JOURNAL OF BACTERIOLOGY, Sept. 1999, p. 5843–5846 Vol. 181, No. 18
0021-9193/99/$04.00+0

Identification of a Functional *fur* Gene in *Bradyrhizobium japonicum*

IQBAL HAMZA, RICHARD HASSETT, AND MARK R. O'BRIAN*

Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York 14214

Received 4 May 1999/Accepted 7 July 1999

The recent identification of the iron response regulator (Irr) in *Bradyrhizobium japonicum* raised the question of whether the global regulator Fur is present in that organism. A *fur* gene homolog was isolated by the functional complementation of an *Escherichia coli fur* mutant. The *B. japonicum* Fur bound to a Fur box DNA element in vitro, and a *fur* mutant grown in iron-replete medium was derepressed for iron uptake activity. Thus, *B. japonicum* expresses at least two regulators of iron metabolism.

Studies on the regulation of iron homeostasis in bacteria have focused on Fur, a global transcriptional regulator that represses target genes when bound to iron. The Irr (iron response regulator) protein from the bacterium *Bradyrhizobium japonicum* mediates iron control of heme biosynthesis and affects iron transport (9). Irr has low homology to Fur, and although it is not a functional homolog (9), its identification led us to ask whether *B. japonicum* also expresses a functional *fur* gene.

A *B. japonicum* genomic expression library was screened for clones that repressed the *fiu*::*lacZ* reporter gene fusion in *Escherichia coli fur* strain H1780 as discerned by the formation of white or light blue colonies in the presence of 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml and 100 μM $FeCl_3$. Three overlapping library clones, pSKBJF1, pSKBJF9, and pSKBJF22, complemented the *fur* strain both on plates and in liquid cultures (Fig. 1). pSKBJF22 repressed less well, perhaps because the complementing gene (see below) was more distal from the plasmid-borne *lacZ* promoter. A 830-bp *Apa*I-*Sau*3AI subclone on pSKBJF800 including the overlapping region was also sufficient to complement the *fur* strain (Fig. 1). This fragment contained an open reading frame encoding a protein with homology to characterized Fur proteins from numerous organisms (Fig. 2). Among the proteins shown experimentally to be Fur, the *B. japonicum* protein was most homologous to that of *E. coli*, showing 39% identity and 49% similarity to *E. coli* Fur. Southern blot analysis indicated a single *fur* gene in the *B. japonicum* genome (data not shown). The complementation and homology indicate that the cloned *B. japonicum* DNA encodes a structural and functional homolog of Fur. A *fur*-like gene of *Rhizobium leguminosarum* was isolated (6) encoding a product with 77% similarity to *B. japonicum* Fur, indicating that the *R. leguminosarum* gene is a functional *fur* gene as well. Previous work indicates that the *B. japonicum* Irr is functionally distinct from bacterial Fur despite their modest homology to each other (9). Consistent with this, the plasmid-borne *irr* gene did not functionally complement the *E. coli fur* strain as did the *B. japonicum fur* gene (Fig. 1B).

Complementation analysis showed that the *B. japonicum* Fur (BjFur) had repressor activity in vivo (Fig. 1); thus, we examined its DNA-binding activity in extracts from cells that overexpressed Fur by gel mobility shift assays with a double-stranded DNA probe that includes a "Fur box" consensus sequence recognized by *E. coli* Fur (EcFur) and other characterized Fur proteins (4, 5, 11). In addition, the assays were carried out in the presence or absence of Mn^{2+}, which has

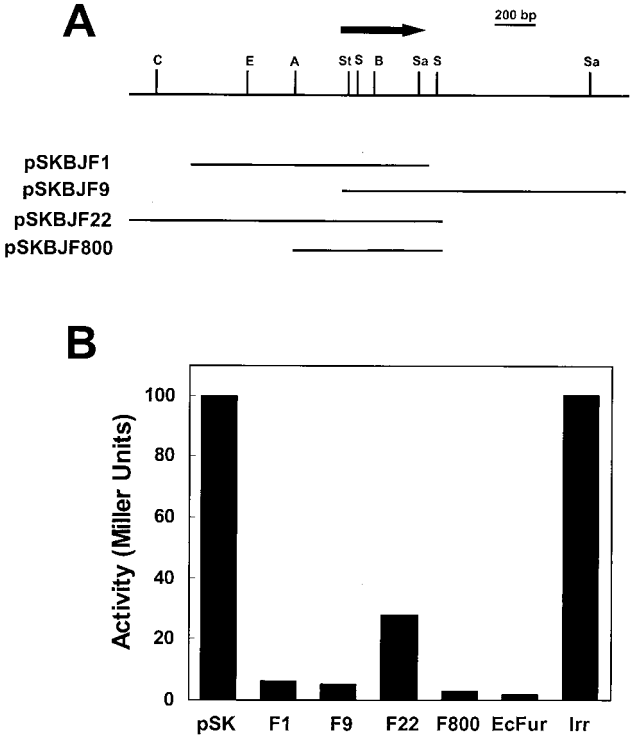

FIG. 1. Complementation of *E. coli fur* strain H1780 for in vivo repression of *fiu*::*lacZ* gene fusion by *B. japonicum* library clones. (A) *B. japonicum* genomic region represented by library plasmids pSKBJF1, pSKBJF9, and pSKBJF22, which complemented the *fur* strain on plates as described in the text. pSKBJF800 is an *Apa*I-*Sau*3AI-digested subclone of pSKBJF22. The arrow represents the open reading frame encoding the *fur* homolog. Restriction sites: A, *Apa*I; B, *Bgl*II; C, *Cla*I; E, *Eco*RI; Sa, *Sac*I; S, *Sph*I; St, *Sty*I. (B) Repression of β-galactosidase activity in *fur* strain H1780 by *B. japonicum* library clones pSKBJF1 (F1), pSKBJF9 (F9), pSKBJF22 (F22), and pSKBJF800 (F800). Cells containing the library vector pBluescript SK(+) (pSK) were the control for unrepressed activity. The positive control was pMH15fur (EcFur), which contained the *E. coli fur* gene. Also shown is the result for *B. japonicum irr* gene on pSKSBIrr (Irr).

* Corresponding author. Mailing address: Department of Biochemistry, 140 Farber Hall, State University of New York at Buffalo, Buffalo, NY 14214. Phone: (716) 829-3200. Fax: (716) 829-2725. E-mail: mrobrian@buffalo.edu.

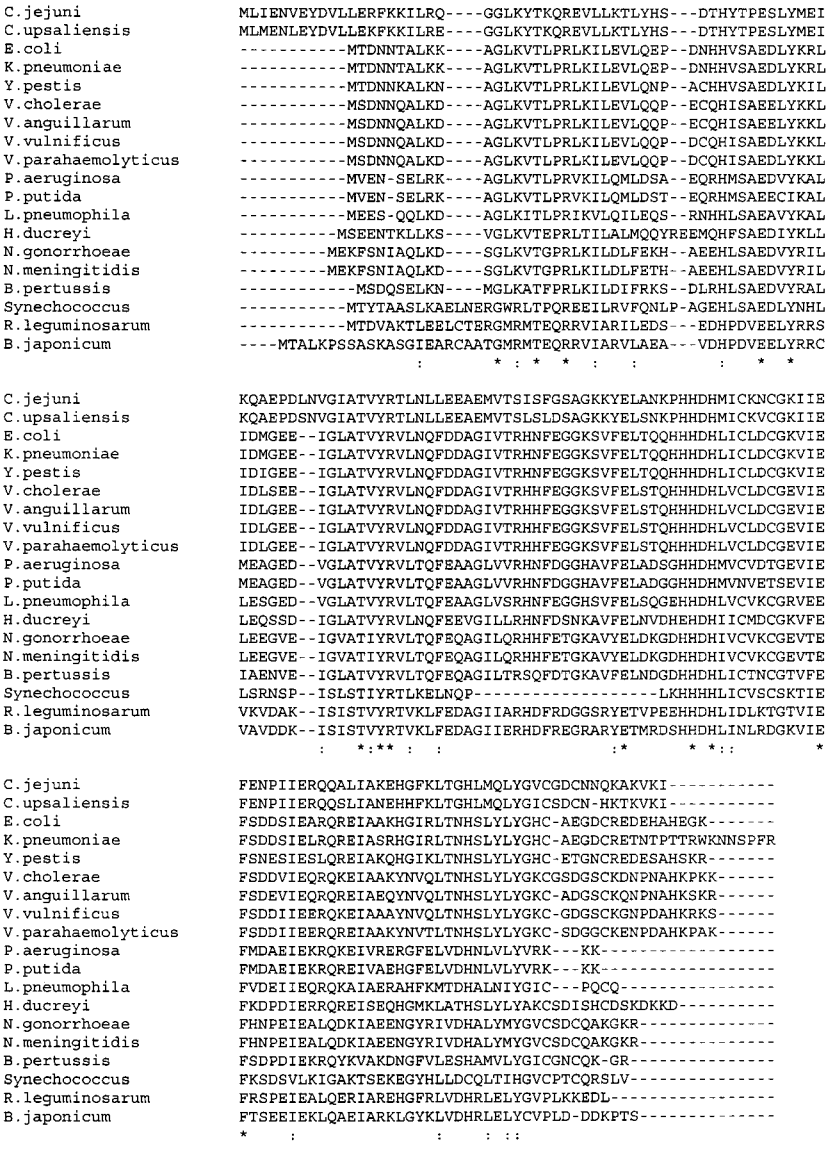

FIG. 2. Deduced amino acid sequence of the *B. japonicum* Fur protein (bottom sequence) and alignment with other Fur proteins. Asterisks and colons denote amino acids that are identical or similar, respectively, at that position for all sequences. The sources of the proteins used, along with GenBank accession numbers, are as follows: *E. coli* (X02589), *Klebsiella pneumoniae* (L23871), *Yersinia pestis* (Z12101), *Vibrio cholerae* (M85154), *Vibrio anguillarum* (L19717), *Vibrio parahaemolyticus* (AB003752), *Vibrio vulnificus* (L06428), *P. aeruginosa* (L00604), *Pseudomonas putida* (X82037), *Neisseria gonorrhoeae* (L11361), *Neisseria meningitidis* (L19777), *Bordetella pertussis* (U11699), *Haemophilus ducreyi* (U37224), *Campylobacter jejuni* (AF052056), *Campylobacter upsaliensis* (L77075), *Synechococcus* sp. PCC 7942 (L41065), *Legionella pneumophila* (U06072), and *R. leguminosarum* (Y13657).

been shown to substitute for Fe^{2+} as a cofactor of Fur in vitro (5). Mn^{2+} is used because Fe^{2+} is readily oxidized in air to Fe^{3+}, which is not functional as a Fur cofactor. The results show that BjFur bound to the Fur box consensus element in the presence of metal (Fig. 3A) but not in its absence (Fig. 3B). EcFur showed metal-dependent DNA-binding activity as well (Fig. 3). However, in the absence of metal, the DNA-binding activity of EcFur produced a complex that was different from that observed in the presence of metal. Binding of *E. coli* apo-Fur to operator DNA with lower affinity in a gel mobility shift assay has been documented previously (1). Unlike BjFur, the *B. japonicum* Irr protein did not bind to the iron box element under any condition (Fig. 3), consistent with the conclusion that Irr is not functionally equivalent to Fur.

E. coli fur has a Fur box element in its promoter and is modestly autoregulated, whereas the *Pseudomonas aeruginosa fur* gene does not (11). No recognizable Fur box element was found upstream of the *fur* gene, and an *Apa*I-*Sty*I fragment that included 268 bp of DNA upstream of the *fur* open reading frame did not bind to BjFur in a gel mobility shift assay (data not shown). Thus, the *B. japonicum fur* gene does not appear to be autoregulated.

Many bacterial species, including *B. japonicum* (8, 9), express an inducible high-affinity iron uptake activity in response to iron limitation as a means of scavenging the metal. Fur proteins repress bacterial iron transport genes under iron-replete conditions; thus, a *fur* mutant of *E. coli* is derepressed for iron transport (for a review, see references 2 and 10). A *B. japonicum fur* null mutant of wild-type strain USDA I110 was constructed by the insertional inactivation of the *fur* gene of

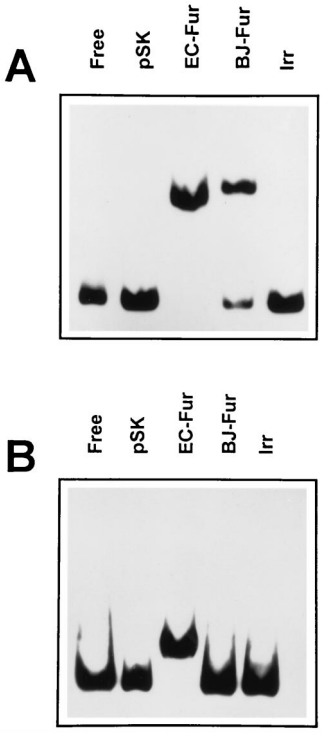

FIG. 3. Metal-dependent binding of BjFur to the Fur box consensus sequence. Gel mobility shift assays were carried out in the presence (A) or absence (B) of 100 μM $MnCl_2$. The DNA used in the assays was radiolabeled with ^{32}P and contained the Fur box consensus sequence 5′-GATAATGATAATCATTA TC-3′. The protein samples used were extracts of *E. coli fur* strain H1780 containing plasmids pBluescript SK(+) (pSK), pMH15fur (EC-Fur), pSKBJF800 (BJ-Fur), or pSKSBIrr (Irr). The DNA was also run in the absence of extract (Free).

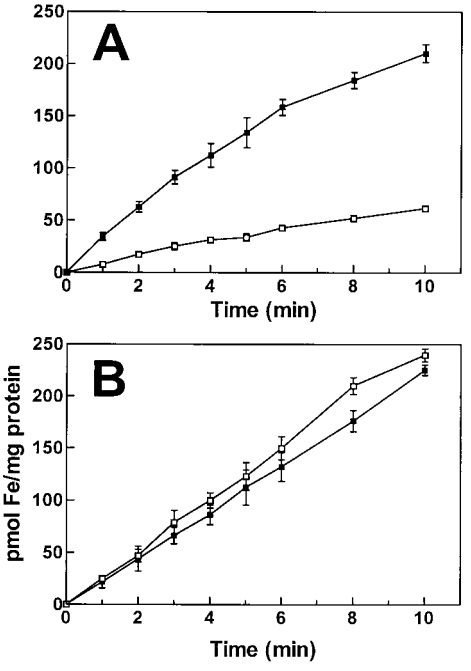

FIG. 4. Iron uptake activity in *B. japonicum* parent strain I110 (open squares) and *fur* strain GEM4 (closed squares). Cells were grown to mid-log phase in high-iron (A) or low-iron (B) medium, washed, and prepared as described in the text. At time zero, ^{59}Fe (0.1 μM final concentration) was added to the cells. Aliquots were removed at various times, collected, and counted as described in the text. Each point represents the average of three separate samples; standard deviation bars are shown. As controls, 1- and 8-min samples of cells that remained on ice throughout the time course were taken, and no uptake was observed (data not shown). The data shown are typical of two independent experiments.

parent strain I110 with an Ω cassette to construct strain GEM4. Ferric citrate uptake activity was carried out as described previously (9). The *fur* strain showed similar iron uptake activity as the wild type when grown in iron-limited medium (Fig. 4). However, the mutant expressed a three- to fourfold higher initial rate of iron uptake activity than the parent strain when grown in high-iron medium. This modest derepression was similar to that observed for an *E. coli fur* mutant (10). A phenotype for the mutant strain showed that the cloned *fur* gene is functional in *B. japonicum*. The uptake activity of the *fur* strain grown in high-iron medium reached saturation by 10 to 15 min (Fig. 4 and data not shown), whereas activity induced in the wild type by iron deprivation is unchanged for at least 90 min (9). Irr is not expressed in high-iron medium (9); thus, it is possible that the iron transport system normally induced under iron limitation in the wild type was not fully expressed in the *fur* strain grown in high-iron medium.

The identification of a functional *fur* gene in *B. japonicum* shows that Irr does not replace Fur, but rather both regulators function in that organism to regulate iron metabolism. Genes encoding Fur-like proteins in addition to bona fide Fur have been recently identified in *E. coli* and *Bacillus subtilis*; these proteins are involved in the maintenance of zinc homeostasis (Zur) (7, 12), or in a manganese-dependent response to oxidative stress (PerR) (3). Thus, there appears to be a family of functionally diverse Fur-like proteins, including Zur, PerR, and Irr, that are all involved in metal-dependent regulation but are distinct from Fur. These findings underscore the need for experimental evidence to correctly identify *fur* homologs obtained by nucleotide sequence information.

Nucleotide sequence accession number. The nucleotide sequence of the *Apa*I-*Sau*IIIA insert of pSKBJF800 was deposited in GenBank under accession no. AF052295.

We thank K. Hantke and M. Vasil for *E. coli* strains and plasmids.
This work was supported by National Science Foundation grant MCB-9722974 to M.R.O.

REFERENCES

1. **Althaus, E. W., C. E. Outten, K. E. Olson, H. Cao, and T. V. O'Halloran.** 1999. The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. Biochemistry **38:**6555–6569.
2. **Braun, V., and V. Hantke.** 1991. Genetics of bacterial iron transport, p. 107–138. *In* G. Winkelmann (ed.), Handbook of microbial iron chelates. CRC Press, Inc., Boca Raton, Fla.
3. **Bsat, N., A. Herbig, L. Casillas-Martinez, P. Setlow, and J. D. Helmann.** 1998. Bacillus subtilis contains multiple Fur homologs: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol. Microbiol. **29:**189–198.
4. **Calderwood, S. B., and J. J. Mekalanos.** 1988. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. **170:**1015–1017.
5. **de Lorenzo, V., F. Giovannini, M. Herrero, and J. B. Neilands.** 1988. Metal ion regulation of gene expression. Fur repressor-operator interaction at the promoter region of the aerobactin system of pColV-K30. J. Mol. Biol. **203:** 875–884.
6. **De Luca, N. G., M. Wexler, M. J. Pereira, K. H. Yeoman, and A. W. B. Johnston.** 1998. Is the *fur* gene in *Rhizobium leguminosarum* essential? FEMS Microbiol. Lett. **168:**289–295.
7. **Gaballa, A., and J. D. Helmann.** 1998. Identification of a zinc-specific met-

alloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. J. Bacteriol. **180:**5815–5821.
8. **Guerinot, M. L., E. J. Meidl, and O. Plessner.** 1990. Citrate as a siderophore in *Bradyrhizobium japonicum*. J. Bacteriol. **172:**3298–3303.
9. **Hamza, I., S. Chauhan, R. Hassett, and M. R. O'Brian.** 1998. The bacterial Irr protein is required for coordination of heme biosynthesis with iron availability. J. Biol. Chem. **273:**21669–21674.
10. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol. Gen. Genet. **182:**288–292.
11. **Ochsner, U. A., A. I. Vasil, and M. L. Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J. Bacteriol. **177:**7194–7201.
12. **Patzer, S. I., and K. Hantke.** 1998. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol. Microbiol. **28:**1199–1210.