Is the fur gene of Rhizobium leguminosarum essential?

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Abstract

Using primers corresponding to conserved regions of the bacterial regulatory gene fur, a homologue of this gene from the genome of Rhizobium leguminosarum biovar viciae, the nitrogen-fixing symbiont of peas, was isolated and sequenced. The fur gene is normally expressed constitutively, independent of the presence of Fe in the medium, but in one Rhizobium strain it was transcribed at a low level. Attempts to isolate a fur knockout mutant failed, suggesting that the gene is essential for free-living growth. In other bacteria, certain fur mutations confer manganese resistance; however, none of the manganese-resistant mutants of R. leguminosarum which we isolated was corrected by the cloned fur gene. When the cloned R. leguminosarum fur gene was introduced into a fur mutant of Escherichia coli, it caused some Fe-dependent reduction in the amount of siderophore, indicating that it can function heterologously. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Bacteria known as rhizobia induce N₂-fixing nodules on legume roots. Their ability to sequester iron is important since within nodules there is a great demand for this metal. There, the most abundant protein made by the plant is leghaemoglobin and the nitrogenase complex contains Fe, as do its e⁻ donors and the cytochromes required for bacteroid respiration [1,2].

Rhizobia synthesise and excrete siderophores, low molecular mass compounds that sequester Fe³⁺, the most abundant, though insoluble, environmental form of the metal [3,4]. The Fe-siderophore complex is internalised by a dedicated import system. The rhizobial siderophores include catecholates, hydroxamates, anthranilate, rhizobactin and, perhaps, haem [2]. The symbiotic effects of mutations that abolish siderophore production are not uniform. Some non-producers induce functional nodules but others induce nodules that fail to fix N₂ [2]. In these latter cases, the Fix⁻ phenotype may be due not to the siderophore defect per se but to pleiotropic effects, since Yeoman et al. [5] found that mutations in the cycHJKL genes, which are required for the maturation of cytochrome c, are also defective in synthesising the trihydroxamate siderophore vicibactin [6]. It is likely that the respiratory defect causes the Fix⁻ phenotype.
Typically, genes that are involved in Fe uptake are expressed only when Fe is deficient [3,4]. In *Bradyrhizobium japonicum*, the symbiont of soybeans, an outer membrane protein that is made in response to Fe starvation is similar to the hydroxamate receptor, FhuA, of *Escherichia coli* [7]. In many cases, Fe-mediated gene expression is controlled by the ‘global’ transcriptional regulator fur, which is conserved in many bacterial genera. When Fe is replete, Fur, which interacts with Fe, binds to DNA sequences (fur boxes) that overlap the target promoters, repressing their transcription [3,4]. In the absence of Fe, Fur no longer binds, allowing transcription to occur. Fur also affects transcription of genes concerned with traits as varied as toxin production, superoxide dismutase or acid tolerance [8]. At some promoters, Fur is a positive regulator, in response to the cell’s Fe status [8]. In some (e.g. *Pseudomonas aeruginosa* and *Neisseria meningitidis*), but not all (e.g. *E. coli*) bacteria, fur knockout mutants are non-viable [9,10]. However, viable fur alleles have been isolated in *P. aeruginosa*, based on the fact that fur mutant strains are manganese-resistant [9].

There is a gene, *irr*, in *Bradyrhizobium japonicum* resembling fur but which has a ‘mirror image’ function [11] positively affecting iron uptake during Fe limitation. Here we describe a gene with striking similarity in sequence to fur in a strain of *R. leguminosarum* bv. *viciae*, the symbiont of peas, and present evidence that this gene is essential for viability.

2. Materials and methods

2.1. Strains, microbiological techniques and assays

Bacterial growth conditions were as in [12]. Side-rophore production was analysed using the chrome azulon sulfonate (CAS) agar plate method [5,13]. *E. coli* was streaked on LB medium supplemented with FeCl₃ (50 μM) or with no added Fe but with 50 μM dipyridyl. β-Galactosidase activities were determined as described in [14]. For *Rhizobium*, in some cases, 10 μM dipyridyl was added to the media, to reduce the available Fe. Plasmids were transferred to *R. leguminosarum* by conjugation using the helper plasmid pKK2013 [15]. Pea seedlings were inoculated and scored for nodulation and N₂ fixation as in [16].

2.2. DNA manipulations

Routine DNA manipulations were carried out as in [17]. Using a method similar to that in [18], a library of *R. leguminosarum* strain 8401pRL1JI [18] was made by partially digesting genomic DNA with EcoRI, and ligating size-fractionated fragments to the wide host-range cosmid pLAFR1 [19]. After packaging into phage λ, approx. 15 000 primary transfectants were obtained. Ten of these were checked to confirm they had different inserts, each of total size approx. 25 kb. A fragment internal to *R. leguminosarum* fur was amplified by PCR from genomic DNA using primers indicated in Fig. 1. DNA sequencing used the dideoxy chain-terminating method on a Pharmacia ALF sequencer, according to the manufacturer’s instructions.

3. Results and discussion

3.1. Identification of an *R. leguminosarum* fur homologue

The fur genes of several bacteria have some highly conserved regions. Two primers (see Fig. 1) which correspond to two such regions were used for PCR with DNA isolated from *R. leguminosarum* bv. *viciae*. One fragment of about 120 bp was obtained. It was cloned in pUC18 to form pBIO923 and sequenced; it specified a deduced peptide with 37% identity to the corresponding region of *E. coli* Fur.

By colony hybridisations using pBIO923 as a probe, three overlapping plasmids of the *R. leguminosarum* gene bank which contained fur were identified. One of these, pBIO929, was studied further. By further hybridisations, fur was localised to specific restriction fragments, which were cloned into pUC18. From these, the sequence of fur plus 522 bp upstream and 213 bp downstream of it was determined (EMBL accession number Y13657).

The deduced Fur protein of *R. leguminosarum* is most similar (49% identity) to Fur of the closely related *Brucella abortus* but is also similar in sequence to Fur of, for example, *E. coli* (37%), *Pseudomonas aeruginosa* (34%) and *Yersinia pestis* (35%). In the Fur protein of other bacteria, there is a highly conserved sequence, HHDH, towards its C-terminus...
which is important for its function [20] and which is present in *R. leguminosarum* (Fig. 1). Another conserved region of Fur is towards the N-terminus and is represented by GLATVYR in *E. coli*, in which the ‘G’ is required for DNA binding [21]. In *R. leguminosarum* Fur, the corresponding sequence is SISTVYR.
(Fig. 1), in which the highly conserved ‘G’ is replaced by an ‘S’; an ‘S’ also occurs in the corresponding position in Fur of *B. abortus* [22]. Thus, in these bacteria, the configuration of the Fur DNA binding domain may differ from that in *E. coli*. In the absence of a detailed structure of Fur from any bacteria, it is not known what such differences are at a molecular level.

### 3.2. *fur* is linked to *tlyC* and *actA*

In sequencing the *fur* region of *R. leguminosarum*, it was noted that in its vicinity there are two other genes, homologues of which in other bacteria are regulated by *fur*. Approximately 3 kb downstream of *fur* and in the opposite orientation, we sequenced 120 bp of the 3′ end of an ORF whose product is similar (43% identity) to that of *tlyC* of *Haemophilus influenzae* which encodes a haemolysin. Some haemolysin genes of *E. coli* and *ethA* of *Edwardsella tarda* are regulated by *fur* [23]. It remains to be seen if the *tlyC* homologue of *R. leguminosarum* is also regulated by *fur*.

Approximately 1 kb upstream of *fur* was the 3′ end of an ORF whose product was similar (57% identity in the sequenced region) to that of *actA* of *Sinorhizobium meliloti*, mutations in which confer acid sensitivity [24]. In *Salmonella typhimurium*, the acid-tolerant response following phagocyte invasion is abolished in *fur* mutants [25]. Although in *S. meliloti* transcription of *actA* is controlled by a two-component regulator [26], it remains to be seen if in *R. leguminosarum* the *actA* homologue is also controlled by the closely linked *fur*.

### 3.3. Attempts to isolate a *fur* knockout mutant

An attempt was made to make an insertional mutation into *R. leguminosarum* *fur* as follows. The ‘suicide’ plasmid pBIO925 is based on pJQ200SK [27] which is mobilisable but fails to replicate in *Rhizobium*. pJQ200SK also has *gen* and *sacB* genes, the latter being lethal in sucrose-grown cells, due to levan production. Plasmid pBIO951 has a 2.4-kb *ClaI* fragment spanning *R. leguminosarum* *fur*, into which an *Ω* fragment, specifying *spe*′, had been cloned into a *SacI* site within the *fur* coding sequence. pBIO951 was mobilised into *R. leguminosarum*. *Spe*′ transconjugants should arise by plasmid integration at the *fur* locus, either by a single crossover, in which case the transconjugants would be *gen*′ and sensitive to sucrose, or by a reciprocal crossover, generating a *fur* insertional mutation in which *gen*′ and *sacB* of pJQ200SK would be lost.

In a cross with *R. leguminosarum* strain 8002*str* as recipient and *E. coli* with pBIO951 as the donor, selection was made for the transfer of *spe*′, on plates that were either supplemented with 5% sucrose to counter-select *sacB* or not. The frequencies of *spe*′ colonies on the media with and without sucrose were, respectively, approx. 10⁻⁴ and 10⁻⁶ per recipient, showing that single, integrative crossovers were much more frequent than double, ‘*fur* replacement’ events. Further, of 20 transconjugants arising on medium lacking sucrose, 18 were found to be *gen*′ and retain *sacB* as judged by sucrose sensitivity. Also, about 50% of transconjugants isolated initially on medium with sucrose were *gen*′, so, although they had lost *sacB*, they retained some vector DNA.

Genomic DNA was isolated from seven *spe*′, *gen*′, sucrose-resistant derivatives. Following digestion with *ClaI*, the DNAs were probed with a 2.4-kb *ClaI* fragment containing the entire *R. leguminosarum* *fur* gene. With strain 8002*str* itself, a single *ClaI* fragment of 2.4 kb hybridised, as expected. Thus, there is apparently only one copy of *fur* in *R. leguminosarum*. Since the *spe*′ cassette is 1.8 kb and has no *ClaI* sites, a simple double crossover replacement into *fur* would generate a 4.2-kb hybridising fragment. In fact, with all eight mutants, single fragments in the range of 5.8–6.2 kb hybridised, corresponding neither to intact *fur* nor to *fur* with the *Ω* insertion. The *fur* gene has two *Aval* sites separated by 650 bp, between which is located the *Ω* cassette. This internal *Aval* fragment was used to probe *Aval* digests of wild-type DNA and those of the putative *fur* insertional mutants. In all cases, it hybridised to a fragment of 650 bp, corresponding to the intact *fur*, and also to two other fragments of 800 and 1500 bp, these having a combined size predicted for the *fur* gene plus the insertion (the cassette has a single *Aval* site).

It appears that the DNA of pBIO951 had inserted into the *fur* gene but no reciprocal crossovers had occurred. It seems most likely that there was a single crossover at the *fur* locus, followed by deletion of...
part of the inserted DNA containing sacB and gen'.

Taken together, the observations suggest that fur knockout mutants cause a loss of viability in *R. leguminosarum*. This approach of using the sacB counter-selection of derivatives of pJQ200SK containing the spc cassette within cloned *R. leguminosarum* DNA has been used routinely for the isolation of insertions in other genes of this species (J. Lithgow, personal communication).

3.4. Isolation of manganese-resistant mutants

In other bacteria, fur mutants are resistant to Mn²⁺ [9,10]. We isolated Mn²⁺ mutants of *R. leguminosarum* and tested if they had mutations in fur. These were isolated on TY media containing 20 mM MnCl₂ and arose at a frequency of about 4 × 10⁻⁶.

A phenotype associated with fur mutants is over-production of siderophores [3,4,10], identifiable as larger halos on CAS plates. With 120 independent Mn²⁺ mutants picked to CAS plates, the halos were the same size as wild-type. Plasmid pBIO929 (contains cloned fur) was mobilised into 40 of the Mn²⁺ mutants and transconjugants were tested on 20 mM MnCl₂. In all cases, they grew as well as the host mutant strain; thus the cloned fur did not restore manganese sensitivity. As a check, it was shown that introducing pBIO929 did not itself lead to manganese resistance.

3.5. Expression of the fur gene of *R. leguminosarum*

Genes that are regulated by Fur contain a sequence, the fur box [3,4], to which the protein binds. No convincing match to the fur box consensus (5'-GATAATGATAATCATTATC-3') was present within the region 522 bp upstream of fur, suggesting that in *Rhizobium*, as in several other bacteria, fur is not autoregulatory.

To study expression of *R. leguminosarum* fur, a transcriptional fusion was made. A 0.8-kb PstI fragment, one end of which was in the fur coding sequence and the other 522 bp upstream of the fur translational start, was cloned in the correct orientation into the PstI site of the lacZ reporter plasmid pMP220 [28] to form pBIO939. This plasmid was mobilised into several strains of *R. leguminosarum* and the transconjugants were grown on minimal medium which was either supplemented with FeCl₃ (+Fe) or which had no added Fe and to which the Fe chelator dipyridyl had been added (−Fe) before assaying β-galactosidase.

In most of the strains, the wild-type bv. phaseoli 8002, the bv. viciae strains 1062 and 248 and the ‘cured’ strain 8401, which lacks a Sym plasmid, fur-lacZ was expressed constitutively (approximately 200 Miller units), independent of the presence of Fe in the medium. However, with one strain, 8401pRL1JI, expression of the fusion was low, similar to that of the background containing the vector pMP220 alone (about 40 units). This suggested that in the 8401 genetic background, symbiotic plasmid pRL1JI inhibited expression of fur. Therefore, pBIO939 was mobilised into other strains that were identical to 8401pRL1JI except that they had Tn⁵ insertions in different locations in pRL1JI (in nodC, nifA and rhiA). In all cases, expression of fur-lacZ was constitutive (about 200 units). Since these insertions were in different genes, some of which (nod and nif) are not even expressed in free-living *Rhizobium*, this indicates that in our laboratory stock strain 8401pRL1JI, a mutation that affects fur expression had inadvertently arisen, despite its having been used as a ‘wild-type’ strain in many previous studies. Further, it was found that when the fur::lac fusion plasmid was transferred to *R. leguminosarum* bv. viciae field isolate strain 248, the strain in which pRL1JI was originally discovered [29], expression of β-galactosidase was detectable at relatively high level. Given that it was impossible to inactivate fur completely (above), this indicates that the low level of fur expression still found in strain 8401pRL1JI is sufficient to confer viability. It has also been noted that in the laboratory strain 8401pRL1JI, the fluCD operon which is involved in uptake of the vicibactin siderophore is expressed constitutively, whereas in all other near-isogenic strains, fluCD expression is repressed by Fe in the medium (J.B. Stevens, personal communication). Further, the halo on CAS medium that surrounds colonies of the laboratory stock of strain 8401pRL1JI was found to be larger than near-isogenic strains which either lacked pRL1JI or which had different allelic forms of it. It remains to be seen if the same chance mutation in 8401pRL1JI is responsible for the aberrant effects on the expres-
sion both of fur and of fluCD. Whatever this mutation might be, it is unlikely to be in fur itself since the strain is no more resistant to Mn$^{2+}$ than the other near-isogenic strains and the various phenotypes were not corrected by the introduction of the cloned fur gene of R. leguminosarum.

3.6. Introduction of R. leguminosarum fur into a fur mutant of E. coli

Plasmid pBIO949 contains a 2.4-kb Clal fragment that includes the whole of R. leguminosarum fur, cloned in pJQ200SK. A fur deletion mutant (strain JRG2653) and its wild-type parent (JGR2652) of E. coli (kindly supplied by Simon Andrews) were transformed with pBIO949, selecting for kan$^\beta$ transformants. These were plated on LB medium containing CAS and which had either been supplemented with 50 $\mu$M FeCl$_3$ or been stripped of available Fe by adding 50 $\mu$M dipyridyl. As expected, the wild-type control stained much more brightly on low Fe than Fe-replete medium and, in this background, pBIO949 had no observable effect on the staining on either medium. Thus, the R. leguminosarum fur gene did not appear to exert a dominant phenotype. In the $\Delta$fur mutant, the colonies excreted large amounts of CAS-staining material even when Fe was present in the medium, however. In the mutant containing the cloned R. leguminosarum fur gene, this CAS-staining material was significantly reduced by approximately 40% compared to a control in which the vector plasmid pLAFR1 had been introduced. Thus, it seems that the R. leguminosarum fur gene causes a reduction in siderophore production in response to Fe in the medium; however, the effect is not as great as with fur of E. coli itself. It is not known if this is due to poor expression of R. leguminosarum fur or if the Fur protein of this bacterium is less effective at repressing E. coli fur-regulated promoters. In either event, though, this observation supports the view that the fur homologue in R. leguminosarum shares some of the regulatory functions of its homologues in other bacteria.

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References


