The manganese-responsive repressor Mur of *Rhizobium leguminosarum* is a member of the Fur-superfamily that recognizes an unusual operator sequence

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The manganese uptake regulator Mur of *Rhizobium leguminosarum* is a close homologue of the global iron regulatory protein Fur. Mur represses the *sitABCD* operon, which encodes a Mn^{2+} transport system, specifically in response to Mn^{2+} but not Fe^{2+} . In previous work the authors mapped the 5' ends of two *sit* operon transcripts, termed TS1 and TS2, which were co-ordinately regulated by Mn^{2+} -Mur, but this paper now shows that only TS1 is a primary transcript. DNase I protection analyses showed that purified Mur bound, with similar affinity, to two sites in the regulatory region of *sitABCD*, but only when Mn^{2+} was present in the reaction buffer. These Mn^{2+} -Mur-binding sites, termed MRS1 and MRS2 (Mur-responsive sequence), were closely related in sequence to each other and were separated by 16 bp, spanning the transcription initiation site TS1. The extent of the protected DNA was 34 and 31 bp for MRS1 and MRS2, respectively, which is in accord with other members of the Fur family. The DNA sequences recognized by Mn^{2+} -Mur are wholly different from conventional Fur boxes, but some similarities to a recognition sequence for the Fur regulator from *Bradyrhizobium japonicum* were noted. Transcription analysis of the *R. leguminosarum* *mur* gene showed its expression to be independent of Mn^{2+} -Mur. Thus, Mur is a sequence-specific DNA-binding protein that responds *in vitro* to manganese, and thus can occlude RNA polymerase access to the *sitABCD* promoter. Moreover, Mur recognizes a DNA sequence atypical for the Fur superfamily and, like Fur from *B. japonicum*, defines a new subclass of Fur-like transcriptional regulators.

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INTRODUCTION

The rhizobia comprise a group of symbiotic α -proteobacteria that can induce N_2 -fixing nodules in the roots of leguminous plants. Recent studies show that these bacteria regulate gene transcription in response to the availability of iron very differently to the model γ -proteobacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Andrews *et al.*, 2003; Johnston, 2004). In particular, the ferric uptake regulator Fur plays a pivotal role in iron homeostasis, repressing the transcription of >100 genes in both *E. coli* and *P. aeruginosa* (Andrews *et al.*, 2003). In the presence of Fe^{2+} , *E. coli* Fur binds to conserved sequences, known as *fur* boxes, near the promoters of the genes that it represses (Andrews *et al.*, 2003; Hantke, 2001). Since one of the genes (*ryhB*) that Fur represses in Fe-replete conditions encodes a small, negatively

acting regulatory RNA, this accounts for the fact that Fur can appear to be an activator of some genes in *E. coli* (Massé & Gottesman, 2002). Although the structure of the *P. aeruginosa* Fur protein has been determined (Pohl *et al.*, 2003), there is still uncertainty as to the molecular structures involved when Fur, complexed with Fe^{2+} , binds to the *fur* boxes.

However, a very different protein, called RirA, appears to be the major Fe-responsive transcriptional repressor in at least two rhizobial species, namely *Rhizobium leguminosarum* (Todd *et al.*, 2002, 2005) and *Sinorhizobium meliloti* (Viguier *et al.*, 2005). These two species both have a protein with amino acid sequence similarity to *bona fide* Fur, and in *R. leguminosarum* this protein can complement an *E. coli fur* mutant and it can bind to Fur boxes (Wexler *et al.*, 2003). Nonetheless, in *Rhizobium* and *Sinorhizobium* this homologue appears to be relegated to a minor role involved in the uptake of manganese, not iron (Chao *et al.*, 2004;

Abbreviations: DP, 2,2'-dipyridyl; Fur, ferric uptake regulator; MRS, Mur-responsive sequence; Mur, manganese uptake regulator.

Díaz-Mireles *et al.*, 2004; Platero *et al.*, 2004). We have renamed this Fur-like protein Mur, because it regulates the *sitABCD* operon, which encodes an ABC-type transporter for Mn^{2+} , repressing its transcription under conditions of Mn^{2+} sufficiency. A similar role for Mur of *S. meliloti* in the regulation of its *sitABCD* operon was also demonstrated. Furthermore, as noted previously (Wexler *et al.*, 2003), *Mesorhizobium loti* has no Fur-like protein in its deduced proteome, nor, interestingly, does it have a *sitABCD* operon.

It had been shown by gel shifts (Díaz-Mireles *et al.*, 2004) that *R. leguminosarum* Mur can bind to the *cis*-acting regulatory region of *sitABCD*, most probably to two versions of a conserved sequence, termed the MRS motif, which differs significantly in sequence from canonical Fur boxes (Díaz-Mireles *et al.*, 2004; Escolar *et al.*, 1999; Baichoo & Helmann, 2002).

The Fur-like protein (Fur_{Bj}) of *Bradyrhizobium japonicum*, which is more distantly related to *Sinorhizobium* and to *Rhizobium*, also has some unusual characteristics. Although Fur_{Bj} regulates another regulatory gene, *irr*, in response to Fe availability (Hamza *et al.*, 2000), the sequence to which it binds has little or no similarity to that of a conventional Fur box (Friedman & O'Brian, 2003).

Here, we identify the *R. leguminosarum* Mur-binding site, confirming that Mur requires Mn^{2+} to bind *in vitro* and that its cognate DNA-binding sequences do not resemble those described for conventional Fur.

METHODS

Strains, media and growth conditions. The *R. leguminosarum* wild-type strain 3841, whose genome has been sequenced (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/r_leguminosarum), was used as the source of genomic template DNA for PCR amplifications. Strain J251 is the immediate parent of the *mur* mutant J325, containing a *Spc^R* cassette in the *mur* gene (Wexler *et al.*, 2001). The Met⁻ *E. coli* strain 803 (Wood, 1966) is especially effective as a recipient for the introduction of large (e.g. pLAFRI-based) plasmids by transformation and was used as the routine *E. coli* host. *R. leguminosarum* was grown routinely in complex (TY) medium or in minimal (Y) medium (Beringer, 1974) prior to assaying β -galactosidase activities. To generate metal-depleted media, the chelator 2,2'-dipyridyl (DP) (30 μ M) was added. In metal-supplemented media, $MnCl_2$ was added at 50 μ M.

Plasmid construction. The *sit-lacZ* transcriptional fusion plasmid pBIO1552 was made by PCR amplification of the 504 bp DNA region that extended from the end of the putative deaminase gene *adm* directly 5' of the *sitABCD* operon to within the *sitA* gene using primers *Sit-EcoRI* (5'-CGCGAATTCGTGGAAGCGCGGCTCCGAC-AGAAG-3') and *Sit-SphI* (5'-CGCGCATGCTTGGTGATCGATTCCACGATCGCGG-3') and genomic DNA isolated from *R. leguminosarum* 3841 as template in the reactions (the relevant *EcoRI* and *SphI* sites are underlined). The amplified fragment was digested with *EcoRI* and *SphI* and cloned into the wide-host-range *lacZ* promoter-probe plasmid pMP220 (Spaink *et al.*, 1987) to form pBIO1552. The DNA regions spanning either TS1 or TS2 were individually cloned into pMP220 as 304 bp and 222 bp DNA fragments, respectively, after PCR amplification using oligonucleotides *Sit-EcoRI* and the *SphI*-containing MRS2R (5'-ACATGCATGCTTGACGACCCTTGTTCGACTATT-3') to make

pBIO1480 (TS1) and *Sit-SphI* and the *EcoRI*-containing MRS1F (5'-CCGGAATTCGAAACAAGGGTCGTCAAGCATTTTTCG-3') to make pBIO1479 (TS2). Starting with pBIO1552 as template DNA, the putative -10 RNA polymerase recognition sequence of transcript start 1 (TS1) was mutated from AATAGT to AAGCTT (generating a *HindIII* restriction site) using the Stratagene Ex-Site PCR-based site-directed mutagenesis kit and oligonucleotides TS1-*HindF* (5'-GCTTCGAAACAAGGGTCGTCAAAGCAT-3') and TS1-*HindR* (5'-TTGTGCAATTAAGAATGAATTGCAAC-3'). The nucleotide sequences that constitute the restriction site are underlined. The resulting plasmid was named pBIO1528. Similarly, the predicted -10 RNA polymerase recognition sequence of the putative TS2 promoter was mutated from TTCGACT to ATCGATT (generating a *Clal* site) using oligonucleotides TS2-*ClalF* (5'-ATCGATTCTCCGCGGCTTTGGCAATTCGGTC-3') and TS2-*ClalR* (5'-CAAGTTGCG-ATTCAGAAATGATTTCG-3'). The nucleotide sequences that constitute the restriction site are underlined. The resulting plasmid was named pBIO1526.

The inserts of all the plasmids formed by cloning PCR products were ratified by DNA sequencing.

In vivo and in vitro genetic manipulations. Plasmid conjugation, isolation and transformation were as described by Wexler *et al.* (2001). Primer extension analyses of the *mur* and the *sitABCD* transcripts were done as described by Sawers & Böck (1989) using 25 μ g total RNA isolated from *R. leguminosarum* J251 or appropriate derivatives using a Qiagen RNeasy kit. In some cases the strain from which the RNA was isolated contained the corresponding cloned gene, in order to amplify the signal. The primers used were 0.2 pmol ³²P-labelled oligonucleotide Mur-1 (5'-CATGCCGCGTTTCG-GTGCAAGCTCC-3') or *sit*-PE (Díaz-Mireles *et al.*, 2004) (5'-GGTGTCTGTGGGCGAGCGGG-3') as appropriate. The 5' end of the *mur* transcript was precisely located by running in parallel a DNA sequence ladder generated using plasmid pBIO939 (de Luca *et al.*, 1998) as template and labelled Mur-1 oligonucleotide as the sequencing primer. The DNA sequence ladder used with the *sit* operon transcript was generated using plasmid pBIO1457 (Díaz-Mireles *et al.*, 2004). DNA sequencing employed a T7 DNA sequencing kit as described by the manufacturer (USB).

DNase I footprinting. A 318 bp DNA fragment spanning the transcription start site and regulatory sequences of *sitABCD* was amplified using oligonucleotides *sit*-PE (see above) and *sit*-ds (5'-GGTGCCTGTTCGATGAGGTTGACCG-3'), with pBIO1552 DNA as template. To demonstrate binding of Mur to the coding and anti-coding strands, 50 pmol of either *sit*-ds or *sit*-PE, respectively, was labelled with 50 μ Ci (1850 kBq) [γ -³²P]ATP prior to inclusion in the PCR reaction. The reaction conditions were essentially as described by Tucker *et al.* (2004). A 0.5 μ g aliquot of labelled DNA fragment was mixed with 2.5 μ l of 10 $\times$ binding buffer (100 mM Tris/HCl, pH 8.0, 300 mM KCl), 10 μ g bovine serum albumin, 0.01 unit poly(dI-dC) (Roche), purified Mur protein freshly diluted in 1 $\times$ binding buffer (Díaz-Mireles *et al.*, 2004) to a final concentration of between 0 and 10 μ M, 1 μ l 2.5 mM $MnCl_2$ (where specified), in a final volume of 25 μ l. After incubating the mixture for 30 min at 25 °C, 1 μ l (0.2 units freshly diluted in 1 $\times$ binding buffer and 50% w/v glycerol) DNase I (Amersham Biosciences) was added and after 15 s the reaction was stopped by adding 100 μ l stop solution (50 μ g proteinase K ml⁻¹, 20 mM EDTA, 0.5%, w/v, SDS, 0.2 mg glycogen ml⁻¹) and 100 μ l 2-propanol. The DNA was precipitated at -20 °C for 30 min and after centrifugation and washing with cold 80% (v/v) ethanol, the air-dried pellet was resuspended in 8 μ l formamide dye. Reaction products were separated on a denaturing sequencing gel of 6% (w/v) acrylamide and 7 M urea. The locations of the Mur-binding sites were determined using radioactively labelled ϕ X174 DNA digested with *HinfI*.

Other methods. β -Galactosidase enzyme assays on *R. leguminosarum* cells, grown in minimal Y medium, were done essentially as described by Rossen *et al.* (1985). Experiments were repeated three times using independent cultures.

RESULTS AND DISCUSSION

Expression of the *R. leguminosarum mur* gene is not subject to autoregulation

To determine if *R. leguminosarum mur* is regulated in response to manganese, the expression of the *mur-lacZ* derivative pBIO939 was examined in *R. leguminosarum* wild-type strain J251 containing this plasmid, grown in metal-depleted (DP-treated) medium or in medium supplemented with 50 μM $MnCl_2$. The β -galactosidase activity in the former was 153 (standard error 3) Miller units, and in the latter, 96 (2) units. Thus, *mur* is expressed at a low level and is slightly reduced by the presence of Mn^{2+} . When this *mur-lacZ* fusion plasmid was present in strain J325, the results were similar in the wild-type [159 (2) units in metal-depleted and 103 (2) in Mn -replete media], showing that *mur* expression is not autoregulated.

To locate the *mur* transcriptional initiation site, total RNA was isolated from strain J251 containing pBIO939 after growth in TY medium. Primer extension analysis revealed one major 5' end at an adenosine, 74 bp 5' of the *mur* translation initiation codon (Fig. 1). The 5' end of a further, very weak transcript was observed at a cytosine 15 bp 5' of the major initiation site.

The Mur-regulated *sitABCD* operon is transcribed from a single promoter

Previously, Díaz-Mireles *et al.* (2004) mapped the 5' ends of two *sitABCD* transcripts, termed TS1 and TS2, separated by 57 bp (Fig. 2a). Both transcripts were co-ordinately regulated in response to Mn^{2+} and a functional Mur regulator. However, it was not established if TS1 and TS2 emanated from two separate promoters or if one was derived from the other, particularly since substantial degradation products were also noted (Díaz-Mireles *et al.*, 2004). To distinguish these possibilities, two new *sit-lacZ* fusion plasmids were made. One of these, pBIO1480, spanned the TS1 site, and the other, pBIO1479, contained the TS2 site, plus 70 bp of 5' sequence, which did not extend significantly beyond the TS1 site. A third plasmid, pBIO1552, contained both the TS1 and the TS2 regions (see Fig. 2a). Each of these plasmids was mobilized into wild-type *R. leguminosarum* (strain 251) and into the Mur^- mutant strain J325. The transconjugants were grown in Mn^{2+} -replete and Mn^{2+} -depleted medium before assaying β -galactosidase activities. As expected, the strain with pBIO1552 (intact *sitABCD* regulatory region in the fusion), was expressed at high levels in Mn^{2+} -depleted medium but was repressed by the addition of Mn^{2+} in the wild-type background, this repression being lost in the Mur^- mutant (Table 1). Similarly, in the strains with pBIO1480, the *sitA-lacZ* fusion was expressed at high levels in the Mn -deficient medium, in both the J251 and the J325 backgrounds and, in the former, the transcription of the fusion was subject to Mn^{2+} -dependent repression. Indeed, for reasons that are not clear, the β -galactosidase activities in strains with pBIO1480 were higher than those with the

Fig. 1. Identification of the *mur* gene transcription initiation site. (a) Lane 1, RNA isolated from wild-type *R. leguminosarum* J251 containing pBIO939 was analysed by primer extension as described in Methods. The DNA sequence at the transcription initiation site is shown on the left. The major, gene-proximal transcription initiation site is 74 bp upstream of the translation initiation codon of the *mur* gene. (b) The locations of the transcription initiation sites in the *mur* gene regulatory sequence are shown as angled arrows. The adenosine residue of the major transcription start site is identified as position +1. A further weak transcription initiation site is located at a cytosine 15 bp upstream of the major site and is depicted by a dotted, angled arrow. The AUG (ATG) translation initiation codon of the *mur* gene is underlined and italicized and the horizontal arrow signifies the direction of transcription of the *mur* gene.

Fig. 2. Transcription of the *sitABCD* operon occurs from the TS1 promoter. (a) Representation of the intergenic region upstream of the *sitABCD* operon, extending into the 5' end of the *sitA* gene. The *adm* gene is located 307 bp upstream of the ATG translation initiation codon of the *sitA* gene (Díaz-Mireles *et al.*, 2004). The DNA fragments used to construct *sit-lacZ* fusion plasmids are shown. The black bar represents the 5' portion of the *sit* gene. The 5' ends of the previously mapped (Díaz-Mireles *et al.*, 2004) *sitABCD* transcripts are indicated by angled arrows, labelled TS1 and TS2. The numbering refers to the dimensions in bp of the respective DNA fragments, position 1 corresponding to the 3' end of the *adm* gene. The DNA fragment amplified with oligonucleotides *sit*-PE and *sit*-ds and used in the DNase I protection experiments is shown at the bottom of the panel. (b) Primer extension analysis of various *sit-lacZ* derivatives. RNA was harvested from wild-type *R. leguminosarum* (J251) after growth on medium containing the chelator DP (D) or with 50 μ M $MnCl_2$ (M). Primer extensions used primer *sit*-PE, which was also used for DNA sequencing of the corresponding cloned region. Strains contained pBIO1552 (wild-type regulatory region; WT), pBIO1526 (mutated in the -10 region of TS2; TS2*), or pBIO1528 (mutated in the -10 region of TS1; TS1*). The locations of the 5' ends of the TS1 and TS2 transcripts are indicated.

larger insert, in pBIO1552. Thus, the region spanning the TS2 start is dispensable for normal, Mn^{2+} -responsive gene regulation of *sitABCD* (Table 1). In contrast, the fusion plasmid pBIO1479 yielded no detectable enzyme activity either in the wild-type or the *mur* mutant (Table 1), further

demonstrating the importance of the DNA in the TS1 region for the expression and regulation of *sitABCD*.

It was still possible that TS2 represents a genuine transcript but that DNA further upstream of the 5' end of that cloned

Table 1. Effect of promoter mutations on *sit-lacZ* expression

Strains containing the different *sit-lacZ* fusion plasmids were grown in Mn^{2+} -replete or metal-depleted (DP) medium. β -Galactosidase enzyme activities are presented as Miller units with standard errors in parentheses. ND, Values no greater than the background of *R. leguminosarum* with no plasmids, of ~50 units. Values reported are the mean of three independent experiments.

Strain	Plasmid	DP	$MnCl_2$
J251 (wild-type)	pBIO1552 (native promoter)	7439 (80)	185 (9)
	pBIO1480 (spans TS1)	9812 (158)	106 (18)
	pBIO1479 (spans TS2)	ND	ND
	pBIO1526 (mutated in -10 of TS2*)	2836 (53)	181 (6)
	pBIO1528 (mutated in -10 of TS1*)	ND	ND
J325 (<i>Mur</i> ⁻)	pBIO1552	7775 (133)	5065 (15)
	pBIO1480 (spans TS1)	11890 (37)	4597 (75)
	pBIO1479 (spans TS2)	ND	ND
	pBIO1526	3204 (100)	1857 (85)
	pBIO1528	ND	ND

in pBIO1479 is needed for its synthesis. We used site-directed mutagenesis of each of the -10 regions that corresponded to the TS1 and TS2 transcripts (Table 1) to examine this possibility.

When the region 10 bps 5' of the TS2 putative transcript start was mutated (in plasmid pBIO1526, see Table 1) expression of the *sit-lacZ* fusion was reduced by little more than twofold, with both Mur- and Mn^{2+} -dependent regulation being maintained. The overall twofold reduction in expression of the mutated plasmid relative to the wild-type construct pBIO1552 is possibly attributable to increased instability of the transcript due to the mutation. In contrast, when the -10 sequence of TS1 was mutated (pBIO1528), no expression was detected. Thus, expression of *sitABCD* depends upon the presence of a functional TS1 promoter. However, sequences downstream of the TS1 promoter, which were mutated in pBIO1526, may also augment *sitABCD* expression, perhaps by affecting mRNA stability.

Primer extensions verified that inactivation of the TS1 promoter in pBIO1528 abolished transcription in the fusion derivative, whereas mutating the -10 region of TS2 had only minor effects on the transcription patterns (Fig. 2b).

Taken together, these observations indicate that the smaller TS2 transcript results from processing of the longer TS1 transcript or from reverse transcriptase stalling at the GC-rich sequence in the neighbourhood of TS2 in the primer extension experiments.

The Mn^{2+} -Mur complex interacts specifically with two DNA sites in the *sitABCD* regulatory sequence

In a previous study (Díaz-Mireles *et al.*, 2004), Mur was shown to bind in gel-shift assays to the 1–222 bp and to the 200–504 bp DNA fragments depicted in Fig. 2(a). The DNA sequences around TS1 and TS2 have two putative heptameric inverted repeats, termed MRS1 and MRS2, which were proposed to be recognized by Mur (Díaz-Mireles *et al.*, 2004). We set out to locate the Mur-binding sites precisely by DNase I footprinting. The target was a 318 bp PCR-amplified DNA fragment that extended from position -108 relative to the TS1 transcription initiation and included the first 99 bp of the *sitA* structural gene (see Fig. 2). Two distinct sites of interaction were seen both on the anti-coding (Fig. 3a) and the coding strand (Fig. 3b), and these sites overlap the previously identified MRS1 and MRS2 motifs. Binding was saturated with 5 μ M Mur protein, since increasing Mur to 10 μ M did not affect the protection pattern observed (data not shown). Both the MRS1 and MRS2 sites appeared to have similar affinity for Mur, suggesting that the binding to both sites is unlikely to be cooperative.

It is notable that Mur interacted with the DNA if Mn^{2+} ions were present; but no specific interaction with the coding strand was noted with up to 10 μ M Mur when Mn^{2+} was

omitted from the binding reaction (Fig. 3c). However, a single hypersensitive site between the MRS1 and MRS2 sites was noted in these experiments, suggesting interaction of metal-free Mur with this region of the DNA, but not with either MRS1 or MRS2.

The extent of DNA sequence protected from DNase I digestion by Mn^{2+} -Mur was 34 bp for MRS1 and 31 bp for MRS2, suggesting that the 14 kDa Mur protein might bind as a tetramer, which is generally observed for other Fur family members (Pohl *et al.*, 2003; Friedman & O'Brian, 2003, 2004). However, this must be verified biochemically. MRS1 and MRS2 are separated by 16 bp and the almost central location of the transcription initiation site means that when the *sitABCD* promoter is bound by Mn^{2+} -Mur, binding of RNA polymerase would be prevented (Fig. 4a).

The MRS1 and MRS2 motifs are very similar to each other, with 21 bp being identical (Fig. 4b). This conserved sequence encompasses a perfect heptameric inverted repeat sequence in the case of MRS1 (TGCAATT-N₇-AATTGCA) and a similar, but imperfect repeat (TGCAAAT-N₇-AATCGCA) for MRS2, as identified previously (Díaz-Mireles *et al.* (2004). This motif also occurs in the likely regulatory region of the *sitABCD* operon of *S. meliloti* (Chao *et al.*, 2004). Interestingly, though, *S. meliloti* has only one version of the MRS motif, its location being equivalent to that of MRS1 in *R. leguminosarum* (Díaz-Mireles *et al.*, 2004). Notably, this motif is not found 5' of the *R. leguminosarum mur* gene, consistent with the lack of *mur* autoregulation.

The Fe^{2+} -dependent Fur protein from *B. japonicum* (Fur_{Bj}) binds, *in vitro*, to a 19 bp recognition sequence in the promoter region of the *irr* gene of this species (Friedman & O'Brian, 2003). This sequence not only differs from the MRS motifs reported here but also is very different from 'typical' Fur recognition sequences (Baichoo & Helmann, 2002; Escolar *et al.*, 1999).

The DNA recognition sequence of conventional Fur, for example from *E. coli*, *P. aeruginosa* and *Bacillus subtilis*, is 19 bp, to which two Fur dimers are thought to bind (Baichoo & Helmann, 2002; Fuangthong & Helmann, 2003). The two Fur dimers are proposed to recognize heptameric inverted repeats with a 7-1-7 organization, two of which, offset by 6 bp, occur in the conventional Fur box (see Fig. 4b). The Zur and PerR regulators are also members of the Fur superfamily and are proposed to bind similar 7-1-7 sequences (Fuangthong & Helmann, 2003). The DNA recognition sequences for Fur, PerR and Zur differ from each other in two key base pairs, allowing selective and exclusive binding by the cognate regulator, suggesting that the DNA-recognition helices of at least some members of the Fur superfamily must be highly discriminatory (Fuangthong & Helmann, 2003).

The Mur-binding sequence has some similarity to the Fur_{Bj}-binding site. This is confined to two direct repeat sequences of 6 bp – TTGCAA/C for the *R. leguminosarum mur*

Fig. 3. Interaction of Mur with the *sit* operon regulatory region. In these DNase I protection experiments, different concentrations of purified Mur were incubated in the presence of 100 μM MnCl_2 with the anti-coding (a) and coding (b) strands of the *sitABCD* regulatory region and in the absence of MnCl_2 with the coding (c) strand. The regions of Mn^{2+} -Mur complex interaction with the DNA are delineated by vertical bars and labelled MRS1 and MRS2 (Díaz-Mireles *et al.*, 2004; and see Fig. 4). The regions protected by Mur were determined from analysis of several independent experiments, a typical one of which is shown. The DNA size markers, generated by digesting ϕX174 DNA with *Hinf*I, are shown in the left lane of each panel. The sizes of the individual markers, in base pairs, are shown at the left of the panels. The final concentrations of Mur in the reactions shown for (a) and (b) were: lane 1, 0 μM Mur; lane 2, 0.1 μM ; lane 3, 0.5 μM ; lane 4, 1.0 μM ; lane 5, 5 μM . In (c), Mur was added at 0 μM (lane 1), 1 μM (lane 2), 5 μM (lane 3) and 10 μM (lane 4). For reference, in (c) the striped bars indicate the corresponding locations of MRS1 and MRS2.

recognition sequence and TTGCA/GT/G for Fur_{Bj} (Fig. 4b). Fur_{Bj} and Mur_{RI} can both bind to canonical *E. coli* or *P. aeruginosa* Fur boxes (Friedman & O'Brian, 2003; Wexler *et al.*, 2003) and the cloned Mur of *R. leguminosarum* can partially correct the regulatory defect of an *E. coli* Fur[−] mutant in response to Fe²⁺ but not to Mn²⁺ availability (Wexler *et al.*, 2003; E. D.-M., unpublished observations). However, their normal functions in *R. leguminosarum* and *B. japonicum* are substantially different from that of the conventional Fur Fe-responsive transcriptional regulator. In *R. leguminosarum*, Mur responds to Mn²⁺ and not to Fe²⁺ in its ability to repress the *sitABCD* Mn²⁺-uptake operon, the role of the global Fe-responsive regulator having been taken over by the very different RirA protein (Todd *et al.*, 2002, 2005). Furthermore, as shown here, the only known recognition sequence of Mur in *R. leguminosarum* itself has essentially no similarity to a canonical Fur box. The sequences of Mur in *R. leguminosarum*, *S. meliloti* and its homologues in other closely related genera (*Agrobacterium*,

Bradyrhizobium, *Brucella*, *Bartonella* and *Caulobacter*) are closely related to each other and form a clade that is more closely related in sequence to *bona fide* Fur of *E. coli* than to the other members of the Fur superfamily, Irr, PerR and Zur. It appears that the progenitor of the *fur* genes of *R. leguminosarum* and *S. meliloti* evolved to respond to a different metal (manganese) and to recognize a different DNA sequence, and is no longer needed for iron homeostasis. In the case of *B. japonicum*, its Fur still responds to iron but its role may be more limited in terms of the range of genes that it regulates and, certainly, its recognition site, too, is very different from the conventional Fur box, although it has some similarity to the Mur-binding sites of *R. leguminosarum*. We cannot exclude the possibility that Mur regulates other promoters in *R. leguminosarum*, perhaps in response to other divalent cations. However, growth of *R. leguminosarum* in the presence of 10 μM Fe³⁺, Zn²⁺, Co²⁺, Cu²⁺ or Ni²⁺ did not affect the levels of expression of the *sit-lacZ* reporter (E. D.-M., unpublished).

Fig. 4. Schematic representation of the Mn^{2+} -Mur-binding sites in the *sitABCD* operon regulatory region. (a) The DNA sequences protected from DNase I digestion by Mn^{2+} -Mur are shown by bars. The numbering relates to the transcription initiation site (TS1), which is designated +1 and is in bold and depicted with an angled arrow. The translation initiation codon of the *sitA* gene is underlined. (b) Alignment of MRS1 and MRS2 Mn^{2+} -Mur-binding sites of the *R. leguminosarum sitABCD* operon with the Fur-binding site of the *sitABCD* operon from *Sinorhizobium meliloti* (Sm) (Chao *et al.*, 2004) and the Fur-binding site in the *irr* promoter from *B. japonicum* (BjFur) (Friedman & O'Brian, 2003). Conserved nucleotides between all four binding sites are underlined. A potential inverted repeat sequence (7-N7-7) is highlighted by a set of convergent arrows above MRS1. The consensus Fur box is shown at the bottom of the figure for comparison, with the 7-1-7 inverted repeats offset by 6 bp highlighted by convergent arrows above and below the sequence (Baichoo & Helmann, 2002). The 19 bp 'core' sequence is shown in bold type.

To date there have been few molecular genetic studies on the Fur-like proteins of other α -proteobacteria (Park *et al.*, 2001). It will be of interest to see the extent to which they resemble those of the rhizobia or other lineages of bacteria in their functions. It will also be intriguing to determine, from a structural point of view (Pohl *et al.*, 2003), how these rhizobial regulatory proteins discriminate such different *cis*-acting sequences compared to those of the much-studied *E. coli* Fur and, at least in the case of Mur described here, how they recognize different DNA motifs in response to the availability of different transition metals.

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