Knockout of cyc2 in *A. ferrooxidans* to reduce sulfuric acid production in acid mine drainage

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At sites of abandoned metal mines, water is polluted by dangerously high concentrations of sulfuric acid that corrode infrastructure and damage natural habitats, ultimately endangering aquatic life. This is due to the phenomenon of acid mine drainage (AMD), in which the sulfide minerals within metal ores are exposed to air and water, leading to formation of sulfuric acid in local bodies of water. Due to AMD, these nearby water supplies become polluted with high metal concentrations and develop a low pH. *Acidithiobacillus ferrooxidans* is an acidophilic bacterium that thrives in abandoned metal mines. Its metabolic pathways play a major role in expediting sulfuric acid production through the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}). As part of its iron-oxidizing/O_2-reducing supercomplex, *A. ferrooxidans* contains iron:rusticyanin reductase, an enzyme which catalyzes this ferrous-oxidizing reaction. By removing the cyc2 gene that encodes for this protein, we expect that *A. ferrooxidans* can survive as a species without contributing to the spread of AMD. In order to inactivate cyc2, we intend to use the gene knockout method developed by Datsenko and Wanner. However, it is unknown whether or not cyc2 knockout will prove cytotoxic, or whether the knockout bacteria would be able to continue to produce ferric ions via alternative pathways. Even if the cyc2 gene is removed, there may be little to no substantial change in the amount of sulfuric acid, since *A. ferrooxidans* is only one of many acidophiles that contribute to AMD. Nonetheless, we propose the knockout approach will disrupt the iron oxidation mechanism of *A. ferrooxidans* involved in AMD, while preserving its bacterial population.

Keywords Acid mine, AMD, acidophile, gene knockout, sulfuric acid

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Watch a video introduction by the authors at https://youtu.be/GTX-ux9WIQQ

Background

As the main source of water pollution within the mid-Atlantic region of the United States, acid mine drainage (AMD) is incredibly damaging to the environment. For example, AMD from the Berkeley Pit, a former copper mine, resulted in the deaths of over 4,000 migrating snow geese (Guarino, 2016). Acid mine drainage occurs when sulfides, common in most metal ores, are exposed to air and react with water to form sulfuric

acid in bodies of water, reducing a neutral pH of 7 to values as low as 2. As a result, dangerously low pH (< 6.5) makes the water inhospitable to aquatic life, and can pollute the area further by dissolving harmful metalloids – such as Arsenic – that are present in the surrounding environment (Rambabu et al., 2020; University of Colorado Boulder, n.d.). AMD is particularly common in abandoned metal mines due to the presence of pyrite, also known as "fool's gold," which contains sulfide minerals (Abandoned Mine Drainage, n.d.). AMD treatment options, such as the addition of alkaline substances to neutralize acid, flooding mines, and relocation of waste, are expensive, with costs reaching hundreds of millions of dollars (What Can be Done, n.d.; Meagher, 2017). Hence, our team decided to address

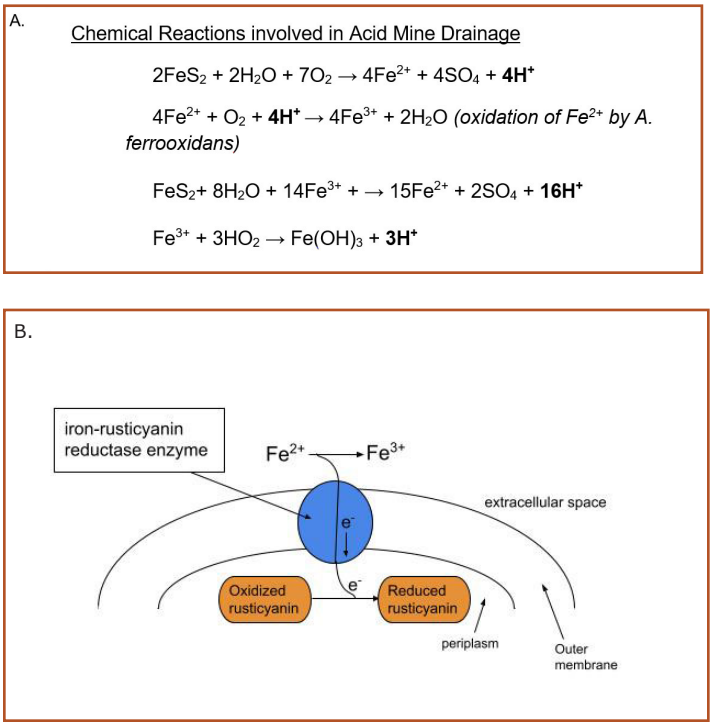

Figure 1. *(A) Sequence of chemical reactions that take place in sites affected by acid mine drainage, leading to increased acidity of the surrounding water. (B) The iron:-rusticyanin reductase enzyme in the outer membrane catalyzes the reduction of rusticyanin proteins using electrons from ferrous ions.*

AMD through a method of bioremediation. Current approaches that utilize the bacteria that influence AMD include killing these bacteria, as well as offering them a more preferable source of energy. We looked into the chemical reactions associated with AMD to discern the impact of the surrounding bacteria. As seen above (Figure 1A), pyrite (Fe_2S) can react with environmental water and oxygen to produce sulfuric acid (H_2SO_4). However, production of sulfuric acid is expedited by ferric ions (Fe^{3+}), arising from oxidation of ferrous ions (Fe^{2+}), since the former promote aqueous oxidation of pyrite, releasing 16 molar equivalents of hydrogen ions (H^+). As shown in Figure 1B, one source of oxidation of ferrous ions is the acidophile, *Acidithiobacillus ferrooxidans*, through its enzyme iron:rusticyanin reductase, or Fe(II):rusticyanin oxidoreductase (Valdés et al., 2008; Brett & Banfield, 2003). Because of the drastic increase in H^+ production that will accompany the resulting ferric ions, *A. ferrooxidans* bacteria undoubtedly contribute to the harmful effects of AMD in bodies of water.

Systems level

By utilizing Datsenko and Wanner's (2000) gene knockout method to inactivate the cyc2 gene of *A. ferrooxidans*, our team intends to delete the organism's iron:rusticyanin reductase and disrupt the iron-oxidizing process (Caspi et al., 2013; The UniProt Consortium, 2020). Although this protocol has primarily been used on E. coli (Datsenko & Wanner, 2000), recent research regarding *A. ferrooxidans* – particularly the complete sequencing of its genome and engineering of strains that overexpress the membrane protein rusticyanin – will aid in application of the gene knockout method (Liu et al., 2011; Zhang et al., 2019). As depicted in Figure 2, we will first replace the cyc2 gene with an ampicillin resistance gene enabling selection of clones with the target gene successfully removed. A second transformation with FLP recombinase will delete the ampicillin resistance gene, producing a strain of *A. ferrooxidans* without its cyc2 gene, therefore rendering its iron-oxidizing pathway useless.

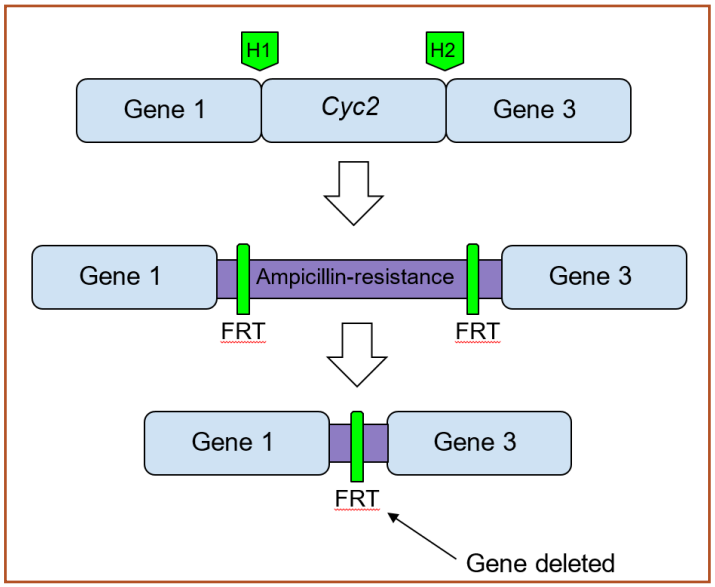

Figure 2. *The gene knockout method replaces the cyc2 gene for an ampicillin-resistant gene using FRT sites, and then deletes the ampicillin-resistant gene, resulting in the deletion of the cyc2 gene. H1 and H2, DNA homology regions; FRT, FLP recognition target.*

Device level

The fundamental components of the design (Figure 3) are made up of the genetically modified *A. ferrooxidans* bacteria and its modified iron oxidizing metabolic pathway, lacking the iron:rusticyanin reductase enzyme.

When the level of the iron:rusticyanin reductase enzyme present in the bacteria is reduced, the production of Fe^{3+} by the iron-oxidizing metabolic pathway should decrease, leading to a reduction in sulfuric acid production and the chemical processes involved in AMD. To achieve this aim, we selected FLP recombinase proteins, expressed by a helper plasmid, to delete the targeted region of the genome.

Parts level

Since the cyc2 gene codes for the iron:rusticyanin reductase enzyme, the gene knockout method will be applied to this gene (The UniProt Consortium, 2020), using the process illustrated in Figure 2. To apply the gene knockout method, PCR will be used to create an ampicillin resistance gene containing flanking FRT (FLP recognition target) sites, and 36-nt DNA homology regions (H1 and H2), at each of its ends. H1 and H2 will be homologous to the DNA flanking the cyc2 gene on *A. ferrooxidans* to mark where the recombination should occur (Datsenko & Wanner, 2000).

By transforming the bacteria with a lambda red recombineering plasmid, the ampicillin resistance gene, and the DNA from *A. ferrooxidans* including the homologies, the plasmid will replace the cyc2 gene

with the ampicillin resistance gene; the success of this replacement can be checked by streaking the bacterial colonies onto solid media containing ampicillin. Finally, ampicillin resistant clones will be transformed with FLP recombinase, which will recognize the FRT sites and "knock out" the ampicillin resistance gene, ultimately leading to an *A. ferrooxidans* genome lacking its cyc2 gene.

Safety

The exact methods and safety specifications of enacting the mechanism outlined in this design brief are currently uncertain. Given that the outlined design affects bacteria that may end up in various bodies of water, it is essential that, before implementation into the proposed environment, there is rigorous safety testing of modified bacteria in a controlled setting. While the proposed mechanism is intended only to stop production of the ferric ions involved in AMD, any potential unintended effects caused by the genetically modified bacteria will have to be closely monitored in experimental trials.

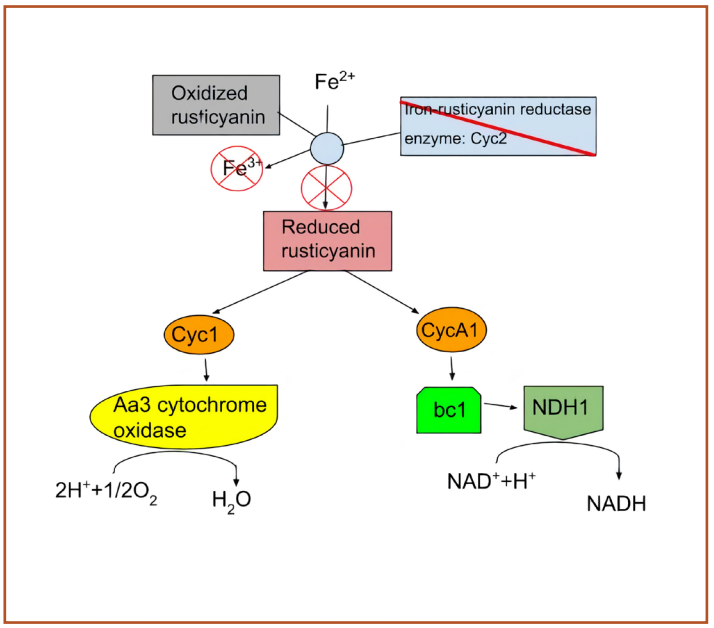

Figure 3. *The engineered iron-oxidizing metabolic pathway of A. ferrooxidans, lacking the iron-rusticyanin reductase enzyme.*

Prior to disposal after safety trials, the modified bacteria should be treated with chlorine. Further considerations

may include potential adverse effects caused by the modified bacteria to workers in operational metal mines; however, this design brief's intended focus is primarily on abandoned metal mines.

Discussion

The proposed system would reduce the amount of ferric ions produced by *A. ferrooxidans*, and therefore limit the production of sulfuric acid. This would decrease the acidity of the affected water and substantially lessen the impact of AMD on freshwater ecosystems. By modifying an organism that already exists in the environment, *A. ferrooxidans*, the risk of introducing an entirely new organism to the ecosystem is removed. This reduces the chance of unexpected consequences. *A. ferrooxidans* is also a highly-researched species, and one of the most well-known acidophiles, making it an ideal subject for such an experiment. However, the knockout method of gene removal has not previously been used on *A. ferrooxidans*, so it is unknown if the method will be effective. It is also possible that the removal of the cyc2 gene will result in death of the bacteria, or make the bacteria less fit within the acidic environment, perhaps leading the species to disappear from AMD-affected sites completely. In order to effectively limit sulfuric acid production, the majority of the *A. ferrooxidans* bacteria within the ecosystem would have to lack the cyc2 gene. For this to occur, the engineered bacteria that are introduced into the population would have to

outcompete, or grow at a faster rate than, the existing *A. ferrooxidans* bacteria.

As cyc2 codes for the single iron:rusticyanin reductase enzyme, it is also possible that its removal will not significantly impact the organism's larger iron-oxidizing enzyme complex. The complex may be able to function without the iron:rusticyanin reductase enzyme, which would mean additional genes would have to be knocked out from the bacteria to achieve the desired goal. Additionally, even if the knockout method were fully successful and *A. ferrooxidans* no longer produced ferric ions, the level of impact this would have on AMD is unknown. Lastly, since sulfide minerals naturally react with water and oxygen to form sulfuric acid, it is possible that engineered strains of *A. ferrooxidans* might not counter the effects of acid mine drainage to an extent sufficient to clean up bodies of water. In that case, it may be necessary to also target the chemical processes that can consume sulfide minerals or free-moving H^+ ions, to prevent any increase in acidity using strategies other than eliminating the microbial iron-oxidation step of AMD.

Next steps

The first step in developing the proposed system would be to test the effectiveness of the gene knockout method on *A. ferrooxidans*. If the knockout method is not effective on *A. ferrooxidans*, a new method of gene modification would need to be selected. To test the effectiveness of the gene knockout method, a controlled environment, replicating the factors in an abandoned metal mine that contribute to AMD, would have to be created. The rate at which a control group of unaltered *A. ferrooxidans* expedites sulfuric acid production within this model environment would need to be tested; these results should be compared to the rate at which the modified *A. ferrooxidans* bacteria (the test group) expedites sulfuric acid production within the model. If the test group does not have a different rate of sulfuric acid production from the control, it is likely that the knockout approach was not effective.

If the gene knockout method is effective at reducing sulfuric acid production in AMD by a statistically significant margin, the next steps for further research would involve creating a method by which the engineered *A. ferrooxidans* bacteria could outcompete existing bacteria, or outgrow the wild type strain until the engineered bacteria are dominant in the ecosystem. This potential design would enable the engineered *A. ferrooxidans* bacteria to be applied on a large scale, and be used as a self-sustaining long-term solution for AMD. Since the various experiments mentioned above may involve more advanced testing and equipment than found in high school biology laboratories, a collaboration between the BioBuilder group and a college or biotech company may be necessary.

Author contributions

P.L.S. drew the graphics and figures; and identified several methods of addressing the issue of acid mine drainage, helping narrow down the focus to *A. ferrooxidans*. K.R. researched the chemical pathways involved in sulfuric acid production, identified the role of *A. ferrooxidans* in producing sulfuric acid, and located the cyc2 gene that codes for iron:rusticyanin reductase. P.E.S. identified the experiments needed to support the design, and proposed next steps for further application of the engineered bacteria. All authors researched the steps necessary to effectively apply gene knockout in *A. ferrooxidans*.

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