AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

BIOTECHNOLOGY

Glutathione Synthetase Overexpression in *Acidithiobacillus ferrooxidans* Improves Halotolerance of Iron Oxidation

Yuta Inaba,[a] Alan C. West,[a] Scott Banta[a]

[a]Department of Chemical Engineering, Columbia University, New York, New York, USA

ABSTRACT *Acidithiobacillus ferrooxidans* is a well-studied iron- and sulfur-oxidizing acidophilic chemolithoautotroph that is exploited for its ability to participate in the bioleaching of metal sulfides. Here, we overexpressed the endogenous glutamate-cysteine ligase and glutathione synthetase genes in separate strains and found that glutathione synthetase overexpression increased intracellular glutathione levels. We explored the impact of pH on the halotolerance of iron oxidation in wild-type and engineered cultures. The increase in glutathione allowed the modified cells to grow under salt concentrations and pH conditions that are fully inhibitory to wild-type cells. Furthermore, we found that improved iron oxidation ability in the presence of chloride also resulted in higher levels of intracellular reactive oxygen species (ROS) in the strain. These results indicate that glutathione overexpression can be used to increase halotolerance in *A. ferrooxidans* and would likely be a useful strategy on other acidophilic bacteria.

IMPORTANCE The use of acidophilic bacteria in the hydrometallurgical processing of sulfide ores can enable many benefits, including the potential reduction of environmental impacts. The cells involved in bioleaching tend to have limited halotolerance, and increased halotolerance could enable several benefits, including a reduction in the need for the use of freshwater resources. We show that the genetic modification of *A. ferrooxidans* for the overproduction of glutathione is a promising strategy to enable cells to resist the oxidative stress that can occur during growth in the presence of salt.

KEYWORDS bioleaching, genetic engineering, glutathione, halotolerance

Acidophilic chemolithotrophic bacteria are critical catalysts in the bioleaching industry as these microorganisms facilitate the solubilization of metals from ores in a less environmentally damaging and energy-intensive manner than traditional pyrometallurgical methods (1). In particular, the production of ferric iron plays a significant role in accelerating the dissolution of metal sulfides as it is a strong oxidant (2, 3). Bioleaching technologies have been successfully applied in copper and gold mining; however, many existing mines operate in arid regions, such as Australia or Chile, where water resources are becoming a greater concern (4).

As freshwater becomes more limited for mines, adapting processes can lead to the accumulation of anions in the leach liquor resulting from the intrusion of chloride ions into the water supply from brackish water, dissolution of silicate minerals, and additional recycling of process water (5, 6). The presence of these anions can have a mixed effect on leaching. Small amounts of chloride can have a positive effect on the extraction of copper from the refractory copper sulfide chalcopyrite (7, 8). The enhancement of copper recovery upon chloride addition is attributed to the reduction of surface passivation of the mineral (9). Furthermore, there are potential new bioleaching applications such as waste metal recycling and corrosion that could benefit from the inclusion of chloride species, permitting unique reaction chemistries (10). However, due to the

Citation Inaba Y, West AC, Banta S. 2021. Glutathione synthetase overexpression in *Acidithiobacillus ferrooxidans* improves halotolerance of iron oxidation. Appl Environ Microbiol 87:e01518-21. https://doi.org/10.1128/AEM.01518-21.

Editor Robert M. Kelly, North Carolina State University

Address correspondence to Scott Banta, sbanta@columbia.edu.

Received 30 July 2021
Accepted 2 August 2021
Accepted manuscript posted online 4 August 2021
Published 28 September 2021

large transmembrane pH gradient and the positive membrane potential in acidophiles, many anions, other than sulfate, can easily permeate the cell. The influx of anions causes the cytoplasm to acidify, ultimately causing cell death if the cell is unable to respond to the stress (11). In most species of acidophiles, the toxicity of chloride is exacerbated at increasingly lower pHs (12).

Since chloride is known to have a deleterious effect on the better-studied acidophiles, some recent efforts have focused on prospecting for new species of acidophilic halophiles that can promote bioleaching under chloride concentrations approaching that of seawater (13–15). The discovery of these new *Acidihalobacter* spp. has spurred the investigation of the genetic mechanisms responsible for their halotolerance (16, 17). It has been shown that these bacterial species have adopted a biphasic salting-out approach to increase their internal osmolarity by transporting potassium into the cytoplasm and synthesizing compatible solutes such as proline, ectoine, and periplasmic glucans (18). While the use of these halotolerant species could be promising in future bioleaching processes, their characterization is in the early stages, and more studies are needed to better understand their suitability for bioleaching. On the other hand, the *Acidithiobacillus* and *Leptospirillum* spp. are widely known, well-characterized acidophiles due to their important role in industrial bioleaching operations (19). These species are represented in mines around the world, but they are limited in their salt tolerance (14). Overall, iron oxidation systems appear to be more sensitive to anions than sulfur oxidation systems, making it difficult to achieve efficient generation of ferric iron when anions are found in leaching liquor (20).

Acidithiobacillus ferrooxidans is a key iron- and sulfur-oxidizing bacterium that has well-studied characteristics, available genomic information, and genetic tools and microbiology techniques that have been developed for genetic modifications (21–25). The interplay between pH and chloride stress makes it difficult to indicate a single inhibitory concentration of chloride; however, at around pH 1.8 (around the optimal growth pH of *A. ferrooxidans*), this concentration is reported to be on the order of 100 mM (14, 16). Compared to other acidophilic halotolerant species, *A. ferrooxidans* has limited mechanisms for adapting to high salt concentrations. While the abundances of protein folding chaperones, stress proteins, and periplasmic solute binding proteins increase in response to salt stress, *A. ferrooxidans* does not produce significant osmoprotectants for prolonged stress conditions (16, 17, 26). Despite its low endogenous resistance to chloride, it is becoming an excellent chassis to apply synthetic biology approaches to overcome challenging conditions in novel bioleaching applications (27). As the mechanisms of halotolerance in acidophilic halotolerant species have been uncovered, this offers new opportunities to develop genetically engineered strains of *A. ferrooxidans* that would thrive under even harsher conditions (17). Furthermore, previous studies have reported that genes associated with glutathione in *A. ferrooxidans* respond to various forms of oxidative stress, suggesting that this pathway could be a good candidate for genetic engineering (28–31), and it has been shown that exogenously provided glutathione was effective in reducing the intracellular reactive oxygen species (ROS) content in an acidophilic microbial consortium containing *A. ferrooxidans*, which suggests that glutathione was an effective ROS scavenger in this system.

Here, we report the investigation of the effects of the overexpression of the two genes that form the glutathione biosynthesis pathway in *A. ferrooxidans* in an attempt to improve the halotolerance of the bacterium by improving its ability to better cope with oxidative stress induced by chloride entry. By increasing the production of glutathione within the cells, we find that the iron oxidation activity is more resistant to chloride stress at a lower medium pH or higher chloride concentration that is inhibitory to wild-type (WT) *A. ferrooxidans*. The formation of intracellular ROS was also measured to better understand how glutathione enables enhanced iron oxidation in these bacteria.

RESULTS

Construction of plasmids and strains overexpressing glutathione biosynthesis genes. The two genes for synthesizing glutathione had been previously identified in the sequenced genome of *Acidithiobacillus ferrooxidans* (21). The glutamate-cysteine

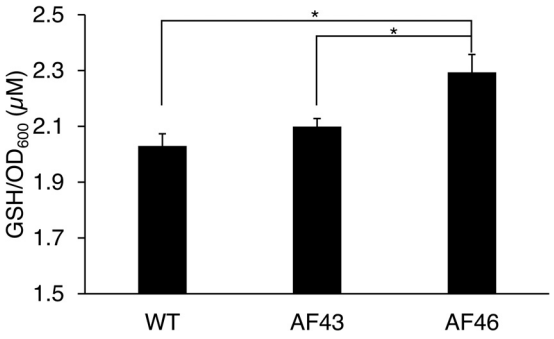

FIG 1 Overexpression of the glutathione synthetase gene *gshB* in AF46 increases the glutathione concentration compared to the WT and AF43. The glutathione (GSH) concentration from the cell lysates was obtained in triplicate, and error bars indicate standard deviations. Statistical significance was calculated using ANOVA and *post hoc* tests (*, $P < 0.05$).

ligase (*gshA* [AFE_3064]) and glutathione synthetase (*gshB* [AFE_3063]) sequences were placed behind the *tac* promoter on a pJRD215-based plasmid to generate plasmids pYI43 and pYI46, respectively. These two plasmids were conjugated into *A. ferrooxidans* to successfully generate the AF43 strain expressing *gshA* and the AF46 strain expressing *gshB*.

Effect of glutathione biosynthesis genes on intracellular glutathione levels. The overexpression of the glutathione biosynthesis genes was expected to increase the production of glutathione in *A. ferrooxidans*. After measuring the extracted concentration of glutathione in the strains, analysis of variance (ANOVA) showed that AF46 had a significantly higher concentration of glutathione than AF43 or the WT (Fig. 1). As AF46 produced more glutathione, we predicted that this strain would be able to better respond to the oxidative stress caused by chloride than the wild-type strain.

Preliminary screening of halotolerance. To initially test the effect of the overexpression of increased intracellular glutathione levels in *A. ferrooxidans*, the engineered strains and the WT were grown under various NaCl concentrations and pHs in a 96-well format to determine if enhanced iron oxidation was observed. The qualitative endpoint assay demonstrated that the halotolerance of *A. ferrooxidans* was sharply impacted within a narrow pH range where iron oxidation could occur as was detected at up to 320 mM NaCl at an initial pH of 2.1 and up to 180 mM NaCl at an initial pH of 1.9. However, iron oxidation was not detected beyond 40 mM NaCl at an initial pH of 1.7 (see Table S1 in the supplemental material). Furthermore, while AF46 appeared to have improved tolerance to chloride over the WT, AF43 seemed to have no increase in chloride tolerance under all pH conditions. These results suggest that the higher glutathione concentration in the cells resulted in improved salt tolerance.

Effect of NaCl concentration and pH on iron oxidation of engineered strains. Since increasing the NaCl concentration or lowering the pH of the medium has adverse effects on ferrous iron oxidation by *A. ferrooxidans*, we further investigated each of these effects on WT, AF43, and AF46 cultures. While keeping the NaCl concentration at 50 mM, the effects of the starting pHs were compared. As shown in Fig. 2, when the initial pH was lowered incrementally, the strains took longer to complete the conversion of ferrous iron, indicating that there was an increasing inhibitory effect of chloride under more acidic conditions. AF46 seemed to oxidize ferrous iron slightly more quickly than the WT and AF43 when the starting pH was 2.04, 1.93, or 1.79. However, when the starting pH was lowered to 1.60, ferrous iron oxidation was seen with AF46 only after a prolonged lag. No oxidation by the WT or AF43 was observed even after further incubation, demonstrating that the expression of *gshB* in AF46 extended the pH minimum where iron could be oxidized under chloride stress.

We determined that AF46 had higher chloride tolerance at a fixed pH than the WT as detected in the preliminary assays. A starting pH of 1.9 was used, which resulted in salt tolerance for iron oxidation in a range comparable to those previously reported in

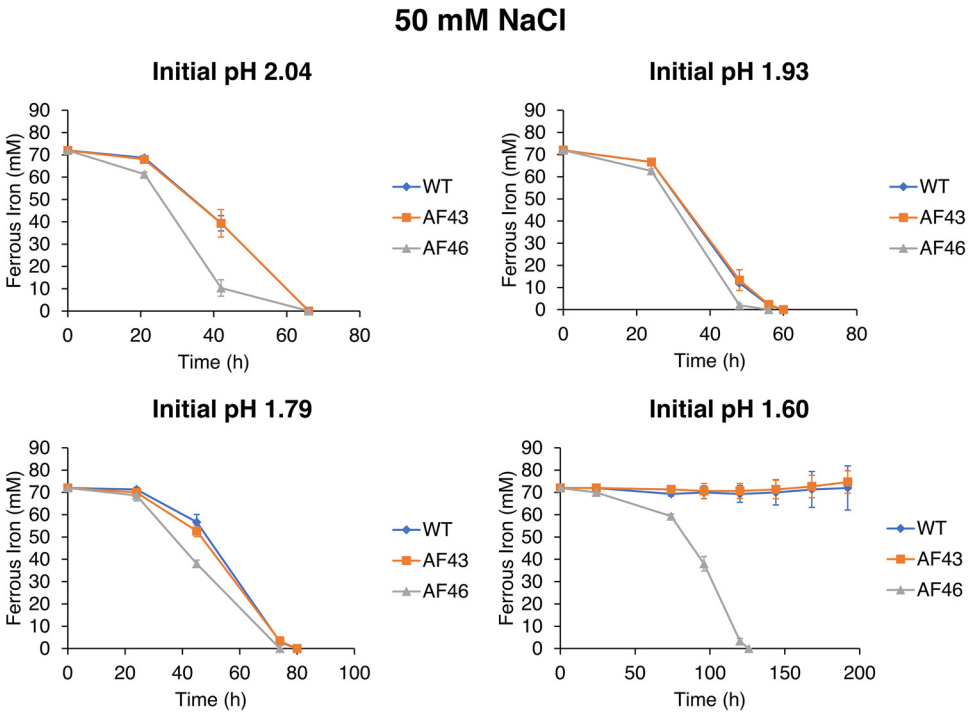

FIG 2 Iron oxidation at a constant salt concentration is strongly inhibited when increasingly acidified over a narrow pH range. The iron oxidation activity of the WT, AF43, and AF46 strains was measured in AFM1 medium with 50 mM NaCl at different initial pHs. Strains were inoculated into cultures in triplicate, and error bars indicate standard deviations.

the literature for *A. ferrooxidans* (16, 32). At 150 mM NaCl, AF46 completed iron oxidation by 144 h, but only a small amount of iron oxidation was detected with the WT, which stagnated at 120 h. This suggests that this salt concentration was near the MIC for this initial pH (Fig. 3). As all of the ferrous iron was fully oxidized by AF46 under these conditions, a higher NaCl concentration of 200 mM NaCl was also tested. Surprisingly, we found that while AF46 could begin to oxidize iron at this salt level, about 50% of the ferrous iron was oxidized before iron oxidation activity halted (Fig. 3). The WT was completely inhibited by 200 mM NaCl. Combined, these results demonstrated that the engineered AF46 strain was capable of iron oxidation under a higher level of chloride stress than the WT.

Impact of glutathione on respiration-induced ROS formation. As increased respiratory activity has been shown to be a response to cytoplasmic acidification in acidophiles, we evaluated whether the differences in iron oxidation halotolerance corresponded to ROS production in the three strains (33). The ROS content in AF46 was significantly higher than those in the WT and AF43 initially (Fig. 4). We found that upon exposure to 100 mM NaCl, the ROS contents of all three strains decreased over the first hour of incubation to similar levels. While this salt concentration at the initial pH of 1.9 should be subinhibitory for iron oxidation, these results indicate that the initial salt shock induced similar responses. Interestingly, during the entire period of incubation, the ROS content for the WT and AF43 remained near this lowered ROS level. While AF46 had a decrease in ROS to a level similar to those of the two other strains after 1 h of incubation, the ROS content increased at 25 h and approached the ROS levels found in the strain at the start of incubation by 72 h. The ROS levels in AF46 were significantly higher than those in the WT and AF43 at 25, 45, and 72 h. These results indicate that the overexpression of *gshB* allows a higher respiration rate and, thus, iron oxidation under conditions of oxidative stress induced by chloride in *A. ferrooxidans* after the osmotic shock observed upon the initial salt introduction.

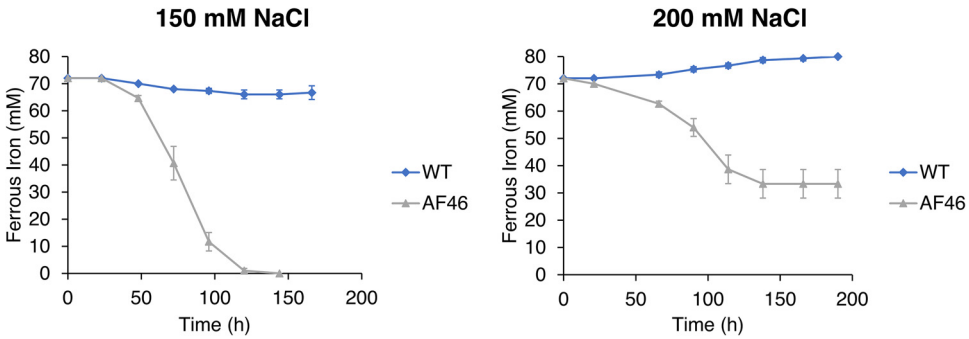

FIG 3 AF46 has increased maximum halotolerance to NaCl compared to the WT. The iron oxidation activity of the WT and AF46 strains was measured in AFM1 medium with 150 mM (left) and 200 mM (right) NaCl at an initial pH of 1.9. Strains were inoculated into cultures in triplicate, and error bars indicate standard deviations.

Sensitivity to exogenous H_2O_2 exposure. Since the glutathione biosynthesis enzymes are localized in the cytoplasm, we evaluated whether AF46 would have an increased tolerance to exogenous sources of oxidative stress where cellular damage would occur starting from the periplasm. High levels of ROS could be generated in acid mine drainage environments, so we treated the WT and AF46 with 1 mM H_2O_2 for 3 h, where iron oxidation activity had been previously shown to be inhibited with an extended lag phase (30). There was no growth advantage displayed by AF46 compared to the WT, and both strains displayed iron oxidation after 48 h (Fig. S1). As such, glutathione overexpression does not seem to be effective for all forms of oxidative stress that *A. ferrooxidans* could be exposed to in the environment. This surprising result suggests that *A. ferrooxidans* may utilize different mechanisms of dealing with oxidative stress in the cytoplasm and periplasm.

DISCUSSION

The iron oxidation activity of *Acidithiobacillus ferrooxidans* has been reported to have low but varied tolerances to NaCl, which has resulted from the different pH values of the media that have been applied for its growth (5, 14). Microorganisms with iron-oxidizing ability tolerant of saline conditions have been noted as a desirable trait for future bioleaching applications due to the importance of ferric iron as an oxidant to solubilize other metals (34). However, many acidophiles have been shown to tolerate lower concentrations of chloride as the medium pH is decreased (12). In this work, we demonstrate the strong impact of medium acidity on the halotolerance of iron oxidation in *A. ferrooxidans* at the low pH values optimal for growth and commonly used to

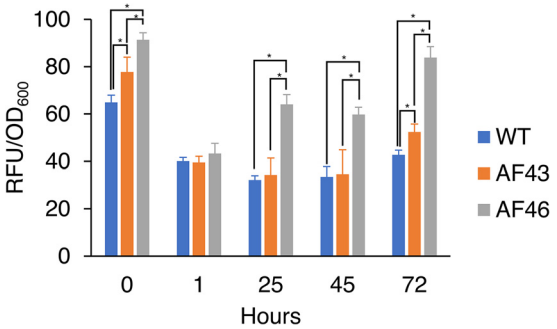

FIG 4 Intracellular ROS levels in the WT, AF43, and AF46 strains exposed to NaCl. Cells were inoculated into AFM1 medium with 100 mM NaCl at an initial pH of 1.9, and the ROS content was quantified by measuring the fluorescence of the ROS indicator at various lengths of incubation. Strains were inoculated into cultures in triplicate, and error bars indicate standard deviations. Statistical significance was calculated using ANOVA and *post hoc* tests (*, $P < 0.05$). RFU, relative fluorescence units.

limit ferric iron precipitation. We show that concentrations of chloride that have been indicated previously to be below the MIC for *A. ferrooxidans* can become inhibitory over a narrow pH range, indicating that it may be important to report the halotolerance of acidophiles as a function of pH (16). Further work is needed to better understand if the sharp dependence of halotolerance on pH affects other acidophilic species in a similar fashion.

To address this observation, we have also engineered *A. ferrooxidans* to overexpress glutathione biosynthesis genes, with the goal of augmenting the native response to oxidative stress elicited by NaCl in this species. The response to anionic stress in acidophiles involves a cascade of steps where the initial chloride entry into the cell causes cytoplasmic acidification through disruption of the positive membrane potential (33). Increased respiration rates are required to maintain pH homeostasis by pumping out excess protons and reducing oxygen. This likely results in more oxidative conditions, as increased intracellular ROS were found to be generated in *Leptospirillum ferriphilum* (33). While acidophilic bacterial species have been reported to have different mechanisms to respond to oxidative stress, *Acidithiobacillus* spp. have a number of unique genes related to glutathione biosynthesis and glutaredoxins (28). These genes involved with glutathione have been reported to be expressed during the early biofilm formation process on pyrite and also protect the cell from oxidative stress induced by cationic heavy metals (31, 35, 36). Although the *gshA* gene has been revealed to be feedback inhibited by glutathione in *Escherichia coli*, we have demonstrated that the expression of glutathione synthetase increased intracellular glutathione levels over glutamate-cysteine ligase (37). This suggests that the regulation of these glutathione biosynthesis genes may be different from that of other studied species as the *gshA* gene from *A. ferrooxidans* was able to sufficiently complement an *E. coli* strain lacking its native gene (38, 39). Although the overexpression of only single genes was tested, the simultaneous overexpression of both genes as a single operon may further increase the glutathione concentrations within the cell.

The inhibitory effects of anions were previously demonstrated to be more pronounced on iron oxidation than on sulfur oxidation in *A. ferrooxidans* (20). *A. ferrooxidans* is not known to have genes related to the production of hydroxyectoine and ectoine, which are utilized as osmoprotectants in *Acidihalobacter* and *Leptospirillum* spp., and produces limited amounts of trehalose when exposed to osmotic stress (26). It is possible that producing these osmoprotectants in *A. ferrooxidans* could be an alternative strategy to improve halotolerance that does not focus on the damage caused by anions. Because of the limited role of compatible solutes in *A. ferrooxidans*, we had hypothesized that glutathione would be a key compound that would enable engineered strains to better respond to the oxidative damage and ROS induced under high-salt conditions. As predicted, the increased production of glutathione in AF46 allowed iron oxidation under conditions of lower pH or added chloride concentrations compared to the WT (40). The role of additional glutathione levels was observed as AF43 did not display any advantages in iron oxidation. We verified that the acid concentration and salt tolerance are inversely related, as higher acid concentrations lowered the chloride resistance of all strains tested. Surprisingly, we found that ROS levels were decreased when all strains were exposed to NaCl, implying that *A. ferrooxidans* is normally unable to increase respiration rates when faced with chloride stress. These results support a previous proteomics report that a reduction in rusticyanin abundance is observed in *A. ferrooxidans* when grown under high-salt conditions and suggest that the additional glutathione content may limit the reduction in the expression of rusticyanin (16). In AF46, we found significantly increased ROS levels after 25 h, suggesting that instead of working to directly lower the concentration of ROS, glutathione may limit macromolecular damage within the cell, even under elevated-ROS conditions. The delay in this response seems to indicate that additional glutathione has no role in enabling the cell to minimize chloride entry and the resulting cytoplasmic acidification.

While increasing glutathione production was effective in enhancing the response of

A. ferrooxidans to chloride-induced oxidative stress, we showed that this did not improve the tolerance of the cells to exogenous hydrogen peroxide, which can be generated from surface reactions on metal sulfides (30). Based on our results, overexpressing genes responsible for other molecular mechanisms that have been identified in biofilm cells, such as globin, peroxiredoxin, or alkyl-hydroperoxidase, may allow *A. ferrooxidans* to have increased resistance to bioleaching environments that operate under high pulp densities. The continued investigation of these endogenous proteins and systems under physiologically relevant conditions will be useful in generating other robust strains of *A. ferrooxidans* for use in novel bioleaching operations.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were sourced from Sigma-Aldrich, and enzymes and reagents for DNA manipulation were purchased from New England BioLabs (NEB) unless otherwise noted. A type of dispersed sulfur (catalog number S789400; Toronto Research Chemicals) was used in SM4 medium for the sulfur source, as mentioned below (41). All primers used in this study were obtained from Integrated DNA Technologies. Western blot supplies were sourced from Thermo Fisher Scientific.

Bacterial strains and culturing. *Acidithiobacillus ferrooxidans* ATCC 23270 and *Escherichia coli* S17-1 were obtained from the ATCC. All *A. ferrooxidans* cultures were initiated with a starting OD_{600} (optical density measured at 600 nm) of 0.001, corresponding to a cell density of 8.3×10^6 cells/ml. All 100-ml cultures were incubated at 30°C and shaken at 140 rpm. *A. ferrooxidans* was maintained for use in experiments by weekly subculture into 100 ml of AFM1 medium [0.8 g/liter $(NH_4)_2SO_4$, 0.1 g/liter HK_2PO_4, 2.0 g/liter $MgSO_4 \cdot 7H_2O$, 5 ml/liter trace mineral solution (MD-TMS), and 20.0 g/liter $FeSO_4 \cdot 7H_2O$] at pH 1.8. Cells were harvested by centrifugation at 5,000 $\times$ *g* for 7 min at room temperature. Harvested cells were washed at least three times with AFM1 basal salts [0.8 g/liter $(NH_4)_2SO_4$, 0.1 g/liter HK_2PO_4, 2.0 g/liter $MgSO_4 \cdot 7H_2O$, and 5 ml/liter MD-TMS] at pH 1.8 to remove any iron and precipitates. Harvested cells were kept in 10 ml of AFM1 medium and maintained viability for 1 to 2 weeks stored at 4°C.

Genetic manipulations and confirmation of transformed strains. The pYI11 plasmid was modified to have an in-frame His tag using Q5 site-directed mutagenesis with primer pair pYIHis-F/pYIHis-R, as previously described (10), to generate pYI39. This plasmid was then digested with BamHI and KpnI. The *gshA* and *gshB* genes from *A. ferrooxidans* were amplified from genomic DNA prepared using the NucleoSpin tissue kit (TaKaRa Bio) with primer pairs pYI43-F/pYI43-R and pYI46-F/pYI46-R, respectively, and purified. The PCR fragments were combined with the digested pYI39 plasmid using NEBuilder HiFi DNA assembly according to the recommended protocol to generate pYI43 and pYI46. All constructs were transformed into *E. coli* DH5α and verified by DNA sequencing. DNA sequencing was performed by Genewiz. The primers used for plasmid construction are listed in Table S2 in the supplemental material.

Plasmids pYI43 and pYI46 were conjugated into *A. ferrooxidans* by the previously described filter-mating and conjugation protocols using the donor strain *E. coli* S17-1, with some modifications (10). After the mating step, possible transconjugant colonies on S204 solid medium plates were grown in AFM1 medium. The *A. ferrooxidans* transconjugants that oxidized iron were then screened in SM4 liquid selection medium to isolate strains overexpressing *gshA* or *gshB*. After verifying that the mutants had persistent kanamycin resistance in SM4 medium, the presence of the overexpressed proteins in the mutants was determined by Western blotting. Cell lysates of each strain were produced by sonicating the suspension of cells resuspended in phosphate-buffered saline at pH 7.4 in ice water for 6 min using a 3-s repeating pulse at a power of 4.5 W in a Misonix 3000 sonicator. The cell lysates of the *A. ferrooxidans* strains were separated by SDS-PAGE in a Novex NuPAGE 4 to 12% Bis-Tris gel. The separated proteins were transferred onto a Novex 0.45-μm nitrocellulose membrane with a semidry blotting unit using the method recommended by the manufacturer. The blotted membrane was blocked using Blocker Fl fluorescent blocking buffer and treated with mouse 6$\times$His tag monoclonal antibody (His.H8) at a 1:1,000 dilution. The primary-antibody-treated membrane was then treated with a goat anti-mouse IgG(H+L) cross-adsorbed secondary antibody, Alexa Fluor 488, at a 1:1,000 dilution. The fluorescently labeled proteins were detected using a Gel Doc XR$^+$ imager (Bio-Rad) and are shown in Fig. S2, where the expected molecular weights of the His-tagged *gshA* and *gshB* proteins are 50.3 and 35.3 kDa, respectively. The clone overexpressing *gshA* is referred to as AF43, and the clone overexpressing *gshB* is referred to as AF46. The bacterial strains and plasmids used in this study are listed in Table 1.

Preliminary iron oxidation halotolerance assays. Iron oxidation in the presence of chloride was conducted in AFM1 medium with the addition of the indicated concentrations of NaCl. The media were acidified with concentrated sulfuric acid to the desired initial pH. Mixtures for iron oxidation assays investigating the effect of the NaCl concentration and initial pH using 96-well plates were incubated at 30°C without agitation. Iron oxidation by the cells in the wells was noted by the color change of the clear medium to orange-brown, indicating that the ferrous iron had been oxidized to ferric iron.

Cultures evaluating the impacts of chloride stress and hydrogen peroxide stress on iron oxidation. For investigating the impact of chloride stress on the strains in culture, cells were inoculated into 100 ml of AFM1 medium at the indicated NaCl concentrations and initial pHs. For investigating the impact of hydrogen peroxide stress, 1 ml of cells at an OD_{600} of 0.1 was washed with AFM1 basal salts at pH 1.8. After washing several times, hydrogen peroxide was added to the cells at a concentration of 1 mM, and the mixture was incubated for 3 h at 30°C without agitation. The incubated cells with

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Escherichia coli		
ATCC 47055	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome (also known as S17-1)	ATCC
Acidithiobacillus ferrooxidans		
ATCC 23270	Type strain	ATCC
AF43	ATCC 23270 with pYI43	This study
AF46	ATCC 23270 with pYI46	This study
Plasmids		
pYI11	pJRD215 empty vector with a *tac* promoter and *rrnB* terminator	10
pYI39	pYI11 with an in-frame C-terminal His tag	This study
pYI43	pYI39 with *gshA* from *A. ferrooxidans*	This study
pYI46	pYI39 with *gshB* from *A. ferrooxidans*	This study

hydrogen peroxide were directly inoculated into a 100-ml culture of AFM1 medium at pH 1.8. The ferrous iron concentration of the cultures was measured by titrating 1 ml of the sampled medium mixed with 10 μl of a ferroin indicator using a 0.1 M cerium sulfate solution and noting the color change of the solution, indicating that the reduced iron species had been oxidized.

Measurement of intracellular glutathione and intracellular ROS. To measure intracellular glutathione, WT, AF43, and AF46 cells were grown, and harvested cells were washed in AFM1 basal and then in phosphate-buffered saline at pH 7.4 and resuspended into a 5% (wt/vol) solution of 5-sulfosalicylic acid dehydrate. The suspended cells were sonicated to disrupt the cells, and the cell lysate supernatant samples were prepared and assayed as recommended by the manufacturer for use with the glutathione colorimetric detection kit (Invitrogen). A linear standard for total glutathione concentrations from 0 to 6.25 μM was used to obtain glutathione concentrations in the cell lysates from the measured absorbance.

To measure intracellular ROS levels, all strains initially grown, harvested, and washed were placed into AFM1 medium with 100 mM NaCl at an initial pH of 1.9. Sampled cells were washed in phosphate-buffered saline at pH 7.4, and a 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) indicator dissolved in dimethyl sulfoxide was then added at a final concentration of 10 μM. Samples were incubated in the dark at room temperature for 30 min, and the samples were then washed with phosphate-buffered saline to remove the excess indicator. The fluorescence was measured at an excitation wavelength of 495 nm and an emission wavelength of 522 nm and normalized by the cell density.

Statistics. All error bars represent 1 standard deviation from the mean. Statistical analyses using ANOVA and *post hoc* tests were conducted using Excel. *P* values of <0.05 were considered to be statistically significant.

Data availability. The nucleotide sequences of pYI39, pYI43, and pYI46 have been deposited in GenBank under the accession numbers MZ420731, MZ420732, and MZ420733, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This work was supported by the U.S. Army Research Office (W911NF-18-1-0239) and ARPA-E grant DE-AR0001340 from the U.S. Department of Energy.

We have no competing interests to declare.

REFERENCES

1. Johnson DB. 2014. Biomining—biotechnologies for extracting and recovering metals from ores and waste materials. Curr Opin Biotechnol 30:24–31. https://doi.org/10.1016/j.copbio.2014.04.008.
2. Schippers A, Sand W. 1999. Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. Appl Environ Microbiol 65:319–321. https://doi.org/10.1128/AEM.65.1.319-321.1999.
3. Rohwerder T, Gehrke T, Kinzler K, Sand W. 2003. Bioleaching review part A. Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. Appl Microbiol Biotechnol 63:239–248. https://doi.org/10.1007/s00253-003-1448-7.
4. Banerjee I, Burrell B, Reed C, West AC, Banta S. 2017. Metals and minerals as a biotechnology feedstock: engineering biomining microbiology for bioenergy applications. Curr Opin Biotechnol 45:144–155. https://doi.org/10.1016/j.copbio.2017.03.009.
5. Zammit CM, Mangold S, Jonna VR, Mutch LA, Watling HR, Dopson M, Watkin ELJ. 2012. Bioleaching in brackish waters—effect of chloride ions

on the acidophile population and proteomes of model species. Appl Microbiol Biotechnol 93:319–329. https://doi.org/10.1007/s00253-011-3731-3.
6. Dopson M, Halinen A-K, Rahunen N, Boström D, Sundkvist J-E, Riekkola-Vanhanen M, Kaksonen AH, Puhakka JA. 2008. Silicate mineral dissolution during heap bioleaching. Biotechnol Bioeng 99:811–820. https://doi.org/10.1002/bit.21628.
7. Bevilaqua D, Lahti H, Suegama PH, Garcia O, Benedetti AV, Puhakka JA, Tuovinen OH. 2013. Effect of Na-chloride on the bioleaching of a chalcopyrite concentrate in shake flasks and stirred tank bioreactors. Hydrometallurgy 138:1–13. https://doi.org/10.1016/j.hydromet.2013.06.008.
8. Noguchi H, Okibe N. 2020. The role of bioleaching microorganisms in saline water leaching of chalcopyrite concentrate. Hydrometallurgy 195:105397. https://doi.org/10.1016/j.hydromet.2020.105397.
9. Carneiro MFC, Leão VA. 2007. The role of sodium chloride on surface properties of chalcopyrite leached with ferric sulphate. Hydrometallurgy 87:73–82. https://doi.org/10.1016/j.hydromet.2007.01.005.
10. Inaba Y, West AC, Banta S. 2020. Enhanced microbial corrosion of stainless steel by *Acidithiobacillus ferrooxidans* through the manipulation of substrate oxidation and overexpression of *rus*. Biotechnol Bioeng 117:3475–3485. https://doi.org/10.1002/bit.27509.
11. Alexander B, Leach S, Ingledew WJ. 1987. The relationship between chemiosmotic parameters and sensitivity to anions and organic acids in the acidophile *Thiobacillus ferrooxidans*. Microbiology (Reading) 133:1171–1179. https://doi.org/10.1099/00221287-133-5-1171.
12. Falagán C, Johnson DB. 2018. The significance of pH in dictating the relative toxicities of chloride and copper to acidophilic bacteria. Res Microbiol 169:552–557. https://doi.org/10.1016/j.resmic.2018.07.004.
13. Huber H, Stetter KO. 1989. *Thiobacillus prosperus* sp. nov., represents a new group of halotolerant metal-mobilizing bacteria isolated from a marine geothermal field. Arch Microbiol 151:479–485. https://doi.org/10.1007/BF00454862.
14. Rea SM, McSweeney NJ, Degens BP, Morris C, Siebert HM, Kaksonen AH. 2015. Salt-tolerant microorganisms potentially useful for bioleaching operations where fresh water is scarce. Miner Eng 75:126–132. https://doi.org/10.1016/j.mineng.2014.09.011.
15. Khaleque HN, Kaksonen AH, Boxall NJ, Watkin ELJ. 2018. Chloride ion tolerance and pyrite bioleaching capabilities of pure and mixed halotolerant, acidophilic iron- and sulfur-oxidizing cultures. Miner Eng 120:87–93. https://doi.org/10.1016/j.mineng.2018.02.025.
16. Dopson M, Holmes DS, Lazcano M, McCredden TJ, Bryan CG, Mulroney KT, Steuart R, Jackaman C, Watkin ELJ. 2017. Multiple osmotic stress responses in *Acidihalobacter prosperus* result in tolerance to chloride ions. Front Microbiol 7:2132. https://doi.org/10.3389/fmicb.2016.02132.
17. Khaleque HN, González C, Shafique R, Kaksonen AH, Holmes DS, Watkin ELJ. 2019. Uncovering the mechanisms of halotolerance in the extremely acidophilic members of the *Acidihalobacter* genus through comparative genome analysis. Front Microbiol 10:155. https://doi.org/10.3389/fmicb.2019.00155.
18. Wood JM. 2015. Bacterial responses to osmotic challenges. J Gen Physiol 145:381–388. https://doi.org/10.1085/jgp.201411296.
19. Rawlings DE. 2005. Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. Microb Cell Fact 4:13. https://doi.org/10.1186/1475-2859-4-13.
20. Harahuc L, Lizama HM, Suzuki I. 2000. Selective inhibition of the oxidation of ferrous iron or sulfur in *Thiobacillus ferrooxidans*. Appl Environ Microbiol 66:1031–1037. https://doi.org/10.1128/AEM.66.3.1031-1037.2000.
21. Valdes J, Pedroso I, Quatrini R, Dodson RJ, Tettelin H, Blake R, II, Eisen JA, Holmes DS. 2008. *Acidithiobacillus ferrooxidans* metabolism: from genome sequence to industrial applications. BMC Genomics 9:597. https://doi.org/10.1186/1471-2164-9-597.
22. Peng JB, Yan WM, Bao XZ. 1994. Plasmid and transposon transfer to *Thiobacillus ferrooxidans*. J Bacteriol 176:2892–2897. https://doi.org/10.1128/jb.176.10.2892-2897.1994.
23. Wang H, Liu X, Liu S, Yu Y, Lin J, Lin J, Pang X, Zhao J. 2012. Development of a markerless gene replacement system for *Acidithiobacillus ferrooxidans* and construction of a *pfkB* mutant. Appl Environ Microbiol 78:1826–1835. https://doi.org/10.1128/AEM.07230-11.
24. Kernan T, Majumdar S, Li X, Guan J, West AC, Banta S. 2016. Engineering the iron-oxidizing chemolithoautotroph *Acidithiobacillus ferrooxidans* for biochemical production. Biotechnol Bioeng 113:189–197. https://doi.org/10.1002/bit.25703.
25. Inaba Y, Banerjee I, Kernan T, Banta S. 2018. Transposase-mediated chromosomal integration of exogenous genes in *Acidithiobacillus ferrooxidans*. Appl Environ Microbiol 84:e01381-18. https://doi.org/10.1128/AEM.01381-18.
26. Galleguillos PA, Grail BM, Hallberg KB, Demergasso CS, Johnson DB. 2018. Identification of trehalose as a compatible solute in different species of acidophilic bacteria. J Microbiol 56:727–733. https://doi.org/10.1007/s12275-018-8176-2.
27. Gumulya Y, Boxall NJ, Khaleque HN, Santala V, Carlson RP, Kaksonen AH. 2018. In a quest for engineering acidophiles for biomining applications: challenges and opportunities. Genes (Basel) 9:116. https://doi.org/10.3390/genes9020116.
28. Cárdenas JP, Moya F, Covarrubias P, Shmaryahu A, Levicán G, Holmes DS, Quatrini R. 2012. Comparative genomics of the oxidative stress response in bioleaching microorganisms. Hydrometallurgy 127–128:162–167. https://doi.org/10.1016/j.hydromet.2012.07.014.
29. Orell A, Navarro CA, Arancibia R, Mobarec JC, Jerez CA. 2010. Life in blue: copper resistance mechanisms of bacteria and archaea used in industrial biomining of minerals. Biotechnol Adv 28:839–848. https://doi.org/10.1016/j.biotechadv.2010.07.003.
30. Bellenberg S, Huynh D, Poetsch A, Sand W, Vera M. 2019. Proteomics reveal enhanced oxidative stress responses and metabolic adaptation in *Acidithiobacillus ferrooxidans* biofilm cells on pyrite. Front Microbiol 10:592. https://doi.org/10.3389/fmicb.2019.00592.
31. Xia J, Wu S, Zhang R, Zhang C, He H, Jiang H, Nie Z, Qiu G. 2011. Effects of copper exposure on expression of glutathione-related genes in *Acidithiobacillus ferrooxidans*. Curr Microbiol 62:1460–1466. https://doi.org/10.1007/s00284-011-9881-9.
32. Rivera-Araya J, Huynh ND, Kaszuba M, Chávez R, Schlömann M, Levicán G. 2020. Mechanisms of NaCl-tolerance in acidophilic iron-oxidizing bacteria and archaea: comparative genomic predictions and insights. Hydrometallurgy 194:105334. https://doi.org/10.1016/j.hydromet.2020.105334.
33. Rivera-Araya J, Pollender A, Huynh D, Schlömann M, Chávez R, Levicán G. 2019. Osmotic imbalance, cytoplasm acidification and oxidative stress induction support the high toxicity of chloride in acidophilic bacteria. Front Microbiol 10:2455. https://doi.org/10.3389/fmicb.2019.02455.
34. Kaksonen AH, Deng X, Bohu T, Zea L, Khaleque HN, Gumulya Y, Boxall NJ, Morris C, Cheng KY. 2020. Prospective directions for biohydrometallurgy. Hydrometallurgy 195:105376. https://doi.org/10.1016/j.hydromet.2020.105376.
35. Vera M, Krok B, Bellenberg S, Sand W, Poetsch A. 2013. Shotgun proteomics study of early biofilm formation process of *Acidithiobacillus ferrooxidans* ATCC 23270 on pyrite. Proteomics 13:1133–1144. https://doi.org/10.1002/pmic.201200386.
36. Zheng C, Zhang L, Chen M, Zhao XQ, Duan Y, Meng Y, Zhang X, Shen RF. 2018. Effects of cadmium exposure on expression of glutathione synthetase system genes in *Acidithiobacillus ferrooxidans*. Extremophiles 22:895–902. https://doi.org/10.1007/s00792-018-1046-3.
37. Murata K, Kimura A. 1990. Overproduction of glutathione and its derivatives by genetically engineered microbial cells. Biotechnol Adv 8:59–96. https://doi.org/10.1016/0734-9750(90)90005-v.
38. Ohtake Y, Watanabe K, Tezuka H, Ogata T, Yabuuchi S, Murata K, Kimura A. 1989. Expression of the glutathione synthetase gene of *Escherichia coli* B in *Saccharomyces cerevisiae*. J Ferment Bioeng 68:390–394. https://doi.org/10.1016/0922-338X(89)90092-5.
39. Powles R, Deane S, Rawlings D. 1996. The gene for γ-glutamylcysteine synthetase from *Thiobacillus ferrooxidans* has low homology to its *Escherichia coli* equivalent and is linked to the gene for citrate synthase. Microbiology (Reading) 142(Part 9):2543–2548. https://doi.org/10.1099/00221287-142-9-2543.
40. Suzuki I, Lee D, Mackay B, Harahuc L, Oh JK. 1999. Effect of various ions, pH, and osmotic pressure on oxidation of elemental sulfur by *Thiobacillus thiooxidans*. Appl Environ Microbiol 65:5163–5168. https://doi.org/10.1128/AEM.65.11.5163-5168.1999.
41. Inaba Y, Kernan T, West AC, Banta S. 2021. Dispersion of sulfur creates a valuable new growth medium formulation that enables earlier sulfur oxidation in relation to iron oxidation in *Acidithiobacillus ferrooxidans* cultures. Biotechnol Bioeng 118:3225–3238. https://doi.org/10.1002/bit.27847.