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Iron homeostasis in the *Rhodobacter* genus

Sébastien Zappa and **Carl E. Bauer***
Department of Molecular and Cellular Biochemistry, Indiana University, Simon Hall, 212 S Hawthorne Dr, Bloomington, IN 47405, U. S. A

Abstract

Metals are utilized for a variety of critical cellular functions and are essential for survival. However cells are faced with the conundrum of needing metals coupled with e fact that some metals, iron in particular are toxic if present in excess. Maintaining metal homeostasis is therefore of critical importance to cells. In this review we have systematically analyzed sequenced genomes of three members of the *Rhodobacter* genus, *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2 to determine how these species undertake iron homeostasis. We focused our analysis on elemental ferrous and ferric iron uptake genes as well as genes involved in the utilization of iron from heme. We also discuss how *Rhodobacter* species manage iron toxicity through export and sequestration of iron. Finally we discuss the various putative strategies set up by these *Rhodobacter* species to regulate iron homeostasis and the potential novel means of regulation. Overall, this genomic analysis highlights surprisingly diverse features involved in iron homeostasis in the *Rhodobacter* genus.

I. INTRODUCTION

The origin of oxygenic photosynthesis can be traced to ~2.9 billion years ago when cyanobacteria-driven photosynthesis created a Great Oxidizing Event that enriched atmospheric oxygen. Prior to photosynthetic oxidation of Earth, most iron was in a reduced ferrous state that is biologically available as it has a solubility of 0.1M at pH7. This form of iron is thought to been present in deep biotopes until ~1.8-1 billion years ago (Van Der Giezen and Lenton, 2012; Planavsky et al., 2011). Beyond that time the presence of atmospheric oxygen effectively oxidized most surface and oceanic iron to a ferric state that has an extremely low solubility (10^{-18}M at pH 7) (Andrews et al., 2003). Consequently, the oxidation of Earth must have caused a crisis of iron availability necessitating that cells evolved a diverse array of ferrous and ferric iron uptake systems.

Iron is an important cofactor in many enzymes where it can form mono- or di-iron centers, or more complex iron-sulfur clusters. Iron is also bound to protoporphyrin IX to form heme that has an important role as a gas and electron carrier. Enzymes that utilize iron are involved in major biochemical processes such as photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, the trichloroacetic acid cycle, oxygen transport, gene regulation and DNA biosynthesis. Iron is also an important actor in cellular events such as virulence, biofilm formation and quorum sensing (Vasil, 2007; Steele et al., 2012; Wen et al., 2012). The role of iron in so many systems indicates that Life evolved enzymes that utilized iron when it was readily available and as a result had to invent biochemical pathways to maintain iron homeostasis when Earth's oxidation caused iron to become scarce. Extremely rare are the examples of organisms that solved the iron

*To whom correspondence may be addressed: Carl E. Bauer, Department of Molecular and Cellular Biochemistry, Indiana University, Simon Hall MSB, 212 S Hawthorne Dr, Bloomington, IN 47405, U. S. A.; tel.: 001-812-855-6595; fax: 001-812-856-5710; bauer@indiana.edu.

availability issue by circumventing the need for iron. This includes the lactobacilli and the Lyme disease agent (Archibald, 1983; Weinberg, 1997; Posey, 2000).

Bacteria have developed iron uptake systems to both the ferrous, ferric forms of elemental iron. They also evolved iron scavenging pathways by excreting and transporting siderophores that function as iron chelators. Bacteria have also developed means of transporting heme that is synthesized by other organisms as a salvage pathway (Wandersman and Delepelaire, 2004; Andrews et al., 2003; Braun and Hantke, 2011). These diverse iron transport systems must be tightly regulated, as excess cellular iron is toxic. Free iron can generate free hydroxyl radicals through Fenton's chemistry (equations 1, 2, 3) that have deleterious effects on fatty acids and other biomacromolecules (Touati, 2000; Chiancone et al., 2004).

$$\mathrm{Fe}^{2+} + O_2 \rightarrow \mathrm{Fe}^{3+} + O_2{}^- \quad \text{(Eq. 1)}$$

$$2O_2{}^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Eq. 2)}$$

$$\mathrm{Fe}^{2+} + H_2O_2 \rightarrow \mathrm{Fe}^{3+} + \mathrm{OH}^- + {}^{\bullet}OH \quad \text{(Eq. 3)}$$

Challenged with balancing a need for iron with iron's toxicity, cells must maintain a tightly regulated iron homeostasis that controls the dynamic equilibrium between import, export and the storage of iron in proteins. Purple nonsulfur bacteria are facultative phototrophs distributed among the α-and β-subclasses of proteobacteria. They have an extremely versatile metabolism that utilizes iron in ways that allows growth under multiple environmental conditions. The use of iron by purple nonsulfur bacteria can be exemplified by such processes as: i) Aerobic respiration where terminal cytochrome oxidase cbb_3 and b_{260} use heme as a cofactor; ii) Respiratory and photosynthesis electron transport where heme containing cytochromes c_y, c_2 and bc_1 shuttle electrons to photosystem reaction centers as well as to respiratory terminal oxidases; iii) Enzymes such as coproporphyrinogen III oxidase that contains a Fe-S cluster involved in synthesis of heme; iv) Enzymes involved in bacteriochlorophyll synthesis that utilize iron-sulfur clusters (Sirijovski et al., 2007; Sarma et al., 2008); v) Purple nonsulfur bacteria are also capable of anaerobic oxidation of ferrous iron to facilitate phototrophic growth (Widdel et al., 1993; Ehrenreich and Widdel, 1994; Croal et al., 2007; Caiazza et al., 2007; Poulain and Newman, 2009). These are just a few representative examples of the many processes used by this group of bacteria that rely on the use of iron as a cofactor, and that illustrate their heavy need for this metal. In this chapter, we will discuss what is known about iron homeostasis in the *Rhodobacter* genus, focusing on iron homeostasis genes present in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2 (formerly known as *R.* sp. SW2 (Saraiva et al., 2012)). Despite numerous studies on photosynthesis, respiration and general physiology, there have been surprisingly few studies on their iron needs and how they regulate cellular iron homeostasis.

II. FERROUS IRON UPTAKE

A. FEO SYSTEM

The Feo iron transport system is widespread among bacteria and thus appears to be a major route of ferrous iron acquisition (Cartron et al., 2006; Perry et al., 2007). Since the first description of the Feo system in *Escherichia coli* in 1987 (Hantke, 1987), it has been shown to be involved in many iron-related phenotypes such as magnetosome formation (Rong et

al., 2012) and virulence (Fetherston et al., 2012). The Feo system is present in many bacterial and in some archaeal genomes. Interestingly, the uptake Feo system has significant sequence similarity to eukaryal G-proteins and thus has been referred to as a “living fossil” of this family of eukariotic GTPases (Hantke, 2003).

Genes coding for the Feo system are present in each of the three genomes considered in this study, namely *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2. *R. sphaeroides* and *R. ferroxidans* contain only one *feo* gene cluster while *R. capsulatus* exhibits two clusters. It is not unusual to find two (or more) *feo* loci in one organism. In such cases, it is hypothesized that one of the Feo systems is specialized in manganese uptake and when proven to be involved in this process is thus called Meo (Cartron et al., 2006; He et al., 2006; Dashper et al., 2005). However, duplicate Feo systems can also be specialized in two different iron-related pathways, such as magnetosome formation and oxidative stress management (Rong et al., 2012).

In the analyzed *Rhodobacter* genomes, a putative four-gene operon is present in each strain, consisting of two *feoA* genes followed by *feoB* and *feoC* loci (*feo*A_1A_2BC) (table 1). Other cases of multiple *feoA* genes in a *feo* operons have been reported (Cartron et al., 2006). Another putative operon, named *feo2AB*, is unique to *R. capsulatus* SB1003 that displays only one *feoA* gene followed by an *feoB* (table 1). In ~80% of the genomes where a *feo* locus is present, it consists of a small *feoA* gene followed by a larger *feoB* gene in an *feoAB* operonal organization. Occasionally an additional *feoC* ORF is also present, particularly in the γ-proteobacteria phylum, which forms an *feoABC* operon. Alignment of translated *feo*A_1, *feo*A_2 and *feo2A* genes from these three *Rhodobacter* species highlights a higher sequence similarity/identity for $FeoA_1$ representatives with homologues from the other *Rhodobacter* species than to gene paralogues present in their own genome (table 2). For example, *R. capsulatus* $FeoA_2$ shows 69.51% and 68.29% similarity with $FeoA_2$ from *R. sphaeroides* and *R. ferroxidans*, respectively. This is contrasted by $FeoA_2$ from *R. capsulatus* exhibiting only 40.22% and 36.56% homology with $FeoA_1$ and Feo2A, that is present in its own genome (table 2). The same pattern occurs when comparing FeoB from these three species: FeoB sequences, translated from the *feo*A_1A_2BC operons, exhibit more homology with other species than to FeoB present in other operons (table 2). Taken together, gene organization and sequence homology indicate that the *feo*A_1A_2BC operon may be a general features of the *Rhodobacter* genus, while the *feo2B* operon may be specific to *R. capsulatus*.

Even though no experimental evidence exists for the role of Feo gene products in *Rhodobacter* species, it is likely that the general model for the Feo system will prevail. If this is the case then FeoB codes for a membrane bound ferrous iron permease with weak GTPase activity that is enhanced by FeoA. FeoA has a SH3 domain that is thought to be involved in protein-protein interactions. In addition, *feoA* mutants have been shown to have hampered ferrous iron uptake (Kammler et al., n.d.; Kim et al., 2012). In operons that contain FeoC, this protein is hypothesized to be a transcription factor involved in controlling the *feoABC* operon. However experimental confirmation of the role of FeoC is still lacking (Fetherston et al., 2012; Guo et al., 2011). Multiple control of the FeoB permease by two different FeoA GTPase enhancers, a local transcription factor FeoC and a global regulator such as Fur might be related to potential toxicity of iron and the need to maintain finely tuned regulation (Kammler et al., n.d.; Cartron et al., 2006). A closer look at the FeoB protein sequences from *Rhodobacter* species confirm the presence of the GTPase domain as 4 out of the 5 G-motifs are conserved (Dashper et al., 2005). The only major divergence may be Feo2B from *R. capsulatus* where the G1 motif exhibits a GPPNCG sequence instead of GNPNCG. The hydrophobic C-terminal domain of FeoB, which consists of two GATE motifs in opposite orientation in the membrane, is similar to the Ftrp1 yeast iron permease. Good conservation of the *Rhodobacter* Gate motifs occurs especially with the presence of

two key Cys residues located in the segment IV of Gates 1 and 2, which are potentially involved in iron binding. Once again, the only striking substitution is observed for Feo2B from *R. capsulatus* where a consensus PC is changed into QC in the Gate 2 segment IV. Preliminary transcription data with *R. capsulatus* show that both Feo systems are controlled by iron availability, but in a different manner. Indeed, while $FeoA_1A_2BC$ is overexpressed ~70- and 200-fold under mild and harsh iron stress, respectively; Feo2AB is overexpressed 3-fold and repressed ~4-fold under the same respective conditions (Zappa and Bauer, in preparation). Generation of a dendrogram based on FeoB sequence alignment highlights the occurrence of two major clades: one containing the *Rhodobacter* FeoB sequences with the exception of *R. capsulatus* Feo2B (fig. 1). Interestingly, two FeoB loci from *Porphyromonas gingivalis* are distributed in each clade. In *P. gingivalis* FeoB1 was shown to be involved in iron uptake, while FeoB2 is shown to be the major manganese transporter (Dashper et al., 2005; He et al., 2006). *R. capsulatus* Feo2B may therefore be involved in manganese homeostasis.

B. EFEUOB(M) SYSTEM

The Elemental Ferrous iron (EfeUOB) system was recently identified as a highly specific to ferrous iron transporter (Grosse et al., 2006; Cao et al., 2007). Orthologues of *E. coli* EfeOUB, formerly called YcdNOB, can be found in many bacterial genomes {(Rajasekaran, Nilapwar, et al., 2010b). Ferrous iron uptake systems similar but distinct to EfeUOB (called EfeUOB-like) have also been identified in various bacteria such as the P19-Ftr1P system in *Campilobacter jejuni* (van Vliet et al., 1998), FetMP in *E. coli* (Koch et al., 2011) and FtrABCD in *Bordetella* species (Brickman and Armstrong, 2012), making the EfeUOB-type transporter a widely utilized iron uptake strategy among microorganisms. Among the *Rhodobacter* genomes, *R. capsulatus* display an EfeUOB operon but no EfeUOB-like system (table 1, fig. 2a). In *R. sphaeroides* and in *R. ferroxidans* SW2 there is a surprising absence of EfeUOB and EfeUOB-like operons. Moreover, *R. capsulatus* also encodes a putative EfeU-EfeO fusion protein (fig. 2b). Although very unusual, a similar EfeU-EfeO fusion has been reported previously (Rajasekaran, Nilapwar, et al., 2010b).

In *E. coli*, transcription of the *efeUOB* operon is known to be induced under iron starvation, low pH or in the presence of exogenous copper. The transcription factor Fur and the phosphorelay CpxAR are involved in the iron-and pH-dependent expression, respectively (Cao et al., 2007). Interestingly, expression occurs under aerobic conditions (Cao et al., 2007). In addition to *E. coli* a few other studies indicate that *efeUOB* homologues from other species are controlled by iron availability. For example, *efeUOB* homologues from *Bacillus subtilis*, (Baichoo et al., 2002; Ollinger et al., 2006), *Neisseria menigitidis* (Grifantini et al., 2003), the magnetotactic bacterium strain MV-1 (Dubbels et al., 2004) and *Magnetospirillum magneticum* AMB-1 (Suzuki et al., 2006) are known to be regulated in response to iron.

Only a few experimental studies on the function of *efeUOB* proteins have been reported so a large part of its understanding comes from sequence analyses. The EfeU protein is homologous to the yeast iron permease Ftr1p, with seven transmembrane helices (TMH). Two of these helices, TMH-I and TMH-IV, contain a iron transport REXXE motif (Grosse et al., 2006; Rajasekaran, Nilapwar, et al., 2010b). These motifs are conserved in the *R. capsulatus* EfeU domain of the EfeU-EfeO fusion protein (fig. 2b).

EfeB is a periplasmic homodimeric heme containing DyP-type peroxidase (Sturm et al., 2006; X. Liu et al., 2011). EfeB has also been proposed to act as a deferrochelatase, providing iron to *E. coli* by extracting iron from heme (Létoffé et al., 2009). However, a study with the related protein YfeX could not confirm such a role (Dailey et al., 2011). The *R. capsulatus* EfeB sequence shows the presence of a conserved TAT signal (Bendtsen et

al., 2005) indicating that it is likely exported to the periplasm (fig. 2b). Moreover, alignment with other DyP-type peroxidases (not shown) show conservation of residues involved in heme binding: D235, H330, R347 (Sugano, 2009).

The role of EfeO (COG 2822) is even less clear. EfeO proteins can present different domain organization with the most common one consisting of a N-terminal cupredoxin domain followed by a C-terminal peptidase M75 domain. However EfeOs comprised of only a cupredoxin domain or a peptidase M75 domain are also frequently found in genomes (Rajasekaran, Nilapwar, et al., 2010b). Although nomenclature is still being undefined the trend is to name cupredoxin-containing members as EfeO and cupredoxin-less members that consist of a solo peptidase M75 domain as EfeM (Rajasekaran, Nilapwar, et al., 2010b; Rajasekaran, Mitchell, et al., 2010a). The *R. capsulatus* putative operon shows two representative of the EfeO/M family (COG 2822), bringing the gene organization to an *efeUOBM* operon where *efeU* and *efeO* are fused (table 1, fig. 2a). The first one is fused to the EfeU domain mentioned above and has the signature of a EfeO protein, *i.e.* showing both the cupredoxin and M75 domains (fig. 2b). On the other hand the second copy (orf 03067) is a typical M75 containing only EfeM. Motifs potentially involved in copper and iron binding (EWE, EEREN) are conserved in the EfeUO cupredoxin domain (fig. 2b). The EfeM peptide also contains the putative HXXE iron binding sequence while EfeUO M75 domain does not, highlighting probable functional differences between these two EfeO/M like proteins (Rajasekaran, Nilapwar, et al., 2010b; Rajasekaran, Mitchell, et al., 2010a). Finally, EfeO/M are thought to be periplasmic proteins (Sturm et al., 2006; Rajasekaran, Nilapwar, et al., 2010b) as a signal peptide signature sequence is predicted to be present on EfeM (Petersen et al., 2011). The cellular location of EfeO is less clear, nevertheless, export of the EfeO domain in the periplasm could be achieved during folding of EfeUO but this has to be confirmed experimentally.

A proposed mechanism of ferrous iron uptake by the EfeUOB system is based on homology with the yeast permeation/ferroxidation Ftr1p/Fet3p system (Stearman et al., 1996; Kosman, 2003; Rajasekaran, Nilapwar, et al., 2010b). Briefly, ferrous iron in the periplasm binds to the M75 domain of EfeO and is subsequently oxidized to ferric iron by the copper center of cupredoxin domain. The ferric iron is then transferred first to the EfeO cupredoxin domain and then to the permease EfeU. The copper center is finally regenerated by the EfeB peroxidase (Rajasekaran, Nilapwar, et al., 2010b). A similar mechanism could be possible in *R. capsulatus*, involving both the EfeO domain of EfeUO and EfeM instead of a unique EfeO. Moreover, according to the hypothetic mechanism, EfeU and EfeO are interacting. The fusion of these proteins in *R capsulatus* is compatible with such a hypothesis. In summary, *R. capsulatus* is unique among *Rhodobacter* species in that it seems to have an intact ferrous iron uptake system with analysis of the sequence indicating that it likely functional (fig. 2a and 2b).

III. FERRIC IRON UPTAKE

The major ferric iron uptake system involves mediation by siderophores. This iron uptake pathway has been extensively studied and reviewed (Köster, 2001; Krewulak and Vogel, 2008; Sandy and Butler, 2009; Chu et al., 2010; Hider and Kong, 2010; Krewulak and Vogel, 2011). Briefly, siderophores are small molecules secreted by bacteria, fungi and graminaceous plants that can solubilize ferric iron in aerobic environments, due to their high binding affinity for Fe(III) (10^{-20} M). Siderophore transporters consist of a tonB-dependent outer membrane ferrisiderophore receptor and an ABC transporter cassette (a periplasmic siderophore binding protein, a permease and an ATPase). Such organization is very well conserved among bacteria and archaea with similar systems used to import heme and vitamin B_{12}. The specificity of the outer-membrane receptor is usually very high compared

the specific of the ABC cassette. This sometimes enables the cross-use of the same ABC cassette to uptake different siderophores that have been imported into the periplasm by different outer-membrane receptors.

According to *Rhodobacter* genome annotations, *R. capsulatus* and *R. sphaeroides* display an *exbB-exbD-tonB* operon while *R. ferroxidans* does not (table 3). The outer membrane bound ExbB-ExbD-TonB complex enables transfer of protomotive force from the cytoplasmic membrane to the outer-membrane. As such, it drives the energy needed to import the ferrisiderophore from its outer-membrane receptor to the periplasm where the cytoplasmic membrane bound ABC transporter then imports the ferrisiderophore into the cytoplasm. Given that the ExbB-ExbD-TonB complex is absent in *R. ferroxidans* it is not surprising that no siderophore uptake system is annotated in the *R. ferroxidans* SW2 genome. On the other hand, 8 and 5 complete siderophore uptake systems are present in the *R. capsulatus* SB1003 and *R. sphaeroides* 2.4.1 genomes, respectively. Looking closer at the sequences and the genomic environment indicates that the respective TonB-dependent uptake systems orf 3358 to orf 3362 and orf 2102 to orf 2105 in *R. capsulatus* and *R. sphaeroides*, respectively, are more likely to be involved in vitamin B_{12} uptake rather than siderophore transport. These strains still potentially encoding 7 and 4 siderophore uptake systems as detailed in table 3. Interestingly, no siderophore synthesis gene cluster is present in either genome, meaning that *R. capsulatus* and *R. sphaeroides* likely scavenge siderophores from other species (termed xenosiderophores) to fulfill their iron needs. Such situation is not unusual as some species even rely on xenosiderophores to provide enough iron for growth (D'Onofrio et al., 2010). This situation happens between bacteria as well as between bacteria and fungi (Kosman, 2003). Moreover, it has been shown that bacteria can use “improbable siderophores” such as α-keto acids instead of classical siderophores such as catecholates and hydroxamates (Reissbrodt et al., 1997).

Once a ferrisiderophore complex had entered the cytoplasm, its fate is not well known. One of the most studied siderophore processing events is the involvement of the enterobactin esterase in *E. coli*. This enzyme hydrolyses enterobactin, producing trimers of dihydroxybenzoylserine that weakens the bond with ferric iron. Enterobactin esterase also seems to act as an enterobactin-specific reductase that reduces the ferric iron into a soluble ferrous iron which triggers iron release from the siderophore (Andrews et al., 2003; Rudolph et al., 2006). Importantly, two putative esterases both located in siderophore uptake gene clusters are annotated in the *R. capsulatus* genome, orf 00110 and orf 01050. The latter is actually annotated as an enterobactin (enterochelin) esterase. The presence of this gene is very unusual among α-proteobacteria (Rudolph et al., 2006).

IV. IRON ABC TRANSPORTERS

Some bacteria also contain metal-ABC transporters that exhibit specificity for iron. Unlike classical siderophore-based iron uptake systems, the metal-ABC transporters are not dependent on an outer-membrane receptor for iron or siderophore transport into the periplasmic space. The best characterized version of this class of transporters is the FbpABC system studied in *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Campilobacter jejuni*, *Bordetella pertussis*, *Marinobacter* species, *Vibrio cholerae*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* (called sometimes AfuABC) (Chin et al., 1996; Khun et al., 1998; Paustian et al., 2001; Tom-Yew et al., 2005; Wyckoff et al., 2006; Brickman et al., 2011; Strange et al., 2011; Amin et al., 2012). This transporter is specific to ferric iron and consists in a Fe(III)-binding periplasmic protein, a membrane permease and an ATPase. Also, in *Neisseria* and *Bordetella*, this transporter also undergoes tonB-independent uptake of endogenous siderophores or xenosiderophores (Brickman et al., 2011; Strange et al., 2011). Similar transporters have been characterized in *Serratia marcescens* and

Haemophilus influenzae under the respective names SfuABC and HitABC (Angerer et al., 1992; Sanders et al., 1994). The cyanobacterium *Synechocystis* sp. PCC6803 also contains this transport system, with the particularity of having two Fe(III)-binding proteins instead of one (Katoh, Hagino, Grossman, et al., 2001b; Katoh, Hagino and Ogawa, 2001a; Badarau et al., 2008).

Present in the annotated *R. sphaeroides* genome is a putative homologue of FbpA, the periplasmic substrate-binding protein of the FbpABC system (Tom-Yew et al., 2005). Looking closer at the genome reveals that this ORF (orf 2913) is not part of an FbpABC operon. On the other hand, a second ORF matching FbpA's COG 1840 appears to share an operonal organization with three ORF's that subunits of an ABC transporter: two inner membrane proteins and an ATPase subunits. These four ORFs (orf 0346 to orf 0349) may thus constitute an FbpABC transport system in *R. sphaeroides*. Such a transport system is also present in *R. ferroxidans* and *R. capsulatus* where a similar gene organization is found (table 4).

Another metal-ABC transporter system, YfeABCD/SitABCD, is thought to be involved in iron uptake, although its specificity between iron and manganese is not clear. Indeed, the YfeABCD system has been shown to transport both Fe(III) and Mn(II) in *Yersinia pestis* and *Photorhabdus luminescens*, being involved in virulence mechanisms in both cases (Bearden and Perry, 1999; Watson et al., 2010). The SitABCD system has also been shown to transport both Fe(II), instead of Fe(III), and Mn(II) in *Sinorhizobium meliloti* and *Shigella flexneri* (Chao et al., 2004; Platero:2004gi Fisher et al., 2009). In an *E. coli* avian pathogenic strains, the transport of Fe(II), Fe(III) or Mn(II) was varying as a function of the strain genetic background (Sabri et al., 2006). Finally, SitABCD in *Salmonella enterica* Serovar *Typhimurium* was shown to be able to uptake both Fe(II) and Mn(II) but with an affinity for Mn(II) that is stronger and physiologically more relevant (Janakiraman and Slauch, 2000; Kehres et al., 2002). An orthologous SitABCD transporter is annotated in the genome of *R. sphaeroides* 2.4.1, but not in *R. capsulatus* SB1003 and *R. ferroxidans* SW2 genomes (table 4). However, there is no sequence signature in the SitABCD system in general and in the *R. sphaeroides* SitABCD cluster in particular, that enables prediction of its involvement in either iron or/and manganese transport. Nevertheless, *R. sphaeroides* does not seem to have an NRAMP-type manganese dedicated transporter MntH, while *R. capsulatus* and *R. ferroxidans* SW2 do contain the MntHR Mn transport system (table 5). Assuming that *R. sphaeroides* has regular manganese needs, and that no other unknown manganese transporters are present, it is reasonable to hypothesize that the SitABCD system in *R. sphaeroides* is dedicated to manganese uptake rather than to iron.

V. HEME IRON USAGE

Heme is often a crucial iron source for pathogens that scavenge it from their host. However, heme uptake is also frequently an iron acquisition mechanism that occurs in beneficial symbiotic bacteria (Nienaber et al., 2001; Runyen-Janecky et al., 2010; Anzaldi and Skaar, 2010; Septer et al., 2011). A major hemin uptake system is the PhuRSTUVW-type heme transporter as described in *Pseudomonas aeruginosa*, where PhuR is a TonB-dependent outer-membrane heme receptor. The PhuT subunit of the PhuRSTUVW heme transporter is the periplamic heme-binding protein while PhuUVW is an ABC transporter and PhuS is a cytoplasmic protein (Anzaldi and Skaar, 2010). Extensive characterization of similar systems has been undertaken in the pathogenic bacteria *Bordetella pertussis*, *Yersinia pestis*, *Yersinia enterolitica*, *Shigella dysenteriae*, *Vibrio cholera*, *Campylobacter jejuni*, *Bartonella quintana* and *E. coli* O157:H7 (Anzaldi and Skaar, 2010) with the transporters names with varying terminology. A homologue has described in the symbiotic Rhizobiales *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* and *Sinorhizobium meliloti* where it

is called HmuQR-HmuTUV, HmuPSTUV and ShmR-HmuPSTUV, respectively (Nienaber et al., 2001; Wexler et al., 2001; Anzaldi and Skaar, 2010; Amarelle et al., 2010; Amarelle et al., 2008).

R. capsulatus displays an orthologous gene cluster where it is annotated as *hmuRSTUV* (loci from orf 00094 to orf 00098). Interestingly, *R. sphaeroides* and *R. ferroxidans* do not seem to code for this heme transporter. Thus, unlike other *Rhodobacter* representatives, *R. capuslatus* is likely to be able to use heme from *de novo* synthesis as well as form exogenous sources. Unlike *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*, the *R. capsulatus* heme uptake gene cluster does not have a nearby TonB-ExBD subunit. It may therefore rely on a homologous gene located on a different region of the genome (table 3) or contain an unidentified protein that provides this function. Also, the *R. capsulatus* Hmu system lacks the HmuP regulator that has been identified in *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* that functions as a co-activator of this heme uptake system along with Irr (Amarelle et al., 2010; Escamilla-Hernandez and O'Brian, 2012). A BLAST analysis on the *Rhodobacter* clade indicates possible candidates for an HmuP transcription factor in *R. capsulatus* (orf 01112) and in *R. sphaeroides* (orf 6006) but not in *R. ferroxidans*. However, it should be cautioned that these putative HmuP homologues have poor sequence conservation, and no genome context with heme transporters, so their actual function needs to be experimentally confirmed.

The heme uptake gene cluster in *R. capsulatus* also contains *hmuS* that in other organisms has been shown to be involved in heme degradation. The mechanism of action of HmuS orthologues is not clear but seems eclectic: i) Some HmuS were shown to enzymatically degrade heme; ii) Some seem to operate via a non-enzymatic process with H_2O_2; iii) Some may store and/or traffic heme to a heme oxygenase (Anzaldi and Skaar, 2010; M. Liu et al., 2012; O'Neill et al., 2012; Barker et al., 2012).

Based on COG analysis, it is notable that *R. capsulatus* contains a second putative HmuS locus (orf 03488) that is located next to a putative coproporphyrinogen oxidase III encoding gene. *R. sphaeroides* only has one HmuS homolog (orf 0228), while *R. ferroxidans* has no HmuS annotated. Presence of a putative heme degrading system is thought to be crucial to use heme as an iron source as well as to prevent heme toxicity by ensuring that there is no pool of unbound free heme in the cell(Frankenberg-Dinkel, 2004; Anzaldi and Skaar, 2010). Moreover, a typical heme oxygenase (BphO-like) is not present in either *R. capsulatus* or *R. ferroxidans*, while there are two annotated heme oxygenase genes annotated in *R. sphaeroides*. These are associated with bacteriophytochrome encoding genes (orf 4191, orf 7212). As shown recently in *Pseudomonas aeruginosa*, bacteriophytochrome-associated BphO is not involved in heme degradation from exogenous heme uptake, but only in holo-bacteriophytochrome synthesis from *de novo* synthesized heme (Barker et al., 2012). Atypical IsdG-like heme oxygenases HmuQ and HmuD, were recently characterized in *Bradyrhizobium japonicum* (Puri and O'Brian, 2006; Skaar et al., 2006). A BLAST analysis against the *Rhodobacter* clades reveals the presence of a potential HmuQ/D-like protein in *R. sphaeroides* (orf 0826) however further work is needed to confirm this identity. Finally, as detailed in chapter II.B, the EfeUOB system has been shown to be able to extract iron from heme by a deferrochelatase activity, although such activity is debated (Létoffé et al., 2009; Dailey et al., 2011). Such an activity could provide iron to *R. capsulatus*, the only representative of the *Rhodobacter* genus displaying the *efeUOB* gene cluster (table 1).

A final point regarding heme usage is the presence of “heme exporter” gene clusters in the annotation of the three *Rhodobacter* strains of interest. Closer look reveals they corresponds to CcmABCDG system used for *c*-type cytochrome synthesis. It has been shown with *R. capsulatus* that a mutation that disrupts cytochrome *c* maturation results in massive secretion

of porphyrins (S. W. Biel and A. J. Biel, 1990). The authors did not confirm if a *ccmABCDG* operon mutation was truly causing this phenotype but did speculate that there is a connection between iron homeostasis, heme and cytochrome synthesis (LASCELLES, 1956; S. W. Biel and A. J. Biel, 1990)

VI. MANAGING IRON TOXICITY

As stated in the introduction, free iron can be extremely toxic. Thus, once cellular iron needs are fulfilled, being able to counterbalance the intake to avoid overload is definitely of interest for a cell. There are two ways are available in bacteria to counterbalance iron intake. One mechanism consists in reversing the uptake process by excreting excess iron using iron efflux pumps. The second process involves the binding or sequestration of iron in a dedicated peptide where it is stored in a harmless state. These peptides are called ferritins and/or bacterioferritins.

A. IRON EFFLUX PUMP

The efflux of iron in bacteria is not well characterized although the last decade has highlighted a prominent role of Cation Diffusion Facilitators (CDF). The CDF family is ubiquitous in the three domains of life and grouped together as heavy metal transporters. They are classified into three subfamilies according to their metal substrate specificity: Zn-CDF, Fe/Zn-CDF and Mn-CDF (Montanini et al., 2007; Nies, 2011). Despite having a "favorite" metal substrate, they usually are capable of transporting a wide array of metals (Munkelt et al., 2004; Montanini et al., 2007). One of the most studied representatives is the ferrous iron efflux protein (FieF, also known as YiiP). Its presence was shown to increase *E. coli*'s tolerance to iron and to lower the total iron cellular content (Grass et al., 2005). Although FieF was biochemically well characterized *in vitro* using zinc as a substrate, its main substrate *in vivo* is ferrous iron and, as such, is likely to an important role in iron homeostasis (Nies, 2007). An ORF was annotated as FieF in *R. capsulatus* SB1003 (orf 00522). Using the same COG number (COG 0053), candidates were found in the two other *Rhodobacter* genomes: orf 0463 in *R. sphaeroides* 2.4.1, and orf 1374 and orf 2021 in *R. ferroxidans* SW2. *E. coli* FieF has four metal binding sites named Z1 through Z4 (Nies, 2011; Lu and Fu, 2007). Alignment of the putative *Rhodobacter* FieF sequences shows good conservation at most of these sites, with the exception of orf 2021 in *R. ferroxidans*. Other CDF genes were also found in these *Rhodobacter* genomes, under COG 1230, but their sequences align better with the *E. coli* zinc transporter ZitB then with the iron transporter Fief (data not shown). However, given the relaxed substrate specificity of CDFs, it is worth noting that the *R. capsulatus* ZitB homologue (orf 00089) is located next to the $feoA_1A_2BC$ putative ferrous iron uptake system.

B. STORAGE AND DETOXIFICATION

The ferritin family consists of three types of protein that form distinct phylogenetical clades: the ferritins (Ftn), the bacterioferritins (Bfr) and the *DNA-binding proteins from starved cells* (DPS). Ftn and Bfr have strong structural homology with each consisting of homooligomers of 24 subunits. DPS are comprised of 12-mers of the same subunit (Bou-Abdallah, 2010; Andrews, 2010). Ftn and Bfr form ball-shaped complexes with an outer diameter of ~120Å that can store up to 4500 iron atoms in a ~80Å diameter cavity. The iron in this cavity consists of either amorphous iron with inorganic phosphate or crystalline ferrihydrite. DPS also form a ball like structure only they have an outer diameter of ~95Å that can handle up to ~500 molecules of iron (Bou-Abdallah, 2010; Carrondo, 2003; Andrews, 2010). Cellular iron concentration is estimated to ~10^{-4}M, which is far above the solubility of this metal. This concentration is reached do to the presence of these iron sequestration storage proteins that concentrate and store the metal in non-reactive form

(Theil and Goss, 2009). By isolating the toxic iron from cellular machinery, storing it and releasing upon needs, ferritins functions almost as cellular organelles.

The ferritin family is ubiquitous with Ftn found in all three domains of life while Bfr and DPS are specific to Bacteria and Archaea (Andrews, 2010). Their activity relies on ferroxidase centers consisting in *intra*subunit di-iron centers in Ftn and Bfr and *inter*subunit di-iron center in DPS (Andrews, 2010). These centers channel Fe(II) into the cavity by oxidizing it into Fe(III) which generates the insoluble oxidized form of iron stored inside the complex core. It seems that Ftn and Bfr uses mostly O_2 as the iron oxidant while DPS use H_2O_2 (Bou-Abdallah, 2010; Andrews, 2010). Ftn and Bfr main role is for iron storage while the function of DPS is thought to be as a detoxifier that protects DNA from redox stress generated by the iron mediated Fenton reaction (equations 1–3) (Andrews, 2010). It is quite common for bacteria to have each ferritin types as well as several copies of one type of ferritin. For example, *E. coli* is typical with the presence of two Ftn, one Bfr and one DPS (Andrews et al., 2003; Chiancone et al., 2004). The major difference between Ftn and Bfr is the presence of 12 b-type heme moieties in the Bfr complex (Cobessi et al., 2001; Bou-Abdallah, 2010; Carrondo, 2003; Andrews, 2010). After decades of mystery about the role of the heme groups, it has recently been shown that they are involved in the mobilization of iron back to the cytosol. Thus while ferroxidase centers oxidize the iron during the mineralization process that internalizes the iron into the Bfr, the heme moieties reduce the core iron during its export into the cytoplasm. Ferroxidase centers and heme moieties functions independently (Yasmin et al., 2011).

Analysis of the *Rhodobacter* genomes reveals the presence of Ftn and Bfr proteins (table 6). *R. sphaeroides* has a membrane-bound ferritin (orf 0850). A BLAST analysis on the genome of *R. capsulatus* also reveals the presence of a homologue in this species (orf 03466). Interestingly, no homologues were found in the *R. ferroxidans* genome in the NCBI database but an ORF with the same COG number could be found in the JGI database (orf 0178). It is annotated as rubrerythrin, which is likely to be the ancestral form of the ferritin-like protein family (Andrews, 2010). The annotation is probably incorrect as the three putative Ftn share strong homology (more than 70% identity and more than 80% similarity, as shown in table 7). Indeed, they are all 325 amino acid long while Bfr are in the 160 amino acid range. Conserved domains analysis showed that they all consist in an Ftn-like N-terminal domain of ~140–150 amino acids followed by a C-terminal CCC1 domain of ~120–140 amino acids (not shown). CCC1 domains are involved in iron and manganese transport. In yeast, CCC1 is a vacuole transmembrane protein responsible for iron and manganese accumulation in vacuoles. Finally, these genes share similarity in terms of genomic organization, as seen on figure 3, indicating an ancient gene cluster that was conserved during evolution.

Beside a membrane-bound ferritin, Bfr homologues are present in the genomes of *R. capsulatus* and *R. sphaeroides*, but not in *R. ferroxidans* (table 6). In fact, *R. sphaeroides* displays two Bfr, one on each chromosome, while *R. capsulatus* has only one. While *R. capsulatus* Bfr share almost 80% similarity with each of the *R. sphaeroides* Bfr, the latters show a similarity score to each other that is close to 90% similarity (table 7). This suggests that a single *bfr* gene duplicated in *R. sphaeroides*. Unlike membrane-bound ferritin, Bfr show weaker genomic conservation. One of *R. sphaeroides* Bfr (orf 1446) is organized in a putative operon with a Bfr-associated-ferredoxin (orf 1447), an iron-regulated protein (orf 1448) and a hypothetical protein (orf 6006). Interestingly, the latter shows homology with HmuP, a heme uptake regulator described in *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* as reported in section V. No iron homeostasis genes are found in the vicinity of the other *R. sphaeroides bfr* gene (not shown). Regarding the *R. capsulatus* Bfr, it seems to be part of an operon that contains a hypothetical protein that aligns well with *R. sphaeroides* Bfr-associated-ferredoxin (orf 1447). Association of a *bfr* gene with a ferrodoxin is very

common in bacteria (Rodionov et al., 2006). This connotation may suggest that orf 1446 was the “original” *bfr* in *R. sphaeroides* before being duplicated without the ferredoxin gene.

Some experimental work has been undertaken with the *Rhododacter* Bfr. The first Bfr to be studied was with *R. sphaeroides* although most work has been done on the *R. capsulatus* homologue (Meyer and Cusanovich, 1985). Between 900 and 1000 amorphous iron atoms along with 600 phosphate molecules are contained in each Bfr complex. It is located in the cytoplasm and its expression in “normal” growth medium is stable. Nevertheless, a moderate control by iron is observed as iron starvation induces a decrease in Bfr cellular content, while iron replete conditions promotes accumulation of Bfr in the cell (Ringeling et al., 1994; Penfold et al., 1996). The *Rhodobacter* Bfr structure was the second Bfr structure to be elucidated after that of *E. coli* Bfr (Cobessi et al., 2001).

Regarding iron storage in *R. sphaeroides*, some transcriptional mechanisms are reported. Transcription of Bfr encoded by orf 1546, along with its associated ferredoxin appears to be controlled by iron availability as their mRNA levels increase when iron is depleted. The transcription factor Irr is implicated in this control (Peuser et al., 2012). The other Bfr (orf 3342), which lacks a linked ferredoxin, is almost insensitive to iron levels and shows a weak Irr regulation profile (Peuser et al., 2012). Finally, the Ftn-like MbfA displays weak iron control but a strong Irr-dependence. In fact, as predicted by Rodionov *et al.*, MbfA was shown to be under the direct regulation of Irr (Rodionov et al., 2006; Peuser et al., 2012).

VII. IRON HOMEOSTASIS REGULATORS

The regulation of iron homeostasis involves a complex overlapping set of global regulators as well as more specialized regulators dedicated to the control of specific iron homeostasis genes, such as the control of siderophore synthesis and ferrous iron uptake. Such local regulators are diverse and belong to the following families: i) AraC-type transcription factor (Pradel et al., 1998; Ducey et al., 2005; Hollander et al., 2011; Fantappiè et al., 2011); ii) two component systems (Steele et al., 2012); iii) extracytoplasmic function (ECF) σ factors (Braun, 1997; Koster et al., 1994; Braun et al., 2003); iv) LysR-type transcription factor (Litwin and Quackenbush, 2001; Vanderpool and Armstrong, 2003); v) small RNA regulators (Massé et al., 2007; Metruccio et al., 2009; Huang et al., 2012; Ducey et al., 2009; Smaldone et al., 2012). *Pseudomonas aeruginosa* is an example of organism that combine all the aforementioned regulators as well as several global regulators (Vasil, 2007; Cornelis et al., 2009). Computational analysis showed that *R. sphaeroides* possesses FecRI (orf 4274 and 4275, table 3) where FecI is a homologue of the ECF-type σ factor and FecR is periplasmic regulator of FecI (Rodionov et al., 2006). Based on its genomic environment, the *fecRI* operon may be co-transcribed with a siderophore uptake gene cluster that could be under its control. FecRI is involved in Fe(III)-citrate uptake in *E. coli* but homologues such as PupBI and HurRI have been characterized in other species. They are involved in pseudobactin siderophores and heme uptake, respectively (Koster et al., 1994; Vanderpool and Armstrong, 2003). Genome analysis shows also that *R. capsulatus* has four AraC-like transcription factors located next to siderophore uptake gene clusters (Rodionov et al., 2006). These AraC encoding genes are given table 3.

In many cases, transcription factors dedicated to the control of specific iron homeostasis genes are usually themselves under the control of a global transcription factor (Fantappiè et al., 2011; Pradel et al., 1998; Braun, 1997) or act as co-regulators with a global regulator (Escamilla-Hernandez and O'Brian, 2012). For a long time, the paradigm of iron homeostasis in bacteria was associated with the Ferric Uptake Regulator (Fur) that was first characterized in *E. coli.* Additional studies quickly showed that Fur homologues are widespread in both gram-negative (proteobacteria) and gram-positive (Firmicutes) bacteria,

as well as in some cyanobacteria (Carpenter et al., 2009; Lee and Helmann, 2007). The basic mechanism of Fur regulation consists of transcriptional repression of iron uptake genes under iron replete conditions by Fur that contains a bound Fe(II) (Fe(II)-Fur). However, under conditions of iron limitation, Fur without bound iron (apo-Fur) is incapable of binding these promoters, derepressing the expression of these iron uptake genes (Rudolph et al., 2006; Lee and Helmann, 2007; Carpenter et al., 2009). The regulation by Fur is now known to be more complex since Fe(II)-Fur can also activate genes in an indirect manner, via the derepression of a small regulatory RNA (Massé et al., 2007; Metruccio et al., 2009; Huang et al., 2012; Ducey et al., 2009; Smaldone et al., 2012). Finally, direct transcriptional activation by apo-Fur on a target gene promoter was also observed (Carpenter et al., 2009; Lee and Helmann, 2007).

The Rhizobiale and Rhodobacterale phyla are also known to regulate iron homeostasis genes by the global regulator Irr (Iron response regulator) (Rodionov et al., 2006; Rudolph et al., 2006; Johnston et al., 2007). It has been proposed that upon the appearance of Irr, Fur evolved into a manganese uptake regulator. Consequently the Fur homologues in Rhizobiales and Rhodobacterales have been renamed Mur. [Another evolutionary event is the inclusion of a third iron regulator, RirA, that is present in the Rhizobiales (Rhodobacterales only have Fur/Irr) (Rodionov et al., 2006; Rudolph et al., 2006; Johnston et al., 2007)]. Fur and Irr belong to the same Fur-superfamily of metal regulators and thus share strong sequences homology. Nevertheless, their mechanisms of action are very different. Fur directly binds elemental iron which subsequently affects the ability of Fur to activate or repress gene expression. Like Fur, Irr can both activate and repress iron-dependent gene expression. However, Irr does not bind free iron but instead monitors the iron level indirectly by sensing the level of heme biosynthesis. Specifically, Irr interacts and monitors the activity of ferrochelatase, the last enzyme of the heme synthesis pathway. Irr obtains a heme from ferrochelatase which results in targeting Irr for degradation (Rudolph et al., 2006; Rodionov et al., 2006; Small et al., 2009).

Regarding *Rhodobacter* genomes, members of the Fur-family are represented in each of the sequenced genomes. However, a closer look reveals that: i) Fur is missing in *R. capsulatus*, while present in *R. sphaeroides* and *R. ferroxidans*; ii) Each genome contains a copy of Irr; iii) Each genome also has a Zur encoding gene (table 8). The latter is also a Fur-family member but specialized in zinc uptake (Lee and Helmann, 2007). It is readily identifiable as Zur is consistently located along with a *znuABC* gene cluster that encodes a zinc transporter. Although sharing high sequence similarity, Fur and Irr can be discriminated from their sequence analysis. A main feature distinguishing Fur and Irr is the HHDH Fe(II) binding motif that is a signature of Fur, which becomes a HHH (or HQH) in Irr. This aspartate deletion transforms this site from a Fe(II) to a heme binding site (Rudolph et al., 2006). Thus, while *R. sphaeroides* and *R. ferroxidans* display the classical Rhodobacterale iron homeostasis transcription factor features, *R. capsulatus* has a very unusual set of regulators. Moreover, *R. capsulatus*, along with *Mesorhizobium loti*, is among the very rare α-proteobacteria that also have a manganese MntHR uptake system, most likely acquired through horizontal gene transfer (Rodionov et al., 2006). Based on *in silico* analysis and studies on Rhizobiales, it has been proposed that the acquisition of Irr as global iron regulator may have pushed Fur into a more marginal role where it evolved into manganese homeostasis regulation, as Mur (Rodionov et al., 2006; Johnston et al., 2007). Following this hypothesis, *R. capsulatus* appears as an extreme example of this evolutionary trend.

In addition to genome analysis, some experimental work has been undertaken on the role of Fur/Mur and Irr in *R. sphaeroides* 2.4.1. In this organism, Fur/Mur is involved in iron homeostasis and oxidative stress response upon iron scarcity, where it acts as a repressor. Interestingly, the Δ*fur/mur* mutant shows a more hampered growth profile during manganese

rather than iron limitation. Furthermore the Δ*fur/mur* mutant also has no activation of the putative Mn(II)/Fe(II) uptake system SitABCD, highlighting this operon as a potential target for Fur (Peuser et al., 2011). The role of Irr in *R. sphaeroides* is also not well defined. A Δ*irr* strain does not display much growth deficiency in an iron-depleted medium suggesting that Irr does not have a major function in iron homeostasis. Moreover, Irr in *R. sphaeroides* was shown to bind heme and to activate many genes beyond that of iron homeostasis such as stress response, oxidative phosphorylation, transport, and photosynthesis.

Consequently the effect of Irr on iron homeostasis may be indirect. Oxidative stress also appears to be an important part of the Irr regulon as Δ*irr* is more resistant to this stress (Peuser et al., 2012). Such resistance could be achieved by Irr through indirect control of a catalase and possibly a small RNA involved in singlet oxygen/superoxide response. Direct Irr control was evidenced on *mbfA* and *ccpA*, encoding the membrane-bound ferritin and a cytochrome *c* peroxidase, respectively. Although transcription data showed weak effect, Irr was proven to directly interact with the respective promoter of these genes, confirming computational prediction of Irr regulon in *R. sphaeroides* (Peuser et al., 2012; Rodionov et al., 2006). This computational study of iron and manganese regulons predicted that *mbfA* and *ccpA* are Irr-regulated genes in both *R. capsulatus* and *R. sphaeroides*. Beside Irr-specific regulation, the iron regulon in *R. capsulatus* and *R. sphaeroides* were predicted to consist of 18 and 8 genes, respectively (Rodionov et al., 2006)

From the data on *R. sphaeroides*, neither Fur/Mur nor Irr appears as a master regulator of iron homeostasis in this species. Rodionov *et al.* hypothesize a potential major role of IscR, a regulator of Fe-S cluster synthesis in *E. coli* (Rodionov et al., 2006). While *R. capsulatus* and *R. sphaeroides* genomes have an IscR encoding gene (orf 01853 and 0443, respectively), none could be found in the *R. ferroxidans* genome. Nevertheless, the absence of Fur, and presence of MntR, in *R. capsulatus* is a good hint that it most likely regulates iron homeostasis differently from *R. sphaeroides* and *R. ferroxidans*. Sequence alignments (not shown) of Fur, Irr, Zur sequences show very high similarity and identity, in the order of 60 to 80%, respectively (table 9). But, consistently, *R. sphaeroides* and *R. ferroxidans* displays higher values with each other than with *R. capsulatus*.

VIII. CONCLUSION

Analysis of the genomes of three model *Rhodobacter* species, highlights the diversity of strategies used by these organisms to maintain iron homeostasis (table 10). The two extreme strategies appeared to be *R. capsulatus* and *R. ferroxidans*. On the one hand, *R. capsulatus* presents a whole battery of ferrous, ferric and heme iron uptake (between 10 and 12!) in addition to an iron efflux pump and several storage genes. On the other hand, *R. ferroxidans* displays a more minimalist arsenal with one ferrous iron uptake system, an efflux pump and one storage system. The case of *R. sphaeroides* seems intermediate with a balanced distribution of ferrous and ferric iron uptake systems (between 5 and 7). Also, it is notable that *R. capsulatus* is the only *Rhodobacter* with a clearly identified heme uptake system that has been mostly studied in pathogens and more recently with symbionts. Moreover, *R. capsulatus* is the only sequenced *Rhodobacter* genome that is missing the canonical iron global regulator Fur. Such a paradox suggests novel regulation of iron homeostasis in this organism. Finally, among *Rhodobacter* species, *R. capsulatus* is the only organism displaying a large portion of its genome putatively dedicated to iron uptake. This “iron island” is a ~22kb gene cluster consisting of a ferrous iron transport cassette, a heme uptake system, an ABC transporter and siderophore import system (fig. 4).

Among the question that remains to be solved is the transport of iron through the periplasmic membrane. Indeed, unlike siderophores and heme, which have specific outer-

membrane receptors, the access of elemental iron to the inner-membrane acquisition systems (Feo, Efe or ABC-type) has not been characterized. The paradigm for such a small compound consists in passive diffusion but recent research indicates that porin-like channels in the outer-membrane can transfer specific divalent metal cations to the periplasm (Hohle et al., 2011).

The field of metal homeostasis, and the regulation of iron uptake in particular, is a complex one. Indeed, after decades of research on iron regulation in a well-known organism such as *E. coli*, iron uptake systems are still being discovered (Koch et al., 2011). Decades of studies of Fur are also not enough to suppress debates about its mechanism of action as well as discriminating related representatives such as Mur, Zur, Nur (Lee and Helmann, 2007). In addition, a new layer of complexity is emerging in the field concerning the overlap between iron and manganese homeostasis which are two metals that have crucial and yet opposite effects on oxidative stress (Puri et al., 2010; Jakubovics and Jenkinson, 2001; Horsburgh et al., 2002). Depending on whether cells are challenged with an oxidative stress response or DNA synthesis under iron scarcity, organisms have been shown to be able to switch from an Fe-based enzyme activity to its Mn-based equivalent (Andrews et al., 2003; Andrews, 2011; Lee and Helmann, 2007).The various strategies to maintain metal homeostasis are printed in the genomes of these *Rhodobacter* strains and clearly highlight the diversity of this genus.

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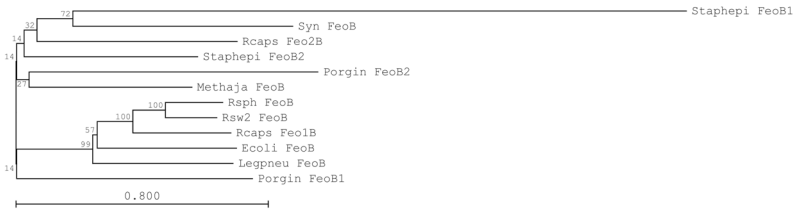

Fig 1.

Dendrogram of FeoB from various organisms, based on protein sequence alignment. Alignment and dendrogram were built using CLC Sequence Viewer software (CLC Bio, Denmark). The bootstrap analysis algorithm was used, with 100 replicates. Bootstrap values are indicated at each knots and substitution rate at the bottom. Sequences were retrieved from the Integrated Microbial Genomes of the DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/w/main.cgi). Ecoli: *Escherichia coli* DH1, Legpneu: *Legionella pneumophila* Paris, Methaja: *Methanocaldococcus jannaschii* DSM 2661, Porgin: *Porphyromonas gingivalis* ATCC 33227, Rcap: *Rhodobacter capsulatus* SB1003, Rsph: *Rhodobacter sphaeroides* 2.4.1, Rfer: *Rhodobacter ferroxidans* SW2, Staphepi: *Staphylococcus epidermidis* ATCC 12228, Syn: *Synechocystis sp*. PCC 6803. Accession numbers: Ecoli FeoB, BAJ45144; Legpneu FeoB, YP_125016; Methaja FeoB, NP_247545; Porgin FeoB1, YP_001929201; Porgin FeoB2, YP_001929425; Rcaps FeoB, YP_003576264; Rcaps Feo2B, YP_003578180; Rsph FeoB, ; YP_351866 Rsw2 FeoB, ZP_05845183; Staphepi FeoB1, NP_763744; Staphepi FeoB2, NP_765669; Syn FeoB, NP_440528.

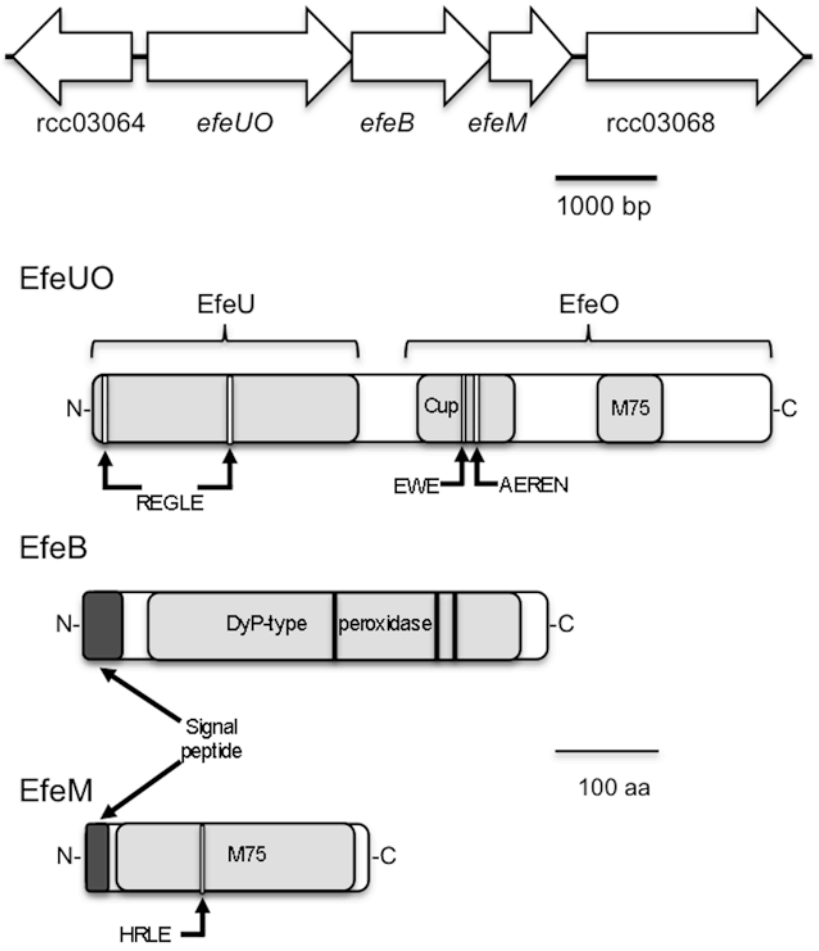

Fig 2.

Genetic organization (a) and protein architecture (b) of *R. capsulatus* EfeUOBM system. Flanking genes orf 03064 and orf 03068 are predicted to encode a membrane protein (HPP family/CBS domain) and a cache sensor protein, respectively. Arrows indicate conserved sequences putatively involved in metal binding. Cup: cupredoxin domain; M75: M75 metallopeptidase domain.

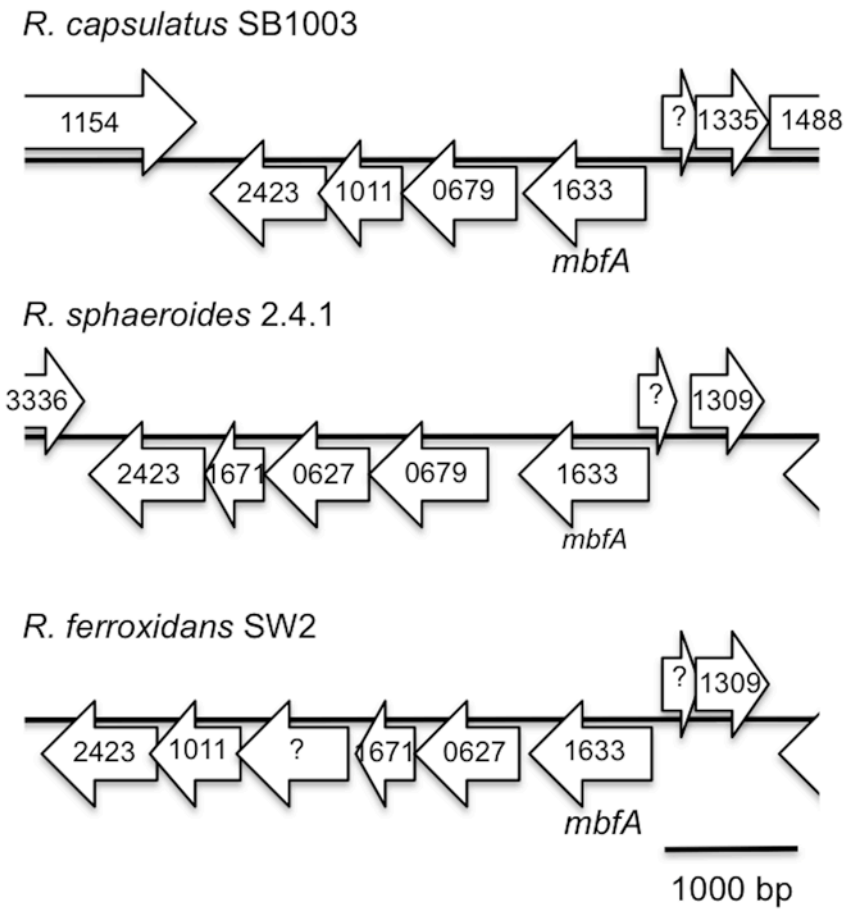

Fig 3.
Genomic environment of membrane-bound ferritin encoding genes, *mbfA*, in *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1, *R. ferroxidans* SW2. COG numbers of each reading frame are indicated.

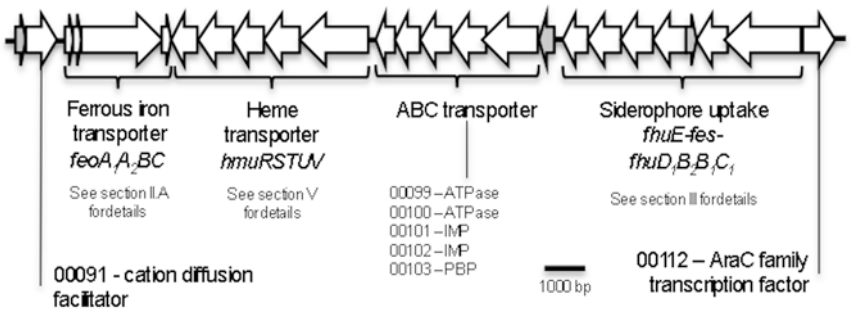

Fig 4.

Putative “Iron island” in *R. capsulatus* SB1003. Genes encoding “hypothetical proteins” are shown in grey. IMP: Inner-membrane protein; PBP: Periplasmic binding protein. ORF number is given with the 5 digits only of the nomenclature of *R. capsulatus* SB1003 (for example, 12345 correspond to locus RCAP_rcc12345 for a chromosomal gene and p12345 to RCAP_rcp12345 for a plasmid gene).

Table 1

Ferrous iron uptake genes identified in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2.

Putative operon name	Gene name	COG	Label *Rcap* SB1003[a]	Label *Rsph 2.4.1*[a]	Label *Rfer* SW2[a]	Product
efeUOB	*efeUO*	2822, 0672	03065	N.D.	N.D.	Ferrous iron permease EfeU
	efeB	2837	03066	N.D.	N.D.	Dyp-type peroxidase family protein
	efeM	2822	03067	N.D.	N.D.	Protein of unknown function DUF451
feoA$_1$A$_2$BC	*feoA$_1$*	1918	00090	6020	3172	Ferrous iron transport protein A
	feoA$_1$	1918	00091	1819	3171	Ferrous iron transport protein A
	feoB	0370	00092	1818	3170	Ferrous iron transport protein B
	feoC		00093	1817	3169	Fe-S dependent transcriptional regulator
feo2AB	*feo2A*	1918	02028	N.D.	N.D.	Ferrous iron transport protein A
	feo2B	0370	02029	N.D.	N.D.	Ferrous iron transport protein B

N.D.: Not detected

[a] *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2 ORFs are accessible in the Integrated Microbial Genomes of the DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) using the following locus tag RCAP_rcc#####, RSP_#### and Rsw2DRAFT_####, respectively, where #### and ##### are the 4 or 5 digits indicated in the table. Regarding *R. capsulatus* numbering, genes on the plasmid are references as RCAP_rcp##### on the JGI database and p##### in this study.

Table 2

Identity and similarity in FeoA, FeoB and FeoC from *R. capsulatus* SB1003, *R. shaeroides* 2.4.1 and *R. ferroxidans* SW2. Similarity is indicated using parenthesis.

FeoA	Rcap $FeoA_1$	Rcap $FeoA_2$	Rcap Feo2A	Rsph $FeoA_1$	Rsph $FeoA_2$	Rfer $FeoA_1$	Rfer $FeoA_2$
Rcap $FeoA_1$	100 (100)						
Rcap $FeoA_2$	19.57 (40.22)	100 (100)					
Rcap Feo2A	15.05 (36.56)	19.57 (36.96)	100 (100)				
Rsph $FeoA_1$	**30.00 (42.22)**	27.59 (36.78)	18.89 (37.78)	100 (100)			
Rsph $FeoA_2$	18.48 (38.04)	**57.32 (69.51)**	19.10 (37.08)	22.99 (35.63)	100 (100)		
Rfer $FeoA_1$	**31.11 (48.89)**	22.09 (38.37)	23.86 (40.91)	**52.44 (68.29)**	22.09 (38.37)	100 (100)	
Rfer $FeoA_2$	20.65 (42.39)	**50.00 (68.29)**	15.73 (35.96)	18.39 (34.48)	**65.82 (78.48)**	19.77 (37.21)	100 (100)

FeoB	Rcap FeoB	Rcap Feo2B	Rsph FeoB	Rfer FeoB
Rcap FeoB	100 (100)			
Rcap Feo2B	30.05 (45.64)	100 (100)		
Rsph FeoB	**55.56 (71.84)**	29.13 (44.64)	100 (100)	
Rfer FeoB	**56.94 (72.73)**	29.99 (46.76)	**71.08 (82.62)**	100 (100)

FeoC	Rcap FeoC	Rsph FeoC	Rfer FeoC
Rcap FeoC	100 (100)		
Rsph FeoC	30.95 (41.67)	100 (100)	
Rfer FeoC	33.33 (44.05)	35.71 (52.38)	100 (100)

Sequences were aligned using the CLC Sequence Viewer software (CLC Bio, Denmark). Similarity and identity scores were calculated based on alignments using the “Ident and Sim” utility (http://www.bioinformatics.org/sms2/ident_sim.html) (Stothard, 2000).

Table 3

Ferric-siderophore uptake genes identified in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2.

Gene product	COG	Label *Rcap* SB1003[a]	Label *Rsph 2.4.1*[a]	Label *Rfer* SW2[a]
ExbB	0811	02375	0920	N.D.
ExbD	0848	02376	0921	N.D.
TonB	0810	02377	0922	N.D.
Uptake element		Label in *Rcap* SB1003[a]	Comments from annotations	
TOMR	4773	00111 (*fhuE*)	Ferrichrome uptake system	
PBP	4607	00108 (*fhuD1*)	Presence of a putative esterase (orf00110)	
IMP	4605	00106 (*fhuB1*)		
	4606	00107 (*fhuB2*)		
ATPase	4604	00105 (*fhu-1*)		
TOMR	4774	01049	Outer membrane receptor for monomeric catechols	
PBP	0614	01047	Presence of a nearby enterochelin esterase (orf 01050) and an AraC-like regulator (orf 01048)	
IMP	0609	01046		
ATPase	1120	01045		
TOMR	1629	01429	Presence of a protein from the major facilitator superfamily: potential role of PBP?	
PBP	N.D.	N.D.		
IMP/ATPase	1132	01426/01427		
TOMR	4774	01433	Enterobactin uptake systeme	
PBP	0614	01434 (*fepB1*)	Presence of a nearby siderophore interacting protein (orf 01438) and two AraC-like regulators (orf 01431 and 01432)	
IMP	0609	01435 (*fepD1*)		
	4779	01436 (*fepG1*)		
ATPase	1120	01437 (*fepC1*)		
TOMR	1629	01445	Enterobactin uptake systeme	
PBP	4592	01444 (*fepB2*)	Presence of a nearby siderophore interacting protein (orf 01438)	
IMP	0609	01443 (*fepD2*)		
	4779	01442 (*fepG2*)		
ATPase	1120	01441 (*fepC2*)		
TOMR	1629	p00051	Presence of one TOMR with two ABC transport systems	
PBP	0614	p00046		
		p00050		
IMP	0609	p00045		
		p00049		
ATPase	1120	p00044		
		p00048		
TOMR	4774	p00112 (*fhuA*)	Ferrichrome uptake systeme	

Gene product	COG	Label *Rcap* SB1003[a]	Label *Rsph 2.4.1*[a]	Label *Rfer* SW2[a]
PBP	0614	p00111 (*fhuD2*)	Presence of a nearby AraC-like regulator (orf p00113)	
IMP	0609	p00110 (*fhuB3*)		
	1132	p00108		
ATPase	1132	p00108		
	1120	p00109 (*fhuC2*)		
Uptake element		**Label in *Rsph* 2.4.1[a]**	**Comments from annotations**	
TOMR	4774	1440	Hydroxamate – type siderophore uptake system orf 1438 is a fused subunit permease	
PBP	0614	1439		
IMP	0609	1438		
	4779			
ATPase	1120	1437		
TOMR	4771	3223	Enterochelin/colicin uptake system	
PBP		N.D.	A periplamic protein, annotated as histidine kinase, is in the same operon (orf 03225): a potential PBP?	
IMP	4606	3220		
	4605	3221		
ATPase	4604	3222		
TOMR	4774	3417	Ferrichrome uptake system	
PBP	0614	3416		
IMP	0609	3415		
	4779	3414		
ATPase	1120	3413		
TOMR	4773	4273 (*fhuA*)	Ferric coprogen or ferric rhotorulic acid uptake system. In the same putative operon of a FecRI system (orf 4274 and 4275)	
PBP	06014	4272 (*fhuD*)		
IMP	0609	4271 (*fhuB*)		
ATPase	1120	7397		

N.D.: Not detected; TOMBR: TonB-dependent outer-membrane receptor; PBP: Periplasmic binding protein; IMP: Inner-membrane protein.

[a] See table 1.

Table 4

Iron-ABC transporter genes identified in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2.

ABC transporter type	Gene name	COG	Label *Rcap* SB1003[a]	Label *Rsph 2.4.1*[a]	Label *Rfer* SW2[a]	Product
FbpABC		1840	01369	0346	1135	ABC transporter, substrate binding protein
		4132	01370	0347	1134	ABC transporter, inner membrane subunit
		1177	01371	0348	1135	ABC transporter, inner membrane subunit
		3842	01371	0348	1135	ABC transporter, ATPase subunit
SitABCD	*sitA*	0803	N.D.	0904	N.D.	ABC Mn^{+2}/Fe^{+2} transporter, periplasmic substrate-binding protein SitA
	sitB	1121	N.D.	0905	N.D.	ABC Mn^{+2}/Fe^{+2} transporter, ATPase subunit SitB
	sitC	1108	N.D.	0906	N.D.	ABC Mn^{+2}/Fe^{+2} transporter, inner membrane subunit SitC
	sitD	1108	N.D.	0907	N.D.	ABC Mn^{+2}/Fe^{+2} transporter, inner membrane subunit SitD

N.D.: Not detected

[a] See table 1.

Table 5

Putative Ferrous iron efflux pumps (COG 0053) identified in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2, with the sequence corresponding to Fe/Zn binding sites dimerization in the *E. coli* FieF (Lu and Fu, 2007; Nies, 2011).

Organism/gene locus	Z1	Fe/Zn binding site Z2 dimerization	Z3/Z4
E. coli K12 FieF[a]	$D_{45}X^3D_{49}$-$H_{153}X^3D_{157}$	$D_{68}DNHX^3H_{75}$	H_{232}-H_{261}-$H_{283}XD_{285}$
Rcaps/orf 00522	$D_{47}X^3D_{51}$-$H_{156}X^3D_{160}$	$D_{70}DDHX^3H_{77}$	H_{236}-H_{265}-$H_{287}XD_{299}$
Rsph/orf 0463	$E_{40}X^3N_{44}$-$H_{148}X^3D_{152}$	$D_{63}ANHX^3H_{70}$	H_{229}-H_{258}-$H_{280}XE_{282}$
Rfe/orf 1374	$D_{50}X^3D_{54}$-$H_{160}X^3D_{164}$	$D_{73}EDHX^3H_{80}$	H_{240}-H_{269}-$H_{291}XD_{293}$
Rfe/orf 2021	$H_{88}X^3D_{92}$-$H_{196}X^3D_{200}$	$S_{111}RTFX^3L_{118}$	H_{247}-L_{303}-$H_{419}XV_{421}$

[a] *E. coli* K12 Fief accession number NP_418350

[b] numbering relative to each sequence is given. X: random amino acid. X^n: succession of n random amino acids.

Table 6

Ferritin-family proteins identified in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2.

Ftn type	Gene name	COG	Label *Rcap* SB1003[a]	Label *Rsph 2.4.1*[a]	Label *Rfer* SW2[a]	Comments
Ftn	*mbfA*	1633	03466	0850	0178	Membrane bound ferritin
Bfr	*bfr*	2193	00913	1546	N.D.	
	bfr2	2193		3342		
DPS		0783	N.D.	N.D.	N.D.	

N.D.: Not detected

[a] See table 1.

Table 7

Identity and similarity in MbfA and Bfr from *R. capsulatus* SB1003, *R. shaeroides* 2.4.1 and *R. ferroxidans* SW2. Similarity is indicated using parenthesis.

MbfA	**Rcap**	**Rsph**	**Rfer**
Rcap	100 (100)		
Rsph	73.54 (81.85)	100 (100)	
Rfer	79.08 (86.15)	73.54 (82.46)	100 (100)
Bfr	Rcap	Rsph Bfr	Rsph Bfr2
Rcap	100 (100)		
Rsph Bfr	67.70 (79.50)	100 (100)	
Rsph Bfr2	66.67 (78.79)	82.42 (87.27)	100 (100)

Sequences were aligned using the CLC Sequence Viewer software (CLC Bio, Denmark). Similarity and identity scores were calculated based on alignments using the “Ident and Sim” utility (http://www.bioinformatics.org/sms2/ident_sim.html) (Stothard, 2000).

Table 8

Iron regulators identified in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2.

Regulator type	Gene name	COG	Label *Rcap* SB1003[a]	Label *Rsph 2.4.1*[a]	Label *Rfer* SW2[a]	Product
Fur-family	*fur/mur*	0735	N.D.	2494	2373	Ferric/manganese uptake regulator
	irr	0735	02670	3179	0819	Iron response regulator
	zur	0735	01134	3569	1799	Zinc uptake regulator

N.D.: Not detected

[a]See table 1.

Table 9

Identity and similarity in Fur/Mur, Irr and Zur from *R. capsulatus* SB1003, *R. shaeroides* 2.4.1 and *R. ferroxidans* SW2. Similarity is indicated using parenthesis.

Fur/Mur	**Rcap**	**Rsph**	**Rfer**
Rcap	No Rcap Fur	-	-
Rsph	No Rcap Fur	100 (100)	-
Rfer	No Rcap Fur	74.64 (84.78)	100 (100)
Irr	Rcap	Rsph	Rfer
Rcap	100 (100)	-	-
Rsph	62.59 (76.87)	100 (100)	-
Rfer	60.00 (73.51)	66.67 (78.23)	100 (100)
Zur	Rcap	Rsph	Rfer
Rcap	100 (100)	-	-
Rsph	63.37 (71.51)	100 (100)	-
Rfer	68.64 (75.15)	73.21 (80.36)	100 (100)

Sequences were aligned using the CLC Sequence Viewer software (CLC Bio, Denmark). Similarity and identity scores were calculated based on alignments using the “Ident and Sim” utility (http://www.bioinformatics.org/sms2/ident_sim.html) (Stothard, 2000).

Table 10

Summary of iron/manganese homeostasis features identified in the genomes of *R. capsulatus* SB1003, *R. sphaeroides* 2.4.1 and *R. ferroxidans* SW2.

Feature type	Feature name	*Rcap* SB1003	*Rsph 2.4.1*	*Rfer* SW2
Fe/Mn uptake	Feo	2	1	1
	Efe	1	0	0
	MntH	1	0	2
	ABC-type	1	2	1
	Siderophore	7	4	0
	Heme	1	0	0
	Total	13	7	4
Efflux pump	FieF	1	1	1
Iron storage	Bfr	1	2	0
	MbfA	1	1	0
Fur-type regulators	Fur	0	1	1
	Irr	1	1	1
	Zur	1	1	1
Manganese regulator	MntR	1	0	2