaching solutions (Brune & Bayer, 2012). However, manipulation and control of microbial consortia is still poorly explored given the lack of details about individual species and the mechanisms associated with each. For example, quorum sensing and sulfur oxidation pathways in these cells are not full elucidated, limiting the effective design of engineered microbial communities (Mamani et al., 2016; Wang et al., 2019).

There is a great deal of potential and interest in the use of synthetic biology to enhance the performance of biomining microorganisms. The genetic tools available for the manipulation of *A. ferrooxidans* allow for overexpression, knockout, and chromosomal integration of genes (Banerjee, Burrell, Reed, West, & Banta, 2017; Inaba, Banerjee, Kernan, & Banta, 2018; Kernan et al., 2016; Yu, Liu, Wang, Li, & Lin, 2014). While interesting phenotypes have been discovered using these tools, engineered strains have not been investigated in simulated leaching conditions. However, it is clear that the genetic engineering of acidophiles holds much promise for future bioleaching studies as complete genome sequences are available for multiple bacterial and archaea species (Kaksonen et al., 2018).

The manipulation of the leaching solution and oxidation kinetics using several different additives have been also proposed, given that chemical modification of biomining systems is relatively simple to implement. Surfactants, such as Triton X-100, were shown to be effective in enhancing copper extraction from chalcopyrite despite their inhibitory effect on *A. ferrooxidans* even at low concentrations (Zhang et al., 2018). In leaching copper from television circuit boards,

the addition of pyrite at a concentration of 50 g/L was shown to improve the extraction of copper by providing additional soluble iron and acid to aid with the process (Bas, Deveci, & Yazici, 2013). Additionally, acid-processed waste newspaper was used as an additive to improve the recovery of copper from chalcopyrite with a mixed consortium through a reductive dissolution mechanism (Panda et al., 2015). These studies demonstrate how optimization of the bioleaching milieu can provide opportunities for low-cost enhancements to biomining processes.

While stainless steels holds little value as a target for metal recovery, stainless steels, especially the 316 grade, are used in the hardware of numerous industries such as mining, paper production, and petroleum processing due to their excellent corrosion resistance (Choudhary, Macdonald, & Alfantazi, 2015; Javaherdashti, 2017). Microbial influenced corrosion (MIC) has been implicated as one of the primary mechanisms involved in the failure of stainless steels, and studies have shown that biofilms formed by anaerobic sulfate-reducing bacteria aggravate MIC (Chen, Frank Cheng, & Voordouw, 2017; Sheng, Ting, & Pehkonen, 2007). Despite the similarities in reaction chemistries involved in biomining and MIC, where metal dissolution is desired in one and undesired in the other, only a few studies have investigated acidophiles for their corrosivity (Dong et al., 2018; Wang, Ju, Castaneda, Cheng, & Zhang Newby, 2014). We recently reported that *A. ferrooxidans* is capable of catalyzing MIC under aerobic conditions. We found that pyrite oxidation in a chloride containing medium could disrupt the passivation layer of stainless steel SS304 to enable thiosulfate-mediated dissolution of the bulk metal (Inaba, Xu, Vardner, West, & Banta, 2019). The more recalcitrant SS316 steel grade involves the addition of molybdenum and increased amounts of nickel compared to the SS304 grade resulting in better protection against pitting corrosion by chloride ions and hindering dissolution of the metal (Kaneko & Isaacs, 2002; Pardo et al., 2008).

Here we report the enhancement of microbial corrosion of stainless steels by optimizing the substrates available for oxidation and by creating a new engineered *A. ferrooxidans* strain with an enhanced capability to oxidize these substrates in a corrosion medium. We investigated the effect of adding elemental sulfur, another low-cost substrate in addition to pyrite, to the corrosion system to improve acid production which serves as the main metal oxidant. In addition to modulating the energy source for *A. ferrooxidans* in the medium, we also considered the effect of initial cell density. Finally, we introduced a plasmid into *A. ferrooxidans* that enables the overexpression of the endogenous rusticyanin (*rus*) gene, which is a periplasmic protein critical for iron oxidation. With these modifications, we demonstrate the enhanced corrosion of SS304 steel and we use these improvements to explore the MIC of the more corrosion-resistant SS316 stainless steel. These results further demonstrate how synthetic biology and the optimization of culturing conditions can be used to enable MIC of industrially processed metals and hardware.

Materials and Methods

Chemicals and reagents

All chemicals were sourced from Sigma-Aldrich (St. Louis, MO) and enzymes and reagents for DNA manipulation were purchased from NEB (Ipswich, MA) unless otherwise noted. All primers used in this study were obtained from Integrated DNA Technologies (Coralville, Iowa). Western blot supplies were sourced from Thermo Fisher Scientific (Waltham, MA). 200-mesh pyrite was provided as a kind gift from Freeport-McMoRan (Phoenix, AZ). SS304 stainless steel shims and SS316 stainless steel shims were obtained from McMaster-Carr (Robbinsville, NJ). *Acidithiobacillus ferrooxidans* ATCC 23270 and *Escherichia coli* S17-1 were obtained as described previously (Kernan, West, & Banta, 2017). All DNA sequencing was performed by

Genewiz (South Plainfield, NJ). The bacterial strains and plasmids used in this study are listed in Table 1.

Media and culturing of *Acidithiobacillus ferrooxidans*

All *A. ferrooxidan*s cultures were initiated with a starting OD_{600}=0.001 (optical density measured at 600 nm), corresponding to a cell density of 8.3×10^6 cells/mL, unless otherwise indicated for corrosion experiments using higher initial cell density of OD_{600}=0.03 (Li, Mercado, Kernan, West, & Banta, 2014). *A. ferrooxidans* was maintained for use in experiments by weekly subculture into 100 mL of AFM3 medium as described previously (Inaba et al., 2019). All cultures were incubated at 30 °C and shaken at 140 rpm. Cells were harvested by centrifugation at 5,000 x g for 7 min. Harvested cells were kept in 10 mL of AFM3 medium (Table S2) and maintained viability for 1-2 weeks stored at 4 °C.

Immersion corrosion experiments were conducted in 100 mL of CM5 medium (Table S2) as described previously (Inaba et al., 2019). All media were sterilized with a 0.2 µm filter (Thermo Fisher Scientific, Waltham, MA). Pyrite and/or colloidal sulfur were added to CM5 medium after filtration using aseptic techniques.

Plasmid construction and genetic manipulation

The empty vector pYI11 was generated by digesting plasmid AF-GFP with NheI and NotI (Kernan et al., 2016). The *tac* promoter and *rrnB* terminator were amplified from pMAL-c4E with primer sets pJRD-tac-F/pJRD-tac-R and pJRD-rrnB-F/pJRD-rrnB-R respectively using Q5 DNA polymerase. The amplified *tac* promoter was purified and digested with NheI and BamHI and the amplified *rrnB* terminator was purified and digested with BamHI and NotI. The two amplicons were ligated into the digested AF-GFP vector. The resulting plasmid was then modified with Q5 Site-Directed Mutagenesis as per the recommended protocol from the manufacturer with primers

pJRD-EcoRI-F/pJRD-EcoRI-R to include an additional EcoRI restriction site. Then, using the Q5 Site-Directed Mutagenesis with primers pJRD-dRSF-F/pJRD-dRSF-R, a small portion of the plasmid backbone before the oriV was deleted to produce pYI11.

To generate pYI37, the pYI11 plasmid was modified to have an in-frame His-tag using the Q5 Site-Directed Mutagenesis with primers pYIHis-F/pYIHis-R. This plasmid was then digested with BamHI and KpnI. The *rus* gene from *A. ferrooxidans* was amplified from genomic DNA prepared using the NucleoSpin Tissue kit (Takara Bio, Mountain View, CA) with primers pYI37-F/pYI37-R and purified. The PCR fragment was combined with the digested plasmid using NEBuilder HiFi DNA Assembly as per the recommended protocol. All constructs were transformed into *E. coli* DH5α and were verified by DNA sequencing. The primers used for plasmid construction are listed in Table S1. The DNA sequences and plasmid maps of plasmid pYI11 and pYI37 are provided in Table S3 and Fig. S4.

Plasmid pYI37 was conjugated into *A. ferrooxidans* using the mating and conjugation protocol previously described using the donor strain *E. coli* S17-1. Three single colonies of *A. ferrooxidans* transconjugants on S204 solid medium plates were screened in the liquid selection SM4 medium (Table S2) (Inaba et al., 2018).

Western blots for detection of transgene expression

After verifying the mutants had persistent kanamycin resistance in SM4 medium, the presence of the overexpressed *rus* protein in the mutants was determined by Western blotting for the three clones designated as YI37-1, YI37-2, YI37-3. The cell lysates of the genetically engineered *A. ferrooxidans* was separated using SDS-PAGE in Novex NuPAGE 4-12% Bis-Tris gel. The separated proteins were transferred onto a Novex 0.45 µm nitrocellulose membrane with a semi-dry blotting unit using the recommended method of the manufacturer. The blotted

membrane was blocked using Blocker Fl Fluorescent Blocking Buffer and treated with mouse 6x-His tag monoclonal antibody (HIS.H8) at a 1:1000 dilution. The primary antibody treated membrane was then treated with goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 at a 1:1000 dilution. The fluorescently labeled proteins were detected using a Gel Doc XR+ Imager (Bio-Rad, Hercules, CA) are shown in Fig. S5 where the expected molecular weight of the His-tagged *rus* protein is 20.8 kDa. As all three cell lysates had the tagged protein. Clone YI37-3 was used for the remainder of the study and referred to as AF37.

Preparation and analysis of metal coupons

For the immersion corrosion tests, the metal shims were cut into 100 mg coupons and weighed using an analytical balance (Accu-124, Fisher Scientific, Hampton, NH). All coupons retrieved after immersion tests were rinsed with distilled water, dried in air, and weighed using an analytical balance.

Analysis of immersion medium

The pH values of the media samples were measured using the pH 700 Benchtop Meter (Oakton, Vernon Hills, IL). The redox potentials of the media were measured using Mettler Toledo InPro3253SG pH probe. The redox potentials (ORP) were calibrated against an ORP standard at E_H = +420 mV against a Standard Hydrogen Electrode (SHE). The combined ferrous iron and thiosulfate concentrations were measured by titrating 1 mL of the sampled media mixed with 10 µL 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 (Thermo

Fisher Scientific, Waltham, MA), and sulfate concentrations were measured using a barium sulfate turbidimetric method as previously described (Inaba et al., 2019).

Statistics

All error bars represent one standard deviation from the mean. Statistical analyses using ANOVA and post-hoc tests were conducted using Matlab and unpaired t-tests were conducted using Excel. P-values less than 0.05 were considered to be statistically significant.

Results

Modulation of steel corrosion by *A. ferrooxidans* using different mixtures of pyrite and sulfur

Previously, we demonstrated that once the passivation layer on the stainless steel was removed by the thiosulfate produced by the oxidation of pyrite, proton attack was responsible for the corrosion of SS304 stainless steel (Inaba et al., 2019). Therefore, the effect of including elemental sulfur as an additional source of acid was investigated. Keeping the total solids constant at 10 g/L in the immersion tests, five different ratios of pyrite to sulfur were tested ranging from conditions with 100% pyrite to 100% sulfur to explore the corrosion of SS304 metal coupons. In the presence of *A. ferrooxidans*, both pyrite and sulfur were oxidized to generate sulfuric acid where the oxidation of pyrite goes through the thiosulfate intermediate in solution before being oxidized to sulfate while the oxidation of sulfur goes directly to sulfate within the cell. After a 336-h incubation for each condition, the mass loss of the coupons and final pH values were measured, where the pH indicates the total acid produced by the oxidation of the substrates. In the control condition of 100% pyrite, 18.3 ± 0.9 mg of SS304 was lost, resulting in a final solution pH of 1.62 ± 0.04. As the pyrite was replaced with increasing amounts of sulfur, a maximum mass loss of

27.4 ± 1.0 mg was observed using 80% pyrite and 20% sulfur. This corresponds to an increase of 50% as compared to the control condition. ANOVA showed that the differences in the mass losses were significant (Fig. 1, Top). The lowest final pH of 1.30 ± 0.08 was also observed using 80% pyrite and 20% sulfur (Fig. 1, Bottom). However, ANOVA indicated that differences between pH values did not reach statistical significance. Further increases in the percentage of sulfur in the solid mixture resulted in smaller mass losses in the SS304 coupons and generally higher final pH values in the solutions. The differences in mean mass losses in the 20% pyrite and 80% sulfur and 100% sulfur conditions were also significant as compared to the control conditions indicating that the addition of excess sulfur impedes the corrosion mechanism and further highlights the importance of having pyrite presence in the culture (Fig. 1, Top).

Pyrite and sulfur induced microbial corrosion of SS316 stainless steel

As the mixture of 80% pyrite and 20% sulfur was successful in enhancing the corrosion of SS304 stainless steel, the CM5 corrosion medium was evaluated on SS316 stainless steel to determine if the modification would be effective against a more corrosion-resistant stainless steel. The biotic tests with *A. ferrooxidans* using the SS316 coupons resulted in a higher mass loss of 5.3 ± 0.6 mg using 100% pyrite and 6.3 ± 1.0 mg using 80% pyrite and 20% sulfur as compared to the abiotic control with a mass loss of 4.7 ± 0.1 mg (Fig. 2), however these differences did not reach statistical significance by ANOVA. Comparing the immersion media in the cultures for the three conditions, the pH and sulfate concentrations displayed a notable divergence. Starting at 168 hours, the biotic condition with 80% pyrite and 20% sulfur had a steeper decline in pH and rapid accumulation of sulfate until the end of the immersion test as compared to the biotic condition with only pyrite and the abiotic condition as shown in Fig. 3. At 336 hours, ANOVA was used to determine that there were statistically significant differences in the final pH and sulfate

concentrations. Post-hoc tests revealed that the three pH measurements recorded were different from each other and the sulfate concentration for the biotic tests with 80% pyrite and 20% sulfur was different from both the biotic case with 100% pyrite and abiotic case. These results indicated that *A. ferrooxidans* was oxidizing the sulfur to acidify the media more rapidly as compared to the cultures with only pyrite. While the two biotic conditions had similar ORP readings that were higher than that of the abiotic controls, the ferrous and thiosulfate concentrations and total iron concentration, which correspond to the oxidation of pyrite, showed no particular trends between the three conditions (Fig. S6).

Effect of initial cell density on corrosion of SS316

Since *A. ferrooxidans* catalyzes the oxidation of ferrous iron to ferric iron driving further pyrite oxidation, we hypothesized that increasing the initial cell density in the culture would cause increased pyrite oxidation through both planktonic and biofilm-forming cells which are capable of regenerating ferric iron (Fowler, Holmes, & Crundwell, 1999). Furthermore, additional cells could attach to the elemental sulfur to promote the production of sulfuric acid. With the production of additional thiosulfate and acid, the corrosion of the metal coupons would be enhanced unless the increased presence of ferric iron in solution caused the rapid degradation of thiosulfate to inhibit MIC.

While previous immersion experiments with *A. ferrooxidans* were started with an initial OD_{600}=0.001, referred to as the low-OD case, a much higher initial OD_{600}=0.03 was chosen for the high-OD experiments. The most corrosive condition of CM5 medium with 80% pyrite and 20% sulfur was used for the comparison. Fig. 4A shows that the additional cells in the culture had the desired effect on the immersion solution. From the early stages of immersion test, the medium was more acidic and at 336 hours, the pH of the solution reached 1.00 ± 0.02 for the high-OD

experiments compared to 1.21 ± 0.04 for the low-OD experiments, which was statistically significant difference by a one-tail two sample t-test (Fig. 4A). Only small amounts of ferrous iron and thiosulfate were detected until 216 hours when the pH fell below that of ~1.3 which is the low pH limit of iron oxidation by *A. ferrooxidans* (Sand, 1989). After this pH crossover point, the ferrous iron and thiosulfate concentrations slowly accumulated until the end of the experiment. To support these observations, the redox potentials for the high-OD case followed this trend where a relatively high ORP, close to the ferric-ferrous iron redox couple of 770 mV, was maintained until 216 hours after which a decline in ORP corresponding to the increase in ferrous iron was measured (Fig. 4A). An increased accumulation of sulfate concentration over the low-OD case was seen during the second half of the experiment (Fig. 4A). After 336 hours, a mass loss of 8.2 ± 0.7 mg was measured from the SS316 coupons as displayed in Fig. 5. This is a 74% increase in mass loss as compared to the abiotic control experiment where only pyrite was added to the corrosion medium (Fig. 2).

Impact of *rus* gene overexpression in AF37 on corrosion

The degradation of pyrite by *A. ferrooxidans* produces small amounts of elemental sulfur, and growth on pyrite or sulfur as energy sources requires different metabolic adaptations in the cells because the chemical composition of the extracellular polymeric substances needed to attach to these substrates differs in electrostatic properties and hydrophobicity (Gehrke, Telegdi, Thierry, & Sand, 1998; Schippers, Jozsa, & Sand, 1996). A key enzyme for growth on ferrous iron, *rus* is known to be downregulated, yet still newly synthesized, in biofilm cells during growth on pyrite and elemental sulfur (Bellenberg, Huynh, Poetsch, Sand, & Vera, 2019; Valenzuela et al., 2008). Through the overexpression of *rus* in *A. ferrooxidans,* we hypothesized that substrate oxidation would be enhanced in this complex medium, which would further intensify the corrosion of

stainless steel. The high initial cell concentration of OD_{600}=0.03 was used. After 336 hours of incubation, the coupons with the engineered AF37 cell line exhibited 15.2 ± 0.9 mg of mass loss which is 85% higher than what was observed with the wild type cells (8.2 ± 0.7), and this was statistically different by a one-tail two sample t-test (Fig. 5). The most notable measured difference in the media was the acceleration of the pH decline over the experimental time span. The cultures with AF37 reached a pH~1.3 approximately 48 hours before the wild type cells reached a similar pH, at which point the ferrous and thiosulfate concentration began to increase (Fig. 4B). At 336 hours, the pH of AF37 cell cultures reached 0.90 ± 0.04 and the sulfate concentration in solution was significantly higher than the measured levels with the wild type cells (Fig. 4B).

Prior research has indicated that *rus* is downregulated when *A. ferrooxidans* are exposed to high chloride environments (Dopson et al., 2017). To demonstrate that AF37 had no increased iron oxidation activity in the presence of chloride, both wild type and engineered AF37 cells were grown in AFM3 medium supplemented with 45 mM KCl which corresponds to the amount of chloride initially present in CM5 medium. No significant differences in the time taken to oxidize all of the ferrous iron was observed between the two strains (Fig. S7).

Discussion

A. ferrooxidans is an important microorganism for biomining given its ability to interact with acid-insoluble and acid-soluble metal sulfides as it expresses oxidation pathways for both iron and sulfur (Rawlings, 2005; Valdes et al., 2008). As bioleaching biotechnology expands to electronic and other wastes as a source of critical metals for future manufacturing, hydrometallurgy offers a path to the urban mining of these raw materials (Işıldar et al., 2019). While many metals can be mobilized by ferric iron produced through the iron oxidation activity of *A. ferrooxidans*,

alloys such as stainless steel require more complex chemistries for dissolution corresponding to unique media compositions necessary to promote these reactions (Inaba et al., 2019). Thiosulfate-based leaching techniques are also promising for precious metal recovery as cyanide lixiviants for gold are highly toxic and environmentally damaging (Xu et al., 2017). To enhance pyrite-based leaching of stainless steel by *A. ferrooxidans,* here we have demonstrated that the manipulation of substrate utilization and genetic manipulation of the cells can substantially improve the corrosion of stainless steels. We find that the supplementation of sulfur and an increase in cell density leads to almost a doubling of the extent of corrosion of SS316 and the overexpression of the endogenous *rus* gene in the cells leads to almost another doubling of the SS316 corrosion.

The initial oxidation of pyrite by ferric iron is known to yield protons, and subsequently more protons are generated as the thiosulfate intermediate is oxidized to sulfate by *A. ferrooxidans* (Schippers & Sand, 1999). Our data shows that the supplement of elemental sulfur was beneficial in increasing the corrosion rate of both SS304 and SS316 under biotic conditions. These results indicate that direct sulfur oxidation is more effective in producing acid, possibly due to the thiosulfate from pyrite being consumed during the removal of the passivation layer from the stainless and the consumption of protons during iron oxidation by *A. ferrooxidans* (Quatrini et al., 2009). However, as expected, sulfur oxidation only acts as an enhancer to this corrosion mechanism as the mass loss observed decreased with increasing sulfur content beyond the 80% pyrite and 20% sulfur ratio as insufficient amounts of thiosulfate were being produced to initiate corrosion on the metal surface. More surprising was the positive effect of increasing initial cell density on augmenting corrosion of the coupons. While the additional cell mass increased the overall corrosion of the coupons, during the initial phase of the immersion the solution became more oxidative with ferric iron being the dominant state of iron. Our previous studies suggested

that maintaining the iron in the reduced state as much as possible was important to prevent the reaction of ferric iron and the thiosulfate to produce tetrathionate which occurs rapidly in solution. However, these observations seem to indicate that this reaction is not a significant part of the mechanism. During the second phase of the immersion experiments, when the pH drops below 1.3, the concentration of the reduced species increased quickly. Previously, this reduction behavior was attributed with the oxidation of sulfur at extremely low pH, but under the conditions used in this study, the consumption of ferric by pyrite seems more likely, given the slow reduction rates using elemental sulfur (Johnson, Hedrich, & Pakostova, 2017). As the concentration of ferric iron decreases in solution, it is expected that the oxidation of pyrite decreases and the oxidation of the sulfur becomes more important due to the accelerating acidification of the medium.

The *rus* operon in *A. ferrooxidans* contains proteins involved in the downhill pathway for electron transfer used in iron oxidation. The operon contains three identified promoters, PI, PII, and P_{rus}, and a fourth promoter upstream of the *acoP* gene has been suggested (Navarro, Von Bernath, Martínez-Bussenius, Castillo, & Jerez, 2016; Yarzabal, Appia-Ayme, Ratouchniak, & Bonnefoy, 2004; Zhan et al., 2019). The multiple promoters transcribing this operon are differentially affected by various environmental cultivation conditions for *A. ferrooxidans*. The expression of *rus* was found to be higher when sulfur-grown cells were exposed to copper, indicating that the overexpression of *rus* can have an impact beyond that of iron oxidation (Almárcegui et al., 2014). Our results here demonstrate that in this system, where considerable quantities of iron, pyrite, sulfur, and chloride are all present, the overexpression of *rus* from the unregulated *tac* promoter in AF37 affected the oxidation of pyrite and/or sulfur rather than the iron oxidation activity stressed by chloride. Further work focused on investigating *rus* expression under

other relevant bioleaching conditions may further elucidate the additional roles this protein plays in determining the oxidation rates of various substrates.

Although the lack of the *lac* repressor in *A. ferrooxidans* makes the *tac* promoter a constitutive promoter, it still remains useful in synthetic biology as the strongest known promoter in the microorganism (Wang, Fang, Wen, Lin, & Liu, 2017). Despite the applicability of *tac*, a inducible expression of *rus* would likely be beneficial to enable responses to specific environmental signals, avoiding the inefficiencies of synthesizing additional quantities of a protein that already occupies 21% of the total periplasmic volume (Li, Painter, Ban, & Blake, 2015). A logical extension of our results would be to pair *rus* expression with the regulatory elements controlling the oxidation of pyrite and/or sulfur. With recent elucidation of the role of quorum sensing in biofilm formation and identification of a potential *lux-box* like elements in *A. ferrooxidans*, the incorporation of the *lux-box* element into the *tac* promoter or utilization of the upstream sequence of *afeI* instead of *tac* could serve to generate a more optimal expression profile to enhance the oxidation rate of pyrite (Banderas & Guiliani, 2013; Mamani et al., 2016). We have also characterized two promoters that have differential expression in the presence of sulfur, and the *tusA* promoter may also elicit favorable expression in this system (Kernan et al., 2017). Future work in testing novel methods for controlling gene expression will lead to the development of genetically modified strains of *Acidithiobacillus* which reduce the time required for metal dissolution and extraction (Gumulya et al., 2018).

In this study, we demonstrate the dissolution of the more corrosion-resistant 316 stainless steel by manipulating substrate oxidation in the absence of the higher chloride concentrations that were previously explored for 304 stainless steel (Inaba et al., 2019). *A. ferrooxidans* can simultaneously oxidize ferrous iron, pyrite, and elemental sulfur to create an extremely corrosive

environment although past literature has suggested the expression of different EPS genes was required to attach to the surfaces of pyrite and sulfur (Barreto, Jedlicki, & Holmes, 2005; Gehrke et al., 1998; Vu, Chen, Crawford, & Ivanova, 2009). These results demonstrate the need to pay attention to corrosion mechanisms when designing biotechnology processes with this acidophilic organism and conversely also further highlights that multiple metabolic pathways within *A. ferrooxidans* can contribute together to bring about the complex chemistry necessary for the corrosion of stainless steel. Through additional genetic engineering, substantial improvements in optimizing the energy pathways in this microorganism can be expected which could then be applied to commercial biomining.

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562 **Tables**

563 Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid	**Description**	**Source or Reference**
Strain		
E. coli S17-1 ATCC 47055	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	ATCC
A. ferrooxidans ATCC 23270	Type strain	ATCC
AF37	ATCC 23270 with pYI37	This study
Plasmid		
AF-GFP	pJRD215 containing GFP driven by *tac* promoter	(Kernan et al., 2016)
pYI11	pJRD215 empty vector with *tac* promoter and *rrnB* terminator	This study
pYI37	pYI11 with His-tagged *rus* from *A. ferrooxidans*	This study

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Figure Legends

Fig. 1. Mixtures of pyrite and sulfur in CM5 medium affects corrosion of SS304 in the presence of *A. ferrooxidans.* (Top) The percent mass loss was measured from the 100 mg SS304 coupons with varying ratios of pyrite to sulfur at the end of the experiments. The mean mass losses determined to be statistically different from the control 100% pyrite case are indicated with an asterisk (*, $p < 0.05$). (Bottom) The final pH of the culture at the end of the immersion tests. All experiments were performed in 100 mL of CM5 medium with the respective addition of pyrite and sulfur at 10 g/L, and *A. ferrooxidans* was introduced initially at OD_{600}=0.001. Measurements were made after 336 hours. Experiments were conducted in triplicate and the error bars represent one standard deviation.

Fig. 2. Optimizing the addition of solid substrates improves corrosion of SS316. Immersion tests compared the abiotic control with 100% pyrite to biotic tests with either 100% pyrite or 80% pyrite and 20% sulfur. Experiments were performed in 100 mL of CM5 medium with the total solids at 10 g/L and *A. ferrooxidans* was introduced at OD_{600}=0.001 as necessary. Mass loss measurements were made after 336 hours. Experiments were conducted in triplicate and the error bars represent one standard deviation.

Fig. 3. Supplementing sulfur to the biotic corrosion system increases solution acidity. The changes in the pH and sulfate concentration in the solution are shown over 336 hours. The circles, triangles, and squares correspond to the abiotic control with 100% pyrite, biotic condition with 100% pyrite, and the biotic condition with 80% pyrite and 20% sulfur respectively. Experiments were conducted in triplicate and the error bars represent one standard deviation.

588 Fig. 4. Effect of higher cell density of wild type and AF37 cells on solution conditions. Panel A
589 corresponds to the incubation with wild type while Panel B corresponds to the incubation with
590 AF37. (Top) The pH and ORP are indicated with squares and diamonds respectively. (Bottom)
591 The ferrous and thiosulfate, total iron, and sulfate concentrations are shown with triangles, crosses,
592 and circles respectively. Experiments were performed in 100 mL of CM5 medium with the
593 addition of pyrite at 8 g/L and sulfur at 2 g/L. Each strain of *A. ferrooxidans* was introduced at
594 OD_{600}=0.03. The solutions were measured for 336 hours. Experiments were conducted in triplicate
595 and the error bars represent one standard deviation.

596 Fig. 5. High cell density and *rus* overexpression was effective for the corrosion of SS316 using the
597 solid mixture of 80% pyrite and 20% sulfur. The percent mass loss was measured from the 100
598 mg SS316 coupons after 336 hours of immersion. Experiments were conducted in triplicate and
599 the error bars represent one standard deviation. Statistical significance was calculated using an
600 unpaired t test (*, $p < 0.05$).