Received: 16 May 2022 | Revised: 28 June 2022 | Accepted: 6 July 2022

DOI: 10.1111/gbi.12515

gebiology WILEY

ORIGINAL ARTICLE

On the trail of iron uptake in ancestral Cyanobacteria on early Earth

Tristan C. Enzingmüller-Bleyl[1] | Joanne S. Boden[2,3] | Achim J. Herrmann[1] | Katharina W. Ebel[1] | Patricia Sánchez-Baracaldo[2] | Nicole Frankenberg-Dinkel[1] | Michelle M. Gehringer[1]

[1]Department of Microbiology, University of Kaiserslautern, Kaiserslautern, Germany

[2]School of Geographical Sciences, Faculty of Science, University of Bristol, Bristol, UK

[3]School of Earth and Environmental Sciences, University of St. Andrews, St. Andrews, UK

Correspondence
Michelle M. Gehringer, Department of Microbiology, University of Kaiserslautern, 67663 Kaiserslautern, Germany.
Email: mgehring@bio.uni-kl.de

Funding information
Deutsche Forschungsgemeinschaft; Royal Society of Biology; University of Bristol

Abstract

Cyanobacteria oxygenated Earth's atmosphere ~2.4 billion years ago, during the Great Oxygenation Event (GOE), through oxygenic photosynthesis. Their high iron requirement was presumably met by high levels of Fe(II) in the anoxic Archean environment. We *found that* many deeply branching Cyanobacteria, including two *Gloeobacter* and four *Pseudanabaena* spp., cannot synthesize the Fe(II) specific transporter, FeoB. Phylogenetic and relaxed molecular clock analyses find evidence that FeoB and the Fe(III) transporters, cFTR1 and FutB, were present in Proterozoic, but not earlier Archaean lineages of Cyanobacteria. Furthermore *Pseudanabaena* sp. PCC7367, an early diverging marine, benthic strain grown under simulated Archean conditions, constitutively expressed *cftr1*, even after the addition of Fe(II). Our genetic profiling suggests that, prior to the GOE, ancestral Cyanobacteria may have utilized alternative metal iron transporters such as ZIP, NRAMP, or FicI, and possibly also scavenged exogenous siderophore bound Fe(III), as they only acquired the necessary Fe(II) and Fe(III) transporters during the Proterozoic. Given that Cyanobacteria arose 3.3–3.6 billion years ago, it is possible that limitations in iron uptake may have contributed to the delay in their expansion during the Archean, and hence the oxygenation of the early Earth.

KEYWORDS
Archean, Bayesian, Cyanobacteria, iron uptake, molecular clock, *Pseudanabaena* sp. PCC7367

1 | INTRODUCTION

Iron is the fourth most abundant element in the Earth's crust and is indispensable for life, constituting an essential component in enzymes involved in nitrogen fixation, pigment synthesis, cellular respiration, and DNA biosynthesis, to name a few (Sestok et al., 2018; Sutak et al., 2020). It is used more commonly in prokaryotes than any other transition metal (Zerkle et al., 2005), and Fe-binding proteins have been preferentially retained in prokaryotic genomes through life's history, highlighting it is essential for cellular biochemistry (Dupont et al., 2006). Under current oxic atmospheric conditions, Fe exists in complex oxides as Fe(III), which are insoluble and kinetically inert. Therefore, low bioavailability of iron in modern oceans has been considered the major factor limiting open ocean primary

Tristan C. Enzingmüller-Bleyl and Joanne S. Boden contributed equally to this work.

productivity (Jiang et al., 2020; Sutak et al., 2020). The uptake of iron by prokaryotes has been extensively studied and yet the understanding of the mechanisms and identification of all the participating receptor components are still unclear (Fresenborg et al., 2020; Qiu et al., 2022). Additionally, most studies investigating iron uptake have focused on iron depleted conditions, usually under the oxidizing environment of our present atmosphere, where mechanisms that dominate utilize siderophores—low-molecular-weight biologic metal chelators that bind free Fe(III) and facilitate its targeted uptake across the prokaryotic cell membranes—dominate (Årstøl & Hohmann-Marriott, 2019; Fresenborg et al., 2020; Kranzler et al., 2011, 2014).

Early Earth had an anoxic, slightly reducing atmosphere which meant that Fe(II) provided the main source of iron for early life (Canfield, 2005; Catling & Zahnle, 2020). Iron bioavailability was significantly altered with increasing oxygenation, with Fe(II) being oxidized to insoluble Fe(III) (Fresenborg et al., 2020; Jiang et al., 2020; Sutak et al., 2020; Xu et al., 2016). Cyanobacteria, the only present-day prokaryotes capable of conducting oxygenic photosynthesis, are largely accepted to have generated the copious amounts of oxygen required to oxygenate not only the atmosphere, but also the oceans (Jiang et al., 2020; Schopf & Kudryavtsev, 2012). While the GOE is timed at approximately 2.3–2.5 Ga (Bekker et al., 2004; Gumsley et al., 2017; Konhauser et al., 2011), signs of large shallow water, phototrophic tidal mats, thought to contain ancient Cyanobacteria, appear to have existed at ~3.2 Ga (Heubeck et al., 2016; Homann et al., 2018) and molecular clocks predict that ancestral Cyanobacteria appeared more than a billion years before the GOE (Boden et al., 2021; Cardona, 2018; Fournier et al., 2021; Oliver et al., 2021).

Cyanobacteria in general contain a higher metal content than chemoheterotrophic micro-organisms (as summarized in reviews by Fresenborg et al., 2020 and Qiu et al., 2022). Cyanobacteria can have 25–350 times more atoms of iron per cell than *Escherichia coli*, depending on strain, cell type, and function (Fresenborg et al., 2020). The redox status of Cyanobacteria is tightly coupled to the light cycle, with genes encoding high-affinity metal transporters for iron, manganese, and copper following a diurnal expression pattern (Botello-Morte et al., 2014; Saha et al., 2016). For iron to enter the cyanobacterium, it must cross the outer cell membrane, pass through the periplasmic space, and be transported across the inner plasma membrane. An overview of iron specific transporters identified in Cyanobacteria is presented in Figure 1. Summaries of iron transporters in Cyanobacteria are presented in Qiu et al. (2022), Fresenborg et al. (2020) and Jiang et al. (2020). Cyanobacterial porins permit the selective passage of compounds through the outer cell membrane, with an iron-specific porin recently being identified in *Synechocystis* sp. PCC6803 (Qiu et al., 2021). Most iron in modern-day aquatic systems is bound to organic ligands—siderophores—and crosses the outer membrane via TonB-dependent transporters (TBDT) energized by the ExbB/D system on the inner cell membrane (Figure 1;

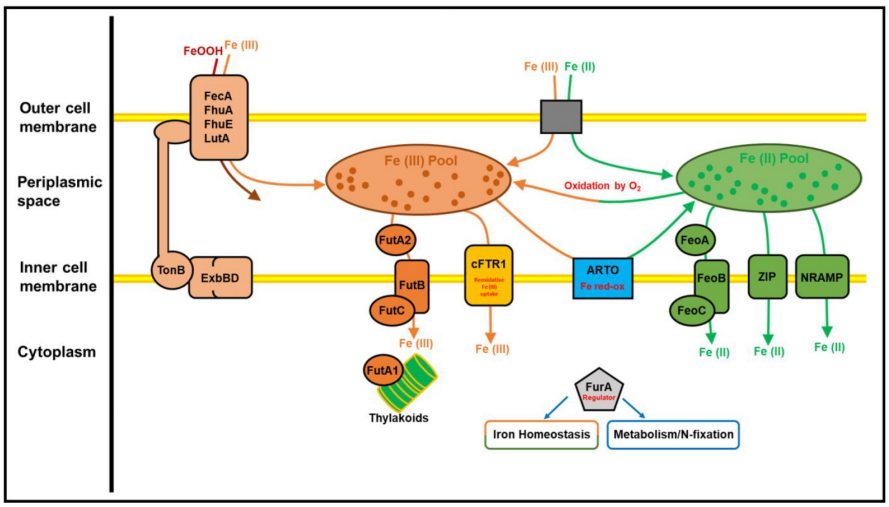

FIGURE 1 Inorganic iron uptake in Cyanobacteria. Uncomplexed iron can enter the periplasmic space via TonB-dependent transporters (TBDTs) shown as the orange rectangle (Jiang et al., 2015; Qiu et al., 2018) or outer membrane porins, indicated by a gray square (Qiu et al., 2021). Cyanobacteria make use of specific transporters to facilitate inorganic iron uptake into the cytoplasm: The FutABC system facilitates Fe(III) uptake into the cell (Brandt et al., 2009; Katoh et al., 2001), while FeoB is the primary transporter for Fe(II) (Katoh et al., 2001; Kranzler et al., 2014). The zinc-iron permease (ZIP) (Morrissey & Bowler, 2012), the natural resistance-associated macrophage protein (NRAMP) homologue (Nevo & Nelson, 2006) and FicI (Bennett et al., 2018) are thought to take up Fe(II) and other divalent cationic metals, indicated in red text. Alternative respiratory oxidases (ARTOs) can mediate the redox state of periplasmic Fe (Berry et al., 2002; Hart et al., 2005; Katoh et al., 2000) and are suggested to play a role in periplasmic iron reduction (Kranzler et al., 2011, 2014). The cytosolic protein, fur a, functions as a transcriptional repressor (Kaushik et al., 2016), regulating iron uptake (González et al., 2012, 2013, 2014, 2016). The permease, cFTR1, takes up Fe(III) possibly by re-oxidation of Fe(II) by oxygen (Xu et al., 2016). Potentially the TonB – ExbB/D complex coordinates with TBDTs to take up iron that is bound to organic ligands (Boukhalfa & Crumbliss, 2002; Jiang et al., 2015; Schätzle et al., 2021). ExbB/D was also found to take up inorganic iron directly (Jiang et al., 2015). Oxygen released during oxygenic photosynthesis can also oxidize the periplasmic Fe(II) pool

Qiu et al., 2022; Fresenborg et al., 2020; Sutak et al., 2020). The synthesis of siderophores is not prevalent in early diverging lineages of Cyanobacteria (Årstøl & Hohmann-Marriott, 2019), but basic siderophore transporters are commonly found in other Cyanobacterial genomes (reviewed by Qiu et al., 2022; Fresenborg et al., 2020). To date, Cyanobacterial siderophore synthesis studies have focused on aquatic strains grown under iron-depleted conditions (Årstøl & Hohmann-Marriott, 2019; Jiang et al., 2020).

Prior to the appearance of free oxygen, Fe(II) transport mechanisms should have provided the main source of iron for Cyanobacteria (Fresenborg et al., 2020; Jiang et al., 2020; Xu et al., 2016). The FeoB transporter functions as a Fe(II) permease, and its cytosolic G-protein domain is considered a precursor of eukaryotic G-proteins (Hantke, 2003). Genes putatively identified as *feoB* analogues are found within many Archaea genomes (Gómez-Garzón et al., 2022; Russum et al., 2021), while pairwise analyses place FeoB in a hierarchical orthologous group that appears at the level of LUCA, with FutB, Ftr1, ZIP, NRAMP, and ExbB/D appearing later in Bacteria (Altenhoff et al., 2018). Cyanobacteria, with their high iron requirements, had to adjust to ever reducing levels of Fe(II) with increasing oxygenation (Fresenborg et al., 2020; Jiang et al., 2020; Qiu et al., 2022). This dramatic change in iron bioavailability may have necessitated the evolution of Fe(III) transporters such as cFTR1 and FutABC within Cyanobacteria (Xu et al., 2016). Given the diversity of iron transporters identified in Cyanobacteria, the expression of iron-specific transporters under iron-replete conditions representing a ferruginous ocean under an anoxic atmosphere is investigated. Whereas most investigations into iron transport in Cyanobacteria have focused on the freshwater, unicellular, *feoB* carrying *Synechocystis* sp. PCC6803 (Fresenborg et al., 2020; Jiang et al., 2020; Qiu et al., 2022 and references therein) and, more recently, the filamentous diazotroph, *Nostoc* sp. PCC7120 (previously *Anabaena* sp. PCC7120) (Schätzle et al., 2021), we focus on the deeply branching strain of *Pseudanabaena* sp. PCC7367. It represents a lineage, which diverged from those leading to *Synechocystis* sp. PCC6803 and *Nostoc* sp. PCC7120 more than 2 billion years ago (Boden et al., 2021; Sánchez-Baracaldo, 2015; Sánchez-Baracaldo et al., 2017; Schirrmeister et al., 2013), so may offer greater insight into possible processes in the former ferruginous oceans of the Archean.

Recently, it was found that *Pseudanabaena* sp. PCC7367 was able to survive repeated nocturnal influxes of Fe(II) under anoxic conditions, whereas another deep branching marine strain, *Synechococcus* sp. PCC7336, did not (Herrmann et al., 2021). Previous analysis of 72 Cyanobacterial genomes by Kranzler et al. (2014; Figure S4) and Qiu et al. (2022), indicated that the genomes of a large number of marine species, including picocyanobacteria and *Pseudanabaena* sp. PCC 7367, do not encode a FeoB protein for Fe(II) uptake. In this study, we expand upon this research by searching for genes encoding additional iron transporters in 125 Cyanobacteria and reconstructing their evolutionary history. These include the zinc-iron permease (ZIP) (Morrissey & Bowler, 2012) also known as ZupT in *Nostoc* sp. 7120 (Fresenborg et al., 2020), the natural resistance-associated macrophage protein (NRAMP) homologue MntH, for transporting Mn(II) and Fe(II) into the cytoplasm (Nevo & Nelson, 2006), the Fe(II) and Co(II) transporter (FicI) (Bennett et al., 2018), the Fe(II) transporter, FeoB (Katoh et al., 2001; Kranzler et al., 2014), and the Fe(III) transporters; namely FutABC (Brandt et al., 2009; Katoh et al., 2001) and the iron permease, cFTR1 (Xu et al., 2016) also known as EfeU in *Nostoc* sp. 7120 (Fresenborg et al., 2020). Furthermore, we screened for siderophore-associated uptake genes encoding TBDTs, TonB, and the ExbB/D complex (Jiang et al., 2015; Qiu et al., 2018; Schätzle et al., 2021). The expression of *cftr1*, the cytochrome c oxidase gene, *cyoC*, and the intracellular iron transcriptional regulator gene, *furA*, in cultures of *Pseudanabaena* sp. PCC7367 grown in an anoxic atmosphere with 0.2% CO_2 was also investigated. Additionally, we employ phylogenetic and Bayesian molecular clock analyses to estimate when iron-specific transporters for Fe(II) (namely FeoB) and Fe(III) (namely FutB and cFTR1) appeared within the evolutionary history of the Cyanobacteria Phylum.

2 | MATERIALS AND METHODS

2.1 | Gene screening

In order to understand the differences in the perceived Fe(II) toxicity between *Pseudanabaena* sp. PCC 7367 and *Synechococcus* sp. PCC 7336 (Herrmann et al., 2021), it was necessary to identify all iron transporters in these, and other basal lineages of Cyanobacterial. To do this, 125 genomes (Supporting data file 1) were screened with protein sequence similarity searches (March 2020 – FeoB, FutB, cFTR1, Fur, May 2021-siderophore associated proteins & March 2022 – FicI & new TonB) using BLASTP (Altschul, 1991, 1993; Zhang et al., 2000) with e-value cut-offs less than 0.001 for the following genes linked to iron transporters identified in *Synechocystis* sp. PCC 6803, namely FutA, FutB and FutC (Brandt et al., 2009; Katoh et al., 2001), FTR1 (Katoh et al., 2000), FeoB (Katoh et al., 2001; Kranzler et al., 2014), ExbB/D TonB (Jiang et al., 2015; Qiu et al., 2018) and the related *E. coli* iron transporters (Altschul, 1991, 1993; Zhang et al., 2000) and *Shewanella oneidensis* FicI transporter (Bennett et al., 2018). The identification of hits of FutABC, cFTR1, FurA, FeoABC, ZIP and NRAMPs found in *Pseudanabaena* sp. PCC 7367 and other deeply branching Cyanobacterial lineages (Sánchez-Baracaldo, 2015) was verified with FeGenie (Garber et al., 2020). The complete bioinformatics search and processing pipeline is illustrated graphically in Figure S7.

2.2 | Phylogenetic analyses

Our initial screen indicated a lack of *feoB* in most basal Cyanobacterial genomes, so the abovementioned gene screen was extended to search for iron transporters in a broader range of genomes representing the full diversity of Cyanobacteria (Boden et al., 2021). Phylogenetic analyses were then employed to investigate how iron transporters evolved in Cyanobacteria. To do this, amino acid

sequences of FeoB, FutB, and Cyanobacterial FTR1 were aligned using MUSCLE (Edgar, 2004) implemented in MegaX version 10.1.8 (Kumar et al., 2018) with the following parameters; gap open −2.9, gap extend 0, hydrophobicity multiplier 1.2, maximum iterations 16, cluster method UPGMA, minimum dialogue length 24. Poorly aligned regions, specifically those with more than 80% gap regions, were removed manually. In order to reconstruct the phylogeny of iron uptake genes, Bayesian phylogenetic trees were generated for FeoB, FutB, and cFTR1 in MrBayes 3.2.7a (Ronquist et al., 2012) using a mixed amino acid substitution model prior, invariant sites, and four categories of a gamma distribution to model changes in substitution rates across different sites of the alignment. Convergence was assessed for two replicate chains using a burn-in of 25%. When the average standard deviation of split frequencies (ASDSF) was ≤0.03, potential scale reduction factors (PSRF) between 1.00 and 1.02 and ESS scores assessed in Tracer v1.6 (Rambaut et al., 2018) ≤ 200, trees were considered converged.

2.3 | Genome tree and molecular clock analyses

To estimate when the iron uptake genes specific for Fe(II), Fe(III), and the cFTR1 permease emerged in Cyanobacteria, the evolutionary history of FeoB, FutB, and cFTR1 was compared with the maximum likelihood phylogeny of Boden et al. (2021). Details of how this phylogeny was produced are present in the original paper (Boden et al., 2021), which incorporates information from 139 proteins, 16S rRNA and 23S rRNA collected from >100 strains representing the entire diversity of Cyanobacteria. If topology of this species tree matched the topology of Bayesian phylogenies of FeoB, FutB, or cFTRA, generated in the present study, then the MRCA of that clade was assumed to have utilized the protein. To find out when those ancestors diversified, we cross-referenced them to the Bayesian molecular clock of (Boden et al., 2021). This was made using information from rRNA (16S and 23S) and 6 soft calibrations from fossils and geological records. For further detail, see (Boden et al., 2021).

2.4 | Culture conditions and experimental setup

Pseudanabaena sp. PCC 7367 (Pasteur Culture Collection, Paris, France) was maintained in the prescribed ASNIII medium and acclimated to the simulated Archean atmosphere in an anoxic chamber atmosphere (GS Glovebox, Germany) of N_2 gas supplemented with 0.2% CO_2, 17:9 hr day–night cycle, 65% humidity, and 25 Photosynthetic Photon Flux Density (PPFD [μmols photons · m^{-2} · s^{-1}]) (Herrmann et al., 2021). Triplicate cultures, inoculated at 0.4 μg Chl a · ml^{-1} from late exponential phase cultures, were set up in acid-washed, sterilized Fernbach flasks containing 600 ml medium equilibrated at the experimental atmosphere. Chl a determination of cell content is routinely used to monitor Cyanobacterial growth and viability. Briefly, Chl a was extracted from a 1.5 ml culture volume on days 1, 3, 6, 9, and 11. Cell pellets were lysed in 90% (v/v) $CaCO_3$ neutralized methanol by bead beating, quantified as described in Herrmann et al. (2021) and plotted to generate a growth curve for *Pseudanabaena* sp. PCC 7367 (Figure S1).

On day 10, Fe(III) was added to the cultures to ensure they were not iron depleted. The following day, an oxygen microsensor (Ox200, UNISENSE, Denmark) was installed to monitor the O_2 levels resulting from oxygenic photosynthesis, in the cultures for the duration of the experiment.

2.5 | Spectrophometric ferrozine iron assay

Fe(II) and Fe(III) levels were monitored periodically by means of the spectrophotometric ferrozine iron assay to confim the availability of Fe(II) at night (Herrmann et al., 2021). Briefly, the cultures in the anaerobic glovebox (GS Glovebox, Germany) were gently resuspended and 2 × 1 ml culture volume was removed under sterile conditions from each biological replicate, added to 2 ml reaction tubes (Sarstedt, Germany), and the particulate matter immediately pelleted by centrifugation at 14,000 × g (Hermle Z 233 M-2) for 1 minute. A volume of 150 μl of the supernatant was diluted 1:1 in anoxic MilliQ H_2O, in the anoxic workstation, in a pre-prepared 96 microwell plate, to dilute the Fe (II) concentration to the detection range of the assay. For the determination of particulate Fe (III), the pellet was resuspended in 1 ml of 1 N HCl (Roth) outside the glovebox.

Assay standards for Fe(II) ranging from 150 μM to 2.34 μM $FeSO_4 \bullet 7H_2O$ (Sigma-Aldrich) and Fe(III) $FeCl_3 \bullet 6H_2O$ (Sigma-Aldrich) were prepared in 1 M HCl. Volumes of 150 μl of each sample or standard were added to a 50 μl volume of buffered ferrozine solution (50% w/v Ammonium acetate, 0.1% w/v Ferrozine (Disodium-4-[3-pyridin-2-yl-6-(4-sulfonatophenyl)-1,2,4-triazin-5-yl]benzosulfonate) (Sigma-Aldrich) in dd, H_2O). In order to determine the amount of Fe (III) in the medium, 150 μl of the samples were added to 50 μl reducing agent (10% [w/v] hydroxyl hydrochloride in 1 M HCl) and incubated for 30 min. in the dark before the addition of 50 μl buffered ferrozine solution. The OD_{562} was measured (Stookey, 1970) after 5 minutes incubation, in a microplate reader (Multiscan FC, ThermoFisher Scientific, USA). The Fe(II) and Fe(III) concentrations were determined off the standard curves ($R^2 = 0.9989$ and $R^2 = 0.9997$, respectively) for the triplicate samples at the timepoints indicated in Figure 2 and Figure S2, respectively.

2.6 | Primer design and validation

Primers for reverse transcription quantitative PCR (RT qPCR) were designed and validated to detect the following genes of *Pseudanabaena* sp. PCC 7367: the Cyanobacterial iron permease, cFTR1 (Pse7367_Rs12485), the ferric uptake regulator, FurA (Pse7367_Rs06445), the cytochrome *c* oxidase (Pse7367_Rs00935), and the reference target gene, *rpoC1* (Pse7367_Rs07505), encoding the RNA polymerase gamma subunit (Alexova et al., 2011). The primer sequences, PCR product length, and primer amplification

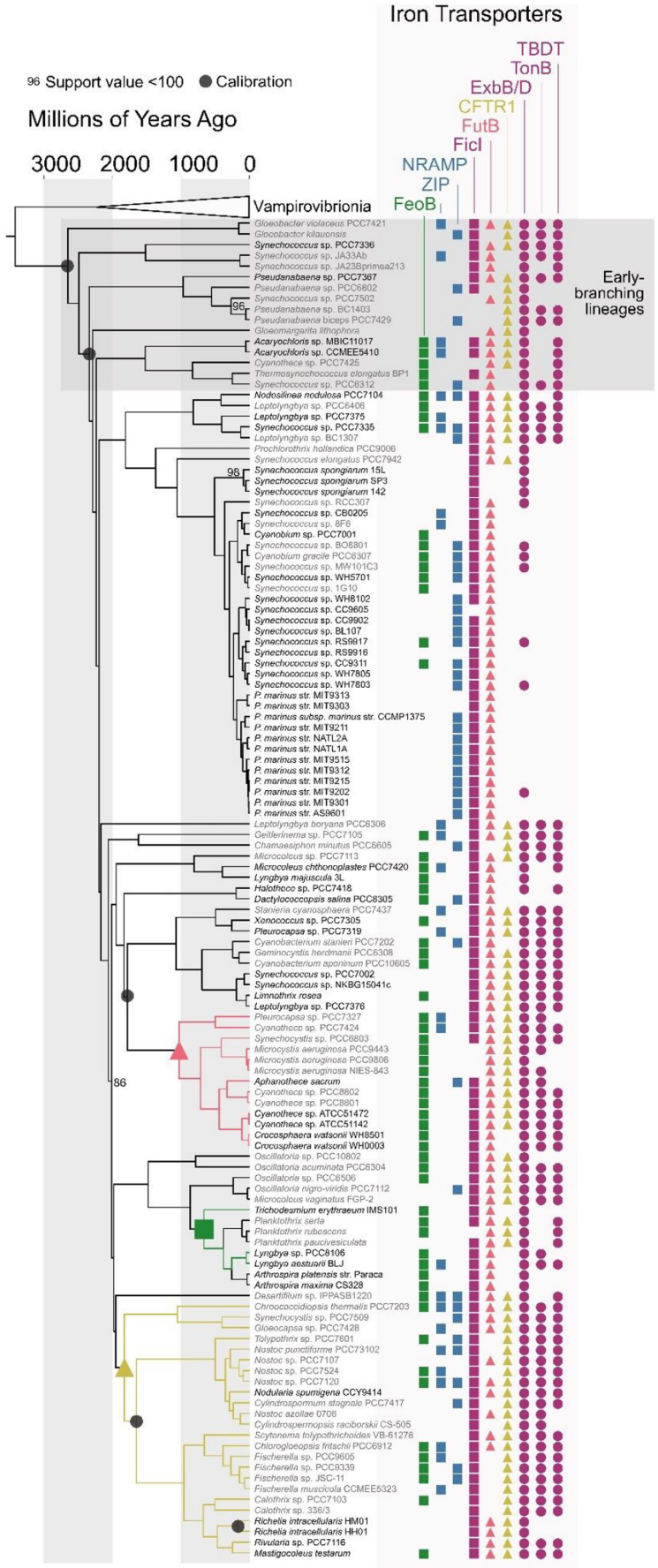

FIGURE 2 Genomic tree of Cyanobacteria indicating the distribution of iron transporters investigated in this study. The inorganic Fe (II) transporters FeoB, ZIP, NRAMP, and FicI (represented by squares), as well as the Fe (III) transporters (represented by triangles), FutB and Cyanobacterial FTR1, are superimposed on a Bayesian molecular clock adapted from (Boden et al., 2021). Since the TonB, ExbB/D, and TBDT system can also play a role in inorganic iron uptake (Qiu et al., 2018) the presence of these three siderophore associated iron uptake transporters are also indicated. Support values for branching relationships represent ultrafast bootstrap approximations (Hoang et al., 2018). These are equal to 100 unless otherwise stated. The annotations for inorganic iron transporters are as follows: FeoB: Green squares; ZIP, NRAMP, and FicI: Blue squares; FutB: Salmon pink triangles; FTR1: Yellow triangles; ExbBD, TonB, and TBDTs: Dark pink circles. The names of Cyanobacteria isolated from marine habitats are colored black, in comparison to strains from freshwater, terrestrial and geothermal springs, which are gray. Deeply branching lineages (Boden et al., 2021; Sánchez-Baracaldo, 2015) are indicated inside the gray box. Black circles represent calibration points described in Boden et al. (2021), Table 1). The first diversification of crown Cyanobacteria was constrained to occur between 2.32 and 2.7 billion years ago based on evidence of the GOE (Bekker et al., 2004) and stromatolitic laminae characteristic of Cyanobacteria (Bosk et al., 2009). The youngest-bound for FeoB, FutB, and FTR1, based on phylogenetic evidence, are indicated with shapes of the relevant colour in the molecular clock (Figure 3)

efficiencies are presented in Table S3. PCR product integrity was confirmed by melt curve analysis using cDNA of *Pseudanabaena* sp. PCC 7367 as template.

2.7 | RNA extraction and synthesis of copy DNA

In order to track the effect of a tidal influx of Fe(II) on the expression of iron transporters in *Pseudanabaena* sp. PCC 7367, the cultures were sampled an hour before darkness and, once the levels of O_2 reached zero, Fe(II) ($FeCl_2$) was added to the experimental cultures to a final initial concentration of 240 μM Fe(II). Further samples for RNA extraction were collected 15 min, 45 min, 2 h, and 7 h after the addition of Fe(II), in the dark period, with a final sample was collected 1 hour after the lights went on. Culture volumes of 45 ml of gently resuspended culture material were decanted into a 50 ml Falcon reagent tube containing 5 ml ice cold stop solution (95% Ethanol: 5% Phenol v/v; Roth, Germany) and gently inverted to prevent further transcription. Cells were pelleted at 4000 × g for 10 min (Eppendorf 5810R, Germany). Throughout the experiment, the cultures were gently agitated by a magnetic stirrer bar set at 150 rpm to facilitate the release of O_2 from the culture medium prior to the addition of Fe(II) (Herrmann et al., 2021). Pelleted cells were drained and stored at −80°C until RNA extraction.

RNA was extracted from the thawed pellets using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions, with a modified cell lysis step (Mironov & Los, 2015). The cell pellets were transferred to a sterile 2 ml tube (Sarstedt, Germany) containing 100 mg RNAse-free 0.1 mm silica beads (Biospec, Germany). RA1 buffer was added (350 μl of RA1 buffer per 100 mg pellet) to the pellet, as well as 1% (v/v) β-Mercaptoethanol (2-Mercaptoethanol, ROTH, Germany). The samples were frozen in liquid nitrogen, allowed to thaw, then were disrupted for 90 sec at setting 6.5 (Fastprep FP120, Thermo systems, USA) followed by an additional freeze/thaw step.

The cell lysates were centrifuged for 1 min at 14,000 × g (Hermle Z233-M2, Germany) to pellet the cell debris and the RNA was extracted from the supernatant using the two column-system of the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Germany). DNA removal was ensured by the on-column DNA digestion according to the manufacturer's description. RNA thus obtained was spectrophotometrically quantified (NanoDrop® Lite, Thermo Scientific, USA) and the quality confirmed by agarose gel electrophoresis, with DNA digestion verified by PCR targeting the housekeeping gene, *rpoC1*.

Extracted and purified RNA was reverse transcribed into first-strand copy DNA (cDNA) using the ProtoScript® II Reverse Transcriptase Kit (NEW ENGLAND BioLabs®Inc, Germany), according to the manufacturer's instructions, using up to 1 μg of RNA template, in RNAse-free microfuge tubes. After cDNA synthesis, the remaining RNA was degraded by the addition of 10 μl 1 M Tris-EDTA and 100 μl of 0.1 M NaOH and incubated at 95 °C for 10 min. The NaOH was neutralized by the addition of 1 M HCl and the cDNA was purified by a PCR-clean-up using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany) according to manufacturer's instructions. The concentration of the newly synthesized cDNA was measured with a NanoDrop® Lite Spectrophotometer (Thermo Scientific, USA), where after the cDNA was stored at −20 °C until use.

2.8 | Quantification of gene expression

The levels of expression of the genes encoding FurA, cFTR1, and cytochrome c oxidase and the housekeeping gene for the gamma subunit of the RNA polymerase, *rpoC1*, were determined via quantitative PCR (qPCR) of the cDNA (Huggett et al., 2013; Nolan et al., 2013). Each 10 μl reaction was prepared with 5 μl 2x iTaq™ Universal SYBR® Green Supermix, 5 pmol of each primer and 10 ng cDNA template. The volume was adjusted to 10 μl with RNase-free water. The reactions were performed in triplicate, on three different days, to evaluate transcript abundance relative to the expression of the housekeeping gene (Lü et al., 2018) using a BIORAD CFX Connect™ Real-Time System thermocycler. Cycling for the qPCR was as follows: activation of the polymerase (50 °C for 10 min), followed by initial denaturation at 95 °C for 5 min and 40 cycles of 95 °C for 10 sec, 20 sec at the primer-pair specific annealing temperature and 72 °C for 10 sec, with a final elongation step at 72 °C for 5 min. Primer T_m and T_a, as well as the product size, are listed in Table 1. Product length was verified by melt curve analysis and the relative gene expression was calculated from the mean fold difference of ΔC_q values of the three biological replicates for each timepoint (Guescini et al., 2008; Huggett et al., 2013; Narum, 2006; Rutledge & Stewart, 2008), in Excel (Excel 365, Microsoft, USA).

2.9 | Statistical analyses

Statistical analyses were done using the two-tailed, heteroscedastic Student's t-test (Excel 365, Microsoft, USA) to determine the influence of Fe(II) on gene expression levels.

3 | RESULTS

3.1 | Iron transporters of *Pseudanabaena* sp. PCC7367 and other deeply branching Cyanobacteria

Initial similarity searches for a FeoB homologue in *Pseudanabaena* sp. PCC7367 indicated that this strain encodes neither the Fe(II) transporter, FeoB, (Table S1; Kranzler et al., 2014 – fig. S4), nor homologues for the standard ZIP and NRAMP metal ion transporters. Instead, it carries genes for Fe(III) specific uptake via FutB, and the iron permease, cFTR1, that transports Fe(III) following Fe(II) re-oxidation (Xu et al., 2016), across the cytoplasmic membrane. (Figure 2). Similarly, the deeply branching marine cyanobacterium, *Synechococcus* sp. PCC 7336, (Boden et al., 2021; Sánchez-Baracaldo, 2015), which is

TABLE 1 Posterior younger-bounds timing the diversification of ancestral Cyanobacteria with genes encoding FeoB, FutB, and cFTR1

Fe transporter	Youngest estimated age/Mya[a]			Strains whose MRCA contained the transporter (based on matching evolutionary histories between the transporter and strains)
	Mean	Upper	Lower	
FeoB	661	1680	237	*Trichodesmium erythreaum* IMS101, *Lyngbya aestuarii* BLJ and *Lyngbya* sp. PCC 8106
FutB	1022	1758	388	*Microcystis aeruginosa* NIES 843, *Microcystis aeruginosa* PCC 9443, *Microcystis aeruginosa* PCC 9806, *Cyanothece* sp. PCC 7424
cFTR1	1822	1965	1700	*Fischerella muscicola* CCMEE 5323, *Fischerella* sp. PCC 9339, *Chlorogloeopsis fritschii* PCC 6912, *Fischerella* sp. PCC 9605, *Rivularia* sp. PCC 7116, *Mastigocoleus testarum*, *Scytonema tolypothrichoides* VB 61278, *Calothrix* sp. 3363, *Calothrix* sp. 3363, *Calothrix* PCC 7103, *Chroococcidiopsis thermalis* PCC 7203, *Gloeocapsa sp.* PCC 7428, *Synechocystis sp.* PCC 7509

Note: These younger-bounds represent the latest (specifically most recent) possible date that each iron transport protein emerged in Cyanobacteria based on phylogenetic evidence. "Upper" and "lower" refer to posterior 95% confidence intervals estimated by the Bayesian molecular clock of Boden et al. (2021).

[a]These ages should not be interpreted as an older-bound on the origin of the proteins in question because our analyses were limited to the phylogenetic information stored in extant proteins of Cyanobacteria. As a result, if any FeoB, FutB, or cFTR1 proteins were encoded in earlier lineages and subsequently lost as a result of extinction or gene loss, their proteins would not be represented here. MRCA: Most recent common ancestor.

incapable of surviving under a simulated anoxic ferruginous ocean (Herrmann et al., 2021), also encoded neither FeoB, nor an NRAMP homologue (Figure 2). Expanding the similarity searches for iron transporters identified that most deeply branching Cyanobacteria do not encode FeoB transporters. They are only encoded in five of 16 genomes, including *Acaryochloris* spp., *Cyanothece* sp. PCC7425, *Thermosynechococcus elongatus* BP1, and *Synechococcus* sp. PCC6312 (Figure 2b; Table S2).

In light of the lack of Fe(II) uptake transporters encoded within deeply branching Cyanobacteria, and the potential for the siderophore associated ExbB/D to take up inorganic iron directly (Jiang et al., 2015), further similarity searches for siderophore-associated uptake genes were conducted. It was confirmed that *Pseudanabaena* sp. PCC 7367 does not produce siderophores (Fresenborg et al., 2020; this study), but does encode a siderophore uptake system. This includes ExbB/D, TonB protein, and the TonB-dependent transporters (TBDTs), *fhuE*, *iutA* and *fhuA* (Table S1; Årstøl & Hohmann-Marriott, 2019; Fresenborg et al., 2020). A similar situation can be observed in many other deeply branching Cyanobacteria because all the genomes tested contained homologues of ExbB/D and several encoded homologues of TBDTs and TonB proteins (Figure 2). We did not investigate their expression under ferruginous conditions because siderophores and their transporters are known to be expressed under iron depleted conditions (Årstøl & Hohmann-Marriott, 2019; Fresenborg et al., 2020). *Pseudanabaena* sp. PCC 7367 also encodes a few porins that permit the nonspecific entry of substances such as metals into the periplasmic space; however, their functionalities are not well characterized (Table S3). The iron selective porin identified in *Synechocystis* sp. PCC6803 is not present in *Pseudanabaena* sp. PCC7367 (Qiu et al., 2021); however, eight potential outer membrane porins were identified (Table S4).

3.2 | Phylogeny of Cyanobacterial iron uptake genes FeoB, cFTR1, and FutB

Considering the above observations, a broader range of genomes spanning the Cyanobacterial tree of life (specifically all of those analyzed in Boden et al., 2021) were screened for the presence of iron transporters (Figure 2, Table S2). Genes encoding FeoB, FutB, and cFTR1 were found in a variety of strains from marine and non-marine habitats (Figure 2), so Bayesian protein phylogenies were generated to describe how these iron transport proteins from different strains of Cyanobacteria are related.

Thermosynechococcus elongatus BP-1 is one of five deeply branching Cyanobacteria genetically capable of synthesizing FeoB (Figure 2). Its genome encodes two FeoB proteins, which are distantly related to each other. One of them (NP_682238.1) shares its most recent evolutionary history with the FeoB proteins of *Oscillatoria* sp. PCC6506, *Desertifilum* sp. IPPASB1220 and *Planktothrix serta* (PP 74), whereas the other shares its most recent evolutionary history with different strains, including nitrogen-fixers (e.g. *Fischerella* spp., *Cyanothece* sp. ATCC 51472 and *Trichodesmium erythreaum* IMS101) and unicellular species, such as *Synechocystis* sp. PCC6803 (PP 100) (Figure S1). This lack of relationship between the FeoB homologues of *Thermosynechococcus* is well-supported (PP 100) and suggests that its two FeoB sequences have different evolutionary origins.

In contrast to FeoB, which was encoded in the genomes of only five of 16 deeply branching strains, the ferric iron transporter, FutB, was identified in 12, most of these strains (Figure 2). Some of these FutB sequences are closely related. For example, the FutB proteins of *Thermosynechococcus elongatus* BP1 and *Synechococcus* sp. PCC6312 are sisters (PP 100). Similarly, FutB proteins of *Gloeomargarita lithophora*, *Synechococcus* sp. JA33Ab, and *Synechococcus* sp. JA23B'a213 are also monophyletic (being derived

from a single common ancestor; PP 100, Figure S2, Figure 5). In contrast, the FutB transporter sequence in *Gloeobacter violaceus* sp. PCC 7421 is distantly related from other deeply branching lineages (PP 75). The FutB transporter sequences for the picocyanobacteria also form a distinctive monophyletic group (PP 100), indicative of a high degree of divergence from other Cyanobacterial FutB proteins.

Similar to FutB, cFTR1 proteins are encoded in the genomes of most deeply branching Cyanobacteria (Figure 2). This includes two *Gloeobacter* spp., which separated from other Cyanobacteria more than 2 billion years ago (Boden et al., 2021). Their FutB homologues are related to those of some other basal lineages, such as *Pseudanabaena* spp., as well as more diverged lineages represented by *Leptolyngbya boryana* PCC6306 and *Chamaesiphon minutus* PCC6605 (PP 78, Figure S3). Like the FeoB paralogs described above, there are also paralogues of cFTR1 in some deeply branching species, such as *Pseudanabaena* sp. PCC 6802 and *Pseudanabaena* sp. BC1403 (Figure S3). When two homologues of a single protein are found in a single genome, it is possible for one of those homologues to evolve a new and novel function.

3.3 | Dating the Cyanobacterial iron transporters FeoB, FutB, and cFTR1

The differing distributions of FeoB, FutB, and cFTR1 proteins among Cyanobacteria could reflect differences in each strain's metal-uptake strategies and environmental history. We therefore searched for congruence between the evolutionary history of each protein and an established molecular clock (Boden et al., 2021) to determine approximately when the *feoB*, *futB*, and *cftr1* genes were introduced into ancestral Cyanobacteria. Similar methods have been utilized previously to map the origin of nitrogen-metabolizing enzymes (Parsons et al., 2021) and oxygen-utilizing enzymes in bacteria (Jabłońska & Tawfik, 2021). It is based on the premise that genes inherited vertically from parental lineages have the same evolutionary history as the species they are found within. A more detailed discussion of how we account for horizontal gene transfer is presented in the Supporting Information. It should also be noted that genes encoding FutB, FeoB, and cFTR1 are present in a variety of non-cyanobacterial phyla. Some of these non-cyanobacterial proteins may share close evolutionary relationships with the Cyanobacterial proteins modelled here.

We found no phylogenetic evidence that genes encoding the Fe(II) transporter, FeoB nor the Fe(III) transporters, FutB and cFTR1, had been inherited from the most recent common ancestor (MRCA) of all extant Cyanobacteria. Instead, congruence between the Bayesian molecular clock of Boden et al. (2021) and the evolutionary history of FeoB indicate that this ferrous iron transporter was inherited from the Neoproterozoic ancestors of *Trichodesmium erythreaum* IMS1 and *Lyngbya aestuarii* BLJ, which lived 661 Mya (95% confidence intervals [CI] range from 1680 to 237 Mya; Table 1, Figure 3). An earlier origin of FeoB would be possible if the sister homologues found in *Limnothrix rosea* and *Halothece* sp. PCC 7418 (PP 100) were inherited from their common ancestor, which existed ~1830 Mya (CIs range from 1954 to 1723 Mya; Table 1).

Similarly, the high efficiency ferric uptake transporter FutB shows evolutionary patterns indicative of inheritance from Mesoproterozoic Cyanobacteria, which lived ~1022 Mya (CIs span 1758 to 387 Mya) (Figure 3). This is based on the relationship of FutB homologues found in the freshwater strains *Microcystis aeruginosa* and *Cyanothece* sp.

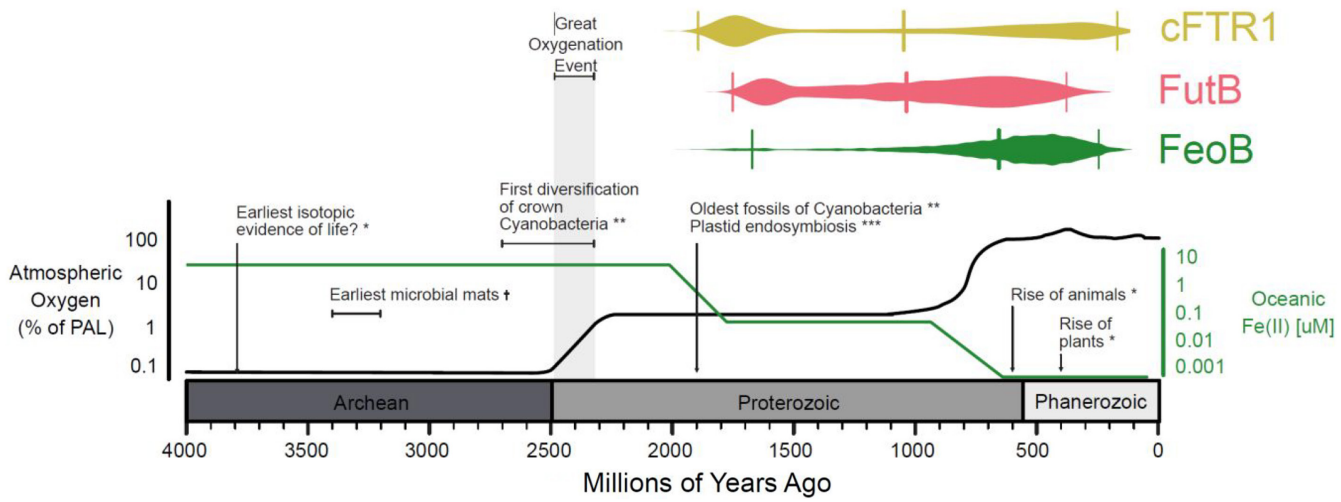

FIGURE 3 Emergence of Cyanobacterial iron transporters in relation to the oxygen record. The dates for younger age bounds on the emergence of the proteins FeoB (green Fe(II) transporter), cFTR1 and FutB (yellow and pink Fe(III) transporters respectively) are superimposed on a plot of atmospheric oxygen content (figure modified from Shih, 2015) plotted as the percentage present atmospheric levels (PAL) of O_2. The dating is based on a Bayesian molecular clock of Cyanobacteria (Boden et al., 2021), so vertical lines on the age distribution for each protein indicate the mean divergence times and lower and upper 95% confidence intervals. The Archean Ocean Fe(II) levels as calculated by Saito et al. (2003) are plotted against the right hand Y axis. * Shih (2015), † Djokic et al. (2021), ** Sánchez-Baracaldo & Cardona (2020); Boden et al. (2021)

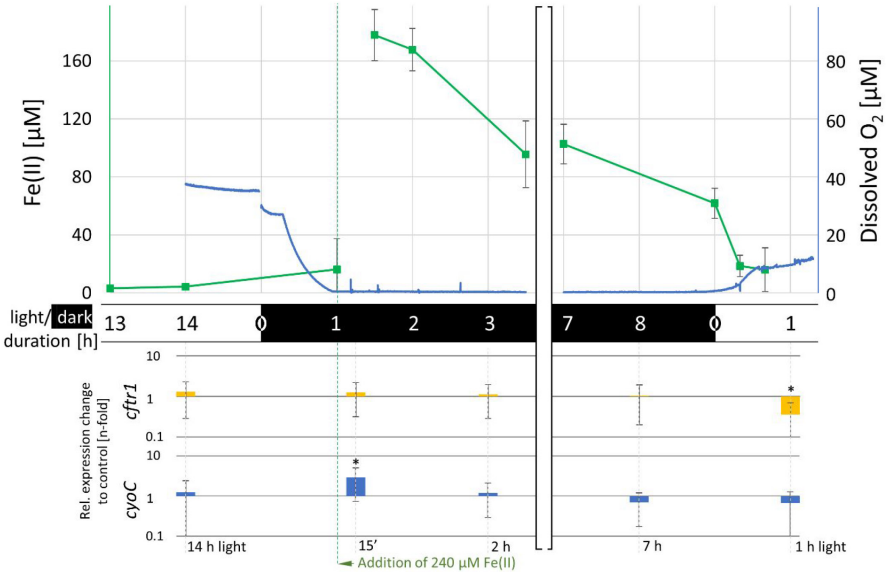

FIGURE 4 Concentrations of dissolved oxygen and Fe(II) in the *Pseudanabeana* sp. PCC 7367 cultures, with relative *cftr1* and *cyoC* gene expression levels over the course of a night cycle. The concentration of dissolved oxygen in the culture medium (blue line), was tracked using an oxygen microsensor, while the Fe(II) concentrations (green line) were periodically determined using the ferrozine assay. The first sample of culture biomass for RNA extraction was taken 1 hour before the dark cycle when oxygen was still present in the medium. The following samples for RNA extraction were taken 15 min, 45 min, 2 h, and 7 h after the addition of Fe(II) to a concentration of 240 µM in the dark (green dotted line), when dissolved oxygen levels reached zero. The last sample for RNA extraction was taken 1 hour after the start of the light cycle. The relative expression of *cftr1* and *cyoC* in the cultures to which Fe(II) was added, is plotted at the corresponding time points in comparison to expression of the control cultures without Fe(II) addition (Figure S6), (n = 3). Stars (*) represent a significant difference to the control culture ($p < 0.05$; Student's t-test, two-tailed)

PCC 7424. Their FutB homologues are sisters (PP 100, Figure S2), as would be expected if they had been inherited from the strain's MRCA. It is notable that *Pseudanabaena* sp. PCC 7367 probably shares a very close relative of the FutB protein that diversified into unicellular diazotrophs and related Cyanobacteria (PP 80).

The other Fe(III)-associated iron transporter, cFTR1 looks to have been inherited from the Paleoproterozoic when the MRCA of *Chroococcidiopsis thermalis* PCC 7203 and heterocyst-formers, such as *Nostoc* sp. PCC 7120, radiated ~1822 Mya (CIs range from 1965 to 1700 Mya; Table 1). An earlier origin of cFTR1 could be possible because the homologues found in *Gloeobacter* spp. are sister to one present in *Pseudanabaena* sp. BC1403. If cFTR1 was present in the crown Cyanobacteria and inherited by *Pseudanabaena* sp. BC1403 and *Gloeobacter* spp., but lost in all other lineages, then this topology could be reminiscent of an ancestral cFTR1 present in Cyanobacteria in the Archean. However, a single horizontal gene transfer event between the *Gloeobacter* lineage and *Pseudanabaena* sp. BC1401 could create the same pattern with a more recent origin, so we can only conclude with certainty that cFTR1 was present in the Paleoproterozoic.

3.4 | Expression *cFTR1* and *cyoC* in a simulated Archean atmosphere

Previous studies suggested that *Pseudanabaena* sp. PCC 7367 was able to withstand nightly influxes of Fe(II) in the Archean ocean, whereas *Synechococcus* sp. PCC 7336 could not (Herrmann et al., 2021). Our bioinformatic investigations indicated that the FeoB transporter, responsible for Fe(II) uptake, was encoded in neither species' genome. *Pseudanabaena* sp. PCC 7367 did, however, encode the FutB and cFTR1 transporters involved in Fe(III) uptake. Expression of *futB* is known to be constitutive and not influenced by Fe(II) availability (Katoh et al., 2001). In contrast, the cFTR1 transporter was demonstrated to preferentially take up Fe(III) in *Synechocystis*, with an increase in its expression observed under iron starvation (Xu et al., 2016). As no alternative respiratory terminal oxidase (ARTO) was identified in *Pseudanabaena* sp. PCC7367 (Figure 1), we decided to investigate the expression of the gene encoding another terminal oxidase, cytochrome *c* oxidase to ascertain whether it possibly influenced by the redox state of environmental iron (Schmetterer, 2016). While normally responsible for generating a proton gradient across the thylakoid membrane through the formation of water in the cytoplasm during respiration, cytochrome *c* oxidase may also be involved in modulating the Fe(II)/Fe(III) pool in the periplasm during respiration (Schmetterer, 2016). Changes in expression of *cftr1* and *cyoC* were monitored in response to an evening influx of Fe(II) under anoxic conditions.

The Fe(II) and oxygen levels in the media were measured over 14 hours and are presented in Figure 4. When oxygen levels dropped to zero, 1 hour after dark, with gentle agitation of the cultures, Fe(II) was added (Figure 4) and its level tracked using the ferrozine assay (Figure 4). Fe(II) was gradually oxidized or taken up overnight, but

was still present at 50 μM when the lights went on, after which it was rapidly oxidized as oxygenic photosynthesis commenced.

The relative expression data show a stable expression of *cftr1* slightly higher than for the control throughout the experiment, with a significant decrease relative to the control an hour after the lights went on. This decrease corresponds to a rapid decrease in Fe(II) in the medium (Figure 4). The expression of cytochrome c oxygenase increased significantly after the addition of Fe(II), decreasing to late daytime levels 2 hours after the addition of Fe(II).

4 | DISCUSSION

The GOE was one of the most significant developments in Earth's history, because in a relatively short period of geological time, the atmosphere changed from an anoxic reducing environment to an oxidizing one. Significantly, soluble Fe(II) was gradually oxidized to barely soluble Fe(III) in the photosynthetically active top layers of aquatic environments, suggesting that early life had to evolve means to overcome iron limitation. Our hypothesis was that ancestral Cyanobacteria, present in the Archaean, would have used Fe(II) transporters, such as FeoB, FTR1, TonB, and ExbB/D prior to the GOE (Fresenborg et al., 2020; Qiu et al., 2022) and that traces of these uptake mechanisms would remain in their genomes and could be elucidated implemeting phylogenetics. FeoB is considered the primary Fe(II) transporter in Cyanobacteria, based on studies of the Cyanobacterial model organism, *Synechocystis* sp. PCC 6803, which preferentially takes up Fe(II), rather than Fe(III) via FutB (Katoh et al., 2001). Previous investigations highlighted differences in growth responses between two unrelated deeply branching strains of Cyanobacteria, *Pseudanabaena* sp. PCC 7367 and *Synechococcus* sp. PCC 7336, in response to a tidal influx of Fe(II) seawater at night (Herrmann et al., 2021). We therefore wanted to compare the genetic potential of these two strains with respect to iron uptake transporters, as well as investigate the expression of the cFTR1 Fe(III) uptake associated gene in *Pseudanabaena* sp. PCC7367 after a tidal influx of Fe(II) in a simulated Archean shallow water marine environment.

Similarity searches to identify FeoB in 125 Cyanobacterial genomes revealed that genes encoding FeoB are missing from a variety of lineages, including *Prochlorococcus* spp., *Synechococcus* spp., *Gloeobacter* spp., *Pseudanabaena* spp., and *Gloeomargarita lithophora* (Figure 2). Many of these lineages arose early in the evolution of the phylum and have been evolving largely independently for hundreds of millions of years (Figure 2). As a result, FeoB may be the dominant iron transporter in *Synechocystis* sp. PCC 6803 (Katoh et al., 2001), but it cannot to be representative of all Cyanobacteria, especially the deeply branching lineages. Specifically, both *Pseudanabaena* sp. PCC 7367 and *Synechococcus* sp. PCC 7336, as well as the previously investigated, more recent lineage *Synechococcus* sp. PCC 7002, do not encode FeoB transporters. While none of these three strains encode the iron permease, ZIP, nor NRAMP, they carry genes for the FicI transporter identified in *Shewanella onidensis* MR-1 (Figure 2); however, its functionality as an Fe(II) transporter remains to be demonstrated in Cyanobacteria. The presence of eight Cyanobacterial putative outer membrane porins encoded on the genome of *Pseudanabaena* sp. PCC 7367 (Table S4) potentially provides a means for inorganic iron and other metals to enter the periplasmic space for transport across the cell membrane.

Localized oxidation of Fe(II) during the day would have provided Fe(III) in the vicinity of *Pseudanabaena* sp. PCC 7367, as demonstrated by the formation of green rust and rust in cultures grown under simulated Archean ocean conditions (Herrmann et al., 2021), even in the relatively reducing environment that existed before the GOE. Freshwater Cyanobacteria species, namely *Gloeobacter violaceus* PCC 7421 and *Chroococcidiopsis thermalis* PCC 7203 cultivated as mats under a micro-oxic atmosphere, demonstrated levels of oxygen exceeding modern-day atmospheric levels (Herrmann & Gehringer, 2019), suggesting that localized Fe(II) would have rapidly been oxidized at the immediate mineral–microbe interface. Both *Pseudanabaena* sp. PCC 7367 and *Synechococcus* sp. PCC7336 encode the Fe(III) transporters, FutABC, cFTR1, as well as the ExbB/D, TonB and TBDTs (Figure 2; Table S2) and do not encode siderophores (Årstøl & Hohmann-Marriott, 2019; Fresenborg et al., 2020). This is in contrast to the previously studied *Synechococcus* sp. PCC7002 (Swanner et al., 2015a, 2015b), which belongs to a lineage that diverged from those leading to *Synechococcus* sp. PCC7336 and *Pseudanabaena* sp. PCC 7367 more than 2 Ga (Sánchez-Baracaldo, 2015) and, in addition to FutB, cFTR1, ExbB/D, TonB, and TBDTs, synthesizes siderophores (Figure 2, Table S2) (Årstøl & Hohmann-Marriott, 2019; Fresenborg et al., 2020), suggesting Fe(III) is its primary source of iron.

Bayesian trees indicated a complex history involving gene duplication and/or other patterns of reticulated evolution, leading to individual strains sometimes harboring more than one gene for a given transporter. For example, two FeoB homologues with different evolutionary trajectories were found in the deeply branching *Thermosynechococcus elongatus* BP1 (Figure S1). Furthermore, the FutB homologue of the cyanobacterium, *Gloeobacter violaceus* PCC 7421 was unrelated to FutB homologues of other basal strains (Figure S2).

To find out approximately when these Fe transporters emerged in Cyanobacteria, the evolutionary history of each iron transporter was compared to an existing genome tree of Cyanobacteria and compared with a previously published molecular clock (Boden et al., 2021). Overall, this revealed that evolutionary histories of the major Fe(II) transporter, FeoB, stem back to the Neoproterozoic, whereas those of oxidized Fe(III) transporters, FutB and cFTR1, trace back earlier, to the Paleoproterozoic (Figure 3) when atmospheric O_2 levels were ~1% of present-day levels. This is surprising because global Fe(II) levels dropped to ~1 nM during the Neoproteozoic Oxygenation Event (Saito et al., 2003), when lineages with the Fe(III) transporters, FutB and cFTR1, were likely already present (Figure 3). Whether the incorporation of the *feo* cluster into the Cyanobacteria lineage coincided with the evolution of reductive iron uptake, whereby Fe(III) reduction to Fe(II) is regulated by the alternative respiratory oxidase (ARTO) on the cell membrane (Kranzler et al., 2011, 2014), is beyond the scope of this study.

ARTOs have been proposed to provide reduced Fe(III) to the periplasmic Fe(II) pool accessed by FeoB (Kranzler et al., 2014). While *Synechococcus* sp. PCC 7336 encodes an ARTO homologue, *Pseudanabaena* sp. PCC 7367 does not (Schmetterer, 2016, this study). As there is some evidence for cytochrome c oxidase to be located on the cytoplasmic membrane in *Trichodesmium thiebautii* (Bergman et al., 1993), its expression in *Pseudanabaena* sp. PCC 7367 after the addition of Fe(II) was determined (Figure 4). Terminal oxidase expression is known to be increased at night during respiration, regulated by the circadian clock in *Synechococcus elongatus* PCC7942 (Ito et al., 2009) and the iron uptake regulator, FurA, in *Anabaena* sp. PCC7120 (González et al., 2012, 2014). If cytochrome c oxidase was involved in Fe(III) reduction, a decrease in its expression after the addition of Fe(II) at night would be expected. Interestingly, expression of the cytochrome c oxidase increased significantly after the addition of Fe(II) before dropping to its original level two hours after Fe(II) addition. Whether this reflects a temporary effect on cellular respiration remains to be determined.

The iron permease of yeast, Ftr1, is tightly coupled to a ferroxidase that oxidses Fe(II) for transport across the cell membrane, whereas prokaryotes were not found to encode ferroxidases to complement their Ftr1 homologues (Banerjee et al., 2022). As 90% of the periplasmic Fe(III) is reduced to Fe(II) during iron uptake (Kranzler et al., 2014), the Fe(III) transporters, namely FutABC and specifically cFTR1, very likely obtain their Fe(III) during the day from re-oxidized Fe(II) after iron reduction (Xu et al., 2016), thereby rendering a specific Ftr1 ferroxidase unnecessary. Expression of the iron permease gene, *cftr1*, in the control cultures of *Pseudanabaena* sp. PCC 7367 remained largely unchanged during the complete 24-hour cycle, with a nonsignificant increase in expression recorded an hour after the lights went on and oxygen levels rose. This upward trend augmented the reduced relative expression of *cftr1* in the experimental cultures exposed to Fe(II) after the start of the light cycle (Figure 4), suggesting a role for O_2 and iron speciation in the regulation of *cftr1* expression.

This is the first time that *cftr1* transcription has been confirmed in a non-*Synechocystis* species, and under anoxic, ferruginous conditions. *Pseudanabaena* and *Synechocystis*, though both Cyanobacteria, are distant relatives, having last shared a common ancestor more than 2 Ga (Figure 2), before evidence for the utilization of cFTR1 appears in the evolutionary tree (Table 1, Figure 3). Perhaps because of this long period of separation, our expression data from *Pseudanabaena* sp. PCC 7367 contradict that observed in *Synechocystis*, whereby *cftr1* expression was induced by iron starvation (Xu et al., 2016). Additionally, the diurnal cycling of the expression of metal transporters as recorded in *Synechocystis* sp. PCC 6803 (Saha et al., 2016) was not observed in this study. Expression of diurnally regulated genes, including some involved in metal transport, were upregulated in *Synechocystis* sp. PCC 6803 an hour before and 2 hours into the light cycle (Saha et al., 2016). As we sampled 2 hours prior to the light cycle, we may not have recorded diurnally induced increased expression at the end of the dark cycle. Further investigations regulating iron speciation and uptake are required to better understand the process of iron acquisition in *Pseudanabaena* sp. PCC 7367 and other early diverging Cyanobacteria under iron-replete conditions. However, our results highlight the potentially different cFTR1 expression strategies between different classes of Cyanobacteria.

Our evolutionary analyses did not find any phylogenetic evidence that Cyanobacteria could use the Fe(II) transporter, FeoB, until the Neoproterozoic (Figure 3). As a result, it is possible that Cyanobacteria were unable to access Fe(II) in the ferruginous Archaean environment. If true, this would suggest that the Archean Cyanobacteria may have been iron limited, thereby reducing their growth rates. Given that FeoB has been proposed to trace back to LUCA (Altenhoff et al., 2018), it is possible that Archaean Cyanobacteria encoded a functional FeoB to import Fe(II), but when Fe(II) levels dropped as a result of the GOE, they lost the gene encoding it to make the most efficient use of their potentially limited resources. When the gene was lost, all phylogenetic remnants of it were extinguished, until *feoB* was regained again via lateral gene transfer in the Neoproterozoic, which may or may not coincide with the establishment of reductive iron transport in the periplasmic space (Kranzler et al., 2011, 2014). Our data are not able to differentiate between these two scenarios, but further study may be able to determine whether the evolutionary history of cambialistic transporters, such as ZIP, NRAMP, and FicI, capable of transporting Fe(II) in addition to zinc, manganese, and cobalt, respectively, traces back to the Archean.

Previous research has found that most Cyanobacterial strains carry genes encoding siderophore uptake transporters (TBDTs) that pass siderophore bound and free inorganic iron species into the periplasmic space (Årstøl & Hohmann-Marriott, 2019; this study Figure 2). Fe(II) can be mobilized from mineral sources by siderophores, specifically desferroxazine (Bau et al., 2013; Kraemer, 2004), produced by several microorganisms in niches with low iron availability. The high levels of oxygen measured in freshwater mats of *Gloeobacter violaceus* PCC 7421 and *Chroococcidiopsis thermalis* PCC7203 (Herrmann & Gehringer, 2019), as well as that recorded in liquid cultures of *Pseudanabaena* sp. PCC7367 (Herrmann et al., 2021) in simulated Archean atmospheres, suggest that there would have been sufficient Fe(III) available for putative ExbB/D uptake in the immediate vicinity within these oxygen-rich niches. Future studies can investigate siderophore mobilization of Fe(II)/Fe(III) under anoxic conditions, both in the presence and absence of siderophores, as well as conducting additional molecular analyses to date the inclusion of ExbB/D in the Cyanobacterial phylum. The presence of other, as yet unidentified iron chelators in Cyanobacteria cannot be excluded, as evidenced by the recent identification of Cyanochelins in some Cyanobacterial species (Galica et al., 2021). The Cyanochelin gene cluster was identified in a single basal species, namely *Synechococcus* sp. PCC 7336, and is only induced under iron-depleted conditions (Galica et al., 2021).

The mechanisms by which ancestral Cyanobacteria acquired Fe(II)/Fe(III) in the ferruginous Archean environment remain

unclear. Under the iron-limiting conditions of today FutB must play an essential role in iron acquisition in *Pseudanabaena* sp. PCC 7367; however, confirmation of its constitutive expression, as reported for *Synechocystis* sp. PCC 6803 (Katoh et al., 2001), especially under conditions of high Fe(II) availability, is required. In conclusion, this study has found phylogenetic evidence for an Fe(II) transporter (FeoB) in Cyanobacteria in the Neoproterozoic, and two Fe(III) transporters (FutB and cFTR1) in earlier Cyanobacteria of the Meso- and Paleo-Proterozoic, but none can be traced back to the Archean. This suggests either that Archean Cyanobacteria were not using FeoB, FutB, or cFTR1 to acquire Fe(II)/Fe(III) before the GOE, or that they lost the genes encoding FeoB after the GOE. Alternative explanations may be that the early marine lineages went extinct, or, if they are extant, have not been sampled yet. Furthermore, this study raises questions as to the influence of trace metal inventories on the evolution of biochemical pathways, particularly with respect to metalloenzymes (Dupont et al., 2006, 2010). Given the recent support for the evolution of oxygenic photosynthesis close to the origin of life (Oliver et al., 2021), basal Cyanobacteria may have had access to Fe(III) from their inception, as the oxygen they emitted oxidized Fe(II) to Fe(III) in their immediate surroundings. Given the propensity of genes encoding TBDT and ExbB/D throughout the Cyanobacterial Phylum (Figure 2), and their potential to scavenge siderophore bound Fe(III) from other community members, future research should estimate their antiquity. Overall, the data presented here have highlighted the need to further investigate iron uptake by Cyanobacteria, especially under the anoxic, ferruginous conditions on early Earth, to obtain a clearer picture of limitations on Cyanobacterial expansion prior to the GOE.

AUTHOR CONTRIBUTIONS

Michelle M. Gehringer, Patricia Sánchez-Baracaldo, Joanne S. Boden, and Achim J. Herrmann conceptualized the project and designed the research experiments. Tristan C. Enzingmüller-Bleyl, Joanne S. Boden, and Patricia Sánchez-Baracaldo performed gene screening and phylogenomic analyses. Tristan C. Enzingmüller-Bleyl, Katharina W. Ebel, and Achim J. Herrmann cultured *Pseudanabaena* sp. PCC7367, quantified gene expression, and conducted laboratory assays. All authors contributed to interpreting the data and writing the manuscript.

ACKNOWLEDGMENTS

This project was funded by the German Research Foundation SPP1833, DFG, Grant numbers: GE2558/3-1 & GE2558/4-1 awarded to MMG, a University of Bristol Graduate Teaching Scholarship awarded to J.S.B. and a Royal Society University Research Fellowship awarded to P.S-B. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The sequence data analyzed in this study are available in the open science framework repository, https://osf.io/7x598/?view_only=715cd38c378446ba8c3f6c924f9be9f5. All other data are included in the published article and its Supporting Information.

ORCID

Achim J. Herrmann https://orcid.org/0000-0001-8533-1842
Michelle M. Gehringer https://orcid.org/0000-0002-4982-2465

REFERENCES

Alexova, R., Fujii, M., Birch, D., Cheng, J., Waite, T. D., Ferrari, B. C., & Neilan, B. A. (2011). Iron uptake and toxin synthesis in the bloom-forming *Microcystis aeruginosa* under iron limitation. *Environmental Microbiology*, *13*, 1064–1077.

Altenhoff, A. M., Glover, N. M., Train, C. M., Kaleb, K., Warwick Vesztrocy, A., Dylus, D., de Farias, T. M., Zile, K., Stevenson, C., Long, J., Redestig, H., Gonnet, G. H., & Dessimoz, C. (2018). The OMA orthology database in 2018: Retrieving evolutionary relationships among all domains of life through richer web and programmatic interfaces. *Nucleic Acids Research*, *46*, D477–D485.

Altschul, S. F. (1991). Amino acid substitution matrices from an information theoretic perspective. *Journal of Molecular Biology*, *219*, 555–565.

Altschul, S. F. (1993). A protein alignment scoring system sensitive at all evolutionary distances. *Journal of Molecular Evolution*, *36*, 290–300.

Årstøl, E., & Hohmann-Marriott, M. F. (2019). Cyanobacterial siderophores-physiology, structure, biosynthesis, and applications. *Marine Drugs*, *17*, 281.

Banerjee, S., Chanakira, M. N., Hall, J., Kerkan, A., Dasgupta, S., & Martin, D. W. (2022). A review on bacterial redox dependent iron transporters and their evolutionary relationship. *Journal of inorganic biochemistry*, *229*, 111721.

Bau, M., Tepe, N., & Mohwinkel, D. (2013). Siderophore-promoted transfer of rare earth elements and iron from volcanic ash into glacial meltwater, river and ocean water. *Earth and Planetary Science Letters*, *364*, 30–36.

Bekker, A., Holland, H. D., Wang, P. L., Rumble, D., 3rd, Stein, H. J., Hannah, J. L., Coetzee, L. L., & Beukes, N. J. (2004). Dating the rise of atmospheric oxygen. *Nature*, *427*, 117–120.

Bennett, B. D., Redford, K. E., & Gralnick, J. A. (2018). MgtE homologue FicI acts as a secondary ferrous iron importer in *Shewanella oneidensis* strain MR-1. *Applied and Environmental Microbiology*, *84*, e01245-17.

Bergman, B., Siddiqui, P. J., Carpenter, E. J., & Peschek, G. A. (1993). Cytochrome oxidase: Subcellular distribution and relationship to nitrogenase expression in the Nonheterocystous marine cyanobacterium *Trichodesmium thiebautii*. *Applied and Environmental Microbiology*, *59*, 3239–3244.

Berry, S., Schneider, D., Vermaas, W. F. J., & Rögner, M. (2002). Electron transport routes in whole cells of *Synechocystis* sp. strain PCC 6803: The role of the cytochrome bd-type oxidase. *Biochemistry*, *41*, 3422–3429.

Boden, J. S., Konhauser, K. O., Robbins, L. J., & Sánchez-Baracaldo, P. (2021). Timing the evolution of antioxidant enzymes in cyanobacteria. *Nature Communications*, *12*, 4742.

Bosk, T., Liang, B., Sim, M. S., & Petroff, A. P. (2009). Morphological record of oxygenic photosynthesis in conical stromatolites. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 10939–10943.

Botello-Morte, L., Bes, M. T., Heras, B., Fernández-Otal, Á., Peleato, M. L., & Fillat, M. F. (2014). Unraveling the redox properties of the global regulator FurA from *Anabaena* sp. PCC 7120: Disulfide reductase activity based on its CXXC motifs. *Antioxidants & Redox Signaling*, *20*, 1396–1406.

Boukhalfa, H., & Crumbliss, A. L. (2002). Chemical aspects of siderophore mediated iron transport. *Biometals*, *15*, 325–339.

Brandt, A.-M., Raksajit, W., Mulo, P., Incharoensakdi, A., Salminen, T. A., & Mäenpää, P. (2009). Transcriptional regulation and structural modeling of the FutC subunit of an ABC-type iron transporter in *Synechocystis* sp. strain PCC 6803. *Archives of Microbiology*, *191*, 561–570.

Canfield, D. E. (2005). The early history of atmospheric oxygen: Homage to Robert M Garrels. *Annual Review of Earth and Planetary Sciences*, *33*, 1–36.

Cardona, T. (2018). Early Archean origin of heterodimeric photosystem I. *Heliyon*, *4*, e00548.

Catling, D. C., & Zahnle, K. J. (2020). The Archean atmosphere. *Science Advances*, *6*, eaax1420.

Djokic, T., van Kranendonk, M., Campbell, K. A., Havig, J. R., Walter, M. R., & Guido, D. M. (2021). A reconstructed subaerial hot spring field in the ~3.5 billion-year-old dresser formation, north pole dome, Pilbara craton, Western Australia. *Astrobiology*, *21*, 1–38.

Dupont, C. L., Butcher, A., Valas, R. E., Bourne, P. E., & Caetano-Anolles, G. (2010). History of biological metal utilization inferred through phylogenomic analysis of protein structures. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 10567–10572.

Dupont, C. L., Yang, S., Palenik, B., & Bourne, P. E. (2006). Modern proteomes contain putative imprints of ancient shifts in trace metal geochemistry. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 17822–17827.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797.

Fournier, G. P., Moore, K. R., Rangel, L. T., Payette, J. G., Momper, L., & Bosak, T. (2021). The Archean origin of oxygenic photosynthesis and extant cyanobacterial lineages. *Proceedings of the Royal Society, B288*, 20210675.

Fresenborg, L. S., Graf, J., Schätzle, H., & Schleiff, E. (2020). *Iron homeostasis of cyanobacteria: Advancements in siderophores and metal transporters. Ch 7, advances in cyanobacterial biology*. Academic Press.

Galica, T., et al. (2021). Cyanochelins, an overlooked class of widely distributed cyanobacterial siderophores, discovered by silent gene cluster awakening. *Applied and Environmental Microbiology*, *87*, e03128-20.

Garber, A. I., et al. (2020). FeGenie: A comprehensive tool for the identification of iron genes and iron gene neighborhoods in genome and metagenome assemblies. *Frontiers in Microbiology*, *11*, 37.

Gómez-Garzón, C., Barrick, J. E., & Payne, S. M. (2022). Disentangling the evolutionary history of Feo, the major ferrous iron transport system in bacteria. *MBio*, *13*, e03512-21.

González, A., Angarica, V. E., Sancho, J., & Fillat, M. F. (2014). The FurA regulon in *Anabaena* sp. PCC 7120: *In silico* prediction and experimental validation of novel target genes. *Nucleic Acids Research*, *42*, 4833–4846.

González, A., Bes, M. T., Peleato, M. L., & Fillat, M. F. (2016). Expanding the role of FurA as essential global regulator in cyanobacteria. *PLoS One*, *11*, e0151384.

González, A., Bes, M. T., Valladares, A., Peleato, M. L., & Fillat, M. F. (2012). FurA is the master regulator of iron homeostasis and modulates the expression of tetrapyrrole biosynthesis genes in *Anabaena* sp. PCC 7120. *Environmental Microbiology*, *14*, 3175–3187.

González, A., Valladares, A., Peleato, M. L., & Fillat, M. F. (2013). FurA influences heterocyst differentiation in *Anabaena* sp. PCC 7120. *FEBS Letters*, *587*, 2682–2690.

Guescini, M., Sisti, D., Rocchi, M. B., Stocchi, L., & Stocchi, V. (2008). A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. *BMC Bioinformatics*, *9*, 326.

Gumsley, A. P., Chamberlain, K. R., Bleeker, W., Söderlund, U., de Kock, M. O., Larsson, E. R., & Bekker, A. (2017). Timing and tempo of the great oxidation event. *Proceedings of the National Academy of Sciences of the United States of America*, *114*, 1811–1816.

Hantke, K. (2003). Is the bacterial ferrous iron transporter FeoB a living fossil? *Trends in Microbiology*, *11*, 192–195.

Hart, S. E., Schlarb-Ridley, B. G., Bendall, D. S., & Howe, C. J. (2005). Terminal oxidases of cyanobacteria. *Biochemical Society Transactions*, *33*(4), 832–835.

Herrmann, A. J., & Gehringer, M. M. (2019). An investigation into the effects of increasing salinity on photosynthesis in freshwater unicellular cyanobacteria during the late Archaean. *Geobiology*, *17*, 343–359.

Herrmann, A. J., Sorwat, J., Byrne, J. M., Frankenberg-Dinkel, N., & Gehringer, M. M. (2021). Diurnal Fe(II)/Fe(III) cycling and enhanced O_2 production in a simulated Archean marine oxygen oasis. *Nature Communications*, *12*, 2069.

Heubeck, C., et al. (2016). Geological constraints on Archean (3.22 Ga) coastal-zone processes from the Dycedale Syncline, Barberton Greenstone Belt. *South African Journal of Geology*, *119*, 495–518.

Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q., & Le Vinh, S. (2018). UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution*, *35*, 518–522.

Homann, M., et al. (2018). Microbial life and biogeochemical cycling on land 3,220 million years ago. *Nature Geoscience*, *11*, 665–671.

Huggett, J., Foy, C. A., Benes, V., Emslie, K., Garson, J. A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R. D., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., Wittwer, C. T., & Bustin, S. A. (2013). The digital MIQE guidelines: Minimum information for publication of quantitative digital PCR experiments. *Clinical Chemistry*, *59*, 892–902.

Ito, H., Mutsuda, M., Murayama, Y., Tomita, J., Hosokawa, N., Terauchi, K., Sugita, C., Sugita, M., Kondo, T., & Iwasaki, H. (2009). Cyanobacterial daily life with Kai-based circadian and diurnal genome-wide transcriptional control in *Synechococcus elongatus*. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 14168–14173.

Jabłońska, J., & Tawfik, D. S. (2021). The evolution of oxygen-utilizing enzymes suggests early biosphere oxygenation. *Nature Ecology and Evolution*, *5*, 442–448.

Jiang, H. B., Lou, W. J., Ke, W. T., Song, W. Y., Price, N. M., & Qiu, B. S. (2015). New insights into iron acquisition by cyanobacteria: An essential role for ExbB-ExbD complex in inorganic iron uptake. *The ISME Journal*, *9*, 297–309.

Jiang, H. B., Lu, X. H., Deng, B., Liu, L. M., & Qiu, B. S. (2020). Adaptive mechanisms of the model photosynthetic organisms, cyanobacteria, to iron deficiency. In *Microbial photosynthesis* (pp. 197–244). Singapore.

Katoh, H., Grossman, A. R., Hagino, N., & Ogawa, T. (2000). A gene of *Synechocystis* sp. strain PCC 6803 encoding a novel iron transporter. *Journal of Bacteriology*, *182*, 6523–6524.

Katoh, H., Hagino, N., Grossman, A. R., & Ogawa, T. (2001). Genes essential to iron transport in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Journal of Bacteriology*, *183*, 2779–2784.

Kaushik, M. S., Singh, P., Tiwari, B., & Mishra, A. K. (2016). Ferric uptake regulator (FUR) protein: Properties and implications in cyanobacteria. *Annales de Microbiologie*, *66*, 61–75.

Konhauser, K. O., Lalonde, S. V., Planavsky, N. J., Pecoits, E., Lyons, T. W., Mojzsis, S. J., Rouxel, O. J., Barley, M. E., Rosière, C., Fralick, P. W., Kump, L. R., & Bekker, A. (2011). Aerobic bacterial pyrite oxidation and acid rock drainage during the great oxidation event. *Nature*, *478*, 369–373.

Kraemer, S. M. (2004). Iron oxide dissolution and solubility in the presence of siderophores. *Aquatic Sciences*, *66*, 3–18.
Kranzler, C., Lis, H., Finkel, O. M., Schmetterer, G., Shaked, Y., & Keren, N. (2014). Coordinated transporter activity shapes high-affinity iron acquisition in cyanobacteria. *The ISME Journal*, *8*, 409–417.
Kranzler, C., Lis, H., Shaked, Y., & Keren, N. (2011). The role of reduction in iron uptake processes in a unicellular, planktonic cyanobacterium. *Environmental Microbiology*, *13*, 2990–2999.
Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*, 1547–1549.
Lü, J., et al. (2018). Selection and validation of reference genes for RT-qPCR analysis of the ladybird beetle *Henosepilachna vigintioctomaculata*. *Frontiers in Physiology*, *9*, 1614.
Mironov, K. S., & Los, D. A. (2015). RNA isolation from Synechocystis. *Bio-Protocol*, *5*, e1428.
Morrissey, J., & Bowler, C. (2012). Iron utilization in marine cyanobacteria and eukaryotic algae. *Frontiers in Microbiology*, *3*, 43.
Narum, S. R. (2006). Beyond Bonferroni: Less conservative analyses for conservation genetics. *Conservation Genetics*, *7*, 783–787.
Nevo, Y., & Nelson, N. (2006). The NRAMP family of metal-ion transporters. *Biochimica et Biophysica Acta*, *1763*, 609–620.
Nolan, T., Huggett, J., Sanchez, E., Sanders, R., Redshaw, N., & Wilkes, T. (2013). *Good practice guide for the application of quantitative PCR (qPCR)*. LGC.
Oliver, T., Sánchez-Baracaldo, P., Larkum, A. W., Rutherford, A. W., & Cardona, T. (2021). Time-resolved comparative molecular evolution of oxygenic photosynthesis. *Biochimica et Biophysica Acta - Bioenergetics*, *1862*, 148400.
Parsons, C., Stüeken, E. E., Rosen, C. J., Mateos, K., & Anderson, R. E. (2021). Radiation of nitrogen-metabolizing enzymes across the tree of life tracks environmental transitions in earth history. *Geobiology*, *19*, 18–34.
Qiu, G.-W., Jiang, H. B., Lis, H., Li, Z. K., Deng, B., Shang, J. L., Sun, C. Y., Keren, N., & Qiu, B. S. (2021). A unique porin meditates iron-selective transport through cyanobacterial outer membranes. *Environmental Microbiology*, *23*, 376–390.
Qiu, G.-W., Koedooder, C., Qiu, B. S., Shaked, Y., & Keren, N. (2022). Iron transport in cyanobacteria - from molecules to communities. *Trends in Microbiology*, *30*, 229–240.
Qiu, G.-W., et al. (2018). Outer membrane iron uptake pathways in the model cyanobacterium *Synechocystis* sp. strain PCC 6803. *Applied and Environmental Microbiology*, *84*, e01512-18.
Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarisation in Bayesian phylogenetics using tracer 1.7. *Systematic Biology*, *67*, 901–904.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*, 539–542.
Russum, S., Lam, K. J. K., Wong, N. A., Iddamsetty, V., Hendargo, K. J., Wang, J., et al. (2021). Comparative population genomic analyses of transporters within the Asgard archaeal superphylum. *PLoS One*, *16*(3), e0247806.
Rutledge, R. G., & Stewart, D. (2008). Critical evaluation of methods used to determine amplification efficiency refutes the exponential character of real-time PCR. *BMC Molecular Biology*, *9*, 96.
Saha, R., et al. (2016). Diurnal regulation of cellular processes in the cyanobacterium *Synechocystis* sp. strain PCC 6803: Insights from transcriptomic, Fluxomic, and Physiological Analyses. *MBio*, *7*, e00464-16.
Saito, M. A., Sigman, D. M., & Morel, F. M. M. (2003). The bioinorganic chemistry of the ancient ocean: The co-evolution of cyanobacterial metal requirements and biogeochemical cycles at the Archean–Proterozoic boundary? *Inorganica Chimica Acta*, *356*, 308–318.
Sánchez-Baracaldo, P. (2015). Origin of marine planktonic cyanobacteria. *Scientific Reports*, *5*, 17418.
Sánchez-Baracaldo, P., & Cardona, T. (2020). On the origin of oxygenic photosynthesis and cyanobacteria. *The New Phytologist*, *225*, 1440–1446.
Sánchez-Baracaldo, P., Raven, J. A., Pisani, D., & Knoll, A. H. (2017). Early photosynthetic eukaryotes inhabited low-salinity habitats. *Proceedings of the National Academy of Sciences of the United States of America*, *114*, E7737–E7745.
Schätzle, H., Arévalo, S., Fresenborg, L., Seitz, H. M., Flores, E., & Schleiff, E. (2021). Functional diversity of TonB-like proteins in the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120. *mSphere*, *6*, e00214-21.
Schirrmeister, B. E., De Vos, J. M., Anotonelli, A., & Bagheri, H. C. (2013). Evolution of multicellularity coincided with increased diversification of cyanobacteria and the great oxidation event. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 1791–1796.
Schmetterer, G. (2016). The respiratory terminal oxidases (RTOs) of cyanobacteria. In *Cytochrome complexes: Evolution, structures, energy transduction, and signaling* (pp. 331–355). Springer.
Schopf, J. W., & Kudryavtsev, A. B. (2012). Biogenicity of Earth's earliest fossils: A resolution of the controversy. *Gondwana Research*, *22*, 761–771.
Sestok, A. E., Linkous, R. O., & Smith, A. T. (2018). Toward a mechanistic understanding of Feo-mediated ferrous iron uptake. *Metallomics*, *10*, 887–898.
Shih, P. M. (2015). Photosynthesis and early earth. *Current Biology*, *25*, R855–R859.
Stookey, L. L. (1970). Ferrozine—A new spectrophotometric reagent for iron. *Analytical Chemistry*, *42*, 779–781.
Sutak, R., Camadro, J.-M., & Lesuisse, E. (2020). Iron uptake mechanisms in marine phytoplankton. *Frontiers in Microbiology*, *11*, 566691.
Swanner, E. D., et al. (2015a). Modulation of oxygen production in Archaean oceans by episodes of Fe(II) toxicity. *Nature Geoscience*, *8*, 126–130.
Swanner, E. D., et al. (2015b). Physiology, Fe(II) oxidation, and Fe mineral formation by a marine planktonic cyanobacterium grown under ferruginous conditions. *Frontiers in Earth Science*, *3*, 60.
Xu, N., et al. (2016). Identification of an iron permease, cFTR1, in cyanobacteria involved in the iron reduction/re-oxidation uptake pathway. *Environmental Microbiology*, *18*, 5005–5017.
Zerkle, A. L., House, C. H., & Brantley, S. L. (2005). Biogeochemical signatures through time as inferred from whole microbial genomes. *American Journal of Science*, *305*, 467–502.
Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology*, *7*, 203–214.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Enzingmüller-Bleyl, T. C., Boden, J. S., Herrmann, A. J., Ebel, K. W., Sánchez-Baracaldo, P., Frankenberg-Dinkel, N., & Gehringer, M. M. (2022). On the trail of iron uptake in ancestral Cyanobacteria on early Earth. *Geobiology*, *20*, 776–789. https://doi.org/10.1111/gbi.12515