Journal of Inorganic Biochemistry 229 (2022) 111721

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

A review on bacterial redox dependent iron transporters and their evolutionary relationship

Sambuddha Banerjee [a,*], Mina N. Chanakira [a], Jonathan Hall [a], Alexa Kerkan [a], Saumya Dasgupta [b], Daniel W. Martin [c]

[a] Department of Chemistry, East Carolina University, Greenville, NC 27858, USA
[b] Department of Chemistry, Amity Institute of Applied Sciences, Amity University Kolkata, WB 700135, India
[c] Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA

ARTICLE INFO

Keywords:
Ftr-mediated iron uptake
Ferroxidase
Novel cupredoxin
FtrABCD evolution

ABSTRACT

Iron is an essential yet toxic micronutrient and its transport across biological membranes is tightly regulated in all living organisms. One such iron transporter, the Ftr-type permeases, is found in both eukaryotic and prokaryotic cells. These Ftr-type transporters are required for iron transport, predicted to form α-helical transmembrane structures, and conserve two ArgGluxxGlu (x = any amino acid) motifs. In the yeast Ftr transporter (Ftr1p), a ferroxidase (Fet3p) is required for iron transport in an oxidation coupled transport step. None of the bacterial Ftr-type transporters (EfeU and FetM from *E. coli*; cFtr from *Campylobacter jejuni*; FtrC from *Brucella*, *Bordetella*, and *Burkholderia* spp.) contain a ferroxidase protein. Bioinformatics report predicted periplasmic EfeO and FtrB (from the EfeUOB and FtrABCD systems) as novel cupredoxins. The Cu^{2+} binding and the ferrous oxidation properties of these proteins are uncharacterized and the other two bacterial Ftr-systems are expressed without any ferroxidase/cupredoxin, leading to controversy about the mode of function of these transporters. Here, we review published data on Ftr-type transporters to gain insight into their functional diversity. Based on original bioinformatics data presented here evolutionary relations between these systems are presented.

1. Introduction

Since the start of life on earth, iron requirement for survival and growth are common themes for most living organisms [1,2]. The biological iron requirement can be explained based on the high abundance of iron in the earth's crust and its facile redox reaction, making it an ideal cofactor for redox proteins [1]. However, in contrast to the higher concentration of iron inside living cells (10^{-4} M) the solubilities of biologically relevant oxidation states of iron (Fe^{2+} $K_{sp} \sim 10^{-6}$, Fe^{3+} $K_{sp} \sim 10^{-18}$) are significantly lower, requiring biological enrichment processes through transport systems [1–4]. In order to avoid precipitation of iron inside the cell it is always sequestered by anionic metabolites (like citrate and phosphate) making the concentration of "free iron" extremely low [1–4]. As can be noted from Fig. 1, in addition to making iron more bioavailable, these inorganic anions/metabolites/proteins significantly alter the reduction potential of aqueous Fe^{2+}/Fe^{3+} couple. This redox modulation of iron is particularly significant since the introduction of O_2 during the great oxidation event (GOE), as the one-electron oxidation of Fe^{2+} to Fe^{3+} in contact with O_2 can create life threatening reactive oxygen species (ROS) in non-enzymatic steps [1–4].

This low solubility and toxicity of iron, despite its absolute requirement, created fascinating iron regulating mechanisms across all kingdoms of life. For example, in mammalian systems, most of the stored iron is recycled with a very small amount being replenished everyday through dietary sources [5–7]. Irrespective, all iron in mammals is tightly sequestered under normal conditions, and release of iron from iron storing cells into the circulation and its transport to the destination cells are regulated by interconnected signaling systems and redox processes [8,9]. Like their hosts, invading pathogens also require iron for survival and virulence, however, upon entering the host system they experience very low levels of this metal for uptake [8,9]. Further, iron concentration is lowered in mammalian host systems using a cascade of reactions that are collectively termed the anemia of inflammation [8–26]. Bacteria, on the other hand, use this low iron concentration as

Abbreviations: ROS, Reactive oxygen species; GOE, Great oxidation event; ET, Electron transfer; IM, Inner membrane; OM, Outer membrane; ILT, Iron and lead transporter; Fd, Ferredoxin; ORF, Open reading frame.

* Corresponding author at: Department of Chemistry, East Carolina University, 552 Science and Technology Building, Room 571, Greenville, NC 27858, USA.
E-mail address: banerjees17@ecu.edu (S. Banerjee).

https://doi.org/10.1016/j.jinorgbio.2022.111721
Received 25 September 2021; Received in revised form 4 January 2022; Accepted 6 January 2022
Available online 10 January 2022
0162-0134/

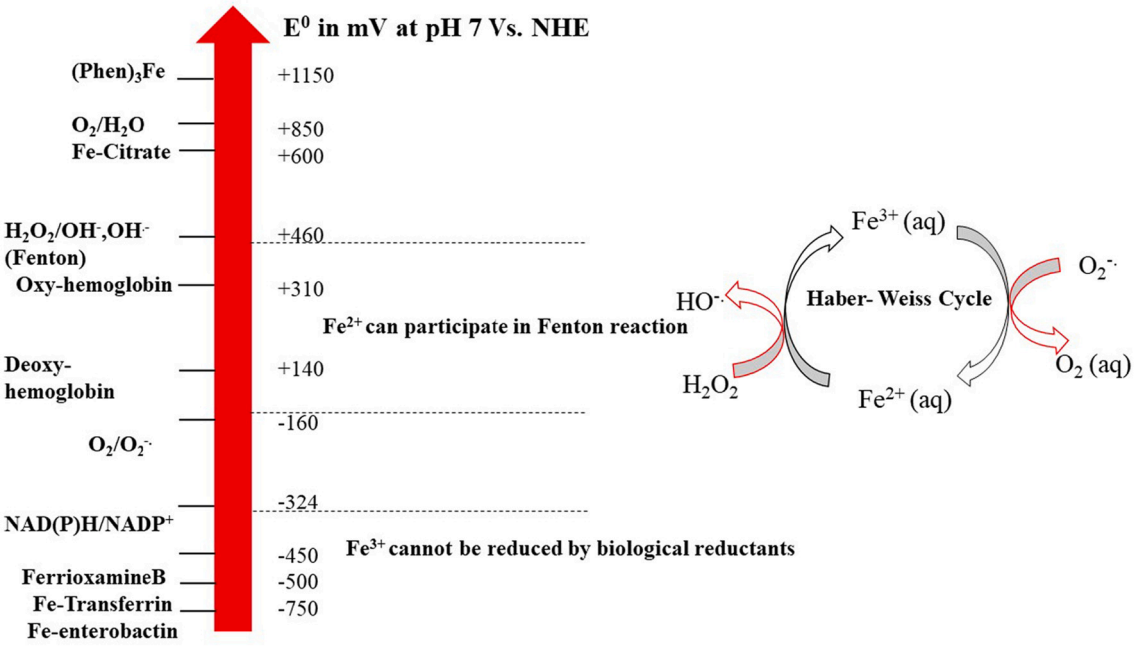

Fig. 1. Bioremediation of aqueous iron precipitation by sequestration of the metal with biological ligands. This reaction subsequently alters iron's redox potential. The dashed lines indicate regions where iron can take part in toxic ROS generating reactions under standard states (i.e. [oxidized O_2 species] = [reduced O_2 species]). However, biological systems seldomly represent standard states. Therefore, in order to accurately measure the reduction potential within a given system, the Nernst equation must be utilized [1–4].

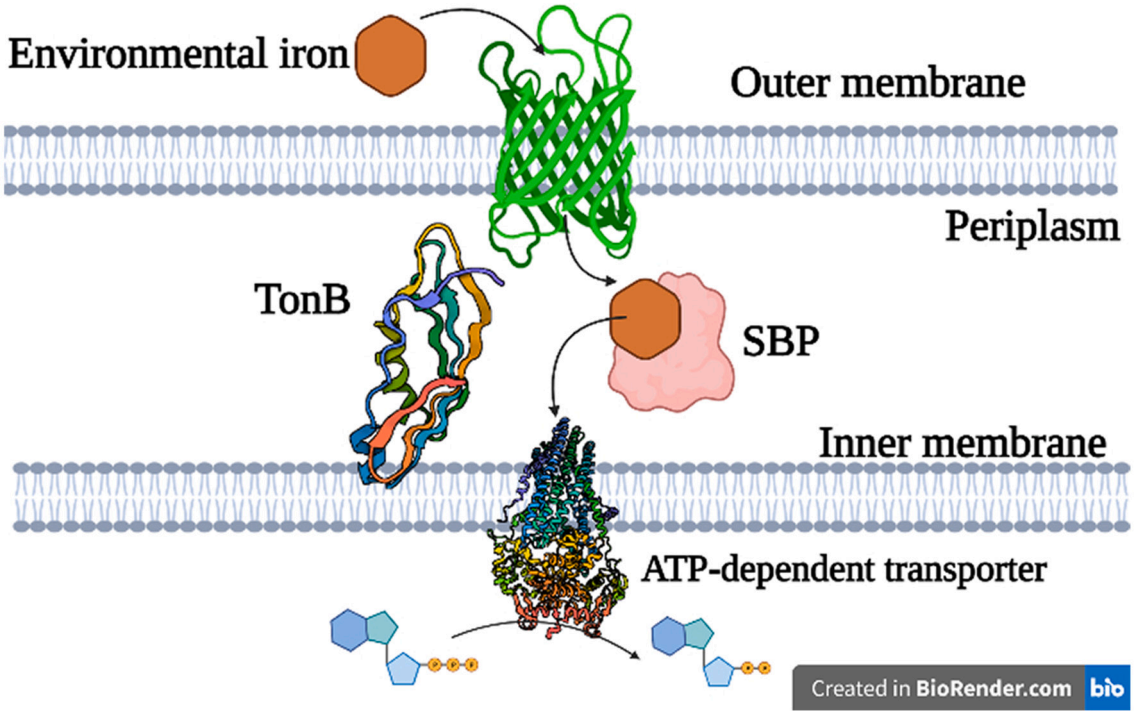

Fig. 2. Environmental iron is received (as Fe^{3+}-siderophore, heme, Fe^{3+} from Tf,) and transported into the periplasm via TonB-dependent outer membrane proteins. Within the periplasm this iron is bound to solute binding proteins (SBP), and then transported into the cytoplasm via ATP-dependent transporters [10–26].

an indication of host colonization therefore, upregulating the expression of dedicated iron uptake machinery [24–26]. It is important to note here that even environmental bacteria which do not grow inside a mammalian host (and do not encounter anemia of inflammation) still encounter low levels of bioavailable iron due to its presence as insoluble iron minerals [10–26]. Therefore, it is not surprising that certain iron uptake mechanisms in environmental and pathogenic organisms utilize similar strategies [24–26]. For example, both mammalian pathogens and environmental microbes produce small Fe^{3+} chelating organic molecules (siderophores), responding to iron limitation in their respective environments, and take up the Fe^{3+}-siderophore complex using dedicated outer membrane transporters [24–26]. Mammalian pathogens

encounter iron in diverse coordination environments, such as bound to proteins directly (transferrin, lactoferrin) or through a prosthetic group (heme containing proteins), as non-transferrin bound iron (NTBI), and Fe^{2+} in low pH and anaerobic/low oxygen conditions [10–26]. As these pathogens absolutely need iron for survival, they employ a plethora of high-affinity uptake systems to internalize any and all forms of this metal that they are exposed to [24–26]. Finally, pathogens often express multiple iron-uptake mechanisms (heme, Fe^{3+}-siderophore, Fe^{2+}) at different stages of infection depending on the changes in host physiology [10–26].

As these iron transporters are essential for the survival of the pathogens inside the hosts, several such uptake systems have been extensively studied and completely characterized. Despite the varied nature of the cargo which these transporters can utilize, these transporters (in Gram negative pathogens) show a basic functional similarity (Fig. 2). The ligand (as Fe^{3+}, Fe^{3+}-siderophore, and heme) enters the periplasm using cognate Ton dependent outer-membrane receptors and is then trafficked to the cytosol using the soluble component of the ATP Binding Cassette (ABC)-transporters (known as periplasmic binding proteins or PBP) and is finally internalized in the cytosol utilizing ATP hydrolysis derived energy [18–23]. Transport of soluble Fe^{2+} across the outer membrane of Gram-negative pathogens, however, does not require any receptor, but its transport into the cytosol takes place using cognate Fe^{2+} transporting ABC-transporters (such as, Feo) [23–26].

In the last couple of decades, a new class of iron transporters (Ftr-type) have been described in both prokaryotic and eukaryotic systems [27–43]. These membrane transporters/permeases are iron inducible, are predicted to form seven trans-membrane α-helices, and all conserve two ArgGluxxGlu (x = any amino acid) motifs. The best characterized of these Ftr-type transporters (Ftr1p) is from yeast and requires a multi-copper ferroxidase (Fet3p) to be functional [35–40]. Based on the experimental findings, the functional mode of this Ftr1p-Fet3p involves the capturing of Fe^{2+} by conserved acidic residues of the ferroxidase Fet3p followed by its oxidation by a Cu from this multicopper oxidase [35–40]. Experimental data shows that this oxidation is coupled with transport of Fe^{3+} through the permease, Ftr1p [35–40]. The conserved ArgGluxxGlu motifs on Ftr1p are required for Fe^{3+} transport and based on the presence of acidic residues are proposed to coordinate to the metal during its translocation to avoid toxicity [35–40]. A similar acidic amino acid rich motif (GluIleGluTyrGlu) conservation is also observed in the plug domain of the outer membrane transporter, TbpA, a Fe^{3+} transporter found in all *Neisseria* species [41]. In vitro studies on the wild-type plug domain of TbpA show direct coordination of Fe^{3+} to this motif [41].

Similar Ftr-type transporters are also observed in several bacterial Gram-negative pathogens (EfeUOB and FetMP from *E. coli*; P19cFtr from *Campylobacter jejuni*; and FtrABCD from *Brucella*, *Bordetella* and *Burkholderia* spp.) [27–34]. In these systems, the inner-membrane permeases (EfeU, FetM, cFtr, and FtrC) are homologous to the yeast Ftr1p membrane permease, conserve the ArgGluxxGlu motifs, and are iron inducible [35–38]. Despite these similarities, none of the bacterial Ftr-systems co-express a multicopper ferroxidase, similar to Fet3p [27–34]. The essential role that the ferroxidase, Fet3p plays in iron utilization in yeast, and the observation that the bacterial systems lacking a ferroxidase are functional suggest either that a) Ftr-type transporters in bacteria can operate without a ferroxidase, or b) there is an uncharacterized redox protein associated with these bacterial Ftr-type transporters [39,40]. Interestingly, a recent bioinformatics study has predicted the *E. coli* EfeO and the *Brucella*, *Bordetella*, and *Burkholderia* FtrB as novel functional cupredoxin homologs, based on a common phylogenetic origin and conservation of acidic residues (which can coordinate to metals) [42]. However, this claim has not been experimentally verified and remains controversial. The controversy arises from the fact that none of these novel cupredoxins contain residues that can coordinate Cu in Type-1 site [43]. Cupredoxins are small single domain electron transport (ET) proteins which have been extensively studied and contains conserved HisHisCys residues acting as Cu ligand in the so-called Type-1 site. Experimental work has shown that in addition to coordinating to the Cu metal, these conserved residues also play crucial roles in the ET process [43] Even if EfeO and FtrB are experimentally shown as novel cupredoxins, the other bacterial Ftr-type permeases (FetM and cFtr) lacking any characterized ferroxidase/cupredoxin will indicate a different model of iron utilization by these transporters [29,30]. To further complicate matters, the existing experimental data for these bacterial Ftr-type transporters are also often confusing, as there is no consensus of the oxidation state of the ligand transported through these systems [27–34].

In this article we use the FtrABCD system to review all known bacterial Ftr-type transporters (FtrC, FetM, cFtr, EfeU) because of the uniqueness of this system [32–34,44]. This four-component Ftr homolog contains a periplasmic iron binding protein (FtrA) that is common between this and the FetMP and P19cFtr systems [32–34,44]. In addition, this is one of the two bacterial Ftr-type transporters that contain a predicted and uncharacterized cupredoxin, FtrB, as well as a polyferredoxin, FtrD, predicted to act as an electron sink [32–34,42] As a result, this four-component system contains all diverse proteins possible for bacterial Ftr-type transporters and is a good functional model for these systems. At the end of this article, we also provided an alternative evolutionary functional model for iron uptake using FtrABC systems, found in several genomes from prokaryotic species, that do not contain the terminal electron acceptor, FtrD.

2. Brief overview of bioinorganic chemistry of ferroxidases and cupredoxins

The essential role that iron plays in biological systems since the beginning of life is not surprising given the high abundance of this metal on earth's crust and the ease of tuning $Fe^{2+}/^{3+}$ redox potential in biological ligand environments (Fig. 1) [45–47]. However, since the introduction of molecular oxygen through GOE, oxidizing soluble Fe^{2+} to insoluble Fe^{3+}, making Cu^{2+} available for biological processes, and the non-enzymatic production of ROS from O_2 with these metals required tight homeostatic control over these biological elements [45–47]. Not surprisingly, Kosman et al have termed the evolutionary processes since the GOE that protect living organisms from the toxic effects of iron, Cu, and O_2 as the "fundamental theme of aerobic life" [40]. One such fundamental detoxification steps involve enzymatically oxidizing four Fe^{2+} ions (to Fe^{3+}) with the help of Cu^{2+} containing enzymes and concomitant four electron reduction of O_2 to water (avoiding ROS production) as seen in reaction scheme 1 (Fig. 4). The biochemical advantage of reaction 1 is that it releases energy as Free energy ($\Delta G = -nFE_{cell}$, where n is the number of electrons involved in the redox process, F is the Faraday constant, and E_{cell} is the potential for the total redox reaction) and this can be used for physiological functions. This class of enzymes that couple Fe^{2+} oxidation with four-electron reduction of oxygen to water, and protect the organism from generation of ROS, is collectively known as ferroxidases.

3. Reaction scheme 1:

$$4Fe^{2+}\ (\text{bioligand}) + 4Cu^{2+}\ (\text{bioligand}) \rightarrow 4Fe^{3+}\ (\text{bioligand}) + 4Cu^{+}\ (\text{bioligand})$$

$$4Cu^{+}\ (\text{bioligand}) + O_2\ (\text{aq}) + 4H^{+}\ (\text{aq}) \rightarrow 4Cu^{2+}\ (\text{bioligand}) + 2H_2O\ (l)$$

Ferroxidase enzymes show β-sandwich folding and contain three distinct Cu ion coordination sites (Fig. 4) (Type-1 through 3) characterized by their unique spectroscopic and electrochemical properties (Fig. 4) [43]. TheType-1 and -2 sites in ferroxidases are mononuclear and contain Cu^{2+} ions in conserved HisHisCys and HisHisTyr/Glu primary coordination shell environments, respectively (Fig. 5) [43]. On the other hand, the Type-3 site, is dinuclear and contains an exogenous O_2 as

Table 1
Compilation of the available bond distance data for oxidized and reduced cupredoxins and their reduction potential data [49–51].

Protein name	Redox partner	Cu Type	Cu-N3 (His46) Å	Cu-S (Cys112) Å	Cu-N3 (His117) Å	Cu-S (Met121) Å	PDB code	E^0 (V vs NHE) (pH)
Azurin	CytochromeC	Type-1						0.310 (7.5)
Cu^{2+}			2.08	2.24	2.01	3.15	4AZU	
Cu^{+}			2.14	2.29	2.01	3.25	1E5Y	
Rusticyanin	Fe^{2+}	Type-1						0.680 (2.0)
Cu^{2+}			2.04	2.26	1.89	2.88	1RCY	
Cu^{+}			2.22	2.25	1.96	2.75	1A3Z	
Plastocyanin	CytochromeF	Type-1						0.380 (7.5)
Cu^{2+}			1.91	2.07	2.06	2.82	1PLC	
Cu^{+}			2.13	2.17	2.39	2.87	5PCY	
hCp [51]	Fe^{2+}	Type-1A/B	–	–	–	–	–	0.448

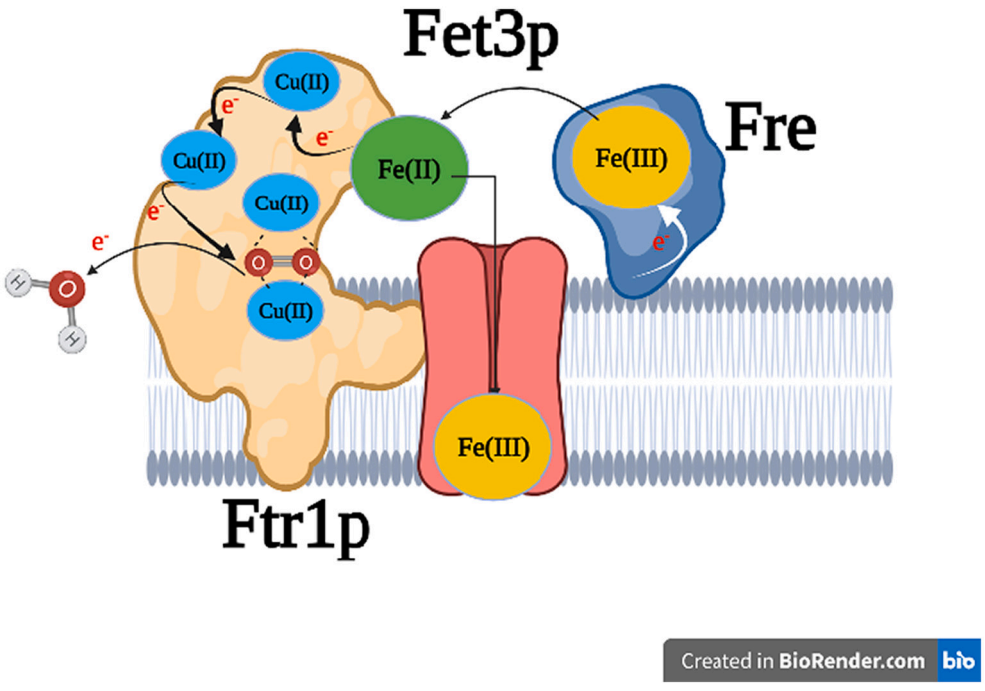

Fig. 3. A schematic representation of yeast Fet3p-Ftr1p-mediated Fe^{2+} uptake. Ftr1p (in orange) is an integral membrane transport protein, whereas Fet3p is a multi-domain MCO (only one domain with three different Cu^{2+}-binding sites are shown). Fe^{2+} (in green) produced by the membrane attached proteins Fre 1, 2, 3, and 4 (not shown in the figure) is sequestered by the MCO, Fet3p close to the type-1 ET site (described by a Cu^{2+} bound by conserved HHC residues) and is oxidized to Fe^{3+} (in yellow) and is transported through Ftr1p. The reduced type-1 Cu site is regenerated by transferring the harvested electrons through type-2 (mono-nuclear) and type-3 (di-nuclear) Cu^{2+} ions and finally reduces O_2 into water. There are several other proteins, such as the extracellular Fre that reduce environmental Fe^{3+} to Fe^{2+} prior to its reoxidation and transport by Ftr1p. Additionally, this iron transport being dependent on an MCO, also relies on Cu transporters, Ctr1p and Ccc2p, and Cu chaperone protein, ATX1p (not shown in the figure). Once inside the cell, the iron is reduced back to Fe^{2+} for its utilization [35–40]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a bridging ligand, in addition to conserved residues coming from the protein structure (Fig. 5) [43]. These unique primary coordination shell environments around the Cu ions as well as the secondary coordination shell effects contribute to their biochemical and physiological functions [48–53]. For example, the strong S(Cys)π-Cu^{2+} (d_{x2-y2}) (from the coordinating Cys) bond in the Type-1 site gives this Cu an intense blue colour and the His and Cys from the primary coordination shell of this site and other essential amino acids from the secondary coordination shell ensure unidirectional electron transport (ET) from Fe^{2+} to the Type-1 Cu (Fig. 4) [48–53].

Examples of ferroxidases that are linked with iron transport are human ceruloplasmin (hCp), and yeast Fet3p. Both proteins contain a specific Fe^{2+} binding site close to the Type-1 Cu and relay electrons through the "biological wire" created through Type-1, Type-2, and Type-3 electron channel (Fig. 4) [48–53]. Similar ET is also observed in single domain proteins containing only Type-1 Cu and are known as cupredoxins [43,48–53]. Unlike the ferroxidases, cupredoxins do not contain the Type-2 and 3 Cu sites and the oxidation of the reductants (Fe^{2+}, other organic metabolites) are not dependent on O_2 reduction [43,48–53]. Lacking the Type-2 and 3 Cu centers, the ET circuit from the reductant to the Type-1 Cu is closed by the involvement of an oxidizing partner (such as Cytochromes, Table 1) which re-oxidizes the Cu ion in the Type-1 site (Fig. 5) [43,48–53]. Although cupredoxins, containing only a Type-1 site, are much smaller than ferroxidases, the primary coordination sphere as well as the structural fold of the Type-1 sites in these two proteins are conserved [43,48–53]. For example, the coordinated Cu ion in the Type-1 sites of these proteins utilize the same amino acid triad, HisHisCys, (with the fourth and axial ligands differing based on the identity of the reductant) and the metal ion is present in a distorted tetrahedral geometry [43,48–53]. This distorted geometry around the Cu ion in the Type-1 site has been attributed to fast ET kinetics due to minimal bond reorganization (Table 1) between the reduced (Cu^{+}) and oxidized (Cu^{2+}) enzymes [43,48–53]. Table 1). Table 1 also provides reduction potentials of Type-1 Cu from different proteins and we note here that apart from rusticyanin, most Type-1 Cu redox potentials fall within a similar range.

The short description above on the bioinorganic chemistry of ferroxidase and cupredoxins show despite the differences in enzyme size, the number and types of Cu ions coordinated in each enzyme, and their ligand specificity the Type-1 Cu sites in these proteins show similar spectroscopic and electrochemical behavior [43,48–53]. These are maintained by the tight conservation of the HisHisCys triad for Cu binding in the Type-1 site as well as the β-sandwich fold that make fast ET reactions possible [43,48–53]. The Ftr system from yeast (Ftr1p) requires a ferroxidase (Fet3p) for transporting iron [35–40]. This being the only characterized Ftr permease in both eukaryotes and prokaryotes,

Table 2
Ftr-type transporters and their associated proteins from eukaryotic and prokaryotic systems.

Name of Ftr-type permease	Organism	Proteins co-expressed (function)	Periplasmic domain	Fe^{2+} oxidation experimentally demonstrated
Ftr1p	Yeast	Fet3p (ferroxidase)	No	Yes
cFtr	*Campylobacter jejuni*	P19 (binds Fe^{3+}) [29–31]	Yes	No
FetM	*E. coli*	FetP (ferrireductase) [29–31]	Yes	No
EfeU	*E. coli, S. subtilis*	EfeO (proposed cupredoxin) [42] EfeB (heme oxidase, deferrochelatase, terminal electron acceptor) [66]	Yes	No
FtrC	*Brucella abortus, Bordetella pertussis, Burkholderia* spp.	FtrA (Binds Fe^{2+}) [71] FtrB (proposed cupredoxin) [42] FtrD (proposed polyferrodoxin) [32–34]	No	No

The table also lists the proposed/demonstrated function of the associated proteins.

the questions that is all Ftr systems require a ferroxidase and is redox dependent are under investigation [28–34]. None of the bacterial Ftr (FetM, cFtr, EfeU, and FtrC) co-express a ferroxidase and there is no experimental evidence so far that these involve a redox reaction for their transport properties [28–34]. In the following sections we will review iron transport through yeast Ftr1p followed by different bacterial Ftr permeases to gain further understanding of this permease and its dependence on the redox step.

4. Ferroxidase dependent iron transport by yeast Ftr1p

As previously mentioned, Ftr-type membrane permeases that transport iron are found in both eukaryotic and prokaryotic systems [27–40]. These transporters are expected to form trans-membrane α-helical domains and are required for iron transport in organisms in which they are found. Of all Ftr-type transporters, the one from yeast, Ftr1p, is well characterized and in this section, we will review iron transport by the eukaryotic Ftr system [27–40]. The yeast Ftr1p mediated iron transport requires the expression of the multicopper ferroxidase, Fet3p [35–38]. Similar oxidation coupled iron transport through Ftr1p-Fet3p type transporters are reported in numerous fungi [35–38,54–57] and notably, these transporters are essential for the wild-type virulence of *Candida albicans* and *Rhizopus oryzae* in mice [58,59,60]. The membrane permease, Ftr1p, from the yeast system consists of seven α-helices with the N-terminus oriented at the external face of the plasma membrane. Residues from this exocytoplasmic domain of Ftr1p (Asp246 and Glu249) from this exocytoplasmic domain of Ftr1p directly interact with Fet3p, and work in concert with glutamate residues in membrane spanning region 3 (Glu85 and Glu89) as well as with arginine and glutamate residues in membrane spanning region 4 (Arg157 and Glu161) of the permease to form a channel that directly transports the Fe^{3+} [35–38]. This oxidation occurs coincident with the transport of Fe^{3+}, as exogenous Fe^{3+} in *fet3p* mutants cannot transport this metal. The benefits of this oxidation-dependent Fe transport are: a) it 'traps' insoluble Fe^{3+} in the transporter by a mechanism analogous to the process of substrate channeling which has been described for some enzymatic complexes [54], and b) by oxidizing the toxic Fe^{2+} before its transport, this mechanism protects the transporter from the detrimental effect of ROS that can be produced by Fe^{2+}. This is the best characterized Ftr mediated iron transport and provide a novel functional model of iron transport through all Ftr-transporters (Fig. 3) [54,55,59,60]. We caution our readers here that although this has been accepted as a model for iron transport in all Ftr-type permeases, the lack of biochemical data showing redox dependence and absence of ferroxidases in bacterial Ftr transporters have made this model controversial.

The multicopper ferroxidase is a multidomain protein and the domain arrangement of different Cu sites in Fet3p is presented in Supplementary Fig. 2. As usual, the Type-1 Cu is coordinated (at domain 3) using the conserved HisHisCys residues and the trinuclear Cu cluster (located in the interface of domain 1 and domain 3) shows the usual Type-2 and 3 Cu coordination (Fig. 5) with an exogenous O_2 bound to the Type-3 site. Close to the Type-1 site in Fet3p, a Glu185 and two Asp283 and Asp409 residues are conserved. These residues have been attributed to iron coordination [39,40,54,55]. Kinetic data on wild-type and Glu185, Asp238, and Asp409 mutants confirm that Asp409 and Glu185 residues take part in Fe^{2+} and electron transfer between the Fe^{2+} and the Cu^{2+} in the Type-1 site [39,40,54,55]. In addition, the Nε2 pyrrole NH groups from the coordinating His489 and His413 in the Type-1 Cu site show strong H-bond interaction with the carboxylate groups in Asp409 and Asp238, and experimental data indicate that these H-bonds take part in outer-sphere electron transport between the coordinated Fe^{2+} and the Type-1 Cu^{2+} center [39,40,54,55]. The above discussion on amino acid conservation and function of Fet3p show that in addition to conserving the Type-1 residues, for the ET to take place, it is also required to conserve acidic residues close to the Cu-binding site which can coordinate Fe^{2+} as well as take part in outer-sphere electron transport. In addition to its primary function as a ferroxidase, Fet3p also has cuprous oxidase activity which is experimentally shown to protect *Saccharomyces cerevisiae* from Cu toxicity [64].

Current functional mode of Ftr1p-Fet3p mediated Fe^{2+} utilization includes environmental Fe^{3+} reduction by ferrireductase proteins (Fre) at the extracellular environment before its capture [62,63], oxidation by Fet3p, and transport of Fe^{3+} through Ftr1p. Other cellular proteins that play important roles in Fet3p/Ftr1p are Cu transporters Ctr1p and Ccc2p and Cu chaperone ATX1p. These proteins are critical for Fet3p assembly and transport to the plasma membrane [61]. The ferric reductases Fre1, Fre2, Fre3 and Fre4 also allow Fet3p/Ftr1p to play a role in the acquisition of free Fe^{3+} or Fe^{3+} complexed with a variety of siderophores, in addition to its role in the direct transport of Fe^{2+} [62,63].

As mentioned already, Ftr-type permeases are also observed in prokaryotes and in the following sections we will review published experimental data bacterial Ftr-proteins and associated components [27–35,44].

5. General discussion on bacterial Ftr-type membrane permeases

In recent decades, several Ftr1p homologs have been identified in bacterial systems (*E. coli* FetMP and EfeUOB; *C. jejuni* P19cFtr; and *Brucella*, *Bordetella*, and *Burkholderia* FtrABCD) (Fig. 6) [27–35,44]. Similar to the yeast Ftr1p permease, these bacterial membrane permeases are iron regulated, take part in iron uptake, and are predicted to form trans-membrane α-helices, like Ftr1p [27–35,44].These also conserve the ArgGluxxGlu (where x can be any amino acid) motifs, like the yeast permease [27–35,44]. As discussed, these motifs from the intermembrane section of Ftr1p are essential for Fe^{3+} transport using the ferroxidase dependent transporter in yeast, and the conservation of the same motifs in the bacterial Ftr permeases led to the prediction that a) these also transport Fe^{3+}, and b) require an oxidation step that coincides with Fe^{3+} transport [34–40]. Conservation of important trans-membrane motifs and the overall secondary structure between these bacterial Ftr permeases and Ftr1p from yeast further supports a similar

Table 3
ITC and DSC data on recombinant *Brucella* FtrA (wild-type and Cu^{2+} binding mutants) compiled with similar ITC data on *E. coli* FetP [30,71].

Recombinant protein name (buffer)	Organism	pH	Metal titrated	N	K_d (μM)	ΔH (kcal/mol)	ΔS (cal/molK)	T_m (°C)
As isolated WT-FtrA (25 mM ACES) [71]	*Brucella abortus*	6.3	None	N/A	N/A	N/A	N/A	75, 84
As isolated WT-FtrA (25 mM ACES) [71]	*B. abortus*	7.3	None	N/A	N/A	N/A	N/A	74, 84
WT-FtrA (25 mM ACES) [71]	*B. abortus*	7.3	Cu^{2+}	0.50	5.3	−3.9	11.05	76, 83
WT-FtrA-Cu^{2+} (25 mM ACES) [71]	*B. abortus*	6.3	Mn^{2+}	0.51	20.0	−1.2	17.5	83, 86
WT-FtrA-Cu^{2+} (25 mM ACES) [71]	*B. abortus*	7.3	Mn^{2+}	1.1	8.4	+0.4	24.6	85, 87
H65A FtrA (25 mM ACES) [71]	*B. abortus*	7.3	Cu^{2+}	0.70	7.6	−4.1	9.6	73, 80
E67A FtrA (25 mM ACES) [71]	*B. abortus*	7.3	Cu^{2+}	1.40	2.05	−5.3	8.4	78, 80
WT-FetP (25 mM Bis-tris) [30]	*E. coli*	7.2	Cu^{2+}	0.915	4.1	−2.18	17.3	Data not available
WT-FetP-Cu^{2+}* (25 mM Bis-tris) [30]	*E. coli*	7.2	Mn^{2+}	PE	PE	PE	PE	Data not available

The DSC data for WT-FtrA show progressive stabilization as the protein binds to metals, which is absent for the mutants, H65A, E67A, although those showed WT Cu^{2+} affinity. None of the FtrA mutants showed Mn^{2+} affinity, and showed lower folding stability indicating loss of function [71]. PE on the table indicates positive enthalpy and the authors did not report any numerical value for the thermodynamic parameters [30].

iron translocation model [27–35,44]. However, some bacterial Ftr-permeases (FetM and cFtr) are considerably longer than the yeast permease and contain a periplasmic domain (PD) which has recently been shown to interact with these system's respective periplasmic proteins [31]. More importantly, although the bacterial Ftr permeases are found as two-, three-, and four-component systems, none contain a ferroxidase that can carry out the expected oxidation [27–35,44]. A recent bioinformatics study has predicted the existence of two novel cupredoxin family of proteins from the EfeUOB and FtrABCD systems which contain uncharacterized Type-1 Cu sites that are not described by the classical HisHisCys residues [42]. These uncharacterized cupredoxins are predicted to carry out the ET reaction in EfeUOB and FtrABCD systems [42], however, there is no experimental data available to date that confirms these claims. Finally, the other two bacterial Ftr-type permease containing systems (FetMP and P19cFtr) does not contain any putative ET proteins and experimental data so far did not show any redox dependence for ligand transport [29–31]. In the following sections, we discuss the available data on these bacterial Ftr-type permeases containing iron transporters. For comparison of all Ftr-type transporters please refer to Table 2 which compiles different protein components from these systems, their (putative) functions, and roles in iron transport through these transporters.

5.1. Two-component bacterial Ftr homologs (FetMP, P19cFtr)

The P19cFtr system is found in *C. jejuni*, an enteric bacterium that resides in the O_2 deplete environment of the gastrointestinal tract [29,31]. This iron-uptake system in *C. jejuni* is Fur (ferric uptake regulator) regulated and the expression of this transporter is upregulated during human infection [29,31,65]. cFtr is the yeast Ftr1p homolog (24% sequence identity) containing the conserved and putative Fe^{3+} binding motif ArgGluxxGlu [29,31]. This inner membrane permease is expected to form trans-membrane α-helices like Ftr1p, however, in contrast to the yeast permease, cFtr contains an additional (~300 amino acid long) PD (Fig. 6) [31]. In a recent study it was shown that the PD from cFtr can recognize and interact with its cognate periplasmic protein, P19 [31]. A similar PD is also found in another bacterial Ftr1p homolog, FetM (from *E. coli*) [31]. FetM shows 30% overall identity with cFtr and shows a soluble ~300 amino acid long PD (Fig. 6), conserves the ArgGluxxGlu motif, and is expected to form trans-membrane α-helices [30,31]. Similar to cFtrPD, the FetM-PD can recognize its cognate periplasmic protein, however, cross-interaction between the periplasmic domains with their non-cognate periplasmic proteins was not observed [31]. Although the exact biological significance of these interactions are yet to be identified, the proposed model of function for these proteins requires the periplasmic protein to interact with their cognate membrane transporters (Fig. 6) [30,31].

5.2. Fe^{2+} transport through FetMP system

As mentioned, FetMP is Fur regulated and takes part in iron transport [30]. FetM, the inner-membrane permease is essential for iron translocation through this system and is part of the divalent iron and lead transporter (ILT) superfamily [30]. On the other hand, the periplasmic FetP enhances transport through this Fet system however, FetM can still transport iron without FetP being present [30]. Although the wild-type *E. coli* (ECA612) and a single copy of *fetMP* complimented strain (ECA458) showed similar growth advantage compared to negative

Table 4
Representative bacteria that contain all FtrABCD genes (Category 1) and FtrABC (Category 2).

Category-1			Category-2		
Bacterial genera (With FtrA, FtrB, FtrC, FtrD)			Bacterial genera (With FtrA, FtrB, FtrC)		
Species	KEGG Code	Genera	Species	KEGG Code	Genera
Brucella abortus	bmf	Alpha	*Morganella morganii*	mmk	Gamma
Iodobacter sp. *H11R3*	iod	Beta	*Oligella urethralis*	our	Beta
Pandoraea pnomenusa 3kgm	ppk	Beta	*Martelella endophytica*	mey	Alpha
Burkholderia mallei ATCC 23344	bma	Beta	*Proteus mirabilis HI4320*	pmr	Gamma
Collimonas fungivorans	cfu	Beta	*Providencia stuartii MRSN 2154*	psi	Gamma
Paraburkholderia aromaticivorans	parb	Beta	*Vibrio mediterrane*	vsh	Gamma
Chromobacterium sp. *ATCC 53434*	chro	Beta			
Herbaspirillum seropedicae	hsz	Beta			
Bordetella pertussis CS	bpc	Beta			
Achromobacter xylosoxidans	axn	Beta			
Azospira oryzae	dsu	Beta			
Rubrivivax gelatinosus	rge	Beta			

The table also includes the KEFF code and the Genera of the bacteria that contains these genes and shows FtrABCD are widespread in all bacterial genera. Several of these species also appeared to contain other bacterial permeases (cFtr, FetM) genes.

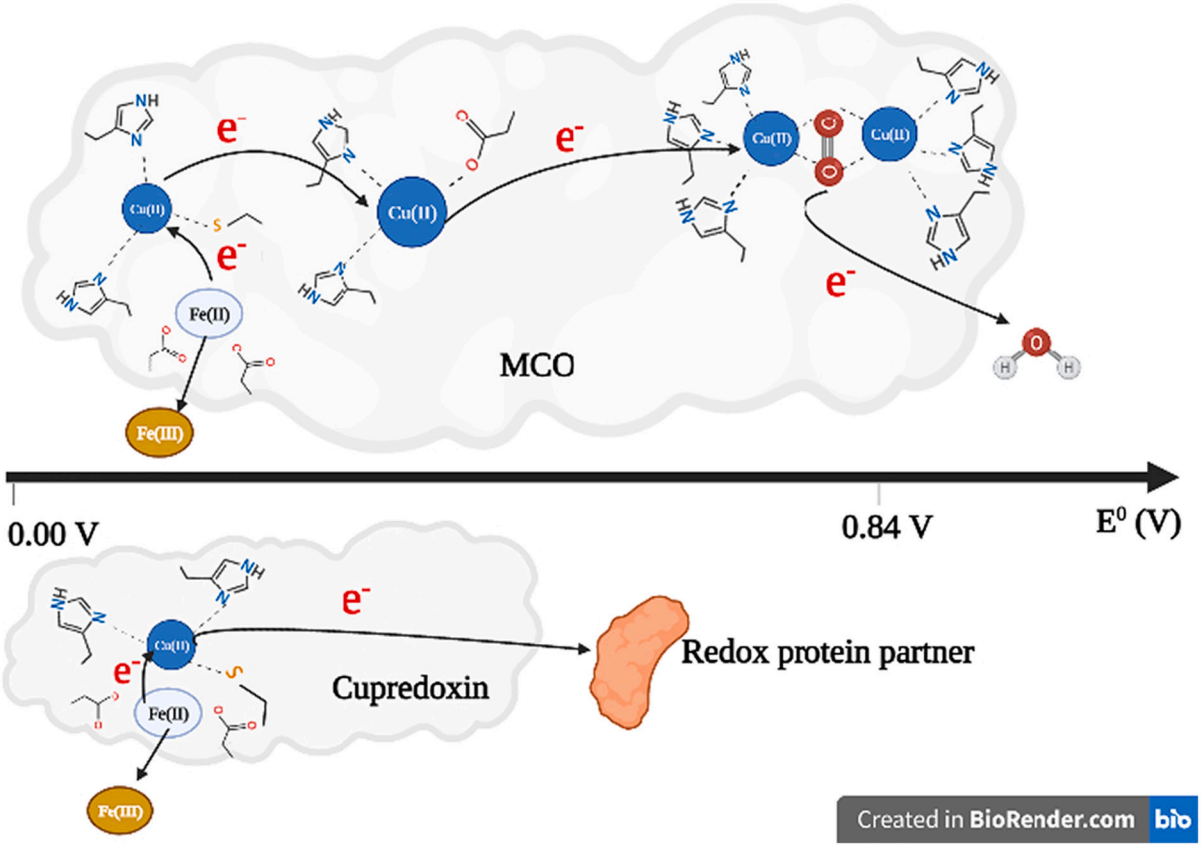

Fig. 4. Mechanism of electron transfer (ET) from Fe^{2+} through the Type-1 Cu site in MCO to O_2 (upper panel) and through a cupredoxin to a redox partner protein (lower panel). The scale in the middle shows a relative value of reduction potentials for Type-1 Cu^{2+} site in MCOs and cupredoxins relative to the four-electron reduction of O_2 to water. As can be noted, the MCOs require O_2 to function whereas there is no direct O_2 consumption for cupredoxin-based ferroxidases [43].

controls in iron limited medium, however, it is important to note, the control strains lacking all known Fe and Mn transporters from *E. coli* (*ΔfecABCDE*, *ΔfeoABC*, *ΔmntH*, *ΔzupT*) did not show complete attenuation [30]. The authors interpreted this as the presence of an unknown iron transporter in *E. coli* [30]. Based on the protein family designation of FetM (ILT), which is different from the oxidase dependent ferrous iron transporters (OfeT), this system is predicted to directly transport Fe^{2+} without requiring a ferroxidase step, as observed in the yeast system [30,31]. This prediction is further experimentally validated by the observation that *E. coli* containing both *fetMP* genes (compared to strain only containing *fetM*) showed ferrireductase activity which was not present when Ag^{+} (a redox inactive Cu^{+} mimic) was added as the substrate to the growth media [30,31]. This indicates that FetP is similar to the Fre ferrireductase found in the yeast system (Fig. 3) which reduces environmental Fe^{3+} prior to its utilization by the Fet3pFtr1p system [30,31] [35–38]. Further, similar to the Fre proteins from yeast, FetP is also not essential for iron transport through FetM, indicating this reduction can take place using other mechanisms. X-ray crystal structure of wild-type FetP shows the presence of a Met rich region (able to coordinate Cu^{+}) next to the Cu^{2+} coordination sites (described by HisHisHisGlu/HisHisHisMet) in the homodimeric FetP. Additionally, X-ray structure also show the Cu^{2+} in a tetrahedral geometry which is known to stabilize the Cu^{+} oxidation state. The identity of the metal coordinating residues and the geometry of the metal were taken as indirect evidence for the ferrireductase function of FetP [29–31]. Although these studies strongly suggest ferrireductase property of this protein, direct biochemical evidence of Fe^{3+} reduction by wild-type FetP is not available. Future biochemical experimentation with wild-type and mutant FetP (lacking the putative Cu^{+} coordinating Met residues) need to be undertaken for further clarification.

With this description of the FetMP system as a Fe^{2+} transporter, the conservation of the ArgGluxxGlu motifs on the permease (which bind to Fe^{3+} in the yeast Ftr1p) seems redundant. Although the Glu residues in these motifs would prefer Fe3+, however, to avoid toxicity and lower solubility of Fe^{2+} (compared to its intracellular concentration), these residues can still provide transient coordination site for the metal. Finally, more experimental work needs to be performed with ArgGluxxGlu mutants on FetM to identify any growth defects in *E. coli* strains containing these mutations.

5.3. Fe^{3+} transport through P19cFtr system

P19 is the periplasmic protein from the P19cFtr system and based on the conservation of the Met86xMet88 (x = any amino acid) motif was predicted to be primarily a Cu^{2+} binding protein, like Cu homeostasis proteins, CopC and CusF [29]. However, experimental data shows that it is required for growth under iron starvation and has been proposed to sequester Fe^{3+} from the fungal siderophore rhodotorulic acid [29,65]. The as-isolated P19 crystal structure shows homodimer formation and Cu^{2+} bound in each monomer in a HisHisHisMet coordination site [31]. On the other hand, the Cu^{2+} reconstituted P19 structure shows Cu^{2+} density in two coordination sites, HisHisHisGlu and HisHisHisMet, indicating flexibility of the Cu^{2+} ligands [29,31]. P19 (and FetP) shows Cu^{2+} dependent Mn^{2+}, Fe^{2+}, and Fe^{3+} affinity in X-ray crystal structure and ITC studies (Table 3) [29–31]. The crystal structures in the presence of $Fe^{2+}/^{3+}/Mn^{2+}$ show the Cu^{2+} coordinating Glu residue exclusively coordinated to $Fe^{2+}/^{3+}/Mn^{2+}$ (see the section on FtrA for more on $Mn^{2+}/Fe^{2+}/^{3+}$ binding to P19 proteins). The authors of these works have hinted to the possibility of a transient carboxylate bridge formation connecting the Cu and Fe ions, which can create an inner sphere electron

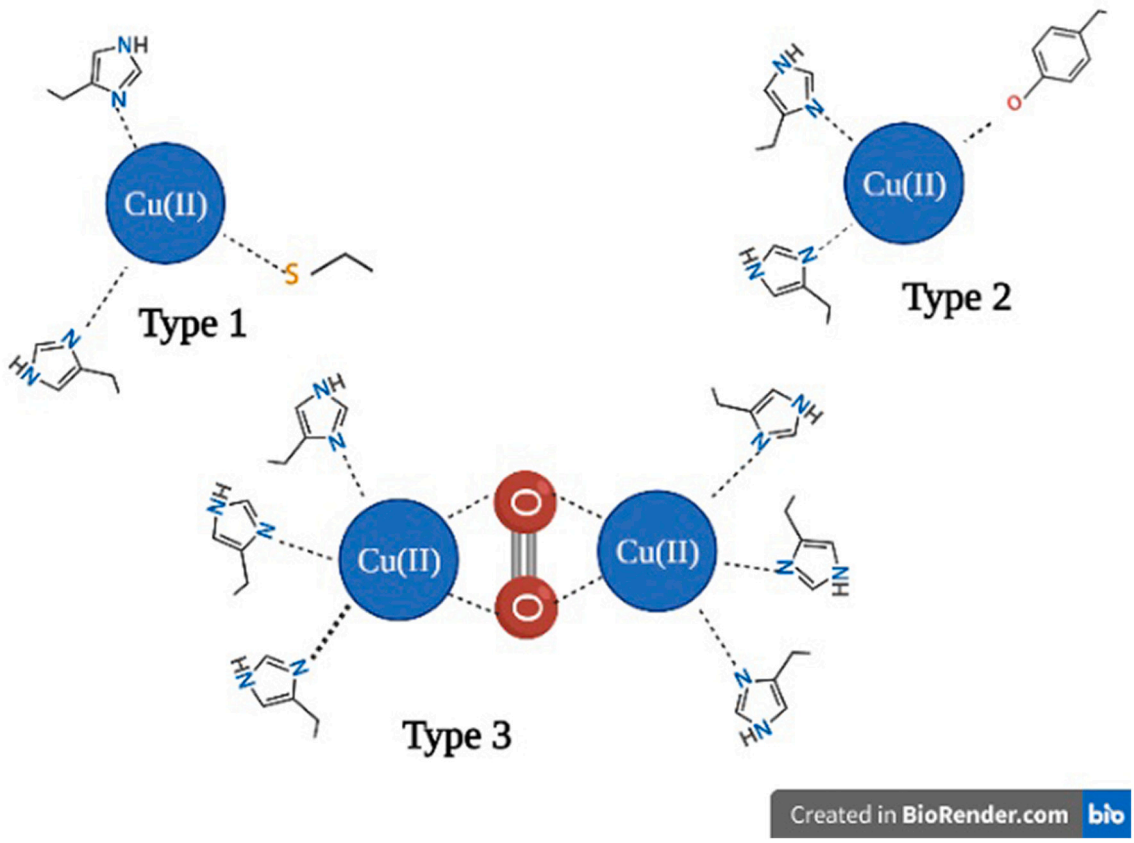

Fig. 5. The conserved inner coordination shell for Type-1, 2, and 3 Cu sites in MCOs. These amino acid residues along with the non-covalent interactions between the amino acids in cupredoxins containing these sites provide characteristic enzymatic, electrochemical, and photophysical properties [43,48].

transfer pathway between the metals [29–31]. However, no experimental evidence is available for such inner-sphere ET using this conserved Glu or the effect of its mutation in the cell growth studies.

The fact that the Fe binding site in P19 is located at the base of an acidic channel, as observed in Fet3p, has been taken as an indication for the ability to undergo redox reactions in P19 proteins [31]. The authors further argued that P19, having a flat structure, can accommodate an uncharacterized redox partner close to its metal binding sites and facilitate the Cu^{2+} mediated Fe^{2+} oxidation. However, this proposed ferroxidase property of P19 has not been experimentally validated [31].

A recent work by Murphy et al showed genes 1649 and 1650 (encoding the cFtr and P19, respectively) in *C. jejuni* 81–176 are followed by downstream (1651–1655) iron-regulated genes in the same open reading frame which are required for growth of this organism using the P19-cFtr system [65]. Similar genes downstream to *fetMP* are also found in *Yersinia pestis* and several other pathogenic bacteria and encode putative ATPase and thioredoxin proteins. Interestingly, the gene 1651 from *C. jejuni* 81–176 encodes the CysxxCys-x(13)-CysxxCys-x(14,15)-Cys (x = any amino acid) motif, a hallmark of some Fre2 (2Fe—2S) and Fre4 (3Fe—4S) type ferredoxins (not shown in Fig. 6), which can act as terminal electron acceptors [65]. Based on this recent work, a modified functional model for P19cFtr mediated iron uptake is predicted to involve simultaneous interaction of P19 with this proposed ferredoxin and cFtr during iron transport [65]. The presence of Fe—S clusters in this system provides indirect evidence of the involvement of redox steps where these Fe—S proteins act as the terminal electron acceptors. However, as P19cFtr lacks a Fet3p type protein, the origin of such redox reaction remains unclear.

5.4. Three-component bacterial Ftr homologs EfeUOB

The three-component EfeUOB from *E. coli* contains an inner membrane permease (EfeU) that conserves the ArgGluxxGlu motif and is expected to form α-helical trans-membrane domains like other Ftr-type transporters (Fig. 6) [27,28]. Additionally, the inner-membrane permease, EfeU, from this system is part of the oxidase-dependent iron transport (OfeT) superfamily of proteins, like the yeast Ftr1P [30]. Experimental work has shown that this conserved motif is essential for iron transport through this transporter in *E. coli* [27,28]. However, the exact role of these motifs on EfeU remains uncharacterized. In vitro studies confirm that the substrate for this transporter from *E. coli* is Fe^{2+}, similar to the yeast system [28]. Experimental and bioinformatics studies have indicated that this Fe^{2+} transporter contains a putative periplasmiccupredoxin, EfeO, which conserves a Cys residue (Cys130) at the N-terminal like other Type-1 Cu containing cupredoxins [42]. Although this protein does not conserve the two conserved Type-1 Cu coordinating residues [42], it shows a highly conserved GluGluArg-GluGln motif which was predicted to show metal coordinating property [42]. Based on these amino acid conservations and a common evolutionary origin with characterized MCOs, *E. coli* EfeO has been classified as a novel cupredoxin (Cup—I), although Cu binding and redox reaction by this protein remain to be investigated [42].

According to the current functional model for EfeUOB from *E. coli*, the proposed periplasmic ferrous oxidase, EfeO, oxidizes Fe^{2+} in a step coupled with its transport through the permease, EfeU (similar to the yeast Fet3p-Ftr1p system) (Fig. 6) [42]. It is important to emphasize that there is no experimental data to support this oxidation coupled permeation of iron through EfeU. The role of the other periplasmic protein, EfeB, from this system is not well defined. In one study, it has been proposed to act as a terminal electron acceptor [42]. Data from another

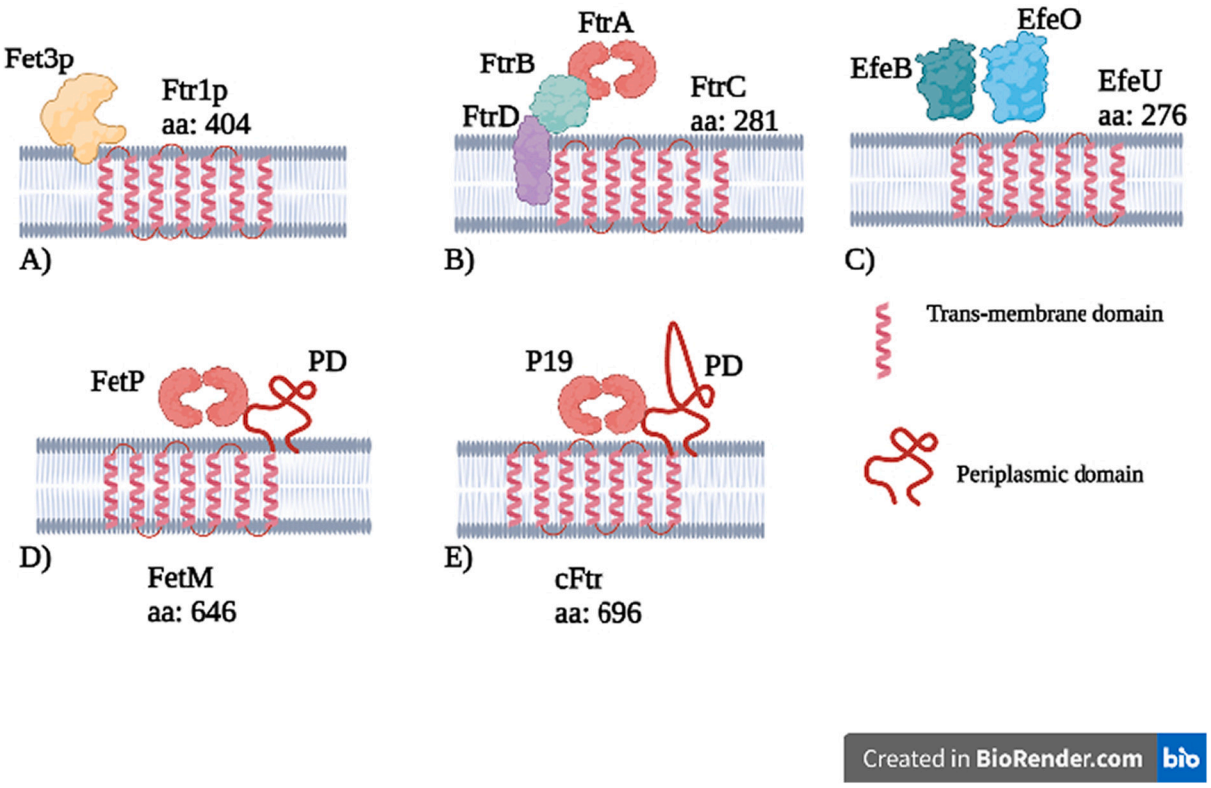

Fig. 6. Schematic representation of A) the yeast two-component Fet3p-Ftr1p, B) the *Brucella*, *Bordetella*, and *Burkholderia* four-component FtrABCD, C) *E. coli* three-component EfeUOB, D) *E. coli* two-component FetMP, and E) *Campylobacter jejuni* two-component P19cFtr iron-uptake systems. As can be seen, all mature membrane permeases conserve six transmembrane helices whereas D) and E) have an additional periplasmic domain (PD) [27–40,65].

study indicates that EfeB is a deferrochelatase and can sequester iron from heme group [66]. The *E. coli* EfeUOB transporter is the only Ftr-type permease containing iron transporter which remains functional under anaerobic conditions. Indicating that O_2 reduction (to water) concomitant to iron oxidation might not be an essential part of transport through these transporters [27]. In other words, this can be taken to indicate that iron oxidation during transport through Ftr-systems can take place with proteins other than ferroxidases. The anaerobic functionality of this *E. coli* EfeUOB is also an indirect support that it requires an alternative terminal electron acceptor, which can be fulfilled by EfeB [42].

A similar EfeOUB system is found in Gram-positive *B. subtilis*, a facultative anaerobe, and is shown to be essential for the transport of both Fe^{2+} and Fe^{3+} [67]. In this system both EfeB and EfeO are membrane attached lipoproteins which can interact with the membrane permease, EfeU but do not show direct interaction with each other [67]. In this system, EfeU and EfeO are required for Fe^{3+} transport under aerobic conditions whereas the presence of all three proteins are required for transport of Fe^{2+} under microaerobic conditions. This study also showed that EfeB can act as a heme-based peroxidase in the presence of hydrogen peroxide and binds to Fe^{2+}. This peroxidase activity is enhanced when recombinant EfeO is added which binds Fe^{3+} and makes the redox process more favorable. Finally, this study also reported higher peroxide and increasing Fe^{2+} sensitivity for the *efeB* mutant. Taking these together, the authors proposed a bifunctional model for the EfeUOB from this organism, where under aerobic condition, it can directly transport Fe^{3+} using EfeU and EfeO. Under a microaerobic environment and in the presence of different concentrations of peroxide and Fe^{2+}, EfeB becomes more important for its protective peroxidase activity (at higher Fe^{2+} concentration) as well as supports an oxidation dependent Fe^{2+} utilization (under lower Fe^{2+} concentration) [67].

6. Reviewing in vivo and biochemical studies on FtrABCD systems

Of the bacterial Ftr homologs identified, FtrABCD from *Brucella*, *Bordetella*, and *Burkholderia* is the only four-component transporter which is also highly homologous to each other [32–34,43]. *Brucella* and *Bordetella* FtrABCD is a Fe^{2+}-specific transporter that functions optimally under acidic conditions which is consistent with the increased solubility of Fe^{2+} at low pH [32,33]. The genes encoding these transporters are also induced in both bacteria independently in response to iron deprivation and acidic pH [32,33]. In the case of *Brucella*, FtrABCD is an essential virulence determinant in experimentally infected mice [32]. The capacity of the brucellae to replicate intracellularly in host macrophages plays a critical role in their capacity to produce chronic infections in their mammalian hosts [68,69]. During the early stages of their intracellular lifecycle in host macrophages, the brucellae reside in acidified compartments known as endolysosomal *Brucella*-containing vacuoles (eBCVs) where Fe^{2+} is likely to be a biologically relevant iron source [68,69]. Similarly, *Bordetella pertussis* and *Bordetella bronchiseptica* are important respiratory pathogens in mammals and colonize in the fluid-covered airway surfaces in humans [70]. This anatomical site has been reported to be acidic, and it is likely that FtrABCD assists *B. pertussis* and *B. bronchiseptica* in iron acquisition during colonization of these acidic microenvironments [32,33]. The importance of transition through acidified vacuoles in the intracellular lifecycle of *Brucella* strongly suggests that Fe^{2+} is a biologically relevant iron source for these bacteria in their mammalian hosts. The significant attenuation displayed by a *Brucella ftrA* mutant in both cultured mammalian cells and experimentally infected mice is consistent with this proposition [32]. On the other hand, experimental data indicate that *Burkholderia* FtrABCD system is responsible for direct Fe^{3+} uptake [44]. The basis for the difference in the iron specificity of this transporter is presently unclear. It is

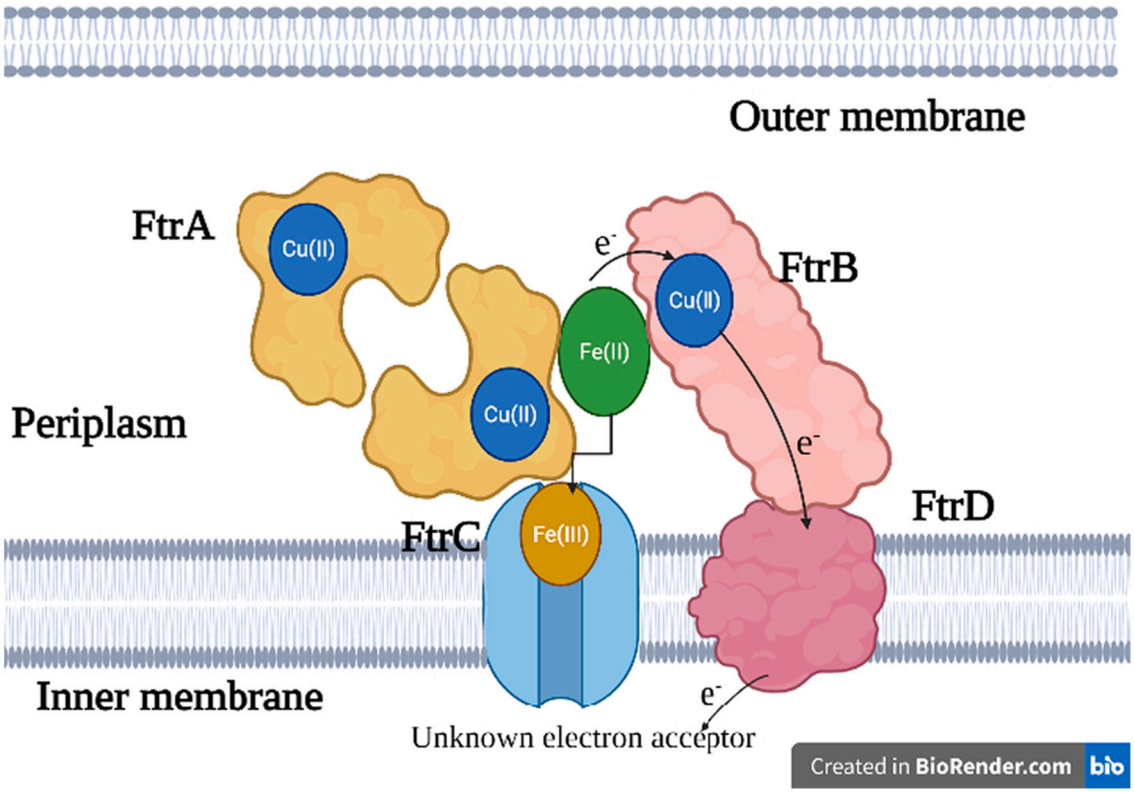

Fig. 7. FtrABCD has also been described in *Burkholderia cenocepacia* [33]. But unlike its counterparts in *Bordetella* and *Brucella*, the *B. cenocepacia* FtrABCD appears to function primarily as an Fe^{3+} transporter. Consistent with this difference, the corresponding *Burkholderia* genes are induced in response to Fe deprivation, but not by exposure to acidic pH [33]. This FtrABCD is also not required for the virulence of *B. cenocepacia* in the *Galleria mellonella* invertebrate infection model. But this relationship has not been examined in cultured mammalian cells or a mouse model of infection. This is an important consideration because iron deprivation is an important component of immune defense in mammals [32–34].

also puzzling on the surface because the four proteins that make up these FtrABCD transporters share extensive homology at the amino acid level and as will be described in the following sections, they are proposed to have equivalent biochemical functions [32–34].

The current working model (Fig. 7) of FtrABCD predicts that periplasmic FtrA is a P19 homolog and binds Fe^{2+} in a Cu^{2+} dependent fashion (Table 3) and provides ferrous iron to the proposed periplasmic cupredoxin [42], FtrB. This proposed cupredoxin, FtrB, oxidizes the metal to Fe^{3+} in a step coupled with its transport through FtrC (Fig. 7) [32,33]. The reduced FtrB then loses electron to the membrane bound predicted polyferredoxin, FtrD, and repeats the redox cycle [32,33]. Based on this model, all four bacterial Ftr proteins are required to interact with each other simultaneously, or in pair-wise fashion, however, these protein-protein interactions have not been experimentally verified. With one notable exception [71] the individual components of the bacterial FtrABCD transporters have not been characterized biochemically. This leaves a significant gap in our understanding of how these systems function at the molecular level.

In the following sections, biochemical and bioinformatics data on *Brucella* FtrA, B, C, and D proteins will be reviewed in detail and will be compared with any available data from their homologs. At present no biochemical data is available on the four proteins from *Bordetella* and *Burkholderia* species, making comparison between different bacterial Ftr proteins difficult. However, based on the extensive homology between the FtrA, B, C, and D proteins from these organisms will suggest similar functional mode for these iron transporters.

7. FtrA

Brucella FtrA is a 182 amino acid protein with a predicted signal sequence which predicts its localization in the periplasmic space [32]. This periplasmic protein is predicted to be a P19 homolog and show Cu^{2+}-dependent Fe binding [32]. The *Bordetella*, *Brucella* and *Burkholderia* FtrA proteins show extensive amino acid conservation with other P19-type proteins. The amino acid residues seen to coordinate Cu^{2+} (HisHisHisGlu/Met for Cu^{2+}) and $Mn^{2+}/Fe^{2+}/^{3+}$ (Glu and Asp) in the crystal structures of these P19-type proteins are conserved in FtrA (His65, Glu67, His118, His115, Asp115) [29]. These residues are found in favorable positions in a wild-type *Brucella* FtrA homology model, when compared to reported P19 protein structures, and mutation of the His118 and His121 residues to non-coordinating alanine-elevated Cu^{2+} binding (see below for details), which proves their roles in Cu^{2+} coordination [71]. Further, superimposition of this homology model on P19 crystal structures shows two conserved Met residues (Met107 and 109) (Fig. 8) on FtrA in similar positions (unpublished data). These Met residues in FetP and P19 crystal structures have been predicted to coordinate to Cu^{+} proposed to provide these proteins with redox function [29–31]. Although redox properties of FtrA (or any other P19-type proteins) have not been experimentally verified, but these hint towards a possible redox role that FtrA can perform.

Biochemical data on recombinant *Brucella* FtrA show that similar to other P19-type protein, wild-type FtrA forms homodimers, shows Cu^{2+} dependent Mn^{2+} (an Fe^{2+} mimic) affinity, and metal binding makes the protein dimer more difficult to unfold (as indicated by higher melting temperature, Table 3) [71]. Further, although FtrA Cu^{2+} binding mutants (His65A, Glu67A, and His151A) show dimer formation, these showed significantly lower melting temperature (T_M) in differential scanning calorimetry indicating lower folding stability (Table 3) as well none of the mutants showed Mn^{2+} binding [71]. Taken together, this data provides direct evidence that the conserved amino acids in FtrA

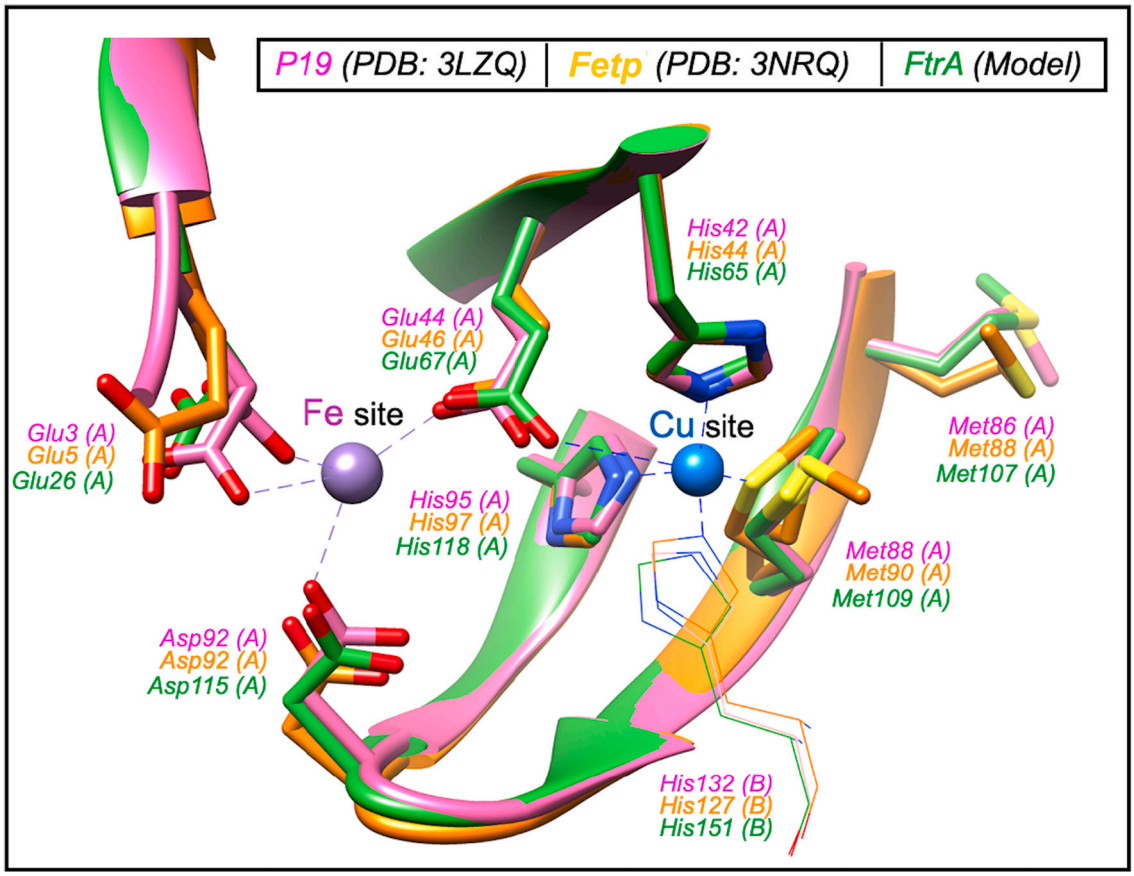

Fig. 8. FtrA homology model showing the conserved Cu^{2+} and Fe^{2+} residues superimposed on the metal binding sites from P19 and FetP crystal structures. The model predicts the metal-binding residues in favorable positions and shows a constellation of Met residues on FtrA close to the Cu^{2+}-binding pocket [29–31,71].

play a more significant role of forming a native dimeric structure which is required for its proposed functionality (Fe^{2+} sequestration) [71]. Interesting differences for Mn^{2+} binding to Cu^{2+}-bound wild-type FtrA are also reported [71]. For example, at acidic pH wild-type FtrA monomer shows a 1:0.5 protein: metal stoichiometry (Table 3), which altered to 1:1 under alkaline pH [71]. The thermodynamics of Mn^{2+} binding to wild-type Cu^{2+}-FtrA also showed variance at these two pH values, changing from exothermic at pH 6.3 to endothermic at pH 7.3 [71]. Based on amino acid homology and the crystal structures of reported P19-type proteins, only two amino acids contribute to Fe^{2+}/Mn^{2+} coordination (Glu67 and Asp115) [33]. Crystal structures of P19-type proteins coordinated to $Fe^{2+/3+}$ show the remaining coordination sites [31]. This is surprising as iron prefers five/six ligands as well as to avoid its precipitation and redox toxicity in contact with water, biological molecules satisfy these coordination sites [2]. The presence of just two conserved residues in these proteins can be interpreted as transient iron interaction with these proteins or the presence of a second periplasmic protein (possibly FtrB) that can provide the additional coordination sites. A fractional Mn^{2+} stoichiometry at an acidic pH (mimicking the periplasmic conditions) [72] can also be interpreted as the formation of a ternary complex at this pH between FtrA and the buffer component, which will be equivalent to a ternary complex in the presence of another periplasmic protein component. This Fe^{2+} sharing between FtrA and FtrB also supports the current model of function of FtrABCD (Fig. 7) which predicts interaction between all four components for the putative ferroxidase function by FtrB and transport by FtrC [32]. The ternary complex formation between Mn^{2+}/Fe^{2+}, FtrA, and a protein/anion component remains to be experimentally verified. Similar Cu^{2+} and Mn^{2+} binding data are also available for *E. coli* FetP and show similar metal affinity and are provided on Table 3 for comparison [30].

8. FtrB

Brucella FtrB is a small periplasmic protein (132 amino acids) which is co-expressed with FtrA, C, and D under low iron and pH conditions [32]. SignalP predicts a 43 amino acid N-terminal signal sequence, and periplasmic localization of mature FtrB using the Sec sequence (unpublished data). In cell studies have shown that FtrB from *Bordetella* and *Burkholderia* is required for iron transport through this system, indicating an essential role played by this protein. A previous bioinformatics study categorized FtrB as a novel cupredoxin (CupII) based on its common evolutionary relationship with other cupredoxin proteins as well as conservation of certain metal-binding residues (His and Asp residues and ArgLysGluLysVal and GluxGlu motifs) [42]. Multiple sequence alignment of FtrB with known cupredoxins show conservation of one histidine residue (His121) which is seen to coordinate Cu^{2+} in other cupredoxins (Fig. 9A). However, none of the other two characteristic Type-1 Cu coordinating residues [43] are present on FtrB. This in addition to not having any biochemical data yet showing wild-type FtrB can bind Cu^{2+}, has made this designation controversial.

Homology modeling with *Brucella* FtrB sequence (without the signal sequence) (Fig. 10A) and our preliminary X-ray data on recombinant wild-type Brucella FtrB (unpublished data) show beta-sandwich with 7 strands arranged in a Greek-key beta-barrel. This FtrB homology model was superimposed on characterized cupredoxins, azurin, and rusticyanin crystal structures to obtain insight into the putative Cu-binding site in this so-called CupII protein. The superimposition shows three coordinating amino acid residues from the FtrB model (Asp118, His121, and Met81) (Fig. 9A) which can provide an alternative Cu^{2+} binding site. Of these, the Asp118, and His121 are the conserved Cup-II residues [42], and the M81 residue is completely conserved in other virulent *Brucella*

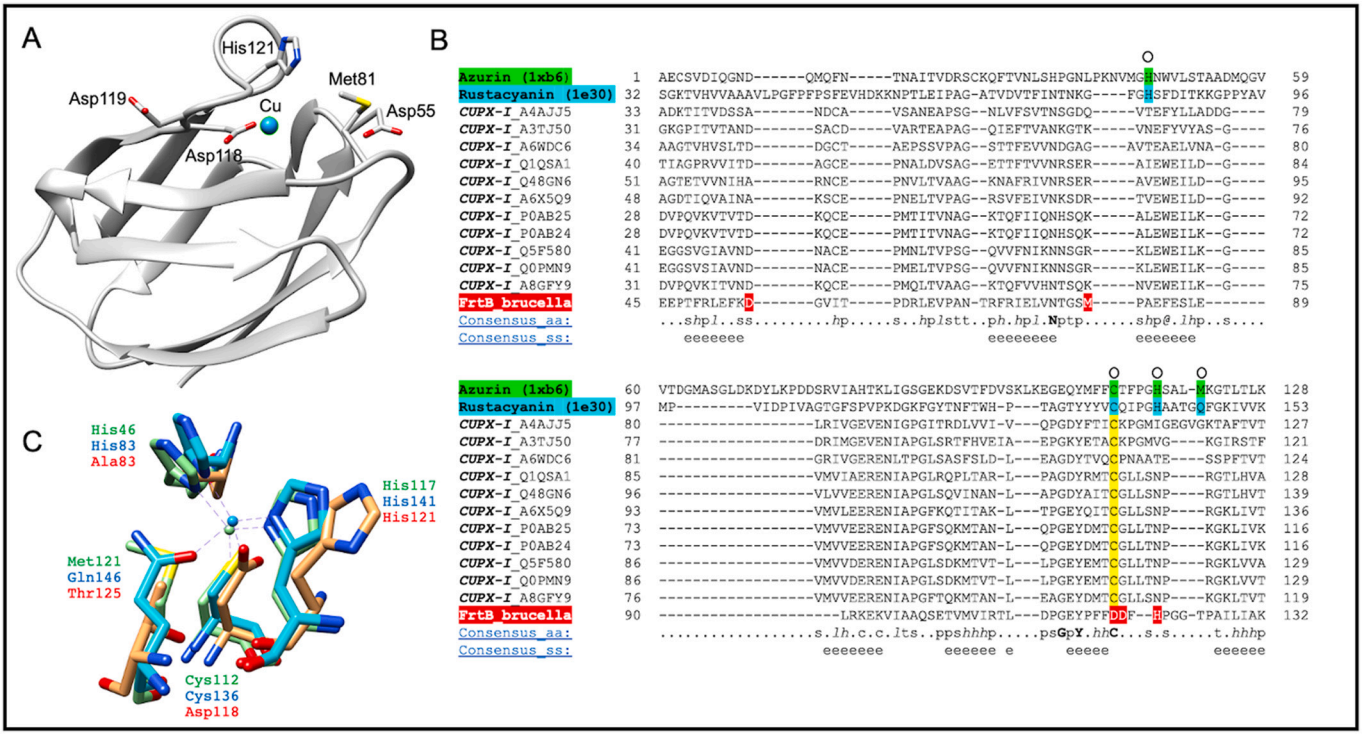

Fig. 9. A) The homology model of mature *Brucella* FtrB (Templet: 4HCF, GQMP: 0.64, RMSDa: 0.84, Cov.: 0.978). The proposed Cu^{2+}-binding site show two proposed CupII conserved residues and a methionine (M81) which is conserved in three other virulent *Brucella* strains (unpublished work). B) A structure-based sequence alignment of *Brucella* FtrB with Type-1 cupredoxins (CupX-1) sequences is presented with two template X-ray crystallographic structures of Azurin (PDB ID: 1XB6) and Rusticyanin (PDB ID: 1E30) The Cu-binding sites of Azurin and Rusticyanin are indicated with the symbol 'o'. C) Structural superimposition of the Cu^{2+}-binding sites of *Brucella* FtrB (orange), Azurin (green) and Rusticyanin (cyan). The Cu^{2+}-binding residues are also indicated in different colors: Red (*Brucella* FtrB), Blue (Rusticyanin) and green (Azurin). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strains (*B. suis*, *Brucella ovis*, *B. canis*, and *Brucella melitensis*) (alignment not shown), indicating some important roles played by these residues. This model also predicts two aspartate residues (55 and 119) in the surface-exposed region of the structure (Fig. 9A), which are completely conserved in FtrB from other virulent *Brucella* strains (*B. suis*, *B. ovis*, *B. canis*, and *B. melitensis*). Interestingly, similar surface-exposed carboxylate side chains are shown to play important Fe^{2+} binding close to the Type-1 Cu site in Fet3p and is suggestive of a similar function in *Brucella* FtrB.

As can be shown in Fig. 9B that the conserved Cys residues in these known cupredoxins is replaced by a conserved Asp118 residue in *Brucella* FtrB. The Cu^{2+}-S(cys) bond in all known proteins with a Type 1 Cu^{2+} site gives rise to its intense blue colour, which is absent in this modeled structure (Fig. 9B and C). In addition to giving the Type-1 Cu site its characteristic blue colour, the H-bonding between the coordinated Cys and neighboring amide protons are shown to modulate the reduction potential of the coordinated Cu^{2+} ion and *Brucella* FtrB lacking the Cys residue would not have the opportunity of such ET pathway (Fig. 9B) [47]. Of the other two characterized Type-1 Cu coordinating residues (HisHis), only the His121 is conserved in *Brucella* FtrB, and in the homology model it is part of a loop positioned away from the Cu^{2+} binding site (Fig. 9A). Instead of another His residue, *Brucella* FtrB model shows a non-coordinating amino acid (Ala83) (Fig. 9B). Lastly, the variable fourth and axial ligands in azurin and rusticyanin (Met 121 and Gln 146) [43] are replaced by a Thr residue in favorable position to have Cu^{2+} coordination (Fig. 9B) in this FtrB model.

As mentioned, despite these dissimilarities, with characterized cupredoxins, FtrB has been predicted to serve as a novel cupredoxin [42]. However, neither the Cu^{2+} binding by the conserved residues from the bioinformatics (see above) work or the model provided on this article nor the Fe^{2+} oxidation step by FtrB have been experimentally determined. Based on the results of in cell studies and bioinformatics prediction, the current model of function of the FtrABCD system involves sequestration and delivery of Fe^{2+} in the periplasm by FtrA to FtrB, which is then oxidized and transported through FtrC (Fig. 7) [33,34,44]. Currently, there is no experimental evidence that suggest that these proteins interact with each other during iron transport and as such needs to be investigated. Lastly, the *Burkholderia* FtrABCD system has been shown to transport Fe^{3+} directly, and at least in that case the role of FtrB as a ferrous oxidase remain contested [44].

9. FtrC

FtrC (30.4 kDa) is a putative membrane protein of the oxidase-dependent ferrous iron transporter (OFeT) family [73], and is similar to the Ftr1p iron transport permease protein of *S. cerevisiae* [74] and the *E. coli* ferrous iron transporter EfeU [66,67]. OFeT family transporters have been shown to be important for Ftr1p and EfeU function in iron binding and translocation [28,38,54].

Similar to the other bacterial Ftr homologs, FtrC conserves two ArgGluxxGlu motifs and are predicted to form transmembrane helical domains and are predicted to play similar roles as observed in yeast Ftr1p [32–34,38,54]. However, the roles of these motifs in iron transport through FtrC need to be investigated. This is intriguing because experimental evidence suggests that the bacterial FtrC proteins, like Ftr1p, require the participation of accessory Fe-binding proteins to carry out their Fe permease activities [32,33]. The current model for FtrABCD in *Bordetella* and *Brucella* is that FtrA directly transfers Fe^{3+} to FtrC after its oxidation by FtrB [32]. But as noted in the previous section, this model does not fit with the proposed role of FtrABCD as an Fe^{3+}-specific transporter in *Burkholderia* [34,43]. However, as mentioned, experimental data suggest that bacterial Ftr systems show diversity in their

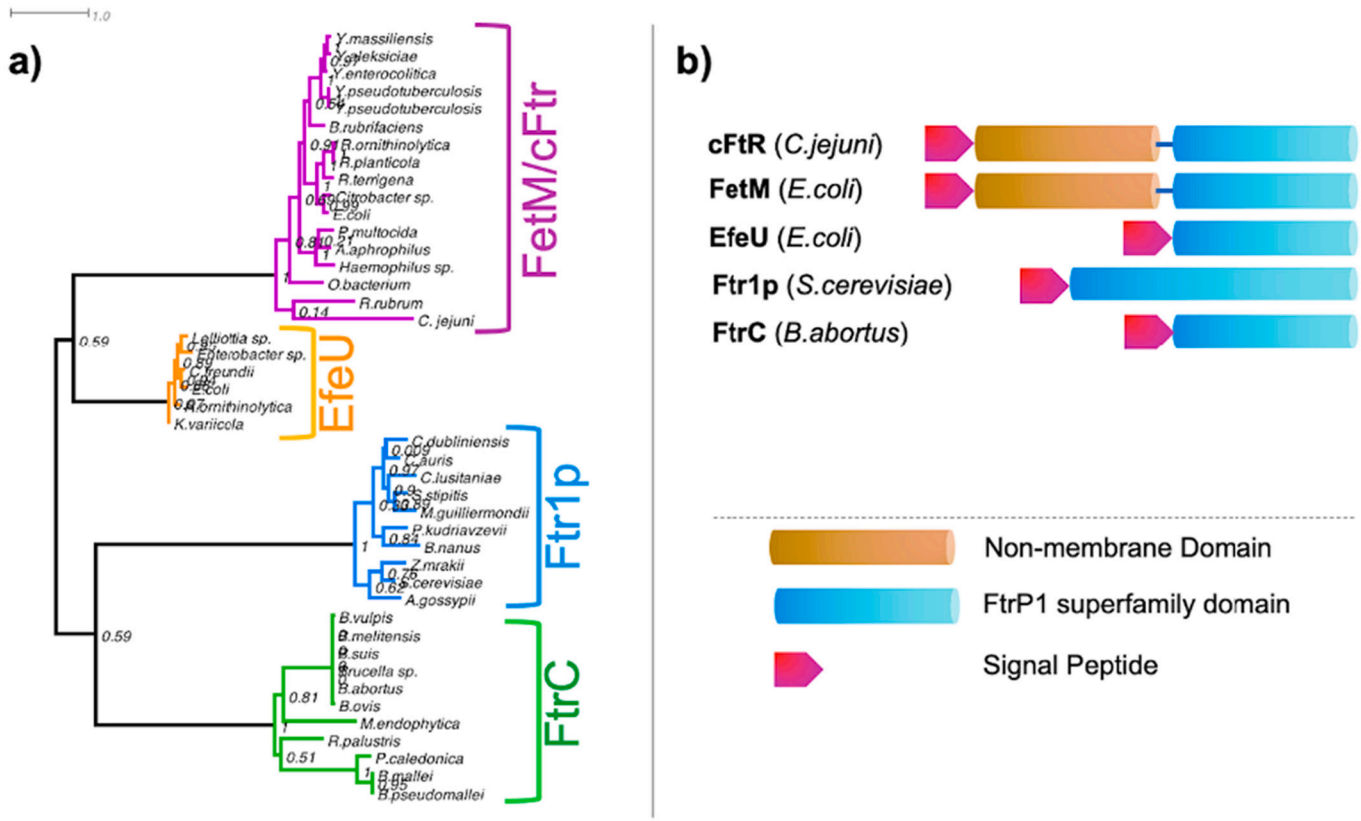

Fig. 10. a) Unrooted phylogenetic tree of bacterial permeases (*E. coli* FetM, *Campylobacter jejuni* cFtR, *E. coli* EfeU, *B.abortus* FtrC) with *Saccharomyces cerevisiae* Ftrp1. B) Schematic diagram of domain organization of *E. coli* FetM, *C. jejuni* cFtR, *E. coli* EfeU, *B.abortus* FtrC and *S. cerevisiae* Ftrp1. All bacterial Ftr homologs show a common ancestry with the yeast Ftr1p [42]. The tree also shows that of the bacterial Ftr homologs, FtrC is closer to the yeast Ftr1p permease compared to other bacterial permeases.

ligand specificity, (FetM transporting Fe^{2+} and *B. subtilis* EfeU transporting Fe^{3+}) and do not require participation of ferroxidase proteins. This provides evidence for Ftr-type transporters having alternative functional models which can be found in other bacterial systems. More extensive comparative biochemical and genetic studies of the contributions of the individual components of the *Bordetella*, *Brucella*, and *Burkholderia* FtrABCD transporters will be necessary to resolve these questions. The next section of this Review will also present evolutionary insight into the FtrC protein and compare it to other bacterial homologs of the yeast Ftr system.

10. FtrD

FtrD is an integral membrane protein that shares predicted Fe—S center-based 'ferredoxin' domains with the NapH protein of *Wolinella succinogenes* [75]. The NapH protein transfers electrons in an anaerobic respiratory chain, and similarly, the ferredoxin (4Fe—4S) domains of the *Bordetella* and *Brucella* FtrD proteins have been proposed to serve as an electron sink and accept the electrons acquired by the proposed ferroxidase, FtrB (Cup-II) during Fe^{2+} oxidation (Fig. 7) [33,34]. FtrD is hypothesized to transfer the electrons to an unknown electron acceptor within the cytoplasm, resetting the entire FtrABCD system [33,34]. For the sake of comparison, this functional model of FtrABCD predicts that FtrD performs the same role as EfeB in *E. coli*, and the Type-3 Cu site in Fet3p [42]. In the yeast Ftr system, the Type-3 Cu ions bind to O_2 and act as an electron sink, whereas the presence of FtrD in this system provides an oxygen- independent alternative model that relies on the proposed cupredoxin (FtrB). The evolutionary advantages of these O_2-independent ferroxidase (mediated by FtrBD) steps need to be verified but seems functionally advantageous as these can avoid accidental ROS production.

11. Bioinformatics studies on FtrABCD

To gain further insight into the evolutionary history of the four-component bacterial Ftr homolog, we carried out phylogenetic analysis. Our data shows that the FtrABCD gene is abundant in selected genome from bacterial species and might also encode other bacterial Ftr-type permease (FetM, cFtr) genes. Bioinformatics data also shows that many bacterial species retain genetic information of three proteins (FtrABC) in their genome, which poses a question about the importance of FtrD in these systems. Before we discuss this data in detail, we want to caution our readers that experimental work needs to be performed to establish if the FtrABCD encoding genes in these bacteria are transcribed/translated and have iron uptake functionality.

A phylogenetic analysis on FtrC and other bacterial Ftr-type permeases (EfeU, cFtr, and FetM) with the yeast Ftr1p protein was performed and the result is presented in Fig. 10. As can be seen, (Fig. 10A) FtrC appears in a different phylogenetic clad than the other bacterial Ftr-type permeases. Further, from an evolutionary point of view, the FtrC protein is more closely related to the yeast Ftr1p as these appeared in the same clad. It was already known that although all Ftr-type permeases are iron regulated, are predicted to form trans-membrane helices, and conserve ArgGluxxGlu motifs, FetM and cFtr had different domain arrangement compared to EfeU and FtrC (Fig. 6). When the different evolutionary origins of Ftr-type permeases are compared with their domain organization (Fig. 10b), it seems the absence of periplasmic domains from Ftr-type permeases (*E. coli* EfeU, *Brucella*, *Bordetella*, and *Burkholderia* FtrC, and yeast Ftr1p) do not guarantee the same evolutionary relationship (Fig. 10a). It is tempting to use this difference in

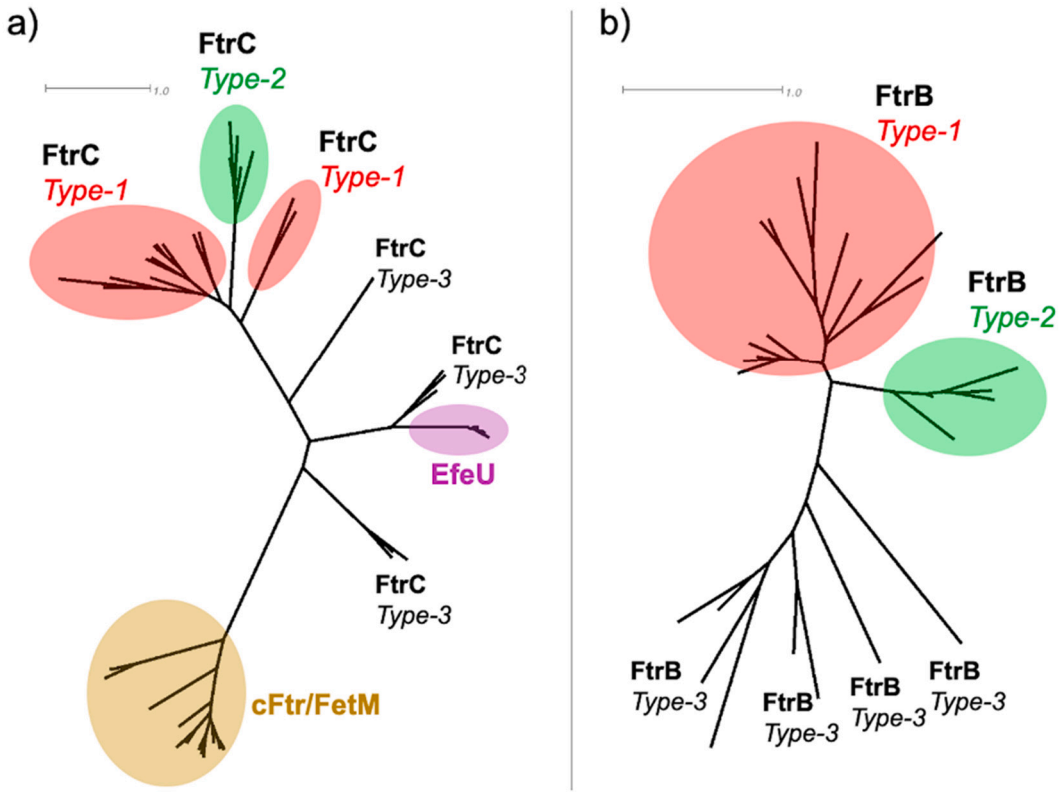

Fig. 11. a) Unrooted phylogenetic tree of bacterial permease sequences (cFtR, FetP, EfeU) along with FtrC sequences from Category1–3 bacteria (See Table 3 and Supplementary Table 1A and C). B) Unrooted phylogenetic tree of bacterial FtrB sequences from Category 1 and 2 bacteria (See Table 3). These trees show that FtrC from Type-1 bacteria appear on a different clade than the same protein from Category 2 and 3 bacteria that lacks FtrD. A similar trend is observed for FtrB, indicating the presence (or absence of FtrD) affects the evolution of the permease and ferroxidase.

evolutionary origins between bacterial Ftr systems as a justification for the functional differences (as discussed above) between these proteins. However, it is important to note that these evolutionary differences can also arise as *E. coli* and *C. jejuni* are enteric pathogens whereas *Brucella*, *Bordetella*, and *Burkholderia* are respiratory pathogens and encounter different growing environments. Based on the absolute iron requirement and different niche these bacteria grow in, we interpret this difference in evolutionary relationship between all Ftr-type proteins as having a common ancestry but showing divergent evolution based on their growth niche.

A genome-wide search for FtrABCD encoding genes was carried out across the bacterial species to investigate the presence of these genes in an open reading frame (ORF). A complete projection of these data among bacterial taxa/genera shows that in addition to *Brucella*, *Bordetella*, and *Burkholderia*, all four genes encoding FtrABCD proteins are also present (in an ORF) in other β-proteobacterial and α-proteobacterial genomes, as well as in few other proteobacterial classes (Category 1 bacteria) (Table 4). A further follow-up analysis showed a second category of bacteria that contains genes encoding FtrABC in a single operon (Category 2 bacteria) but no gene corresponding to FtrD was found (Table 4). We also observed another bacterial group where random occurrence of FtrA, FtrB, or FtrD genes were observed (data not shown). The occurrence of these genes adjacent to each other in both Category 1 and 2 bacteria suggests a much broader occurrence of FtrABCD proteins in bacteria (Table 4), however, transcriptional and proteomics data are not available from these systems. Additionally, experimental data from Bordetella and Burkholderia FtrABCD confirm that presence of all four proteins are required for these four-component systems to be functional. Based on that, the appearance of three (or one) of the four proteins can indicate loss of functionality and/or a different mechanism of function.

Some bacteria belonging to Category 1 also showed genes encoding other bacterial Ftr-type permeases (cFtr, FetM, and EfeU). The simultaneous presence of multiple bacterial *ftr*-like genes in the Category 1 bacteria could indicate two evolutionary models for iron uptake using Ftr-like systems in these organisms:

- o Bacterial Genomes of bacterial species are in a continuous mode of uptake or deletion of different permeases, thus achieving an appropriate mechanism of iron transport machinery.
- o Bacterial *ftr*-like permease (like *cftr*, *fetM*, *efeU*) genes share a common evolutionary origin and have evolved from a common ancestor.

To investigate the evolutionary likelihood of the above two models, a more robust phylogenetic tree was constructed (Fig. 11) using representative sequences from bacteria that contain genes for FetM/cFtr/EfeU (no FtrABCD); and selected bacteria from Category 1 (12 sequences), Category 2 (6 sequences), and Category 3 (6 sequences); all containing the FtrC gene (Supplementary Table 1, Sheet C). As Fig. 11a shows, the FtrC sequence from Category 1 bacteria (containing all four FtrABCD genes) appears in a different clad than FtrC sequences from Category 2 and 3 bacteria (lacking FtrD encoding gene). A similar trend is also observed when the FtrB genes from Category 1 and 2 bacteria were analyzed, showing FtrB from the former category has a different evolutionary origin than the latter (Fig. 11b). These differences in FtrC and FtrB when compared to the presence of FtrD encoding genes in the same operons indicate that absence of FtrD encoding gene results in a different evolution of the permease (FtrC) and the proposed ferroxidase (FtrB). The current model for the Fe^{2+} transport using FtrABCD proteins predicts that FtrD, a proposed polyferredoxin (Fd), similar to NapH (an electron-transport chain protein which resides inside the cytoplasmic membrane) acts as the final electron acceptor in the proposed redox

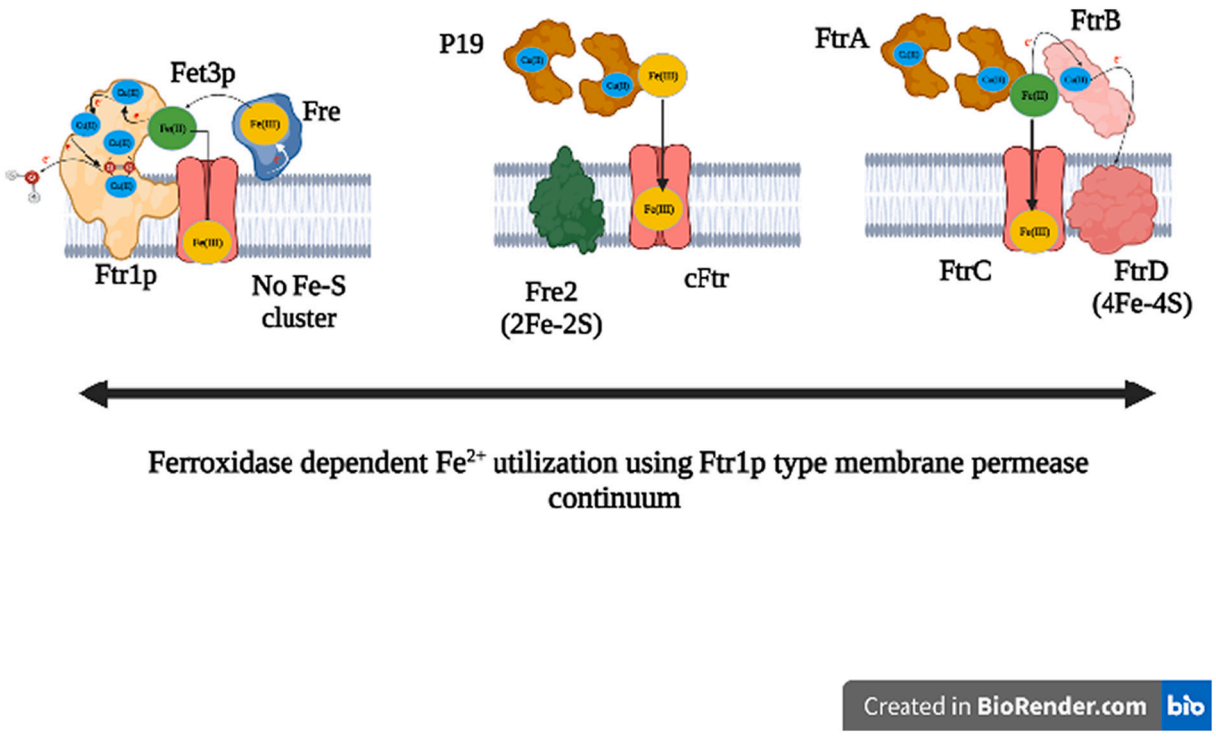

Fig. 12. The proposed evolutionary continuum for Ftr-type permease mediated and ferroxidase-dependent Fe^{2+} utilization in microbes. The left of the figure shows characterized Fe^{2+} utilization using the MCO, Fet3p, and the permease Ftr1p from yeast which does not require the involvement of an Fe—S cluster as the final electron acceptor. The right hand of the proposed model has the FtrABCD system that consists of an ancient 4Fe—4S cluster (FtrD) as the final electron acceptor. The P19cFtr system with a downstream gene encoding a Fre2/4 type protein lies in the middle and does not require O_2 as the final electron acceptor, like the FtrABCD system in the proposed model. This model might explain the use of different proteins/protein domains as the final electron acceptor depending on the availability of free O_2 around the natural niche.

dependent Fe^{2+} transport and resets the system (Fig. 7) [32,75]. The functional role of FtrD can be compared to the Type-3 Cu^{2+} site in Fet3p and Cp (Figs. 3 and 4) which bind to O_2 and reduce it to water. Interestingly, a BLASTP analysis of *Brucella* FtrD among genomes of eukaryotes have shown that *Brucella* FtrD is homologous with iron-sulphur binding 4Fe—4S ferredoxin (Fd) exclusively from the genomes of Green Algae (*Ostreococcus tauri, Monoraphidium neglectum, Chlorella variabilis, Coccomyxa subellipsoidea*). From the evolutionary history of ferredoxins, the 4Fe—4S ferredoxin is predicted to be the ancient version of these proteins, and it is proposed that during evolution, especially with the advent of oxygenic photosynthesis and the Great Oxidation Event, the usage of oxygen-sensitive [4Fe—4S] Fds may have been disincentivized relative to the more O_2-tolerant Ferredoxins [76]. Under this circumstance, functional insight about FtrD genes in bacterial genomes like *Brucella* needs to be explored before drawing any conclusive evolutionary model. However, it is possible that FtrD in the *Brucella*-like genomes (Type-1) may have some primitive functional role as low potential electrical transfer hubs, which might have also been utilized as the 'electron sink' in the corresponding FtrABCD-mediated Fe transport pathway. During the pathway of evolution, by strategic mutation of its FtrC and FtrB counterparts, the FtrABCD-mediated Fe transport pathway had adopted an FtrD-independent pathway (Bac-Type-2), as other Fe transport pathways have. The fact that it has been characterized in three respiratory pathogens exposed to a higher concentration of O_2 is also worth noting. Based on the higher O_2 concentrations that these pathogens encounter, one might expect to see a Type-3 Cu^{2+} binding site as the enzyme resetting point, instead of a 4Fe—4S Fd.

12. Bioinformatics methods

The model structure of *Brucella abortus* FtrB (BAB2_0839) was constructed by using the homology-modeling server SWISS-MODEL (https://swissmodel.expasy.org), based on the template structure of Cupredoxin-like Domain Protein Cupredoxin_1 from *Bacillus anthracis* (PDB ID 4HCF). Structure analysis of proteins were done by UCSF-Chimera (https://www.cgl.ucsf.edu/chimera). Structure based off sequence alignment of protein sequences was performed with PROMALS3D web server (http://prodata.swmed.edu).

Analysis of selected genomes from bacterial species was performed from the KEGG Genome Database for all Ftr proteins [77]. For the analysis, the sequences of FtrA, FtrB, FtrC, and FtrD from the species *B. abortus 2308* were considered as the template: BAB2_0840 (FtrA), BAB2_0839 (FtrB), BAB2_0838 (FtrC), and BAB2_0837 (FtrD). For each protein, an individual BLASTP (prot query vs prot db) [78] search was performed among all prokaryotic complete genomes of the KEGG database. The entire data (500 hits for each) was then mapped among different bacterial taxonomy and was further analyzed with respect to each individual genus. The entire dataset of the BLASTP analysis is summarized in Supplementary Table 1, Sheet-A for each protein along with the tri letter code of organisms of the KEGG Genome Database.

Protein sequences of Fet3p, Ftr1p, FetP, FetM, EfeO, EeeU, P19, cftR1, FtrA, FtrB, FtrC, and FtrD were downloaded from the respective genomes of the species (Supplementary Table 1, Sheet B). The sequence database of FtrB and FtrC were constructed from the selected genomes of bacterial species that resulted from the BLASTP analysis with the respective template proteins of *B. abortus 2308*: bab2_0839 (FtrB), bab2_0838 (FtrC). The proteins are classified into three types (I, II, and III) based on the genomic occurrences of FtrA, FtrB, FtrC, and FtrD. Complete details of the databases are presented in Supplementary Table 1 (Sheet-C).

All phylogenetic analyses presented in this paper were performed by the Maximum-likelihood method using the web-server www.phylogeny.fr [79] by following the methodology of multiple sequence alignment by MUSCLE [80], tree building by PhyML [81], and tree rendering by

TreeDyn [82]. All phylogenetic trees were analyzed and reconstructed with the tree-view software Dendroscope [83].

13. Conclusion

In a review article on cupredoxins, Davidson et al provided a compelling story about the evolution of these Cu^{2+}-coordinating redox-active proteins [43]. Using the near identical three-dimensional structural fold of apo- and holo-cupredoxins, they argued that these proteins, with Greek β-key structure, must pre-date the introduction of Cu^{2+} in biology [43]. The authors further argued that all Type-1 Cu-containing electron transfer proteins (cupredoxins) evolved from a common ancestor (with conserved Greek β-key structures) and gained the Cu^{2+} affinity due to an increase in [Cu^{2+}] during GOE [43]. Further, based on the fact that not all proteins with the Greek-β-key fold coordinate to Cu^{2+}, they proposed that the ET property in cupredoxins might have been added at a much latter stage of evolution by random amino acid mutations [43]. We extend their interpretation to propose that it is possible to identify novel cupredoxins, which do not conserve the known Type-1 Cu-coordinating residues, due to differences in evolutionary pressures. This new proposal will support the finding by Rajasekaran et al. [42], showing that *E. coli* EfeO and *Brucella* FtrB, having a common evolutionary origin with characterized MCOs, constitute two new and novel cupredoxin class (Cup-I and Cup-II). The inclusion of FtrB as a cupredoxin is hypothetical since this biochemical activity has not been experimentally demonstrated yet.

As already mentioned, despite the motif conservation, similarity of the transmembrane domain topology, and roles in iron transport, Ftr-type membrane transporters from eukaryotes and prokaryotes have differences in their ligand preference as well as mode of function [27–40]. The only characterized Ftr (Ftr1p) mediated iron transport from yeast requires the expression of a multicopper oxidase protein (Fet3P) which is absent the bacterial Ftr systems [35–40]. However, unlike the eukaryotic system (Ftr1p-Fet3p), the bacterial Ftr-type transporters are found as two-, three-, and four-component systems, with the accompanying proteins not having been completely biochemically characterized [27–34,44]. The bacterial two- and four-component systems share a second protein, in addition to the Ftr-type permeases (cFtr, FetM, and FtrC), which are collectively known as P19-type (P19, FetP, and FtrA) proteins [29–31]. Based on in vitro data, these proteins are proposed to bind Fe^{2+} in a Cu^{2+}-dependent fashion in the periplasm of the respective bacteria, and such a system is absent from the eukaryotic Ftr-type transport system. The job of these P19-type proteins as Fe^{2+} delivery vehicles is also not consistent between the prokaryotes, as for the two-component systems, these periplasmic proteins are proposed to directly deliver the cargo to the inner-membrane permeases [29–31], whereas for the FtrABCD system, FtrA is expected to deliver the cargo to the proposed cupredoxin, FtrB, for its proposed oxidation [32–34,44]. It is not clear in the current functional model if all four components of FtrABCD need to come together for the proposed ferrous oxidation-and transport to take place (as is shown in Figure) or if the oxidation and transport are decoupled.

In the yeast system, the oxidation and transport take place simultaneously, however [35–40], the lack of FtrB-type proteins in P19-cFtr and FetMP systems hints towards the possibility of iron transport through Ftr proteins in bacteria being decoupled from a possible redox-dependent step [29–31]. The situation is further complicated for the three-component bacterial system, EfeUOB, which can switch between an oxidation dependent and independent mode of function [67]. Based on the data reviewed in this article and the evolutionary data presented here, it is likely that these Ftr-type transporters have evolved function within the specific environment of systems in which they are found.

Based on the evolution data presented here, it is tempting to propose an evolutionary model for these Ftr-type transporters forming a continuum, where the yeast Fet3p-Ftr1p is found on one end that has evolved to utilize a ferroxidase that contains a Type-3 Cu site to play the role of the final electron acceptor and thus does not require a Fe—S cluster as electron sink (Fig. 12). On the other end of this continuum is the FtrABCD systems from Category 1 bacteria, which contain a proposed cupredoxin (FtrB) to oxidize the Fe^{2+} for transport, however, to close this biological "circuit", utilizes an ancient Fe—S cluster (FtrD) as the final electron acceptor. Based on this proposal, all other bacterial Ftr homologs (Category 2, as well as FetMP and P19cFtr) can be found somewhere in the middle of this continuum, with either some evolutionary modification in their FtrC and FtrB genes (category 2 bacteria) or by expressing modern ferredoxins (Fre2 and Fre4, as found in *C. jejuni*). The validity of this model relies on the fact that all Ftr1p homolog containing systems utilize a ferroxidase, and in the bacterial systems, this ferroxidase needs to be identified.

Author statement

In this article we reviewed published experimental and bioinformatics data on bacterial Ftr-type iron transporters and put this in context of the changing geochemistry of the earth and different multicopper oxidases (MCO) that take part in iron uptake in eukaryotes. To our knowledge, this is the first review on such bacterial Ftr systems. Several of these bacterial Ftr transporters are expressed during the virulence stage of the respective pathogens and/or are required for their survival and thus, fall within the scope of this special issue on metals in infection and immunity. At the end of this review article, we also provide new evolutionary data and based on this, we present an evolutionary model connecting the eukaryotic Ftr system with their prokaryotic counterparts. Although these bacterial Ftr-homologs have been known for a decade, little is known about the exact mechanism of iron translocation through these systems, and we believe that the bold questions/models proposed in this article will result in further experiments.

Declaration of Competing Interest

The authors have no conflict of interest in this work.

Acknowledgements

SB is grateful to Dr. Morehead, Chair, Department of Chemistry, East Carolina University for providing laboratory space for this project and the Department of Chemistry, ECU, for general support. The authors are also very grateful to Prof. Roop, Brody School of Medicine, East Carolina University, for his valuable comments and guidance. The authors also acknowledge the Tuscarora people, who are the traditional custodians of the land on which East Carolina University is located.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2022.111721.

References

[1] R.J.P. Williams, FEBS Lett. 586 (2012) 479.
[2] R.R. Crichton, Inorganic Biochemistry of Iron Metabolism, Ellis Horwood, New-York, London, 1991.
[3] J.L. Pierre, M. Fontecave, BioMetals 12 (1999) 195.
[4] A.P. Gumsley, K.R. Chamberlain, W. Bleeker, U. Söderlund, M.O. de Kock, E. R. Larsson, A. Bekker, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 1181.
[5] G. Gao, J. Li, Y. Zhang, Y.Z. Chang, Cellular iron metabolism and regulation, in: Y. Z. Chang (Ed.), Brain Iron Metabolism and CNS Diseases. Advances in Experimental Medicine and Biology, Springer, Singapore, 2019, 1173.
[6] P. Kondaiah, P.S. Yaduvanshi, P.A. Sharp, R. Pullakhandam, Nutrients 2019 (1885) 11.
[7] S. Tomonori, D. Yasuaki, T. Hiroyuki, F. Satoshi, S. Yoshihiro, O. Nobuyuki, Medicine 100 (2021), e24722.
[8] M.I. Hood, E.P. Skaar, Nature 10 (2012) 525.
[9] E.P. Skaar, PLoS Pathog. 6 (2010), e1000949.
[10] J.H. Brock, Cell Biol. 90 (2012) 245.
[11] D. Legrand, J. Mazurier, Biometals 23 (2010) 365.

[12] G. Núñez, K. Sakamoto, M.P. Soares, J. Immunol. 201 (2018) 11.
[13] P.T. Gomme, K.B. McCann, J. Bertolini, Drug Discov. Today 10 (2005) 267.
[14] U. Bilitewski, J.A.V. Blodgett, A.-K. Duhme-Klair, S. Dallavalle, S. Laschat, A. Routledge, R. Schobert, Angew. Chem. Int. Ed. 56 (2017) 14360.
[15] M.F. Cellier, P. Courville, C. Campion, Microbes Infect. 9 (2007) 1662.
[16] E.M. Zygiel, E.M. Nolan, Acc. Chem. Res. 52 (2019) 2301.
[17] T. Ganz, Int. J. Hematol. 107 (2018) 7.
[18] K.N. Raymond, E.A. Dertz, S.S. Kim, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 3584.
[19] D. Malavia, A. Crawford, D. Wilson, Adv. Microb. Physiol. 70 (2017) 85.
[20] D.H. Nies, G. Grass, in: A. Böck, R. Curtiss III, J.B. Kaper, F.C. Neidhardt, T. Nyström, K.E. Rudd, C.L. Squires (Eds.), EcoSal: Escherichia Coli and Salmonella, Cellular and Molecular Biology, American Society for Microbiology Press, Washington, D.C., 2009.
[21] M. Nairz, G. Weiss, Mol. Asp. Med. 75 (2020), 100864.
[22] M. Nariz, S. Dichtl, A. Schroll, D. Haschka, P. Tymoszuk, I. Theurl, G. Weiss, J. Trace Elem. Med. Biol. 48 (2018) 118.
[23] C.K.Y. Lau, K.D. Krewulak, H.J. Vogel, FEMS Microbiol. Rev. 40 (2015) 273.
[24] H. Harigae, Int. J. Hematol. 107 (2018) 5.
[25] Y. Tong, M. Guo, Arch. Biochem. Biophys. 481 (2009) 144.
[26] I.J. Schalk, G.L.A. Mislin, Med. Chem. 60 (2017) 4573.
[27] J. Cao, M.R. Woodhall, J. Alvarez, M.L. Cartron, S.C. Andrews, Mol. Microbiol. 65 (2007) 857.
[28] C. Große, J. Scherer, D. Koch, M. Otto, N. Taudte, G.A. Grass, Mol. Microbiol. 62 (2006) 120.
[29] A.C. Chan, T.I. Doukoy, M. Scofield, S.A. Tom-Yew, A.B. Ramin, J.K. Mackichan, M.E. Murphy, J. Mol. Biol. 401 (2010) 590.
[30] D. Koch, A.C.K. Chan, M.E.P. Murphy, H. Lilie, G. Grass, D.H. Nies, J. Biol. Chem. 286 (2011) 25317.
[31] A.C.K. Chan, H. Lin, D. Koch, G. Grass, D.H. Nies, M.E.P. Murphy, Metallomics 12 (2020) 1530.
[32] A.E.M. Elhassanny, E.S. Anderson, E.A. Menscher, R.M. Roop II, Mol. Microbiol. 88 (2013) 1070.
[33] T.M. Brickman, S.K. Armstrong, Mol. Microbiol. 86 (2012) 580.
[34] A.T. Butt, M.S. Thomas, Front. Cell. Infect. Microbiol. 7 (2017) 460.
[35] R. Stearman, D.S. Yuan, Y. Yamaguchi-Iwai, R.D. Klausner, A. Dancis, Science 271 (1996) 1552.
[36] C. Askwith, D. Eide, A. Van Ho, P.S. Bernard, L. Li, D.M. Sipe, J. Kaplan, Cell 76 (1994) 403.
[37] D.M. De Silva, C.C. Askwith, D. Eide, J. Kaplan, J. Biol. Chem. 270 (1995) 1098.
[38] A. Singh, S. Severance, N. Kaur, W. Wiltsie, D. Kosman, J. Biol. Chem. 281 (2006) 13355.
[39] D.J. Kosman, Advances in Protein Chemistry 60, Academic Press, 2002, p. 221.
[40] D.J. Kosman, Metallomics 10 (2018) 370.
[41] S. Banerjee, C.J. Parker Siburt, S. Mistry, J. Noto, P. DeArmond, M.C. Fitzgerald, L. A. Lambert, C.N. Cornelissen, A.L. Crumbliss, Metallomics 4 (2012) 361.
[42] M.B. Rajasekaran, S. Nilapwar, S.C. Andrews, K.A. Watson, Biometals 23 (2010) 1.
[43] M. Choi, V.L. Davidson, Metallomics 3 (2011) 140.
[44] A. Matthew, L. Eberl, A.L. Carlier, Mol. Microbiol. 91 (2014) 805.
[45] R.E.M. Rickaby, Phil. Trans. R. Soc. A 373 (2015) 20140188.
[46] C.L. Dupont, S. Yang, B. Palenik, P.E. Bourne, Proc. Natl Acad. Sci., U.S.A. 103 (2006) 17822.
[47] K. Hickman-Lewis, B. Cavalazzi, S. Sorieul, P. Gautret, F. Foucher, M. J. Whitehouse, H. Jeon, T. Georgelin, C.S. Cockell, F. Westall, Sci. Rep. 10 (2020) 4965.
[48] A. Burg, D. Meyerstein, Chapter 7 - The chemistry of monovalent copper in aqueous solutions, in: R. Eldik, I. Ivanović-Burmazović (Eds.), Advances in Inorganic Chemistry Vol. 64, Academic Press, 2012, pp. 219–261.
[49] J.J. Warren, K.M. Lancaster, J.H. Richards, H.B. Gray, J. Inorg. Biochem. 115 (2012) 119.
[50] C.S. Ct Clair, H.B. Gray, J.S. Valentine, Inorg. Chem. 31 (1992) 925.
[51] T.E. Machonkin, E.I. Solomon, J. Am. Chem. Soc. 122 (2000) 12547.
[52] M. Eugenia Zaballa, L.A. Abriata, A. Donaire, A.J. Vila, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 9254.
[53] M.C. Machczynski, H.B. Gray, J.H. Richards, An outer-sphere hydrogen-bond network constrains copper coordination in blue proteins, J. Inorg. Biochem. 88 (3-4) (2002) 375–380, https://doi.org/10.1016/s0162-0134(02)00364-1. PMID: 11897353.
[54] E.Y. Kwok, S. Severance, D.J. Kosman, Biochemistry 45 (2006) 6317.
[55] A.B. Taylor, C.S. Stoj, L. Ziegler, D.J. Kosman, P.J. Hart, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15459.
[56] S.M. Jones, E.I. Solomon, Cell. Mol. Life Sci. 72 (2015) 869.
[57] L.F. Larrondo, P. Canessa, F. Melo, R. Polanco, R. Vicuna, Microbiology 153 (2007) 1772.
[58] L. Ziegler, A. Terzulli, R. Gaur, R. McCarthy, D.J. Kosman, Mol. Microbiol. 81 (2011) 473.
[59] E. Tamayo, T. Gallego, C. Aguilar, N. Ferrol, Front. Plant Sci. 5 (2014) 547.
[60] S.C. Stoj, A.J. Augustine, L. Ziegler, E.I. Solomon, D.J. Kosman, Biochemistry 45 (2006) 12741.
[61] R.A. Su-Ju Lin, A. Pufahl, T.V. Dancis, Valeria C. O'Halloran, Culotta., J. Biol. Chem. 272 (2007) 9215.
[62] C.-W. Yun, M. Bauler, R.E. Moore, P.E. Klebba, C.C. Philpott, J. Biol. Chem. 276 (2001) 10218.
[63] C.C. Philpott, O. Protchenko, Eukaryot. Cell 7 (2008) 20.
[64] C. Stoj, D.J. Kosman, FEBS Lett. 554 (2003) 422.
[65] M.M. Liu, C.J. Boinett, A.C.K. Chan, J. Parkhill, M.E.P. Murphy, E.C. Gaynor, Am. Soc. Microbiol. 9 (2018), e01347.
[66] X. Liu, Q. Du, Z. Wang, D. Zhu, Y. Huang, N. Li, T. Wei, S. Xu, L. Gu, J. Biol. Chem. 286 (2011) 14922.
[67] M. Miethke, C.G. Monteferrante, M.A. Marahiel, J.M. van Dijl, Biochim. Biophys. Acta 1833 (2013) 2267.
[68] R.M. Roop II, J.M. Gaines, E.S. Anderson, C.C. Caswell, D.W. Martin, Med. Microbiol. Immunol. 198 (2009) 221.
[69] R.M. Roop II., Animal Health Research Reviews Vol. 13, Cambridge University Press, 2012, pp. 10–20 (1).
[70] D.L. Taylor-Mulneix, S.I. Hamidou, B. Linz, E.T. Harvill, Front. Cell. Infect. Microbiol. 7 (2017) 510.
[71] S. Banerjee, R.J. Garrigues, M.N. Chanakira, J.J. Negron-Olivo, Y.H. Odeh, A. M. Spuches, R.M. Roop II, J.E. Pitzer, D.W. Martin, S. Dasgupta, J. Inorg. Chem. 210 (2020) 11162.
[72] J.C. Wilkis, J.L. Slonczewzki, J. Bacteriol. 189 (2007) 5601.
[73] A.J. Debut, Q.C. Dumay, R.D. Barabote, M.H. Saier Jr., J. Mol. Microbiol. Biotechnol. 11 (2006) 1.
[74] S. Severance, S. Chakraborty, D.J. Kosman, Biochem. J. 380 (2004) 487.
[75] M. Kern, J. Simon, Microbiology 155 (2009) 2784.
[76] I.J. Campbell, G.N. Bennett, J.J. Silberg, Front. Energy Res. 7 (2019) 79.
[77] M. Kanehisa, S. Goto, Nucleic Acids Res. 28 (2000) 27.
[78] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, Nucleic Acids Res. 25 (1997) 3389.
[79] A. Dereeper, V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J.F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J.M. Claverie, O. Gascuel, Nucleic Acids Res. 36 (2008) W465.
[80] R.C. Edgar, Nucleic Acids Res. 32 (2004) 1792.
[81] S. Guindon, J.-F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, O. Gascuel, Syst. Biol. 59 (2010) 307.
[82] F. Chevenet, C. Brun, A.L. Bañuls, B. Jacq, R. Christe, BMC Bioinformatics. 7 (2006) 439.
[83] D.H. Huson, A.F. Auch, J. Qi, S.C. Schuster, Genome Res. 17 (2007) 377.