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Downregulation of the *Escherichia coli guaB* promoter by FIS

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The *Escherichia coli guaB* promoter (P_{guaB}) regulates transcription of two genes, *guaB* and *guaA*, that are required for the synthesis of guanosine 5′-monophosphate (GMP), a precursor for the synthesis of guanine nucleoside triphosphates. Transcription from P_{guaB} increases as a function of increasing cellular growth rate, and this is referred to as growth rate-dependent control (GRDC). Here we investigated the role of the factor for inversion stimulation (FIS) in the regulation of this promoter. The results showed that there are three binding sites for FIS centred near positions −11, +8 and +29 relative to the *guaB* transcription start site. Binding of FIS to these sites results in repression of P_{guaB} *in vitro* but not *in vivo*. Deletion of the *fis* gene results in increased P_{guaB} activity *in vivo*, but GRDC of P_{guaB} is maintained.
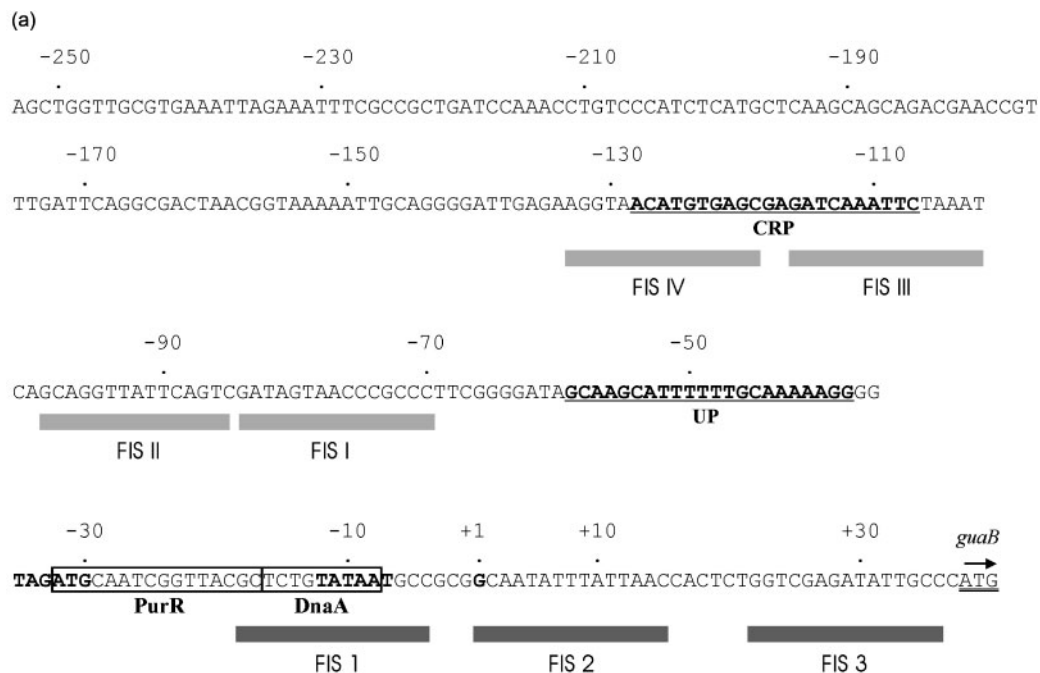
INTRODUCTION

The *Escherichia coli guaB* promoter (P_{guaB}) regulates transcription of two genes, *guaB* and *guaA*, which together constitute the *guaBA* operon. The *guaB* and *guaA* genes encode inosine 5′-monophosphate (IMP) dehydrogenase and guanosine 5′-monophosphate (GMP) synthetase, respectively, and are required for the biosynthesis of GMP from the common purine nucleotide precursor, IMP (Mehra & Drabble, 1981; Tiedeman & Smith, 1984). P_{guaB} is regulated by the cAMP receptor protein (CRP) and a putative CRP binding site is centred near position −117.5 relative to the *guaB* transcription start site (Hutchings & Drabble, 2000). Furthermore, transcription from P_{guaB} is strongly enhanced by an UP element (Husnain & Thomas, 2008). P_{guaB} also contains a putative binding site for PurR that overlaps the core promoter region, and PurR down-regulates expression of *guaB* (Meng *et al.*, 1990; Davies & Drabble, 1996). DnaA binds to a sequence overlapping the core promoter region and also downregulates transcription from P_{guaB} (Tesfa-Selase & Drabble, 1996). It has also been shown that the rate of transcription from P_{guaB} per unit cell mass increases as a function of increasing cellular growth rate (Davies & Drabble, 1996; Husnain & Thomas, 2008). This phenomenon is commonly referred to as growth rate-dependent control (GRDC) (Gourse *et al.*, 1996; Dennis *et al.*, 2004). GRDC of P_{guaB} requires the UP element and sequences located upstream of the UP element (Husnain & Thomas, 2008).

The factor for inversion stimulation (FIS) regulates transcription by binding to highly degenerate 15 bp DNA sequences (Finkel & Johnson, 1992; Ross *et al.*, 1999). At some *E. coli* promoters, FIS activates transcription by contacting the C-terminal domain of the RNA polymerase (RNAP) α subunit (αCTD) (Aiyar *et al.*, 2002; McLeod *et al.*, 2002). FIS can also promote transcription by decreasing the negative superhelicity of DNA (Travers *et al.*, 2001). At other promoters, FIS downregulates promoter activity by binding to a site that overlaps or is located downstream from the RNAP binding site, or by forming a complex assembly with other nucleoid proteins (Gonzalez-Gil *et al.*, 1998; Browning *et al.*, 2000, 2004; Jackson *et al.*, 2004). A previous study identified four putative binding sites for FIS that are located upstream of the P_{guaB} core promoter region (Hutchings & Drabble, 2000). The putative FIS binding sites are centred near positions −77, −92, −109 and −126 relative to the *guaB* transcription start, and were referred to as FIS sites I–IV, respectively (Fig. 1a). FIS contributes to GRDC of the *thrU* and *pdxA* promoters, and is required for growth rate-dependent synthesis of 4.5S RNA, $tRNA_2^{Ser}$ and $tRNA_2^{Thr}$ (Emilsson & Nilsson, 1995; Dong *et al.*, 1996). Moreover, transcription from the *fis* promoter is coupled to cellular growth rate (Mallik *et al.*, 2006). Cellular levels of FIS and FIS mRNA also change with the growth phase, and they increase dramatically upon entry into the mid-exponential growth phase (Appleman *et al.*, 1998; Ali Azam *et al.*, 1999; Mallik *et al.*, 2006).

In this work, we investigated whether FIS plays a role in the regulation of P_{guaB}. We show that the putative FIS binding sites located upstream of the P_{guaB} core elements do not recruit FIS (Hutchings & Drabble, 2000). Moreover, we demonstrate that FIS is recruited to three sites centred near −11, +8 and +29 relative to the *guaB* transcription start site, and all three sites are necessary for full FIS-mediated

(a)

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    -250              -230              -210              -190
     .                 .                 .                 .
AGCTGGTTGCGTGAAATTAGAAATTTCGCCGCTGATCCAAACCTGTCCCATCTCATGCTCAAGCAGCAGACGAACCGT

    -170              -150              -130              -110
     .                 .                 .                 .
TTGATTCAGGCGACTAACGGTAAAAATTGCAGGGGATTGAGAAGGTAACATGTGAGCGAGATCAAATTCTAAAT
                                                       CRP
                                       FIS IV              FIS III

     -90               -70               -50
      .                 .                 .
CAGCAGGTTATTCAGTCGATAGTAACCCGCCCTTCGGGGATAGCAAGCATTTTTTGCAAAAAGGGG
                                                      UP
        FIS II              FIS I

    -30               -10        +1        +10               +30        guaB
     .                 .                  .                   .          →
TAGATGCAATCGGTTACGCTCTGTATAATGCCGCGGCAATATTTATTAACCACTCTGGTCGAGATATTGCCCATG
    PurR           DnaA
              FIS 1              FIS 2              FIS 3
```

(b)

	Location	Category	Protection by FIS	FIS site designation
GcaagcaTttTttgC	-59 to -45	1	−	
GgtcgagAtaTtgcC	+22 to +36		+	Site 3
GatccaaAccTgtcC	-220 to -206	2	−	
GcaatatTtaTtaaC	+1 to +15		+	Site 2
aattAgaAatTtcgC	-239 to -225	3	−	
tcccAtcTcaTgctC	-208 to -194		−	
aggtAacAtgTgagC	-133 to -119		−	Site IV
GatcAaaTtcTaaat	-116 to -102		−	Site III
caatAttTatTaacC	+2 to +16		+	Site 2'
GcaggttAttcagtC	-99 to -85	Other sites	−	Site II
GatagtaAcccgccC	-84 to -70		−	Site I
GctctgtAtaatgcC	-18 to -4		+	Site 1
GnncAaaTttTgnnC				Consensus
...tgttAaaca...				

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 |        |        |
-7        0       +7
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repression. We also show that FIS is not required for GRDC of P$_{guaB}$.

METHODS

Strains and plasmids. All strains were derivatives of the *E. coli* K-12 strain VH1000. Each strain contained a chromosomally integrated transcriptional fusion of *lacZ* to one of three P$_{guaB}$ derivatives: the full-length *guaB* promoter (i.e. strain VH1000G-253), extending from positions −253 to +36 relative to the *guaB* transcription start site [P$_{guaB}$ (−253 to +36)] (Husnain & Thomas, 2008), the P$_{guaB}$ (−253 to +10) promoter (i.e. strain VH1000G-25310, this work), which contains the same upstream end point as the full-length promoter but has a downstream end point at +10, and the P$_{guaB}$ (−69 to +36) promoter (i.e. strain VH1000G-69, this work), which has the same downstream end point as the full-length promoter but has an upstream end point at position −69 relative to the *guaB* transcription start site. Fusions were carried on λ prophage and were constructed using a system based on λimm21 (Simons *et al.*, 1987; Rao *et al.*, 1994). Strain VH1000G-253Δ*fis* was made by introducing the

Fig. 1. Identification of putative FIS sites at P*guaB*. (a) Sequence of the *guaB* promoter from positions −253 to +40 relative to the *guaB* transcription start site. Putative FIS sites I–IV, FIS sites 1–3, a putative CRP binding site centred at position −117.5 and binding sites for PurR and DnaA are indicated (Hutchings & Drabble, 2000). The core promoter elements (−35 and −10 regions), UP element, and the initiating nucleotide are also shown, in bold. Apart from FIS sites I–IV, putative FIS sites that have been shown not to bind FIS in this work are not indicated. For clarity, FIS site 2′ is not shown. (b) Candidate FIS binding sites were identified by comparing the consensus sequence for FIS (Ross *et al.*, 1999; Shultzaberger *et al.*, 2007) with P*guaB* sequences located from positions −253 to +36. The consensus sequence employed is indicated accordingly; alternative bases at each position of the consensus sequence are shown in the second line. Conserved bases at the five positions considered to be critical for FIS binding (positions −7, −3, 0, +3 and +7) are emboldened, and the most frequently occurring base is shown in upper case. 'n' signifies any base. Candidate FIS sites are categorized according to their similarity to the consensus. Category 1 and 2 sites exhibit a 4/5 match at the critical positions, including positions +7 and −7, which are most strongly conserved among FIS sites. However, at category 2 sites, an infrequently occurring base is present at position −3, 0 or +3 (in the case of P*guaB*, such mismatches only occur at position −3). Category 3 sites also exhibit a 4/5 match at the critical positions but the mismatched base occurs at position +7 or −7. Other pertinent sites that do not fulfil the criteria for inclusion in categories 1–3 are shown as 'other sites'. FIS sites that were protected by FIS in DNase I footprinting are as indicated.

fis::*aadA* allele from strain JCB38841 (Ball *et al.*, 1992; Wu *et al.*, 1998) into VH1000G-253 by P1 transduction; strain VH1000G-69Δ*fis* was made by introducing the same allele into VH1000G-69. All plasmids contain P*guaB* derivatives that were inserted as EcoRI–HindIII fragments. Plasmids pBSG-253 and pBSG-133 are derivatives of pBluescript II KS containing the promoters P*guaB* (−253 to +36) and P*guaB* (−133 to +36), respectively. pUCG-253 is a derivative of pUC19 containing P*guaB* (−253 to +36). The plasmid pRLG770 has been described previously (Ross *et al.*, 1990). pRLG770 derivatives containing promoters P*guaB* (−253 to +36), P*guaB* (−133 to +36), P*guaB* (−59 to +36) and P*guaB* (−37 to +36) were constructed previously (Husnain & Thomas, 2008). pRLG770 derivatives pRLG-13321, pRLG-1331 and pRLG-25310 contain promoters P*guaB* (−133 to +21), P*guaB* (−133 to +1) and P*guaB* (−253 to +10), respectively.

DNase I footprinting. The EcoRI–XhoI DNA fragment in pBSG-253 was purified following electrophoresis in a 6 % acrylamide gel (Meng *et al.*, 2000), labelled at the downstream (XhoI) end with [γ-^{32}P]ATP [>7000 Ci (2.59 × 10^{14} Bq) mmol^{-1}, MP Biomedicals] and subsequently purified according to a published procedure (Husnain & Thomas, 2008). The EcoRI–XhoI fragment in pBSG-133 was labelled similarly at the upstream end. Labelled DNA fragment (4 nM) was incubated at room temperature for 30 min in a volume of 20 μl containing 20 mM HEPES (pH 8.0), 5 mM MgCl$_2$, 50 mM potassium glutamate, 1 mM DTT, 20 μg ml^{-1} sonicated calf thymus DNA (GE Healthcare) and 5 % (v/v) glycerol, in the absence or presence of purified FIS protein. Purified FIS was a generous gift from T. Gaal and R. L. Gourse (University of Wisconsin–Madison). DNase I footprinting and DNA fragment separation were performed exactly as described previously (Husnain & Thomas, 2008). Footprints were visualized using a FujiFilm FLA3000 phosphorimager.

Electromobility shift assay (EMSA). A DNA fragment containing P*guaB* (−253 to +36) was amplified by PCR from pUCG-253, using primers pUC19(for) (5′-ACGTTGTAAAACGACGGCCAG-3′) and pUC19(rev) (5′-GCGCGGATCCATGACCATGATTACGCCAAGCT-3′). A DNA fragment containing the *rrnB* P1 promoter with FIS site I (positions −87 to +50 relative to the *rrnB* P1 transcription start site) and an *rrnB* P1 promoter derivative that did not contain a FIS site (positions −37 to +52 relative to the *rrnB* P1 transcription start site, and containing non-*rrnB* P1 sequences upstream to position −92) were PCR amplified from plasmids pRLG1616 and pRLG4720, respectively, using a forward primer with the sequence 5′-GTAT-CACGAGGCCCT-3′ and reverse primer RLG1620 (5′-GCGCTACG-GCGTTTCACTTC-3′), both of which are vector-specific (Newlands *et al.*, 1991; Ross *et al.*, 1998; Meng *et al.*, 2001). PCR products were digested with HindIII, and purified following electrophoresis in a 6 % acrylamide gel (Meng *et al.*, 2000). Fragments were labelled at the

HindIII end using [α-^{32}P]dATP [3000 Ci (1.11 × 10^{14} Bq) mmol^{-1}, MP Biomedicals] and DNA polymerase I Klenow fragment. Labelled DNA (final concentration 0.4 nM) was incubated at room temperature for 30 min in a volume of 10 μl containing 20 mM HEPES (pH 8.0), 5 mM MgCl$_2$, 50 mM potassium glutamate, 1 mM DTT, 10 % (v/v) glycerol and 20 μg ml^{-1} sonicated calf thymus DNA (GE Healthcare), in the absence or presence of different concentrations of FIS. Samples were loaded (under tension) onto a 6 % acrylamide gel (37.5 : 1 acrylamide : bis acrylamide) containing 7.5 % (v/v) glycerol while running at ~15 V cm^{-1}, and gels were run for ~1 h at 4 °C. Radiolabelled DNA was visualized using a FujiFilm FLA3000 phosphorimager.

Measurement of transcription *in vitro*. Multiple-round transcription reactions were performed as described previously, using super-coiled pRLG770 derivatives containing P*guaB* fragments (Husnain & Thomas, 2008). As a control, transcription was also measured from an *rrnB* P1 promoter derivative that did not contain any FIS sites (plasmid pRLG4238; Estrem *et al.*, 1998). FIS (250 nM) was incubated with DNA in reaction buffer at room temperature for 30 min. Transcription was initiated at 30 °C with 10 nM *E. coli* RNAP holoenzyme (Epicentre), and reactions were allowed to proceed for 20 min.

Measurement of transcription *in vivo*. Strains containing a chromosomally integrated P*guaB*-*lacZ* transcriptional fusion were employed in the measurement of promoter activity *in vivo*. Cells were inoculated from dense starter cultures into media that supported different cellular growth rates, as described previously (Husnain & Thomas, 2008). The β-galactosidase activity was determined following disruption of cells by sonication (Miller, 1972). To measure promoter activity at different stages of the growth cycle, cells were grown overnight in M9 minimal medium with 0.4 % (w/v) glucose, 0.8 % (w/v) Casamino acids and 5 μg thiamine ml^{-1}, and inoculated into fresh medium to an OD$_{600}$ of ~0.01. Growth was monitored at OD$_{600}$, and the β-galactosidase activity was measured at different points on the growth curve after cells were permeabilized with chloroform-SDS (Miller, 1972).

RESULTS

Identification of putative FIS sites at P*guaB*

The 15 bp consensus sequence for FIS [5′-$\underline{G}$nn(c/t)($\underline{A}$/g)(a/t)(a/t)($\underline{T}$/A)(t/a)(t/a)($\underline{T}$/c)(g/a)nn$\underline{C}$-3′] contains five highly conserved positions (underlined) that are the most significant for the recruitment of FIS (Finkel & Johnson,

1992; Hengen *et al.*, 1997; Ross *et al.*, 1999; Shultzaberger *et al.*, 2007; Shao *et al.*, 2008). At each highly conserved position (hereafter referred to as a 'critical' position), an upper-case letter indicates the most strongly conserved base at that position, and a lower-case letter indicates a conserved base that is non-consensus. We employed the MatInspector program (Genomatix) to identify putative FIS sites at P_{guaB} that matched the consensus in at least four out of the five critical positions (Quandt *et al.*, 1995). This was achieved by performing a sequence alignment of a modified consensus sequence (5′-GnnnRnnWnnYnnnC-3′, where R=A or G; W=A or T; Y=T or C; n=any base) with P_{guaB} DNA sequences located between positions −253 and +36, and also with the reverse complement of this DNA sequence.

Using this approach, no sites were identified that contained the most highly conserved base at all five critical positions. However, we identified nine candidate FIS binding sites that contained the most highly conserved base at 4/5 critical positions (Fig. 1b). All of these sites contained the consensus A or T base at the central position (position 0) and the conserved T residue at position +3. These sequences were subdivided into three categories. Category 1 sites contain bases that match the consensus at 4/5 critical positions, including the outer bases (positions −7 and +7) that are most strongly conserved among FIS sites and which are presumed to be bound by the D helices of FIS (Shultzaberger *et al.*, 2007). The remaining critical position (position −3) contained the alternative purine base G that occurred less frequently at that position. Two candidate FIS sites were identified that fell into this category (Fig. 1b). Category 2 sites differ from category 1 sites in having a less frequently occurring C or T residue at position −3. Two additional sites fell into this category. Category 3 sites also contain bases that match the consensus at 4/5 critical positions. However, the mismatches occur at one of the highly conserved bases that are located at the outermost positions. Five sequences were classified as category 3 sites

(Fig. 1b). Two of them correspond to the previously identified putative FIS sites III and IV located upstream of P_{guaB} (Hutchings & Drabble, 2000). Putative FIS sites I and II were not identified by this analysis as they harbour bases that match the consensus at only 3/5 of the critical positions, although they do include non-consensus but frequently occurring bases at the remaining two critical positions (positions −3 and +3) (Fig. 1b).

Analysis of FIS binding to P_{guaB}

EMSA was employed to determine whether FIS can bind to a DNA fragment containing the *guaB* promoter. As a comparison, binding of FIS to the *rrnB* P1 promoter, which is known to bind FIS under physiological conditions, was also analysed. The *rrnB* P1 promoter fragment employed contained the promoter-proximal FIS site, i.e. FIS site I (Ross *et al.*, 1990; Bokal *et al.*, 1995). The minimum concentration of FIS required to observe FIS–DNA interactions at either P_{guaB} (−253 to +36) or the *rrnB* P1 fragment by EMSA was 50 nM. Increasing the FIS concentration to 300 nM resulted in the formation of three different complexes between FIS and P_{guaB} (Fig. 2). At this concentration of FIS, a larger fraction of the *rrnB* P1 promoter fragment was bound by FIS, but there remained only a single FIS–DNA complex, and no FIS–DNA complexes were observed at an *rrnB* P1 promoter derivative that lacked a FIS site (Fig. 2). At a FIS concentration of 500 nM, an additional FIS–DNA complex was observed at both P_{guaB} and *rrnB* P1 harbouring FIS site I. As a complex was also observed with the promoter fragment that did not contain a FIS site, it is likely that the additional FIS–DNA interactions observed at 500 nM FIS are non-specific (Fig. 2). These results indicate that FIS binds to at least three sites at or near P_{guaB} under similar conditions to those in which FIS specifically binds to *rrnB* P1, and thereby suggest that FIS is likely to bind to these sites under physiological conditions.
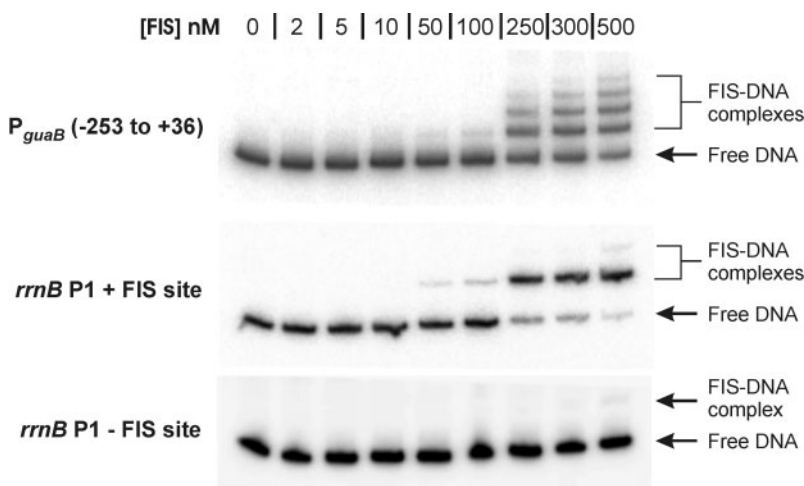
Fig. 2. Analysis of FIS binding to P_{guaB} by EMSA. EMSA was employed to compare the relative binding affinity of FIS for a DNA fragment containing P_{guaB} (−253 to +36) with an *rrnB* P1 promoter derivative containing FIS site I ('*rrnB* P1+FIS site') and *rrnB* P1 containing no FIS sites ('*rrnB* P1−FIS site'). The concentration of FIS in each binding reaction is indicated above the corresponding gel lane.
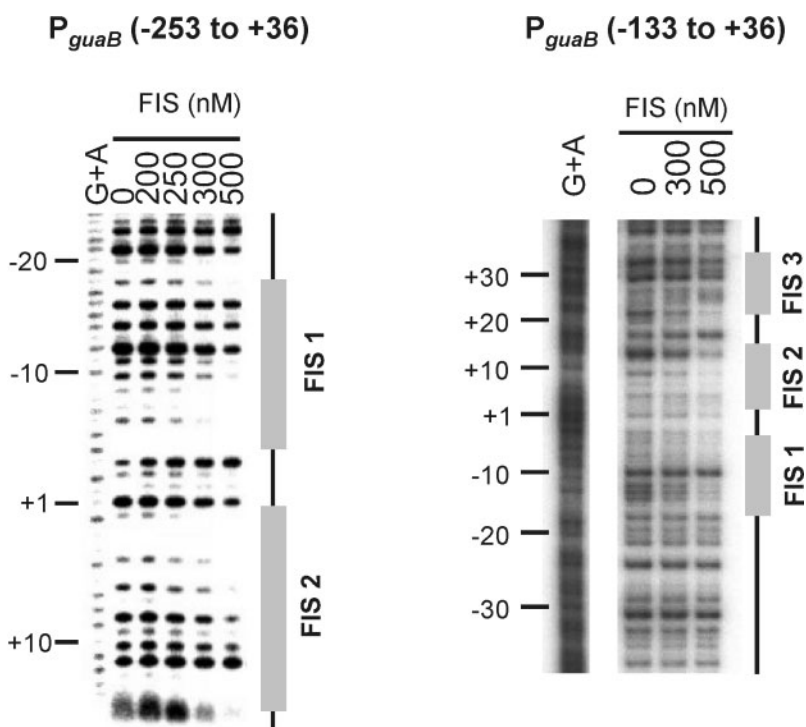
Mapping the location of FIS binding sites at P*guaB* by DNase I footprinting

To determine the location of any FIS binding sites at P*guaB*, a DNA fragment extending from positions −253 to +36 of P*guaB* [i.e. P*guaB* (−253 to +36)] was radiolabelled at the downstream end, and DNase I footprinting was performed in the presence or absence of purified FIS. The results show that increasing the concentration of FIS up to 500 nM resulted in increased protection at two sites centred near positions −11 and +8 (FIS site 1 and FIS site 2, respectively) (Fig. 3). DNA fragments corresponding to P*guaB* sequences downstream of position +16 were not visible on this gel. To determine whether FIS bound to sequences downstream of position +16, a DNA fragment extending from positions −133 to +36 [i.e. P*guaB* (−133 to +36)] was radiolabelled at the upstream end for DNase I footprinting. The results show that, in addition to the protection observed at FIS sites 1 and 2, a third site (FIS site 3) centred near position +29 was also bound by FIS. FIS sites 2 and 3 were identified by comparison with the consensus as being more likely to recruit FIS (i.e. FIS site 3 is a category 1 site, and FIS site 2 is a category 2 site) (Fig. 1b). However, protection of other category 1 and category 2 FIS sites that were identified by bioinformatic analysis was not observed. Interestingly, FIS site 1 contains mismatches to the consensus at two critical positions, and therefore was not identified by the bioinformatic analysis (Fig. 1b). FIS did not bind to any of the previously predicted FIS sites (FIS sites I–IV). It should be noted that another FIS site (site 2′) is located overlapping site 2, with its centre shifted by one base pair downstream of the centre

of site 2 (Fig. 1). As sites that contain both an A at position −4 and a T at position +4 are bound by FIS much less efficiently than are sites that lack a G at position −7 (or a C at +7) (Shao *et al.*, 2008), it is possible that site 2′ may be preferred by FIS over site 2.

Role of FIS in the regulation of transcription from P*guaB* in vitro

To determine the role of FIS in the regulation of P*guaB*, multiple-round transcription reactions were performed in the presence or absence of 250 nM FIS. Transcription was measured from P*guaB* (−253 to +36) and shorter derivatives [P*guaB* (−133 to +36), P*guaB* (−59 to +36), P*guaB* (−37 to +36), P*guaB* (−133 to +21), P*guaB* (−133 to +1) and P*guaB* (−253 to +10)] (end points as indicated). Addition of FIS to the transcription reaction resulted in ~8–10-fold repression of transcription from P*guaB* (−253 to +36). Under the same conditions, there was no repressive effect of FIS on transcription from the *rrnB* P1 promoter (Fig. 4a, b). This indicates that the repression of P*guaB* afforded by FIS at a concentration of 250 nM was due to a site-specific FIS–DNA interaction. Deletion of sequences upstream of the P*guaB* UP element [i.e. P*guaB* (−59 to +36)] did not lead to any significant change in the degree of repression afforded by FIS, confirming that putative FIS sites I–IV do not play a role in the regulation of P*guaB* by FIS. Deletion of the P*guaB* UP element [P*guaB* (−37 to +36)] gave rise to an undetectable level of transcripts from P*guaB* in the presence of FIS, which meant that the fold repression afforded by FIS could not be

Fig. 3. Mapping the location of FIS sites at P*guaB* by DNase I footprinting. A DNA fragment containing P*guaB* (−253 to +36) radiolabelled at the downstream end (relative to the *guaB* transcription start site), and a DNA fragment containing P*guaB* (−133 to +36) labelled at the upstream end, were employed in DNase I footprinting in the presence of different concentrations of FIS (as shown). Nucleotide positions are shown relative to the *guaB* transcription start site, and lanes containing the G+A ladder are indicated accordingly.
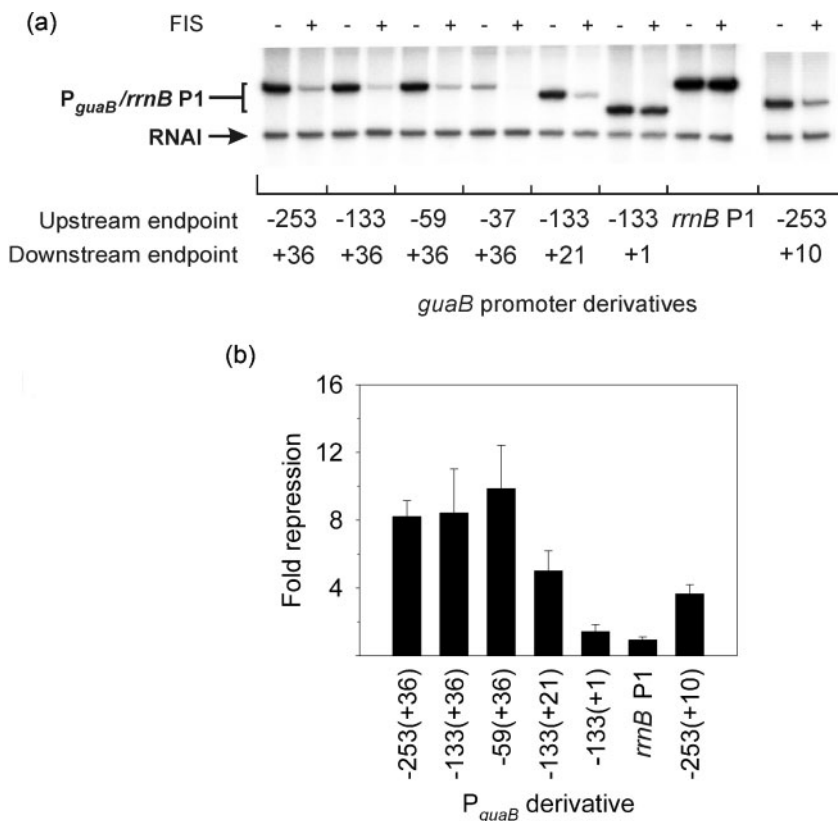
Fig. 4. Role of FIS in regulation of transcription from P_{guaB} *in vitro*. (a) Multiple-round *in vitro* transcription was employed to measure transcription from P_{guaB} derivatives P_{guaB} (-253 to +36), P_{guaB} (-133 to +36), P_{guaB} (-59 to +36), P_{guaB} (-37 to +36), P_{guaB} (-133 to +1), P_{guaB} (-133 to +21) and P_{guaB} (-253 to +10) in the presence (+) and absence (−) of 250 nM FIS. Promoter endpoints are as indicated. P_{guaB} (-133 to +21) lacks FIS site 3, and P_{guaB} (-133 to +1) lacks FIS sites 2 and 3. As a control, transcription was also measured from an *rrnB* P1 promoter derivative that did not contain any FIS sites. All promoters were cloned in pRLG770 and supercoiled DNA was used for the assays. The vector-encoded replication repressor, RNAI (~110 nucleotides), is also indicated. (b) The repression afforded by FIS at each promoter is shown. The fold repression by FIS was calculated by dividing the activity in the absence of FIS by the activity in its presence. Values are the mean (with standard deviation) of three independent experiments. The activity of P_{guaB} (-37 to +36) in the presence of FIS was too low to quantitate and hence a value for the fold repression was not obtained.

determined. Deletion of FIS site 3 [i.e. P_{guaB} (−133 to +21)] led to decreased repression by FIS (approximately sixfold repression), and deletion of both FIS site 2 and FIS site 3 [i.e. P_{guaB} (−133 to +1)] gave rise to an approximately twofold repression by FIS (Fig. 4b). As P_{guaB} (−133 to +1) retains FIS site 1 and is still subject to some degree of repression by FIS, these results indicate that FIS sites 1–3 each contribute to repression of P_{guaB}, and that FIS site 1 can function independently of the other FIS sites. A P_{guaB} derivative harbouring a deletion of FIS site 3 and deletion of the downstream six bases of FIS site 2 [i.e. P_{guaB} (−253 to +10)] was subject to an approximately fourfold repression by FIS, indicating that FIS site 2 had not been completely inactivated (Fig. 4b).

FIS is not required for growth rate-dependent control of P_{guaB}

GRDC of P_{guaB} (−253 to +36) was measured in a wild-type strain background and in a strain that harboured a deletion in the *fis* gene. To determine whether FIS site 3 is important for GRDC of P_{guaB}, activity of P_{guaB} (−253 to +10) was also analysed. In exponentially growing wild-type *E. coli* cells, the activity of P_{guaB} (−253 to +36) increased approximately twofold with every doubling of the growth rate as, shown previously (Fig. 5a, c; Davies & Drabble, 1996; Husnain & Thomas, 2008). In an otherwise isogenic *fis* strain, the activity of P_{guaB} (−253 to +36) was higher than in the wild-type at all growth rates and was

more pronounced at faster growth rates (i.e. an approximately twofold increase in activity was observed at the fastest growth rate) (Fig. 5a). However, the degree of GRDC was similar to that observed in a wild-type strain (i.e. in both cases, a doubling of the growth rate corresponded to an approximately twofold increase in promoter activity) (compare plots of relative activity versus growth rate, Fig. 5c). These results demonstrate that FIS is not required for GRDC of P_{guaB}. Although FIS appears to downregulate transcription from P_{guaB} *in vivo*, deletion of FIS site 3 and part of FIS site 2 did not lead to a significant change in P_{guaB} activity in exponentially growing wild-type cells in comparison to P_{guaB} containing the full complement of functional FIS sites [i.e. a 1.87-fold increase in P_{guaB} (−253 to +10) activity occurred for every doubling of the growth rate in comparison to a 1.84-fold increase for P_{guaB} (−253 to +36) (Fig. 5a, b)]. These results suggest either that FIS site 1 is able to effect full repression *in vivo* (contrasting with the results obtained *in vitro*) or that FIS sites 1–3 may not contribute to repression of P_{guaB} *in vivo* under the conditions employed, and therefore the observed FIS-dependent repression is indirect.

FIS is not required for growth phase-dependent regulation of P_{guaB}

Previous studies indicate that FIS levels are elevated during the mid-exponential growth phase, and they decrease sharply as cells enter stationary phase (Appleman *et al.*,

(a) P$_{guaB}$ (−253 to +36)

(b) P$_{guaB}$ (−253 to +10)
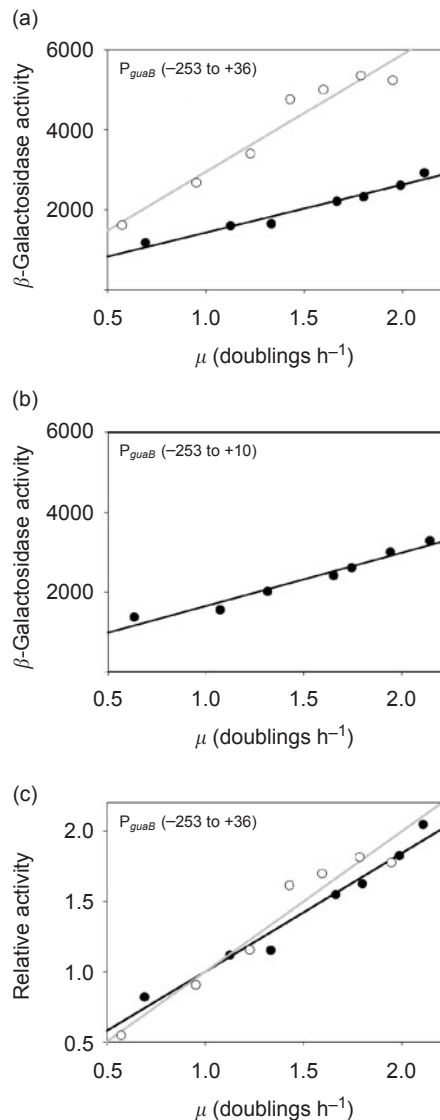
(c) P$_{guaB}$ (−253 to +36)

Fig. 5. GRDC of P$_{guaB}$. (a, c) GRDC of a wild-type strain (●) or a strain containing the fis::aadA allele (○), harbouring a fusion of P$_{guaB}$ (−253 to +36) to lacZ, was analysed. (b) GRDC of a wild-type strain harbouring a P$_{guaB}$ (−253 to +10)-lacZ fusion was also measured. Strains were grown at different cellular growth rates to an OD$_{600}$ of 0.34–0.45, whereupon the β-galactosidase activity was determined. The promoter activity for P$_{guaB}$ (−253 to +36) in the presence and absence of functional fis is given both in Miller units (β-galactosidase activity) and expressed as a ratio to the activity at 1 doubling per hour (relative activity). The magnitude of the gradient in plots of relative promoter activity versus doublings per hour is proportional to the degree of GRDC. Each data point represents the mean promoter activity or mean growth rate. The mean was calculated using data obtained from three independent experiments.

1998). This phenomenon is responsible for the known contribution of FIS to growth-phase-dependent regulation of some promoters (Nilsson et al., 1992; Appleman et al.,

1998; Mallik et al., 2006; Bradley et al., 2007). To test whether FIS-dependent regulation of P$_{guaB}$ varies with the growth phase, transcription from a P$_{guaB}$ derivative with an upstream end point of −69 that contained all three experimentally determined FIS sites [i.e. P$_{guaB}$ (−69 to +36)] was measured at different stages of growth in a wild-type strain, and in a strain that harboured a deletion in the fis gene. This promoter derivative was chosen as it lacks the putative CRP site centred near position −117.5 (Hutchings & Drabble, 2000). It has previously been shown that FIS represses the crp1 promoter and this may alter cellular levels of CRP (Gonzalez-Gil et al., 1998).

In accordance with the results of the GRDC experiment, the activity of P$_{guaB}$ was higher in the fis background than in the wild-type strain throughout the course of the growth cycle. In the wild-type strain, P$_{guaB}$ activity increased by nearly 40 % as cells entered the mid-exponential growth phase (i.e. the promoter activity at an OD$_{600}$ of ~0.15–0.20 was 40 % higher than the activity at an OD$_{600}$ of ~0.012). The increase in activity in a fis strain over the corresponding part of the growth curve was less marked (i.e. there was a ~16 % increase in promoter activity). Upon entry into stationary phase, there was a gradual decrease in the promoter activity in both strain backgrounds (Fig. 6). The results suggest that P$_{guaB}$ is subject to a degree of growth-phase-dependent regulation. However, there was no significant change in the transcription activity profile during the growth cycle when comparing the two strain
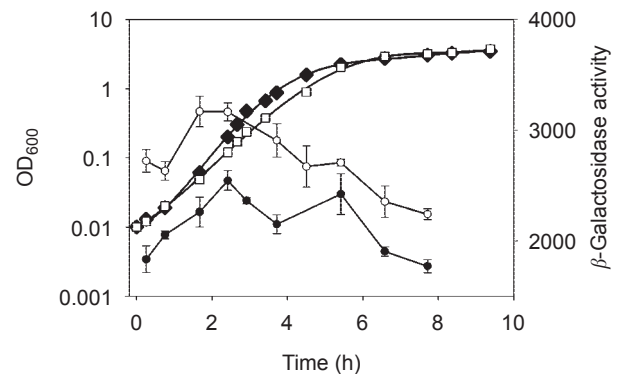
Fig. 6. Growth-phase-dependent regulation of P$_{guaB}$. A wild-type strain or a strain containing the fis::aadA allele (Δfis), each harbouring a fusion of P$_{guaB}$ (−69 to +36) to lacZ, were inoculated from a dense culture into fresh growth medium [M9 minimal medium containing 0.4 % (w/v) glucose, 0.8 % (w/v) Casamino acids and 5 μg thiamine ml^{-1}] to an OD$_{600}$ of ~0.01. Samples were taken during different stages of growth and the β-galactosidase activity was determined. The promoter activity of the wild-type strain (●) and Δfis strain (○) is given in Miller units (β-galactosidase activity). Values presented are the mean ± SD, for three independent experiments. Cell density measurements (OD$_{600}$) for single cultures that are representative of the growth curve for the wild-type strain (◆) and the Δfis strain (□), were plotted on a logarithmic axis.

backgrounds. Furthermore, P_{guaB} activity peaks at the time that FIS levels are expected to be at their highest, and then falls off upon entry into stationary phase when FIS levels fall (Appleman *et al.,* 1998; Ali Azam *et al.,* 1999). These observations suggest that FIS does not significantly influence growth phase-dependent regulation of P_{guaB} under the conditions employed.

DISCUSSION

A previous study identified four adjacent putative FIS binding sites (FIS sites I–IV) located upstream of the UP element at P_{guaB}. These sites were identified by comparison of the upstream P_{guaB} sequence to a published consensus sequence for FIS (Finkel & Johnson, 1992, Hutchings & Drabble, 2000). By employing sequence alignment to compare P_{guaB} sequences to a more representative consensus for FIS, we have identified four candidate FIS sites that bear a closer resemblance to this consensus than FIS sites I–IV (Ross *et al.,* 1999). Two of these sites, sites 2 (or 2′) and 3 (centred near positions +8 and +29 relative to the *guaB* transcription start site, respectively), are protected by FIS in DNase I footprinting experiments. One of the other two candidate FIS sites overlaps the UP element, and the remaining site is located at a distance upstream of the core promoter elements (centred at −213). Neither of these sites recruits FIS as judged by DNase I footprinting. Putative FIS sites I–IV also do not bind FIS. Interestingly, we show that a site centred at position −11, which was not identified as a likely FIS site, also recruits FIS. This suggests that although bioinformatic analyses are useful when searching for sites that are bound by FIS, they may not be useful in identifying some FIS binding sites that exhibit a weak match to the consensus.

At the *tyrT* promoter, FIS binding to sites II and III (centred at positions −91 and −122, respectively) cooperatively affects the binding of FIS to site I centred at −71 bp upstream of the transcription start site (Lazarus & Travers, 1993; Pemberton *et al.,* 2002). As the B-form of DNA has a periodicity of 10.6 bp, this places these three sites on the same face of the DNA helix, with the centres of sites I and II separated by 21 bp. This suggests that cooperative interactions require adjacent FIS dimers to be positioned on the same face of the DNA, two turns of the DNA helix apart. At the *rrnB* P1 promoter, FIS sites I–III are centred at positions −71, −102 and −143, respectively, also placing them approximately on the same face of the DNA helix. However, FIS does not bind to *rrnB* P1 cooperatively in the absence of RNAP, and it is noteworthy that the central positions of these sites are separated by 32 or 42 bp (i.e. not 21 bp). Interestingly, at P_{guaB}, FIS sites 2 and 3 are positioned 22 bp apart (centre to centre) which will also place them on the same face of the DNA helix and a similar distance apart as FIS sites I and II at the *tyrT* promoter. However, the centres of FIS sites 1 and 2 at P_{guaB} are 18 bp apart, making it unlikely that they are located on the same face of the DNA. Therefore, it is possible that FIS binds cooperatively to sites 2 and 3, but it appears less likely that occupancy of sites 2 and/or 3 stimulates binding of FIS to site 1.

Consistent with the location of functional FIS sites, we show that FIS represses transcription from P_{guaB} ~8–10-fold *in vitro*. Deletion of FIS site 3 results in partial relief of repression, and deletion of FIS sites 2 and 3 together further relieves repression *in vitro*. The residual FIS-mediated repression of the *guaB* promoter fragment containing the +1 downstream end point is likely to occur through interactions with site 1, and would suggest that binding of to site 1 does not require cooperative interactions with FIS dimers bound to adjacent sites. The binding of FIS to site 1 is likely to sterically hinder the recruitment of RNAP to P_{guaB}, as observed at the *crp1* promoter (Gonzalez-Gil *et al.,* 1998). The role of FIS sites 2 and 3, which together exert the most influence on transcription from P_{guaB} *in vitro*, is less clear, although it is likely that the role of FIS site 3 is to stimulate binding of FIS to site 2, which in turn may play more of a direct role in repression. Our results suggest that the presence of FIS should decrease RNAP binding to P_{guaB}. However, DNase I footprinting experiments carried out in the presence of both FIS and RNAP were inconclusive (data not shown).

Although our results demonstrate that FIS represses transcription from P_{guaB} *in vitro*, evidence for direct repression by FIS *in vivo* was not obtained (i.e. deletion of FIS sites 2 and 3 did not result in increased P_{guaB} activity in wild-type exponentially growing cells). This is consistent with the results of a chromatin immunoprecipitation (ChIP)-chip analysis carried out under similar conditions, in which FIS binding at P_{guaB} was not detected (see supplementary data in Grainger *et al.,* 2006). However, in a *fis* strain we observed an increase in the activity of the *guaB* promoter in derivatives containing all three FIS sites in the presence (P_{guaB} (−253 to +36)) or absence [P_{guaB} (−69 to +36)] of the putative CRP site centred at −117.5. This rules out the possibility that the effect of deleting *fis* on *guaB* promoter activity is mediated by changes in CRP abundance [FIS has been shown to modulate transcription of *crp* (Gonzalez-Gil *et al.,* 1998)]. However, it is possible that the change in transcription activity of P_{guaB} in a *fis* background occurs as a result of altered regulation of P_{guaB} by a transcription factor other than CRP, for example H-NS or HU (Claret & Rouviere-Yaniv, 1996; Falconi *et al.,* 1996) or by changes in supercoiling (Schneider *et al.,* 1997; Weinstein-Fischer *et al.,* 2000). Another possible explanation is that the potential relief of P_{guaB} repression that occurs upon deleting FIS sites 2 and 3 is masked *in vivo* through an alternative compensatory regulatory mechanism. A less likely explanation, in view of the poor match to the consensus FIS binding site, is that FIS binding to site 1 mediates full FIS-mediated repression *in vivo*.

A previous study has shown that the P_{guaB} UP element, and sequences located further upstream, are required for GRDC of P_{guaB} (Husnain & Thomas, 2008). Our results show that

FIS does not play a role in GRDC at this promoter, thereby implying that a different cellular factor is required for conferring GRDC on P*guaB* (Emilsson & Nilsson, 1995; Dennis *et al.*, 2004; Paul *et al.*, 2004). Experiments are under way to uncover the identity of this factor(s). Our results also suggest that P*guaB* is subject to growth phase-dependent control. However, although levels of FIS protein are also subject to growth phase-dependent control, it does not appear to play an important role in growth phase-dependent control at P*guaB*. Thus, the physiological role of FIS at the *guaB* promoter remains to be elucidated.

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