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Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium *Rhizobium* and other α-proteobacteria

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Abstract The alpha-proteobacteria include several important genera, including the symbiotic N_2-fixing ''rhizobia'', the plant pathogen *Agrobacterium*, the mammalian pathogens *Brucella, Bartonella* as well as many others that are of environmental or other interest—including *Rhodobacter, Caulobacter* and the hugely abundant marine genus *Pelagibacter.* Only a few species—mainly different members of the rhizobia—have been analyzed directly for their ability to use and to respond to iron. These studies, however, have shown that at least some of the ''alphas'' differ fundamentally in the ways in which they regulate their genes in response to Fe availability. In this paper, we build on our own work on *Rhizobium leguminosarum* (the symbiont of peas, beans and clovers) and on *Bradyrhizobium japonicum*, which nodulates soybeans and which has been studied in Buffalo and Zürich. In the former species, the predominant Fe-responsive regulator is not Fur, but RirA, a member of the Rrf2 protein family and which likely has an FeS cluster cofactor. In addition, there are several *R. leguminosarum* genes that are expressed at higher levels in Fe-replete conditions and at least some of these are regulated by Irr, a member of the Fur superfamily and which has the unusual property of being degraded by the presence of heme. *In silico* analyses of the genome sequences of other bacteria indicate that Irr occurs in all members of the Rhizobiales and the Rhodobacterales and that RirA is found in all but one branch of these two lineages, the exception being the clade that includes *B. japonicum.* Nearly all the Rhizobiales and the Rhodobacterales contain a gene whose product resembles *bona fide* Fur. However, direct genetic studies show that in most of the Rhizobiales and in the Rhodobacterales it is a ''Mur'' (a manganese responsive repressor of a small number of genes involved in Mn uptake) or, in *Bradyrhizobium*, it recognizes the operator sequences of only a few genes that are involved in Fe metabolism. We propose that the Rhizobiales and the Rhodobacterales have relegated Fur to a far more minor

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role than in (say) *E. coli* and that they employ Irr and, in the Rhizobiales, RirA as their global Fe-responsive transcriptional regulators. In contrast to the *direct* interaction between Fe^{2+} and conventional Fur, we suggest that these bacteria sense Fe more indirectly as functions of the intracellular concentrations of FeS clusters and of heme. Thus, their ''iron-omes'' may be more accurately linked to the real-time needs for the metal and not just to its absolute concentration in the environment.

Keywords α-Proteobacteria · Fur · Iron · Irr · Manganese · Mur · Rhizobia · RirA · Rrf2 family

Introduction

Bacteria known collectively as the ''Rhizobia'' are famous for their ability to induce nodules on the roots (and, occasionally, stems) of legume plants. Within these nodules, the differentiated, ''bacteroid'' forms fix atmospheric N_2, the resultant ammonia being used as a source of fixed N. This symbiosis provides the bacteria with an exclusive niche and, in return, the plants obtain a personalized N source. The enzyme nitrogenase is an iron protein as are the many electron donors in the nodule; also the host legumes make the abundant O_2-carrying protein, leghemoglobin, another major sink for Fe. It is therefore surprising that the iron biology of rhizobia was long-neglected. Even now, we still do not know the major sources of iron that feed the symbiotic forms of the rhizobia, nor do we know in detail how iron traverses the membranes (one from the plant and one from the bacterium) into the bacteroids (see Johnston 2004).

However, there has been increasing, recent attention to the iron biology of the rhizobia and, although these have not elucidated what is going on in the nodules, they have generated some novel sets of observations. The main surprise is that Fe-responsive gene regulation in the rhizobia, and, by inference, some other close bacterial relatives, is very different from that in other model bacteria, such as *E. coli, Pseudomonas* and *Bacillus.* Indeed, the work on the rhizobia has highlighted the fact that we know very little about Fe-mediated gene regulation in *any* members of the subclass of α-proteobacteria. Again, this lack of knowledge is surprising, since this assemblage includes pathogens on animals (*eg Brucella*, *Bartonella*, Rickettsias), or plants (*Agrobacterium*) and others that are of environmental importance (*e.g. Novosphingobium*, which degrades many xenobiotics). Also, the hugely abundant SAR11 clade of marine bacteria (e.g. *Pelagibacter* spp.) is an α-proteobacteria (Giovannoni et al. 2005). And, of course, several genera of this clade have been analysed in detail for their particular, unusual attributes; e.g. differentiation in *Caulobacter* and photosynthesis in *Rhodobacter*.

Here, we consider the ''iron-omes'' of the rhizobia, the data having been obtained largely from three species of these symbionts. We then make some predictions on the likely regulatory genes and their targets in other α-proteobacteria, based on bioinformatic analyses of their genome sequences.

However, before that, we briefly review the phylogeny of some of the ''alphas'' that feature below.

Brief taxonomic description of the α-proteobacterial sub-class

As shown in Fig. 1, there are several orders of α-proteobacteria. One of these, the Rhizobiales, comprises symbionts and pathogens. There are two major lineages of the Rhizobiales, the ''*Rhizobiaceae*'' and the ''*Bradyrhizobiaceae*''. The first contains *Agrobacterium*, *Bartonella, Brucella*, *Mesorhizobium, Rhizobium* and *Sinorhizobium* and the *Bradyrhizobiaceae* comprise *Bradyrhizobium*, its close relative, the non-symbiotic *Rhodopseudomonas*, plus *Nitrobacter.* The genomes of all these species have been sequenced allowing their iron-omes to be analyzed by comparative genomics (Rodionov et al. 2006). The taxonomic tree of the Rhizobiales is mirrored in the mechanisms of Fe-responsive gene regulation. Thus, *Rhizobium* and the rhizobial genera *Mesorhizobium* and *Sinorhizobium* are more closely related to *Agrobacterium* (a pathogen of plants) and *Brucella* and *Bartonella* (mammalian pathogens) than they are to the rhizobial microsymbiont of soybeans, *Bradyrhizobium*. This

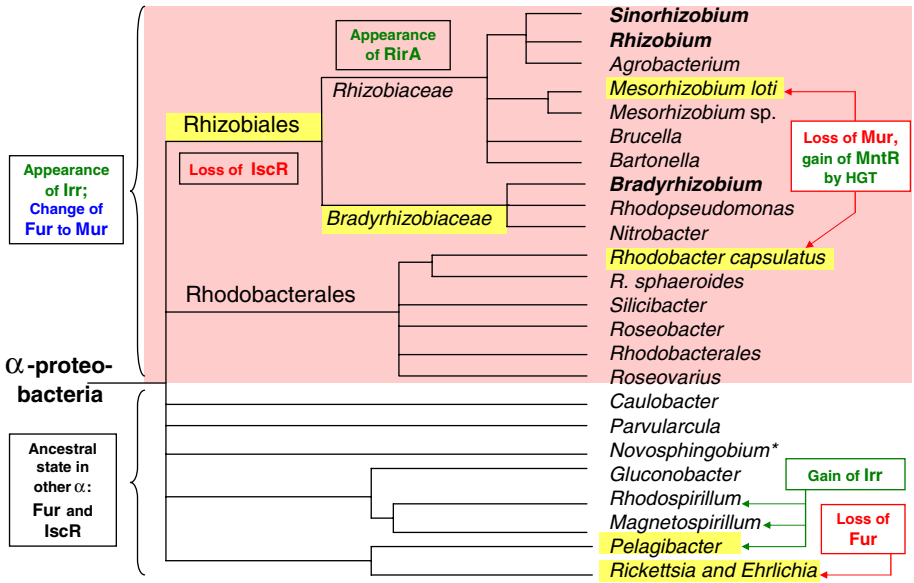

Fig. 1 Phylogenetic tree of the α-proteobacteria, with suggested pathway of loss and acquisition of Fe-responsive regulators. The generally accepted tree that pertains to the phylogeny of the α-proteobacterial genera described in this paper is shown. Those clades in which a proposed loss (in red) or gain (in green) of one of the regulators IscR, Fur, Irr, MntR, Mur and RirA are highlighted in yellow. HGT, horizontal gene transfer. The clades with experimentally charecterized iron regulators are shown in bold.

taxonomic distance is reflected by significant differences in the iron-responsive gene regulation in the two lineages of the Rhizobiales (see below).

To date, most of the *direct experimental* analyses on the α-proteobacteria have been on just three species of the Rhizobiales (*Rhizobium leguminosarum, Sinorhizobium meliloti* and *Bradyrhizobium japonicum*), with some preliminary work having been done on *Brucella* and on a strain of the magnetotactic *Magnetospirillum* sp..

We now summarize these experimental data, beginning with *Rhizobium leguminosarum* and its close relatives *Sinorhizobium meliloti* and *Brucella abortus*, then compare these with those done in other species.

Iron responsive gene regulation in the *Rhizobiaceae and Rhodobacterales*

Just as in other bacteria, the Rhizobial genes involved in iron uptake (involving siderophores, inorganic iron and heme) are expressed at higher levels in Fe-depleted than in Fe-replete media (Johnston 2004). This is not surprising, but what *is* unusual is the identity of the regulatory protein, called RirA, which mediates this differential expression.

RirA

Rather than the well-known global Fe-responsive regulator ''Fur'' (which is considered in detail elsewhere in this volume), the ''global'' Fe-responsive regulator in *Rhizobium* and *Sinorhizobium* was shown directly to be a very different protein, RirA (Todd et al. 2002). By examining the expression of individual genes that are involved in Fe uptake and by pangenomic analyses, it was found that RirA repressed >80 transcriptional units in *Rhizobium* and in *Sinorhizobium* in Fe-replete media (Todd et al. 2005; Viguier et al. 2005; Chao et al. 2005). RirA has no detectable sequence similarity to Fur (or to DtxR, a different global Fe-responsive transcriptional regulator in Actinobacteria). Rather, it belongs to a different protein family called Rrf2.

Like metallo-regulators from the Fur superfamily, Rrf2-like proteins are very widespread in

eubacteria, and the family comprises several branches, with some bacteria having representatives of more than one branch. However, Rrf2 family proteins are still little-studied, with only three other members, Rrf2 itself, NsrR and IscR having been examined directly. Rrf2 regulates the expression of a large cytochrome in *Desulfovibrio* (Keon et al. 1997), NsrR is a nitrite-responsive regulator of genes involved in nitrogen oxide metabolism in *Nitrosomonas*, *E. coli* and other proteobacteria (Beaumont et al. 2004; Bodenmiller and Spiro 2006; Rodionov et al. 2005), and IscR represses transcription of genes involved in the assembly of FeS clusters in proteins (Schwartz et al. 2001; Giel et al. 2006). IscR is itself an FeS cluster protein, which only binds to its target DNA sequences with high avidity when charged with its co-factor. RirA, too, can bind to a FeS co-factor (J. Crack and JDT, unpublished), so this may be a general feature of Rrf2-like proteins.

On the phylogenetic tree of the Rrf2 protein family, the RirA regulators in α-proteobacteria are clustered with the NsrR branch and its members occur in only a tightly related subset of the Rhizobiaceae, a lineage within the Rhizobiales (see above). Interestingly, the more distantly related Bradyrhizobiaceae has no RirA protein, showing that this regulator tracks taxonomic relatedness, not ecological niche (Rodionov et al. 2006).

Not surprisingly, the DNA recognition sites for RirA are significantly different from the well-documented *fur* boxes to which Fur binds (see Andrews et al. 2003). Instead, the promoter regions of RirA-repressed genes have a conserved sequence, the IRO box (Yeoman et al. 2004; Rodionov et al. 2006). Although not formally proved, in +Fe conditions, the RirA protein is likely charged with its FeS co-factor, then binds to the IRO box, inhibiting initiation of transcription (Fig. 2).

Genome-wide searches were made for the IRO motif in the appropriate regions (*i.e.* upstream of genes, in their likely regulatory regions) in other members of the α-proteobacteria. This identified many likely RirA-regulated genes in *Agrobacterium, Brucella*, *Bartonella, Mesorhizobium* as well as (not surprisingly) in *Rhizobium* and *Sinorhizobium* but, significantly, not in *Bradyrhizobium.* So, there is a good match between the incidence of IRO motifs in the genome and the possession of RirA. In many cases, the genes with IRO boxes had clear links with iron, being predicted to be involved in the uptake of the metal or in its intracellular metabolism or storage (Rodionov et al. 2006).

In some cases, RirA participates in a regulatory cascade. Thus, in *R. leguminosarum*, it represses *rpoI*, which specifies a σ factor that transcribes some of the *vbs* siderophore biosynthetic genes (Todd et al. 2002). And, *S. meliloti* RirA represses *rhrA*, which encodes an AraC-type transcriptional activator of the siderophore biosynthetic genes (Viguier et al. 2005).

Fur/Mur

Given that RirA appears to have taken over the role of Fur as the wide-ranging Fe-responsive gene repressor in the Rhizobiaceae, does Fur have any role in these bacteria? This question is of real relevance, since homologues of *bona fide* Fur occur in nearly all known species of these two lineages. These ''Fur'' proteins have been studied directly in *Rhizobium* and in *Sinorhizobium*, and in both cases, it is clear that their ''Furs'' are (a) much less important than in (say) *E. coli* and (b) responsive to manganese, and not iron.

The Fur of *B. japonicum*, which has also been studied, is considered in more detail below.

The Fur-like proteins in *Rhizobium* and *Sinorhizobium* not only have similar sequence to ''classical'' Furs, but they can bind to artificially provided canonical *fur* boxes. Also, they can functionally complement *E. coli* Fur^- mutants (Wexler et al. 2003). The only *known* target for these Fur-like regulators is the *sitABCD* operon, which specifies a Mn^{2+} ABC transporter (Chao et al. 2004; Diaz-Mireles et al. 2004, 2005; Platero et al. 2004). The promoter region of this operon has a Mn-responsive motif, called the MRS, which is the binding site for the Fur-like protein (Diaz-Mireles et al. 2005). These MRS motifs are significantly different from conventional *E. coli fur* boxes although there are some detectable similarities between them. Therefore, in these two genera at least, the Fur protein lost its role as

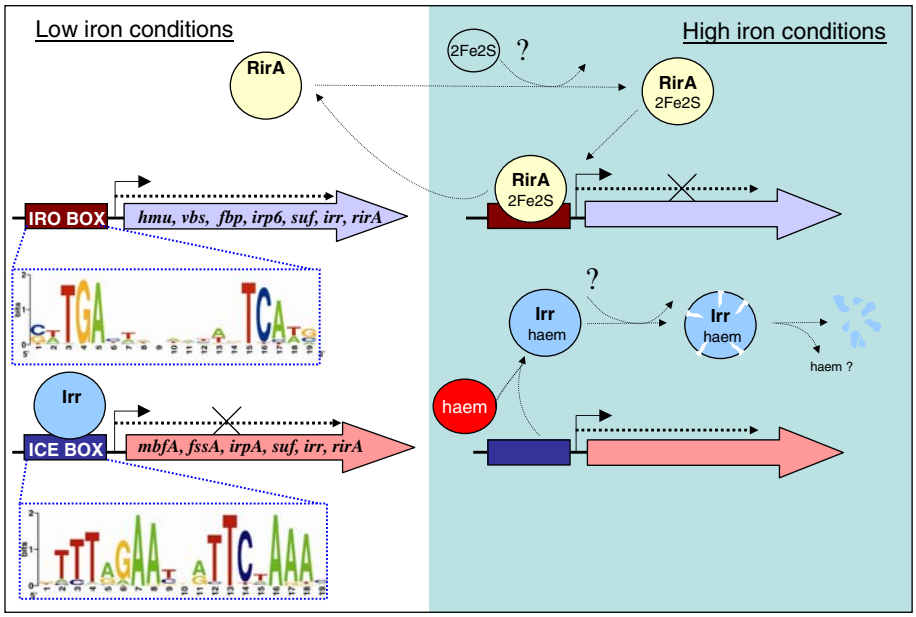

Fig. 2 Summary diagram of the RirA and Irr regulons. The two regulators, RirA and Irr are indicated in yellow and blue circles respectively. In ''Low iron conditions'' (left panel) RirA fails to bind to its cognate IRO boxes that precede the genes indicated in the pale blue arrows. But, in ''High iron conditions'' RirA interacts with an FeS cluster causing it to bind to the IRO boxes, repressing expression of the downstream genes. In contrast, Irr binds to the ICE boxes that precede the genes shown in the pink arrow but only in the ''low iron conditions''. In the ''high iron conditions'' the Irr protein is inactivated, due to the interaction with heme. The consensus sequences of the IRO and ICE boxes are shown

a global Fe-responsive regulator and evolved to sense a different metal and to recognize a different *cis*-acting operator sequence. Another MRS motif in the *R. leguminosarum* genome, was found upstream of the *mntH* gene, which encodes another type of Mn^{2+} transporter, which is in the NRAMP family (Rodionov et al. 2006). There are also MRS motifs in the promoter regions of the *sitABCD* and/or *mntH* genes in many other members of the Rhizobiaceae (*Agrobacterium, Bartonella, Brucella*) and in the Rhodobacterales. Because of this, we renamed this Fur-like protein manganese uptake regulator (Mur).

There are two intriguing exceptions that appear to prove this rule that Mur is a Mn^{2+}-responsive regulator. The genome sequences of two different species of the genera *Mesorhizobium* and *Rhodobacter* show that, for each genus, one the strains (*Mesorhizobium* sp BNC1 and *R. sphaeroides*) has a Fur-like protein and the other (*M. loti* and *R. capsulatus*) does not. Strikingly, the deduced proteomes of the latter two strains include a protein that resembles MntR, a DtxR-like Mn^{2+}-responsive regulator, with no sequence similarity to Fur (Fig. 1). Thus, in these two strains *fur/mur* is lost entirely, with the role of Mn-dependent regulator being undertaken by the MntR protein.

Irr

Though first found in *B. japonicum*, the remarkable Irr regulatory protein has also been studied in *Rhizobium* and *Brucella*, both members of the Rhizobiaceae. Irr and its orthologs form a distinct sub-branch of the Fur superfamily, but occur *only* in members of the Rhizobiales and the Rhodobacterales, and, for reasons that are not clear, the SAR11 bacterium *Pelagibacter ubique*, and the two other genera, *Magnetospirillum* and *Rhodospirillum*, which are closely related to each other. Indeed, Irr is something of a signature gene for

these groups, being absent from all other prokaryotes.

Irr represses a suite of genes in cells grown under Fe-depleted conditions, the mirror image of what occurs with RirA. The Irr protein can bind to conserved sequences, ICE motifs (iron control elements) near the promoters of genes that it regulates, but in ''+Fe'' conditions, the Irr protein is physically degraded (see Fig. 2 and below for more details on this unusual phenomenon) and so cannot function (Martinez et al. 2005; Todd et al. 2006). This degradation is mediated by interaction with heme, whose intracellular concentration increases, in parallel with external Fe availability.

Several genes are regulated in this way by Irr in *Rhizobium*, some of these being of interest in their own right. For example, the *mbfA* gene product (*RL0263*) has an N-terminus in the rubrerythrin/ferredoxin family but has an additional C-terminal domain that is characteristic of inner membrane proteins, a highly unusual location for this type of electron transfer protein (Todd et al. 2006). Like *mbfA*, another *R. leguminosarum* gene, *fssA* (*RL0400*), which is also expressed at higher levels in Fe-replete conditions, is regulated by Irr, and has an ICE motif near its promoter. FssA, which is predicted to be an unusual form of FeS scaffold protein, is also found only in α-proteobacteria among prokaryotes, but, interestingly, close homologues occur in eukaryotes, including humans (Todd et al. 2006). Orthologs of *mbfA* and *fssA* in other Rhizobiales and Rhodobacterales species are preceded by conserved ICE motifs (Rodionov et al. 2006). These core members of the Irr regulon do not overlap with the RirA regulon, which mostly includes various iron uptake genes.

Genes that are regulated by both Irr and RirA

Perhaps the most intriguing of the Fe-regulated genes in *Rhizobium* are those that are regulated by both Irr and RirA. These include the *suf* operon, which is required for the synthesis of FeS clusters and the *bfd-bfr* operon that encodes bacterioferritin. Both of these (and several other) *Rhizobium* operons have ICE and IRO motifs near their promoters, indicating dual regulation by *both* Irr *and* RirA (Todd et al. 2006; Rodionov et al. 2006). For example, in wild type *Rhizobium*, the *suf* operon is expressed at higher levels in +Fe than in –Fe medium. However, in an Irr^- mutant, the reverse is true, indicating that the repression in –Fe media is mediated by this regulator. In contrast, in a $RirA^-$ mutant, the expression in Fe-depleted medium is greater than in the wild type. Taken together, these results show that regulation of the *suf* operon in the wild type is a function of the repressive activities of *both* Irr *and* RirA.

This led Todd et al. (2006) to propose a novel model for Fe-responsive gene regulation in *Rhizobium* and, by analogy (and homology) in its close relatives. This proposes that, whereas *E. coli* (say) senses Fe more or less directly, through the interaction between the metal and Fur, the *Rhizobium* regulators, Irr and RirA sense the metabolic and physiological *consequences* of iron availability, in the form of intracellular concentrations of heme and of FeS clusters (Fig. 2). Thus, at a first approximation, in +Fe medium RirA will be more repressive—due to the accumulation of FeS clusters and Irr will be less so, because the increased heme levels will lead to degradation of this regulator. However, this arrangement can allow fine-tuning, since in any given case in which the external Fe concentration is such-and-such, other parameters (oxygen availability, carbon source, energy demands etc.) may alter the relative levels of intracellular heme and FeS clusters. Thus, the external Fe concentration can be matched, in a subtle way, to the particular demands of the cell at that time and place.

Since some genes are regulated by *both* Irr *and* RirA, the scope for such integration is further enhanced. Also, Irr regulates expression of RirA and vice versa, allowing greater interweaving between the functions of these two mirror-image Fe-responsive regulators (Todd et al. 2006).

Iron regulation in Bradyrhizobiaceae

As mentioned, *Bradyrhizobium* responds to iron rather differently compared to *Rhizobium* and other members of the Rhizobiaceae, and these relate to RirA, Fur/Mur and Irr.

RirA

This widely acting Fe-responsive repressor is, quite simply, absent from *Bradyrhizobium* and its close relatives *Rhodopseudomonas* and *Nitrobacter.*

Fur

Although *Bradyrhizobium* has a *fur*-like gene (Hamza et al. 1999), its product also differs from canonical Furs, though not in the same way as seen with *Rhizobium* and its close relatives, nor is it quite so distinctive, when compared to canonical Fur proteins. For example, a *B. japonicum* Fur$^-$ mutant is unaffected in iron-dependent regulation of the heme uptake *hmu* genes (Nienaber et al. 2001), which are usually members of the Fur regulons in γ-proteobacteria. To date, only two target genes have been found for *Bradyrhizobium* Fur, namely *hemA*, which encodes amino-levulinic acid synthase, the first step in heme biosynthesis and the *irr* gene (see above and below). These genes are regulated, but only slightly, at a transcriptional level in response to Fe, but this regulatory effect is lost in Fur$^-$ mutants (Hamza et al. 1998).

B. japonicum Fur can bind effectively to conventional *fur* boxes when these are supplied ectopically and it can recognize a motif in the regulatory region upstream of *irr* in its ''home'' species, *B. japonicum*. However, this sequence differs from regular *fur* box and resembles the MRS motif of *Rhizobium* (Friedman and O'Brian 2003, 2004; Hamza et al. 1999, 2000). Thus *B. japonicum* Fur is reminiscent of the Mur of *Rhizobium*, which can recognize both atypical and typical *cis*-acting regulatory sequences (see above).

Irr

So, if *B. japonicum* has no RirA and the role of its Fur is limited, what might Irr of that species be doing? The answer is ''quite a lot''. Although there are clear similarities between Irr of *B. japonicum* and that of *Brucella* and *Rhizobium*, there are also some critical differences.

In fact, Irr was discovered in *Bradyrhizobium*, and we know more about this protein than any other Irr. It was identified as a regulator of *hemB*, which is involved in heme biosynthesis and whose transcription is enhanced in Fe-replete conditions (Hamza et al. 1998). However, in an Irr mutant strain, this Fe-responsive control is lost. The mechanism that effects this Irr-dependent regulation is complex, unusual and beautifully fit for purpose.

Thanks to some elegant experiments, done in Mark O'Brian's lab in Buffalo and in the Zurich lab of Hans-Martin Fischer and Hauke Hennecke, we know the following about Irr of *B. japonicum* (In fact, the genome [http://www.kazusa.or.jp/rhizobase/Bradyrhizobium/index.html] of *B. japonicum* strain USDA110 has *two* Irr-like proteins; what follows deals only with one of them, Bll0768. The second Irr, the product of the Blr1216 gene has not yet been studied).

B. japonicum Irr is a DNA-binding protein that recognises conserved ICE motif in the regulatory regions of its target genes. Depending on the location of the ICE motif relative to the promoter, Irr can act positively or negatively in response to iron availability. If the ICE motifs are close to (or overlaps) the promoters, Irr is a repressor. An example of this is the product of the *blr7895* gene (Rudolph et al. 2006), an ortholog of *R. leguminosarum* MbfA, which is also regulated by Irr in *B. japonicum* (see above). Thus, *blr7895* is expressed at higher level in +Fe than in –Fe media, but this regulation is disturbed in the Irr$^-$ mutant (Rudolph et al. 2006; Yang et al. 2006b). In contrast, with the *hmuR* operon, Irr is an *activator*, causing enhanced expression in Fe-depleted cells (Rudolph et al. 2006). This is reminiscent of *Neisseria meningitidis* Fur (Delany et al. 2004), which can act positively or negatively, depending on the location of the *fur* box relative to the particular promoter. Rudolph et al. (2006) successfully predicted the identity of genes that are likely induced or repressed by Irr in *B. japonicum.* In many cases, these were substantiated by microarray experiments, which also showed that Irr regulated many genes in this species, more, indeed, than in *Rhizobium* (Yang et al. 2006b). Ironically though, *hemB* the gene that was first shown to be regulated by Irr, does not have an ICE motif and the way(s) in which this gene is regulated is unknown.

The most striking feature of Irr, however, concerns the way in which it senses iron. In sharp contrast to Fur ''proper'', which interacts directly with ferrous Fe^{2+}, Irr forms a complex with heme, whose intracellular concentration is correlated with external Fe availability (Qi and O'Brian 2002; Qi et al. 1999; Yang et al. 2005). The heme can interact directly with the Irr but this is more efficient if the moiety is delivered by the enzyme ferrochelatase, whose normal role is to insert Fe into the protoporphyrin precursor of heme (Qi and O'Brian, 2002). This rapid and total degradation of the Irr protein, which accounts for its loss of function in Fe-replete cells, involves oxidative damage, being much enhanced by peroxide (Yang et al. 2006a). This highly unusual (at least in bacteria) phenomenon involves at least three different parts of the Irr protein. One of these, the haem-regulatory motif resembles analogous motifs in IRP2, a eukaryotic protein that regulates genes in response to Fe availability and is destabilised when bound to heme (Iwai et al. 1998). Perhaps significantly, Irr of *B. japonicum* and of *Rhodopseudomonas* are the only ones with the HRM motif, although Irrs of the other α-proteobacteria have the other regions that are important for heme-responsive instability (Rodionov et al. 2006).

Iron responsive gene regulation in the ''other'' α-proteobacteria

As noted above, hardly any work has been done on Fe-responsive gene regulation in α-proteobacterial genera other than in the Rhizobiales and Rhodobacterales. However, recent microarray studies on the intriguing bacterium *Magnetospirillum magneticum* revealed genes whose expression is affected (up or down) by Fe availability (Suzuki et al. 2006).

Rodionov et al. (2006) undertook an in silico search for the presence of the iron-responsive transcriptional regulators described above and their cognate, *cis*-acting recognition sequences in all sequenced genera, including those in Fig. 1 (*Caulobacter, Parvularcula, Novosphingobium, Gluconobacter, Rhodospirillum, Magnetospirillum, Pelagibacter*). The findings can be summarized as follows.

(i) They all contain *fur* like genes and, in nearly all cases there are conserved motifs that lie in the regulatory regions of known (or surmised) Fe-regulated genes. These ''quasi-*fur* boxes'' differ from canonical types (e.g. in *E. coli*) but their locations 5′ of Fe-related genes indicate that their Furs are more conventional, responding to Fe.
(ii) They all lack any RirA-like regulatory proteins
(iii) Most of them contain no Irr homologues, the exceptions being the Irr-like protein of *Pelagibacter ubique, Rhodospirillum and Magnetospirillum*. Given the rather strict association between the presence of Irr proteins in the Rhizobiales and the Rhodobacterales, the *P. ubique* Irr, which is 41% identical to that of *Rhodobacter*, genus may have been acquired by lateral gene transfer.
(iv) All α-proteobacteria except Rhizobiaceae have an ortholog of the FeS cluster repressor IscR, which is always encoded within the FeS synthesis operons in proteobacteria. However, the roles of IscR-like proteins in α-proteobacteria have not been examined—might some of them have a wider, Fe-responsive regulatory role?
(v) Finally, the bacteria in the obligate intracellular pathogens *Rickettsia* and *Ehrlichia* lack RirA, Irr, Fur/Mur and Irr, their only gene with the potential for wide-ranging Fe-responsive gene regulation being their versions of IscR. Given the minimalist genomes of these pathogens, it is not surprising that their Fe-omes are also simplified, compared to their free-living relatives.

Concluding remarks

It is clear from the brief outline that at least some α-proteobacteria, namely the Rhizobiales and the Rhodobacterales, regulate their genes in response to iron availability in ways that differ quite dramatically from the ''norm'' of *E. coli* and other model γ-proteobacteria.

Here, we consider the events that might have been responsible for the unusual iron-responsive regulatory mechanisms in these bacteria.

The demise of Fur

As RirA (presumably) became the predominant Fe-responsive repressor, it usurped Fur, which had a much-diminished and/or altered role. *In extremis*, (one strain each of *Mesorhizobium* and *Rhodobacter*), Fur was lost completely, but in other Rhodobacterales, and in the Rhizobiaceae, it morphed into Mur, responding to Mn^{2+} *in vivo* and regulating the manganese uptake genes by recognizing the MRS regulatory sequences. In *Bradyrhizobiaceae*, the shift in function may not have been so marked. However, these Fur proteins retain a ''memory'' of their past history, being able to recognize conventional *fur* boxes when these are provided artificially.

The varied role of Irr

Perhaps the real hallmark regulator in the Rhizobiales and the Rhodobacterales is Irr, a version of the Fur superfamily, which is restricted to these two lineages. Thus, its distribution closely matches that of RirA, although it is impossible to say which of these two regulators arose first. Since Irr is a repressor in all the bacteria in which it is known to occur, this was presumably its initial role. However, in *Bradyrhizobiaceae*, it can activate some genes but repress others, depending on the location of the ICE motif, relative to the particular promoter. Thus, in *Bradyrhizobium*, the mirror-image roles of repression and activation of genes in response to Fe are undertaken by one protein. In contrast, in the *Rhizobiaceae*, there is a division of labor, with RirA and Irr being respectively the repressors in high- and low-levels of Fe.

So, why should the Rhizobiales and the Rhodobacterales have such different systems to regulate their profile of expressed genes in response to Fe availability, when Fur seems to do a good job in many, widely distributed (both taxonomically and environmentally) bacterial types? Our own view is, simply, that the Irr/RirA system only evolved once, relatively recently (in terms of bacterial evolutionary time!) and that, at least for the descendants of the individual bacteria (bacterium?) in which it arose, it was better than Fur. Given the wide range of habitats (e.g. marine, soil, infection of mammals, legume root nodules) that are occupied, successfully, by Rhizobiales and the Rhodobacterales, the Irr/RirA combination is well suited to many niches and not just one particular lifestyle. Its restricted distribution in just one branch of α-proteobacteria may be because it arose so recently that it has not had time to spread widely by horizontal transfer to other taxa, or, perhaps there are other, unknown features of the physiology, biochemistry of the Rhizobiales/Rhodobacterales that are especially suited to the RirA/Irr system.

We described above why it might be that these regulators are, inherently, more effective than Fur. We think that the *indirect* sensing of Fe availability through the levels of intracellular heme (for Irr) and FeS clusters (for RirA) has some advantages, since the cells can integrate their responses to the external levels of Fe more accurately to their real-time physiological demands. This should allow more appropriate adaptive behaviour, especially since some key genes (e.g. *suf, bfr*) can be regulated by *both* Irr *and* RirA.

Finally, we stress the importance of *experimental* work on any ''new'' regulon in hitherto unstudied bacteria. This, of course, applies not only to α-proteobacteria and not only to the response to iron. This may seem self-evident, but, one can see allusions in the literature to ''*fur* boxes'' near the predicted promoters of Fe-regulated genes, yet subsequent direct experimental work showed that these genes are not Fur-regulated.

Concerning future work on the α's, it will be of particular interest to know how the ''other'' clades, outside the Rhizobiales and the Rhodobacterales respond to Fe—conventionally by using Fur, or by some other, unknown mechanisms? Also, what are the roles of the ''second'' Irr proteins that occur in some Rhizobiales and Rhodobacterales and what are the regulators of those genes that are controlled by neither RirA nor Irr in these bacteria? Do small RNAs have any role in the Fe-regulons, as has been shown so clearly in some other bacterial types? Given the availability of genome sequences for so many species, coupled to their genetic tractability, there is no major block to obtaining the answers to

these questions, perhaps in time for the next ''Biometals'' meeting?

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