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Engineering lithoheterotrophy in an obligate chemolithoautotrophic Fe(II) oxidizing bacterium

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Neutrophilic Fe(II) oxidizing bacteria like *Mariprofundus ferrooxydans* are obligate chemolithoautotrophic bacteria that play an important role in the biogeochemical cycling of iron and other elements in multiple environments. These bacteria generally exhibit a singular metabolic mode of growth which prohibits comparative "omics" studies. Furthermore, these bacteria are considered non-amenable to classical genetic methods due to low cell densities, the inability to form colonies on solid medium, and production of copious amounts of insoluble iron oxyhydroxides as their metabolic byproduct. Consequently, the molecular and biochemical understanding of these bacteria remains speculative despite the availability of substantial genomic information. Here we develop the first genetic system in neutrophilic Fe(II) oxidizing bacterium and use it to engineer lithoheterotrophy in *M. ferrooxydans*, a metabolism that has been speculated but not experimentally validated. This synthetic biology approach could be extended to gain physiological understanding and domesticate other bacteria that grow using a single metabolic mode.

Genetic studies have been primarily limited to colony-forming microorganisms that can grow using multiple metabolic strategies. The ability to form colonies on solid medium is advantageous for selection and screening of mutants, while metabolic flexibility allows for viability of mutants missing pathways of interest. However, the vast majority of microorganisms are not readily cultured on solid medium[1] and many exhibit specialist lifestyles using a singular metabolic mode to grow[2–8]. Our overall hypothesis is that synthetic biology can be leveraged to better understand and domesticate environmental microorganisms with novel metabolic capabilities. To begin exploring this hypothesis, we focus on *Mariprofundus ferrooxydans* PV-1[3] that grows only by one metabolic mode, the oxidation of Fe(II) coupled to the reduction of oxygen while fixing carbon dioxide through a metabolism called chemolithoautotrophy.

Diverse neutrophilic chemolithoautotrophic bacteria have been known to oxidize Fe(II) in many circumneutral environments[2,3,7,8]. Understanding the metabolism and physiology of chemolithoautotrophic Fe(II) oxidizing bacteria is of environmental and ecological importance because of their widespread impact in various environments on multiple biogeochemical cycles including iron, carbon, nitrogen, phosphorous and other metals[2,3,7–11]. While a substantial amount of genomic information about chemolithoautotrophic Fe(II) oxidizing bacteria exists[12–14], functional knowledge remains speculative in the absence of metabolic and genetic studies. Since atmospheric concentrations of oxygen rapidly react with Fe(II), chemolithoautotrophic Fe(II) oxidizing bacteria grow under microaerobic conditions[2,3,7–11] where they catalyze oxidation of Fe(II) faster than oxygen. The requirement for this specific niche makes these microorganisms difficult to culture for laboratory studies. Furthermore, chemolithoautotrophic Fe(II) oxidizing bacteria are considered non-amenable to genetic methods because these bacteria do not grow on solid medium, produce low growth yields (10^6–10^7 cells/mL) and accumulate a substantial amount of insoluble iron oxyhydroxide as their obligate metabolic byproduct[2–8]. The inability to grow and form colonies on solid medium prevents the application of traditional genetic methods to select and screen for mutants. Low cell yield along with the presence of insoluble iron oxyhydroxide presents a formidable challenge for DNA transformation and subsequent phenotypic analysis to readily test genetic parts and methods. These bacteria are also generally metabolic specialists which grow by a singular metabolic mode of oxidizing Fe(II) as the energy source while fixing carbon dioxide and respiring low levels of oxygen[2,3,7,8]. The

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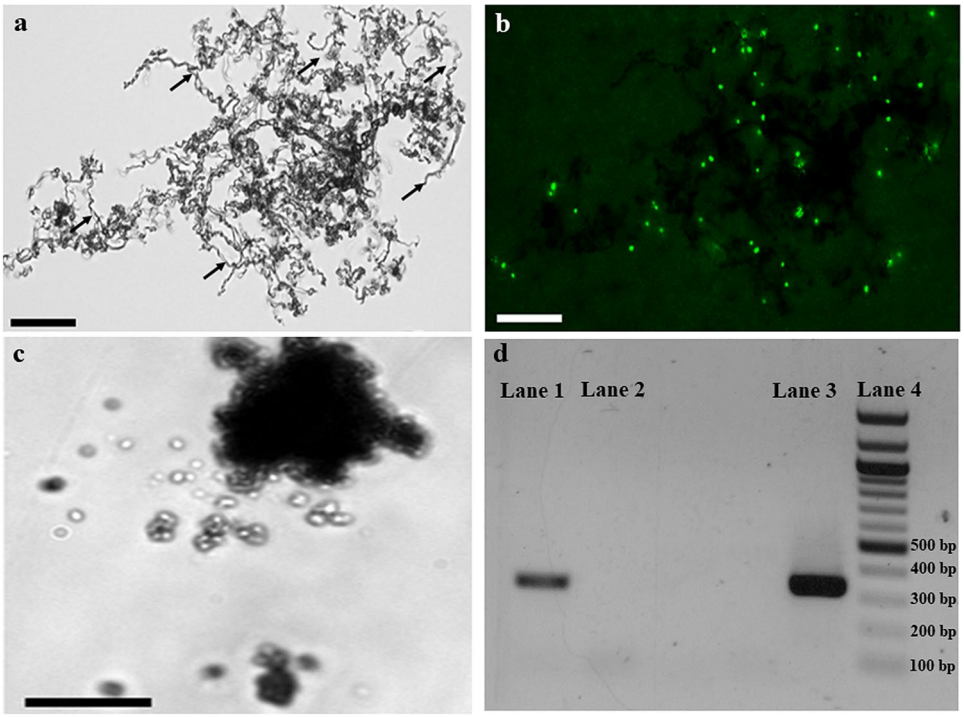

Figure 1. Transformation of *M. ferrooxydans*. Successful transformation of pRK2m3 (empty vector) into *M. ferrooxydans* was confirmed by (**a**) growth in the presence of 200 µg/mL kanamycin as shown by the production of characteristic stalk formation (black arrows) in the bright field micrograph and (**b**) an epifluorescent micrograph of the same field showing cells stained with Syto 9. (**c**) Wild-type cells were unable to grow or produce stalks in the presence of 200 µg/mL kanamycin and only formation of amorphous iron oxyhydroxides was observed. Scale bars indicate 25 µm. (**d**) Electropherogram showing amplification of pRK2m3 specific DNA fragment using DNA extracted from the transformed cells after ten transfers in the presence of 200 µg/mL kanamycin (Lane 1), and purified pRK2m3 (Lane 3) as the template. Template DNA containing DNA extracted from the wild-type cells did not produce any amplification (Lane 2). Lane 4 is a DNA ladder.

single metabolic growth mode prohibits comparative "omics" studies and targeted gene deletions to probe Fe(II) oxidation and carbon flow as mutants defective in these pathways will be unable to grow.

Here we use synthetic biology to study *Mariprofundus ferrooxydans* PV-1, the founding member of the *Zetaproteobacteria*[3] which are thought to be the dominant Fe(II) oxidizers in marine environments[3,11]. We develop genetic methods and tools to transform *M. ferrooxydans* and manipulate its metabolic capacity by expressing foreign genes, yielding an engineered variant capable of using glucose as a carbon source instead of CO_2.

Results and discussion

We developed a conjugation protocol to successfully transform *M. ferrooxydans* using the donor strain *Escherichia coli* WM3064, which is auxotrophic for diaminopimelic acid (DAP)[15]. *M. ferrooxydans* transformed with pRK2m3[16] continued to grow and produce characteristic twisted iron oxide stalks[3] over successive transfers in the presence of kanamycin (Fig. 1a, b). Wild-type cells incubated with kanamycin was unable to grow (data not shown) and only amorphous iron oxyhydroxides were observed (Fig. 1c), likely produced from abiotic Fe(II) oxidation. 16S rRNA gene sequencing confirmed that the transformed culture was *M. ferrooxydans*. After ten transfers, *E. coli* cells were undetectable by microscopy or growth in lysogeny broth (LB) medium augmented with DAP. Maintenance of pRK2m3 in the transformed *M. ferrooxydans* cells was confirmed by amplifying 330 bp of plasmid specific DNA using total extracted DNA as the template (Fig. 1d) and verified by sequencing. These results demonstrate that *M. ferrooxydans* was able to replicate pRK2m3 over repeated transfers under kanamycin selection. With a method for transformation and selection established, we were able to express green fluorescent protein (GFP), encoded by *gfpmut2*[17] and driven by the P_{neo} promoter amplified from upstream the gene encoding kanamycin resistance on pRK2m3. P_{neo} was used because it provided sufficient expression to confer kanamycin resistance in *M. ferrooxydans* transformed with the pRK2m3 vector. Microscopy confirmed production of GFP in the engineered strain (Fig. 2).

We next sought to leverage our ability to introduce and express foreign genes to augment the metabolism of *M. ferrooxydans*. The *M. ferrooxydans* PV-1 genome is predicted to encode genes for glycolysis and the Krebs cycle[12], but lacks genes encoding glucokinase or an apparent glucose transporter. We hypothesized that by introducing the capability to transport and phosphorylate glucose, *M. ferrooxydans* could use it as a carbon and energy source. The genes *galP* and *glk* from *E. coli*, encoding a glucose symporter and glucokinase[18], were cloned into pRK2m3

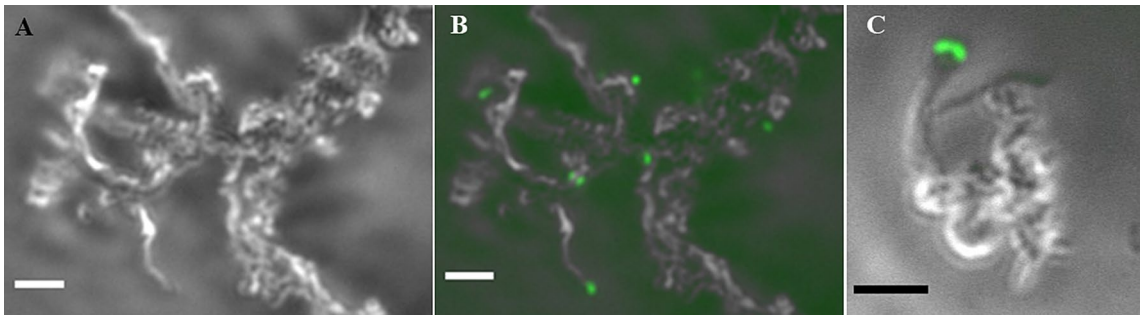

Figure 2. Expression of green fluorescent protein in *M. ferrooxydans*. (**a**) Light micrograph showing the characteristic twisted stalks produced by *M. ferrooxydans* containing a plasmid with *gfpmut2* and grown in the presence of 200 µg/mL kanamycin. (**b**,**c**) Composite images of light and epifluorescent micrographs showing the green fluorescent cells attached to the stalks. Scale bars indicate 5 µm.

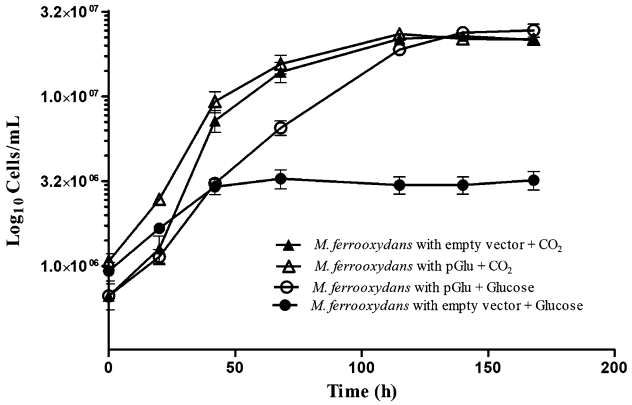

Figure 3. Lithoheterotrophic growth of *M. ferrooxydans* containing pGlu. Growth curves of *M. ferrooxydans* containing pGlu (open symbols) and *M. ferrooxydans* containing empty vector pRK2m3 (closed symbols) grown on either glucose (o, ●) or carbon dioxide (Δ, ▲) as the sole carbon source. Error bars represent standard deviation of three replicates.

to create pGlu where each gene was individually driven by P_{neo} promoters. *M. ferrooxydans* cells transformed with pGlu did not yield viable cells when selected heterotrophically using glucose (data not shown). However, growth was observed when *M. ferrooxydans* cells transformed with pGlu were selected lithoheterotrophically under Fe(II) oxidizing conditions with glucose as the sole carbon source without the addition of carbon dioxide (Fig. 3). *M. ferrooxydans* transformed with an empty pRK2m3 vector was unable to grow with glucose as the sole carbon source (Fig. 3). The presence of pGlu was confirmed by amplifying and sequencing *galP*, *glk* and the kanamycin resistance cassette using total DNA extracted from transformed glucose-grown cells as template (data not shown). Purity of the transformed cells was confirmed by 16S rRNA gene sequencing, microscopy analysis and the absence of bacterial growth in LB medium augmented with DAP.

The rate of Fe(II) oxidation by the engineered lithoheterotrophic strain was slower during glucose-dependent growth compared to the Fe(II) oxidation rate during carbon dioxide-dependent growth (Fig. 4). Interestingly, the engineered strain oxidized less total Fe(II) when grown with glucose compared to carbon dioxide (Fig. 4), despite achieving similar final cell densities (Fig. 3). The increase in cell yield per unit Fe(II) oxidized during growth on glucose of the engineered strain can be theoretically attributed to additional energy production from glycolysis and/or biomass precursors provided by glucose.

The inability to transport organic carbon or glycolytic lesions have previously been hypothesized as the reasons for obligate autotrophy in some microorganisms[19]. However, when these deficiencies were addressed using pGlu in *M. ferrooxydans*, heterotrophic growth was not observed. While the reasons for absence of heterotrophic growth in *M. ferrooxydans* containing pGlu are unknown, we speculate that the cells may either have insufficient flux through glycolysis or that the genes required for glycolysis and anaplerotic reactions are not expressed under the conditions tested. Another possibility could be the inability to convert NADH/NADPH produced by glycolysis into proton motive force (and then ATP). The obligate requirement of Fe(II) as the energy source even while using glucose in the engineered lithoheterotrophic strain provides an important insight into the metabolic functioning of *M. ferrooxydans* where glycolysis seems to be partitioned from energy metabolism. Such a metabolism could be one of the reasons driving obligate lithotrophy in *M. ferrooxydans*. We hypothesize that additional components and alteration of metabolic networks will be required to achieve heterotrophic growth in

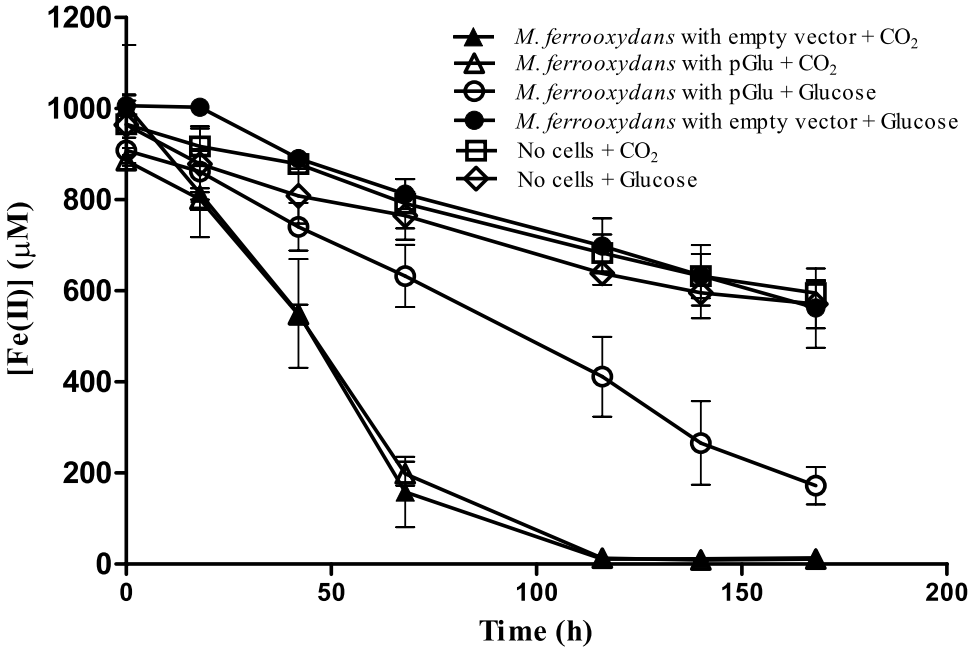

Figure 4. Fe(II) oxidation rate of *M. ferrooxydans* containing pGlu. Fe(II) quantification over time for *M. ferrooxydans* containing pGlu (open symbols) and *M. ferrooxydans* containing empty vector pRK2m3 (closed symbols) grown on either glucose (o, ●) or carbon dioxide (Δ, ▲) as the sole carbon source. Fe(II) was also quantified over time for abiotic treatments in the absence of *M. ferrooxydans* cells, containing either glucose (◊) or carbon dioxide (□) in the medium. Error bars represent standard deviation of three replicates.

M. ferrooxydans, and possibly other obligate chemolithoautotrophs. Although chemolithoheterotrophy, where Fe(II) oxidation provides energy and organic carbon serves primarily as a carbon source, has been speculated in Fe(II) oxidizing bacteria[20], it has not been experimentally validated. Our ability to successfully engineer chemolithoheterotrophy in *M. ferrooxydans* suggests that other microorganisms in the environment may also be capable of this growth strategy.

Our work provides a proof of concept for using synthetic biology to augment metabolism in microbes with limited or unknown metabolic capabilities to enhance their growth capabilities in laboratory conditions. For example, this approach may be applied to a wide range of bacteria that live by only a single metabolic mode. Additional metabolic enhancement of *M. ferrooxydans* may yield a fully heterotrophic strain that is able to grow without iron oxidation. A heterotrophic strain of *M. ferrooxydans* would grow more robustly, without producing iron oxides as a metabolic byproduct, and be amenable to characterization of genes involved in iron oxidation by mutation. Enhancing the metabolic capabilities of metabolic specialists can provide a way to better understand their physiology and provide a blueprint for their domestication.

Materials and methods

Bacterial cultivation and DNA extraction. *M. ferrooxydans* PV-1 was obtained from National Center for Marine Algae and Microbiota culture collection (https://ncma.bigelow.org) and was grown on artificial sea water medium (ASW)[3], buffered to pH 6.5 with 10 mM MES buffer, using Fe(0) or $FeCl_2$ as electron donor. When Fe(0) was used, *M. ferrooxydans* was inoculated into petri plates containing liquid ASW medium and incubated in sealed boxes containing one BD Campy Pack (Catalogue # 4080) to produce a headspace of N_2:CO_2:O_2 (80:15:5). When $FeCl_2$ was used, the culture was grown in 1000 mL serum bottles containing 750 mL of ASW medium with a headspace of N_2:CO_2 (80:20) and sealed with butyl rubber stoppers. Sealed serum bottles containing the medium were autoclaved and 3 mL of filtered ferrous chloride solution (100 mM) was added to obtain final Fe(II) concentration of 400 μM. 10 mL of filtered air was added to introduce oxygen as the electron acceptor. 3 mL of filtered ferrous chloride solution (100 mM), and 10 mL of filtered air were added to the serum bottles at every 24 h. Growth-curve experiments were performed in 25 mL Balch tubes containing 10 mL of the appropriate medium. Balch tubes were sparged with the appropriate gas to remove oxygen and sealed with butyl rubber stoppers. After autoclaving, sealed Balch tubes were added with 100 μL of filtered ferrous chloride solution (100 mM) to obtain final Fe(II) concentration of 1000 μM. 0.5 mL of filtered air was added to introduce oxygen as electron acceptor. For carbon dioxide dependent growth, ASW medium sparged with N_2:CO_2 (80:20) was used. For glucose dependent growth, ASW medium lacking bicarbonate and augmented with 500 μM glucose was sparged with argon gas. To check for glucose dependent growth in the absence of Fe(II), only filtered air was added to the sealed Balch tubes after autoclaving and ferrous chloride was omitted. DNA was extracted from *M. ferrooxydans* cultures using a Qiagen DNeasy PowerSoil kit. *Escherichia coli* WM3064 was grown in LB medium containing 360 μM DAP. 50 μM kanamycin was added to the medium when required.

Transformation of *M. ferrooxydans*. A 750 mL culture of *M. ferrooxydans* grown using $FeCl_2$ was centrifuged at 1500 × rcf for 3 min. The supernatant was then collected and centrifuged at 16,000 × rcf for 10 min. The pellet obtained was washed with ASW-LB medium (9:1 mixture of ASW and LB) and resuspended in 900 µL ASW-LB. One mL of *E. coli* donor strain culture containing approximately 10^9 cells was washed with LB medium and resuspended in 100 µL LB. Donor and recipient cells were mixed and centrifuged at 16,000 × rcf for 10 min, supernatant removed and 5 µL DAP (360 mM) added to the pellet. After incubation at 30 °C for 18 h, the pellet was washed with ASW medium and transformed cells were selected under iron-oxidizing conditions with 200 µg/mL kanamycin (without DAP), while diluting out untransformed *M. ferrooxydans* and *E. coli* cells over successive transfers (each at 1:100 dilution).

Cell and Fe(II) quantification. 200 µL of the sample was collected periodically from the Balch tubes using sterile syringes and needles. 100 µL of the sample was added to 900 µL of 0.5 N HCl to be used for Fe(II) quantification using ferrozine assay[21] performed in microtiter plates. Cells were fixed in 0.8% paraformaldehyde for 2 h, stained with 12.5 mM Syto9 and counted using a Petroff-Hausser counting chamber on an epifluorescent microscope.

Plasmid construction. The pRK2m3 plasmid used in this study confers resistance to kanamycin, is approximately 5 KB in size and contains an origin of transfer (oriT) for conjugative transfer[16]. pRK2m3 is derived from pRK2, which has been shown to be present in low copy in *E. coli* and *Pseudomonas aeruginosa*[22]. A 330 bp pRK2m3-specific DNA fragment was amplified using the following primers: CCATGTCGGCAGAAT GCTTA and TGTAAAACGACGGCCAGT. P_{neo} was amplified from pRK2m3 using pneoF (GATAGAATT CTTGAGACGTTGATCGGCACG) and pneoR (TAGACTCGAGAACACCCCTTGTATTACTGTTTATGT AAGC) primers. To construct the plasmid for GFP expression, *gfp*mut2[17] was amplified from pUA66[17] using gfpF (ACGACTCGAGATGAGTAAAGGAGAAGAACTTTTCACTGGA) and gfpR (TAGAGAGCTCTTATT TGTACAATTCATCCATACCATGGGTA) primers and cloned into pRK2m3 with the P_{neo} promoter driving its expression. To construct pGlu, *galP* and *glk* were amplified from *E. coli* K-12 using galPF (ATTTACTAGTAT GCCTGACGCTAAACAGG) /galPR (ATTCGAGCTCTTAATCGTGAGCGCCTATTTCG) and glkF (ACG ACTCGAGATGACAAAGTATGCATTAGTCGGT) /glkR (TAGAGAATTCTTACAGAATGTGACCTAAGG TCTG) primers respectively. Amplified *galP* and *glk* were cloned under the control of separate P_{neo} promoters in pRK2m3. All the plasmids were transformed into chemically competent *E. coli* WM3064[15] cells, followed by selection on LB plates containing 50 µM kanamycin and 360 µM DAP.

Received: 12 October 2020; Accepted: 4 January 2021
Published online: 25 January 2021

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Acknowledgements

This research was supported by the National Science Foundation Center for Dark Energy Biosphere Investigations (OCE-0939564) post-doctoral fellowship to AJ; and Office of Naval Research Grant N00014-13-10552 and National Science Foundation Grant MCB-1815584 to JAG. This is C-DEBI contribution #560.

Authors contributions

A.J. and J.A.G. designed research; A.J. performed research; A.J. and J.A.G. analyzed data and wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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