Raquel Quatrini · Eugenia Jedlicki · David S. Holmes

Genomic insights into the iron uptake mechanisms of the biomining microorganism *Acidithiobacillus ferrooxidans*

Abstract Commercial bioleaching of copper and the biooxidation of gold is a cost-effective and environmentally friendly process for metal recovery. A partial genome sequence of the acidophilic, bioleaching bacterium *Acidithiobacillus ferrooxidans* is available from two public sources. This information has been used to build preliminary models that describe how this microorganism confronts unusually high iron loads in the extremely acidic conditions (pH 2) found in natural environments and in bioleaching operations. *A. ferrooxidans* contains candidate genes for iron uptake, sensing, storage, and regulation of iron homeostasis. Predicted proteins exhibit significant amino acid similarity with known proteins from neutrophilic organisms, including conservation of functional motifs, permitting their identification by bioinformatics tools and allowing the recognition of common themes in iron transport across distantly related species. However, significant differences in amino acid sequence were detected in pertinent domains that suggest ways in which the periplasmic and outer membrane proteins of *A. ferrooxidans* maintain structural integrity and relevant protein-protein contacts at low pH. Unexpectedly, the microorganism also contains candidate genes, organized in operon-like structures that potentially encode at least 11 siderophore systems for the uptake of Fe(III), although it does not exhibit genes that could encode the biosynthesis of the siderophores themselves. The presence of multiple Fe(III) uptake systems suggests that *A. ferrooxidans* can inhabit aerobic environments where iron is scarce and where siderophore producers are present. It may also help to explain why it cannot tolerate high Fe(III) concentrations in bioleaching operations where it is out-competed by *Leptospirillum* species.

Keywords *Acidithiobacillus ferrooxidans* · Iron uptake, storage and homeostasis · Bioleaching · *Leptospirillum* · Siderophore

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Introduction

Microorganisms are used commercially to extract copper, zinc, uranium, nickel, and cobalt from low-grade or difficult-to-process sulfide ores or mineral concentrates by a process known as bioleaching. Bioleaching has improved the efficiency of the mineral processing industry by lowering overall capital and processing costs and by diminishing environmental concerns associated with the pollution derived from emissions of smelting operations. It is also a cost-effective process for treating ores of marginal value and ore deposits that are remote or difficult to access. Microorganisms have also been used to pre-treat (beneficiate) pyrite or arsenopyrite containing occluded gold in a process termed bio-oxidation. The organisms involved in bioleaching and biooxidation processes and the biological mechanisms whereby they release metals or beneficiate ores have recently been the subjects of comprehensive reviews [45, 50, 54].

One of the most studied microorganisms involved in bioleaching is *Acidithiobacillus ferrooxidans*, formerly called *Thiobacillus ferrooxidans* [33]. *A. ferrooxidans* is a chemolithoautotrophic, γ-proteobacterium that obtains energy and electrons by the oxidation of reduced sulfur compounds to sulfate or Fe(II) to Fe(III). It is a mesophilic, facultative aerobe that fixes atmospheric CO_2 and N_2 to provide cellular C and N. It thrives in extremely acidic conditions (pH 1–2) and is often

confronted with high concentrations of metals including iron. These multiple challenges make it an excellent choice for understanding microbial physiology in extreme environments. Knowledge of its metabolism is also essential for describing its role in the bioleaching process and in the biogeochemical recycling of iron, sulfur, carbon and nitrogen in extreme acid environments. *A. ferrooxidans* is a member of a consortium of microorganisms in bioleaching operations and other naturally low pH environments. Knowledge of its role in the development and maintenance of its associated consortium is important for generating a comprehensive description of its role in mineral leaching and environmentally associated processes.

Recently, a nearly complete genome sequence of the type strain *A. ferrooxidans* ATCC 23270 became available from The Institute for Genome Research (TIGR) and Integrated Genomics (IG). This sequence was annotated and was used to develop preliminary models for nitrogen fixation, hydrogen utilization and sulfur assimilation [2, 62]. Pathways for amino acid biosynthesis have also been identified [56] and preliminary descriptions of metal ion fluxes and Ti plasmid-like genes have also been published [2]. Despite considerable effort, *A. ferrooxidans* remains largely recalcitrant to standard genetic techniques for its manipulation, although promising progress has been made towards developing a conjugation system for genetic exchange with *Escherichia coli* [37]. This deficiency seriously impedes the direct experimental investigation of the metabolism of *A. ferrooxidans* and bioinformatics becomes an important platform for genomics in the absence of genetics.

One aspect of the physiology of *A. ferrooxidans* that has received little attention is how it copes with the high iron loads typically found in its environment and in bioleaching operations. Iron is an essential micronutrient for all organisms, where it plays a central role in redox reactions. Although iron is the most abundant transition metal on Earth, its solubility is very low at neutral pH in aerobic environments. In such environments, Fe(II) is generally not available because it rapidly oxidizes to Fe(III), which precipitates as insoluble ferric ion complexes. Given its limited bioavailability, most microorganisms have been faced with the need to develop specialized uptake mechanisms to scavenge iron from their environment [5]. On the other hand, excess intracellular Fe(II) acts as a catalyst in Haber-Weiss-Fenton chemistry, which leads to the production of reactive oxygen species (Fig. 1). The hydroxyl radical (HO^-) is considered to be the most toxic, damaging lipids, proteins and nucleic acids. A number of enzymes and cofactors function in microorganisms to detoxify oxygen radicals. However, a key method to reduce radical formation is to limit the intracellular availability of iron. By sensing iron levels, limiting its uptake, and sequestering excess iron in storage proteins, organisms have developed tight intracellular homeostatic controls to balance

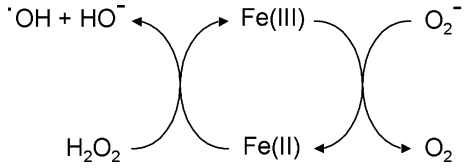

Fig. 1 The Haber-Weiss-Fenton cycle showing the role of iron in the formation of reactive oxygen species. The hydroxyl radical (HO^-) is considered to be the most toxic to biological processes. The left hand side of the scheme is the classical Fenton reaction

intracellular iron levels, guarding cell integrity against the deleterious effects of excess iron [27, 60].

A. ferrooxidans lives in acidic, aerobic conditions where Fe(II) is relatively stable and Fe(III) is much more soluble (>0.1 M) than at neutral pH, exceeding by seven orders of magnitude typical bacterial iron requirements (10^{-8} M) [27]. This poses several interesting questions:

- Has *A. ferrooxidans* evolved novel mechanisms for Fe(II) and Fe(III) uptake not found in neutrophilic microorganisms or does it rely on standard uptake mechanisms adjusted for functioning both at low pH and in the presence of an abundant supply of iron?
- How does *A. ferrooxidans* sense Fe(II) and Fe(III) in its environment given their high concentrations?
- Has *A. ferrooxidans* modified or even abandoned the typical iron storage mechanisms present in most microorganisms? Storage of iron may be less important for *A. ferrooxidans* since it has such an abundant supply.
- Has the extremely high concentration of iron found in its environment necessitated the development of novel mechanisms for the avoidance of oxidative stress due to excess intracellular iron?
- Given that *A. ferrooxidans* also oxidizes extracellular Fe(II) as an energy and electron source, can bioinformatics-based models be built that could explain the regulation of Fe(II) oxidizing and Fe(II) uptake genes?

In this report we develop preliminary models of iron uptake, regulation and storage of iron in *A. ferrooxidans* based on genome analysis, and discuss some of the evidence that begins to address the questions raised above. One of the most surprising findings is that *A. ferrooxidans*, despite having a presumed abundant supply of iron, has at least 11 distinct candidate TonB-dependent Fe(III) siderophore uptake systems, a number that rivals or exceeds the complexity found in well-studied neutrophilic bacteria that must scavenge iron from their environment. We describe the characteristics of these Fe(III) uptake systems. The necessity of their presence and the significance of their activity in the commercial biorecovery of metals and in the growth of *A. ferrooxidans* in the environment are discussed.

Overview of potential iron uptake [Fe(II) and Fe(III)] storage, sensing, and regulation mechanisms found in *A. ferrooxidans*

Iron uptake

Fe(II) uptake

Fe(II) is bioavailable in neutrophilic (or slightly acidic) environments lacking oxygen, and obligate and facultative anaerobes have devised several mechanisms to take up this ion [32, 61, 63]. Since *A. ferrooxidans* lives in extremely acidic conditions it also encounters bioavailable Fe(II) even under aerobic conditions, and it has candidate genes potentially encoding two Fe(II) uptake systems, FeoAB and MntH (Fig. 2).

Interestingly, the *feoAB* gene cluster in *A. ferrooxidans* is associated in an operon-like structure with a predicted gene that potentially encodes a porin-like protein (*porA*). PorA exhibits similarity to OprB, which belongs to a family of porins (COG3659, pfam04966) implicated in the movement of carbohydrates across the outer membrane [55, 67] that can also transport other ions [68], suggesting that PorA may participate in the uptake of Fe(II). The prediction of a Fur box-like sequence upstream of the putative *A. ferrooxidans porA* supports this hypothesis. Recently, a hypothetical gene encoding a porin of the OprB family, which is immediately followed by a FeoAB-type transporter and preceded by a predicted Fur box, was also described in *Geobacter sulfurreducens* [52].

Fe(III) uptake

Most organisms living in neutrophilic and oxic conditions have evolved a variety of iron uptake systems to compensate for iron limitation. Under such conditions, bacteria express high affinity outer membrane receptors that specifically bind and transport a variety of iron-containing molecules thereby increasing iron transport rate. Some groups of bacteria, most notably those that are pathogenic to animals, can obtain iron directly from host iron-binding proteins such as transferrin and lactoferrin [16], or indirectly through heme from hemoglobin by utilizing hemophores [22]. However, most bacteria take up iron via ferri-siderophores [34, 65]. Siderophores are low molecular weight chelating compounds that have high affinity for Fe(III) [65]. They are synthesized and excreted into the environment by many bacteria and fungi. Producers of siderophores usually have cognate receptors for the siderophores. However, many microorganisms have receptors for siderophores that they do not synthesize, allowing them to compete for iron with siderophore producers.

A. ferrooxidans has no genes with significant similarity to those involved in the utilization of heme or other pre-produced sources of iron such as transferrin or lactoferrin. However, it does have 11 candidate genes for several potential siderophore outer membrane receptors (OMRs) (Fig. 2, Table 1), but no genes have been detected that might be involved in siderophore production. This immediately suggests that it can live in environments in which Fe(III) availability is limiting and in which other organisms capable of producing siderophores are present. *A. ferrooxidans* has genes potentially involved in transporting the Fe(III) from siderophore receptors into the cytoplasm (Fig. 2). These include *tonB* and associated *exbB* and *exbD* and genes encoding ABC iron transporters. TonB is an energy transduction protein anchored in the inner membrane of Gram-negative bacteria, and spans the periplasm contacting the siderophore receptor embedded in the outer membrane. TonB also provides the energy for the uptake of the

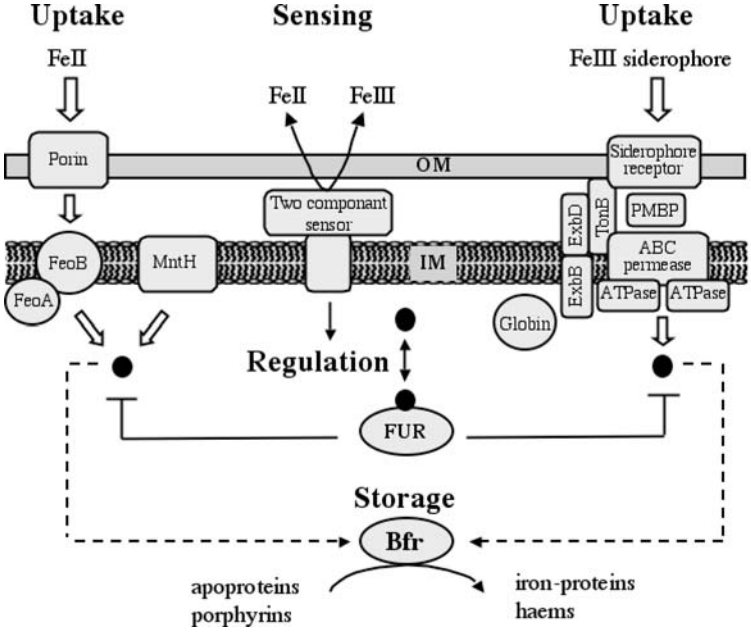

Fig. 2 Potential iron uptake [Fe(II) and Fe(III)], sensing, storage and regulation mechanisms found in *Acidithiobacillus ferrooxidans*. The Fe(II)-responsive regulator Fur, with bound Fe(II), is shown down-regulating the Fe(II) and Fe(III) uptake genes. *Black circle* Fe(II), *dotted line* incorporation of Fe(II) into the iron storage protein bacterioferritin. *OM* Outer membrane, *IM* inner membrane, *PMBP* periplasmic metal-binding protein, *ABC* ATP binding cassette transporter, *Bfr* bacterioferritin

Table 1 Potential outer membrane siderophore receptors (OMRs) found in *Acidithiobacillus ferrooxidans* with their similarity to families of known siderophores

Gene cluster	% Similarity to closest homolog, gene accession number, organism	Siderophore affinity, COG[a]	pI
A	36, AAM41335, *Xanthomonas campestris*	Linear catechol, COG1629	8.2
A	38, AAM38270, *Xanthomonas axonopodis*	Catechol, COG4771	8.6
A	38, ZP00125403, *Pseudomonas syringae*	Dicitrate, COG4772	9.0
A	56, AAM43451, *Xanthomonas campestris*	Hydroxamate, COG4773	8.8
B	50, AAM72768, *Chlorobium tepidum*	Catechol, COG4771	7.3
B	36, AAK24120, *Caulobacter crescentus*	Dicitrate, COG4772	7.3
C	38, AAO54213, *Pseudomonas syringae*	Dicitrate, COG4772	7.1
D	38, CAD86076, *Nitrosomonas europaea*	Linear catechol, COG1629	9.2
E	38, AAM49816, *Sphingobium chlorophenolicum*	Dicitrate, COG4772	6.5
F	39, AAK22197, *Caulobacter crescentus*	Dicitrate, COG4772	8.8
G	38, AAO54213, *Pseudomonas syringae*	Catechol, COG4771	8.9

[a]Clusters of ortholog genes

ferri-siderophore. *A. ferrooxidans* also has candidate genes for a periplasmic metal binding protein (PMBP) and an ABC transporter (ATP binding cassette) that provides energy via ATP, completing the Fe(III) uptake process (Fig. 2).

Iron storage

Once the Fe(III) reaches the cytoplasm it is reduced to Fe(II) and subsequently incorporated into proteins and hemes or is stored for future supply into iron-storage proteins like bacterioferritin and ferritins (Fig. 2). *A. ferrooxidans* has several candidate genes that could accomplish the role of Fe(III) reduction, including the nitric oxide dioxygenase HmpA (EC1.6.99.7; EC1.14.12.17) and the sulfite reductase CysIJ (EC1.8.1.2), as described for other microorganisms [21]. In *A. ferrooxidans*, the HmpA protein is encoded by two independent but juxtaposed genes separating the globin domain from the oxido-reductase domain. *A. ferrooxidans* also has a candidate gene potentially encoding the bacterioferritin storage protein but not the ferritin storage protein.

Iron sensing and regulation

A crucial aspect for the preservation of iron homeostasis is sensitive iron-responsive regulation [17, 26]. In bacteria, a Fe(III)-specific two component regulator (PmrAB) typically regulates a small subset of target genes and two Fe(II)-responsive regulators (Fur and DtxR) control global gene expression. The *A. ferrooxidans* genome harbors candidate genes encoding a sensor kinase similar to BasA/PmrB from *Neisseria gonorhoeae* (AAG24266) and to its cognate response regulator BasR/PmrA (48% and 74% amino acid sequence similarity, respectively; Fig. 2). This system is essential for growth in high concentrations of Fe(III) and for resistance to certain peptidic antibiotics through controlled modification of the lipopolysaccharide in *Salmonella enterica* subsp *typhimurium* [24, 66]. Fe(III)-dependent PmrAB regulation, has been hypothesized to be essential for *Salmonella* survival in extracellular environments [11] and necessary for the survival of *Erwinia carotovora* on excess iron at acidic pH [30].

Three candidate genes belonging to the Fur family of metallo-responsive regulators are also present in *A. ferrooxidans*. One of these genes encodes a protein that exhibits 79% similarity to *E. coli* Fur, including full conservation of functional motifs. It has been demonstrated to function in *E. coli* as a Fur ortholog [49].

Predicted TonB-dependent Fe(III)-siderophore uptake and Fe(III) internalization mechanisms in *A. ferrooxidans*

Diversity of TonB-dependent Fe(III)-siderophore uptake mechanism in *A. ferrooxidans*

Analysis of the *A. ferrooxidans* genome reveals the presence of candidate genes that could encode 11 potential TonB-dependent OMRs, varying widely in their predicted pI (Table 1). There is a significant concordance of secondary structure predictions between *A. ferrooxidans* OMRs and well-defined siderophore receptors, such as FecA and FhuA, from other bacteria. For example, potential signal peptides and conserved two to four N-terminal α-helices and 22 β-strands in the putative β-barrel domain, characteristic of well-defined OMRs, were detected in the potential *A. ferrooxidans* receptors, allowing tentative assignments to be made to known families of siderophore receptors (Table 1).

The frequency of potential OMRs exhibited by *A. ferrooxidans* is similar to that detected in many neutrophilic and pathogenic bacteria; for example, eight different OMRs with affinity for siderophores have been described and characterized in *E. coli* [1] and *Vibrio cholerae* [39, 40, 53, 59]. An analysis of the *Nitrosomonas europaea* [9] genome revealed more than 20 candidate siderophore OMRs, about 32 putative siderophore OMRs were also found in *P. aeruginosa* [15] and as many as 65 in *Caulobacter crescentus* [43]. The diversity of OMRs exhibited by *A. ferrooxidans* might be considered unexpected for an extreme acidophile inhabiting conditions typically rich in soluble iron. There are several possible explanations for this observation. It may be

that they represent non-active pseudogenes that are not required for iron uptake. However, their amino acid sequence conservation, including the conservation of functional motifs, and their organization in operon-like gene clusters argues against this interpretation. Future experimental investigation will be required to examine this option. Another possibility, which we favor, is that they represent functional iron uptake genes with a diverse range of specificities and pIs that allow them to function at different pHs and with different iron sources. *A. ferrooxidans* has long been known to grow at pH 4 in sulfur [48, 64] and, more recently, at pH 5.5 (our unpublished results). At the latter pH in aerobic environments iron begins to become limiting, favoring the selection of active iron uptake systems. The presence of siderophore OMRs but the apparent absence of siderophore biosynthesis systems in *A. ferrooxidans* argues that the organism inhabits aerobic environmental niches in which potential siderophore producers are present, such as are found, for example, in the Rio Tinto [23].

Interestingly, the 11 distinct siderophore OMRs of *A. ferrooxidans* exhibit a wide range of isoelectric points (Table 1). Whereas neutrophilic bacteria like *E. coli* and *P. aeruginosa* have receptors with isoelectric points in the neutral range (pI 6–7), some of the receptors from *A. ferrooxidans* exhibit pIs as high as 9, which may help the OMRs to maintain their integrity at low pH. Supporting this view is the fact that the acid tolerant *Helicobacter pylori* has four receptors for ferric-dicitrate all with pIs above 8. The range of pIs exhibited by *A. ferrooxidans* OMRs would potentially allow *A. ferrooxidans* to import iron over a range of different pHs and to compete with other microorganisms within consortia and biofilms that are known to exist in environmental and bioleaching milieus.

The presence of potential multiple Fe(III) uptake systems might also help to explain why *A. ferrooxidans* is virtually absent after prolonged bio-oxidation of minerals in tank reactors in which the Fe(III) to Fe(II) ratio is very high. In these circumstances *Leptospirillum* strains appear to dominate the microbial population, an observation that has been attributed to their ability to grow in the presence of high concentrations of Fe(III), which selects against *A. ferrooxidans* [51]. For example, in laboratory studies it has been shown that the K_I for inhibition of growth by Fe(III) for *L. ferrooxidans* is 42 mM compared to 3.1 mM for *A. ferrooxidans* [44]. This distinction could reflect differences between *A. ferrooxidans* and *Leptospirillum* in their ability to take up Fe(III) or in their capacity to preserve iron homeostasis.

How might interactions between Fe(III) uptake proteins be maintained in an acidic environment?

Outer membrane and periplasmic proteins, and periplasmic loops of proteins embedded in the inner membrane of *A. ferrooxidans*, presumably exhibit relevant protein-protein contacts and structural motifs that are stable and function at pH 2. Can genome analysis of the pertinent proteins in *A. ferrooxidans* suggest how this might be achieved?

It is known that recognition of Fe(III)-enterobactin by its receptor FepA in *E. coli* is largely dependent upon charge interactions [8, 42], although other contacts may play roles in the adsorption and movement of the siderophore [41]. Charge interactions are affected in an acidic milieu by protonation of amino acid side chains such as Asp, Glu and His. We have noted amino acid changes in the predicted channel facing portions of the β-barrel of *A. ferrooxidans* siderophore OMRs that are expected to reflect adaptation to extreme acidic conditions. The impact of these residues on potential ligand affinity has not been tested experimentally. Sequence and structural similarities of the *A. ferrooxidans* siderophore OMRs with their neutrophilic counterparts extends, in all cases, to the functional N-(PS00430) and C-terminal (PS01156) TonB interaction motifs [3, 46, 47].

How might the uptake of Fe(III) be energized in *A. ferrooxidans*?

Iron transport across the outer membrane is an energy-dependent process that relies on the electrochemical potential (PMF, proton motif force) of the inner membrane and the energy transduction complex TonB-ExbB-ExbD [4]. In the acidic growth environment of *A. ferrooxidans*, the concentration of protons is orders of magnitude higher than in neutral environments and this presumably has strong consequences on the use of PMF as an energy source coupling OMR proteins and the TonB PMF transducing complex.

In neutrophilic organisms, loading of the Fe(III) ligand drives a conformational change in OMRs, which decreases their affinity for their ligands, resulting in a transference of the ligand to TonB [31]. TonB appears to shuttle between the cytoplasmic and outer membranes, extracting the ligand from the OMRs and passing it to a cytoplasmic metal binding protein. This function is energized by PMF harnessed by the integral inner membrane proteins ExbB and ExbD [28, 29, 35].

There are six putative candidate TonB-class proteins predicted in the *A. ferrooxidans* genome and, compared to their neutrophilic counterparts, each exhibits a considerable degree of sequence divergence in the central proline-rich domain. This divergence does not alter the overall proline content (16–21%) of the protein but does include the frequent replacement of acidic residues (Glu) found in neutrophilic organisms with Ala, Ser or basic residues. Given that this central domain appears to be important for the ability of TonB to bridge the periplasmic space by allowing TonB to adopt a rigid extended conformation [36], these substitutions could represent a functional adaptation of the TonB of *A. ferrooxidans* to acid conditions, allowing it to be activated by the ExbB/ExbD complex.

Heteromultimers of ExbB and ExbD are thought to constitute a proton-translocating unit. Both proteins are anchored in the inner membrane and predicted inner membrane embedded loops of the putative ExbB and ExbD of *A. ferrooxidans* are highly conserved whereas the predicted periplasmic spanning portions of ExbD exhibit significant increases in pI compared to their neutrophilic counterparts. This may reflect changes necessary to stabilize the structure and function of these proteins at low pH.

How the PMF across the cytoplasmic membrane activates the transport receptor proteins in the outer membrane is not fully understood and so it is difficult to be sure how the relevant *A. ferrooxidans* proteins function with the enormous pH gradient across the inner membrane at pH 2. Future work, involving comparisons of the predicted tertiary structures of these acidophilic proteins with known crystal structures of their neutrophilic counterparts, will identify sequence and structural differences that could explain how they maintain structural and functional integrity in acid pH. For example, there are solved crystal structures for three siderophore OMRs, FepA [7], FecA [20, 69], FhuA [19, 38], and the ABC transporter protein FhuD [13, 14]. There are also crystal structures at 1.55 Å resolution of the carboxy-terminal domain of TonB [12] and for the periplasmic Fe(III) binding protein PMBP [6, 57, 58].

Internalization of iron across the inner membrane by siderophore-specific ABC transporters is driven by ATP as an energy source [34]. In *E. coli* three different ABC transporters carry out the uptake of hydroxamates, catecholates or dicitrate siderophores, whereas only two such transporters have been recognized in *A. ferrooxidans*. These two ABC transporters are present in one gene cluster (Table 1, cluster B; Fig. 3). All ABC-transporter components, including those with domains in the periplasm, are highly conserved with respect to neutrophilic counterparts. The two ABC permeases showed 55–69% amino acid sequence similarity to prototypic members of pfam01032 family, and one cytoplasmic protein involved

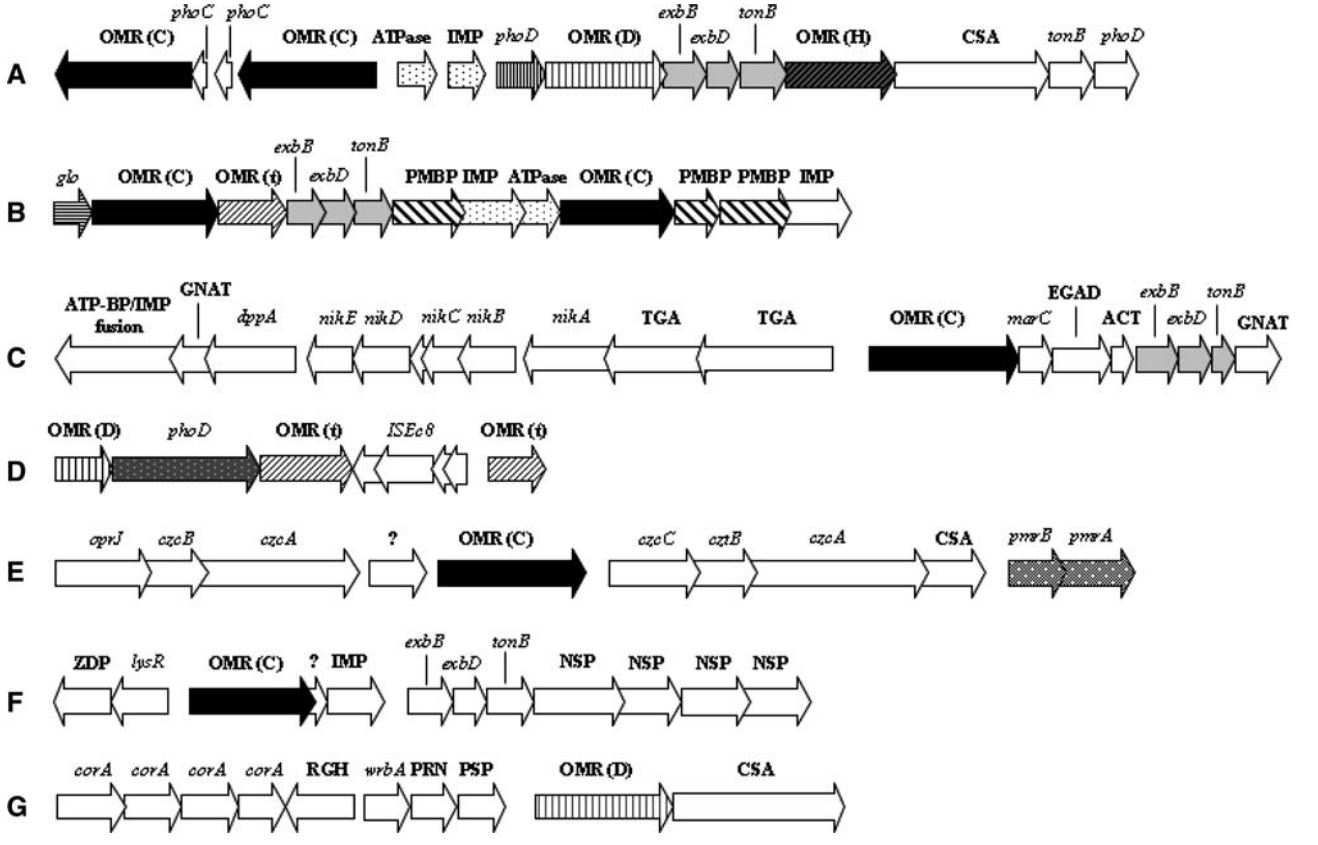

Fig. 3 Proposed genetic organization of *A. ferrooxidans* outer membrane siderophore receptor (OMR) gene clusters A–*G* (gene clusters correspond to those presented in Table 1). *OMR*©) OMR with affinity for catechol siderophores, *OMR(D)* OMR with affinity for dicitrate siderophores, *OMR(H)* OMR with affinity for hydroxamate siderophores, *OMR(t)* OMR truncated gene, *PMBP* periplasmic metal binding protein, *IMP* ABC transporter inner membrane permease, *ATPase* ABC transporter ATP binding protein, *glo* globin-like, *phoC* non-hemolytic phospholipase C (fragment), *phoD* alkaline phosphatase, *GNATN*-acetyltransferase, *dppA* ABC-type heme binding protein, *nikABCDE* Ni(II) ABC-type transporter, *TGA* transglutaminase, *marC* putative channel protein, *EGAD* L-lactate dehydrogenase, *ACT* putative protein containing the amino acid concentration-responsive ACT domain (pfam01842), *ISEc8* insertion sequence, *oprJ* outer membrane lipoprotein, *czcABC* cation efflux system, *CSA* cell surface antigen, *pmrAB* Fe(III)-responsive two component system, *ZDP* Zn-dependent protease, *lysR* transcriptional regulator, *NSP* nucleoside permease, *corA* Mg(II) low affinity uptake transporter, *RGH* ribosylglycohydrolase, *wrbA* tryptophan repressor binding protein, *PRN* pirin, *PSP* predicted secreted protein

in ATP binding was 63% similar to other characterized ATPases of the COG1120 family. Also, two highly conserved PBPs (52–54% similarity to PBP: COG0614) with predicted affinity for Fe(III)-hydroxamate siderophores are present. General primary sequence conservation of these components further supports that a predicted mechanism of siderophore uptake similar to those found in other organisms accumulating Fe(III) in iron-poor conditions may exist in *A. ferrooxidans*.

Genetic organization of genes encoding putative Fe(III) uptake proteins

The 11 predicted genes for OMRs are grouped in seven gene clusters in the *A. ferrooxidans* genome (Table 1, Fig. 3). Some of these clusters include other genes known to be involved in iron transport (clusters A, B), whereas others contain genes that participate in diverse metal-homeostasis responses (clusters C, E). The remaining siderophore OMRs detected in *A. ferrooxidans* appear not to be distributed in iron or metal-related gene contexts.

Cluster A contains two divergent sets of genes that between them include four OMRs predicted to belong to different siderophore-affinity families (Fig. 3a, Table 1). This is also the case for gene cluster B, which contains two OMRs of different specificity. The organization of iron uptake functions in operons, or forming part of pathogenicity islands, is frequent in bacteria. However, the presence of several siderophore OMRs in the same gene cluster is not as frequent [53].

Clusters A and B both include the predicted genes *exbB*, *exbD* and *tonB* for the TonB energy-transducing system, but cluster B also contains three candidate genes for periplasmic metal binding proteins and genes that potentially encode an ABC-type inner membrane iron transporter. Cluster B, therefore, potentially encodes a complete suite of proteins necessary for Fe(III) uptake (Fig. 3b). Interestingly, this possible operon also includes a putative gene for a globin-like protein of unknown function and an upstream Fur box, indicating possible regulation via a Fur-mediated mechanism.

Two other OMRs are encoded in the proximity of TonB systems, while the three remaining OMRs appear isolated from standard iron transporting functions (Fig. 3, clusters D, E, G). The presence of an OMR embedded within the heavy metal resistance efflux system *czc* (cluster E) seems intriguing, as is the presence of an OMR near the Ni(II) transporting system *nikABCDE* (Fig. 3, cluster C) or in the vicinity of the *corA* Mg(II) transporter. Both the NikABCDE and CorA transporter have been reported to participate in low affinity transport of Fe(II) into the cell in the presence of low concentrations of competing specific metals, Ni(II) or Mg(II) [10, 18, 25]. The genetic proximity of these systems with Fe(III)-siderophore OMRs may indicate cross-talk between metal transporting systems at the inner membrane level.

The close proximity of two OMRs of *A. ferrooxidans* to two well-conserved large cell surface antigens (Fig. 3, clusters A, G) is almost unprecedented. Similar proteins (40–60%), of as yet unestablished function, are encoded in many genomes deposited in the databases (e.g., 58% S *Gloeobacter violaceus*—NP_926147) but only in one case (e.g., 56% S *Nostoc punctiforme*—ZP_00109441) does the ortholog appear embedded in a Fe(III)-siderophore transporting operon. The presence in the cell surface antigens of a C-terminal phosphoesterase motif (pfam04185) and a heme D1 binding motif (pfam02239), typically present in cd1 cytochromes, suggests a possible role for these proteins in heme binding or deesterification of PO_4-chelated iron at the cell surface.

Conclusions

Bioinformatic genome analysis for iron uptake, sensing, storage and regulation provides an initial framework for addressing how *A. ferrooxidans* copes with high iron loads at low pH in its natural environment and in bioleaching operations. The multiple siderophore uptake systems found in *A. ferrooxidans* were unexpected and suggest that the microorganism may be capable of living in environments where iron is scarce. So far, only the role of Fur from *A. ferrooxidans* has been investigated experimentally [49] and the bioinformatic genome predictions presented here will help guide experimental analysis, highlighting areas in urgent need of experimental testing. The modeling presented here will also enable researchers to identify possible regulatory connections required for the coordinated control of the components involved in balancing the requirements of iron as a nutrient in very small amounts versus its use as an energy and electron source in much larger amounts.

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