

# A dedicated translation factor controls the synthesis of the global regulator Fis

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**BipA is a highly conserved protein with global regulatory properties in *Escherichia coli*. We show here that it functions as a translation factor that is required specifically for the expression of the transcriptional modulator Fis. BipA binds to ribosomes at a site that coincides with that of elongation factor G and has a GTPase activity that is sensitive to high GDP:GTP ratios and stimulated by 70S ribosomes programmed with mRNA and aminoacylated tRNAs. The growth rate-dependent induction of BipA allows the efficient expression of Fis, thereby modulating a range of downstream processes, including DNA metabolism and type III secretion. We propose a model in which BipA destabilizes unusually strong interactions between the 5' untranslated region of *fis* mRNA and the ribosome. Since BipA spans phylogenetic domains, transcript-selective translational control for the 'fast-track' expression of specific mRNAs may have wider significance.**

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## Introduction

Organisms such as *Escherichia coli* precisely adjust their metabolism to match the available nutrients in the extracellular environment. Thus, in a poor nutrient environment, *E. coli* has a low growth rate but this increases very rapidly when cells are shifted to rich nutritional conditions, thereby allowing the bacterium to compete effectively with other organisms (reviewed by Bremer and Dennis, 1996). Nutritional upshift requires a major reconfiguration of cellular processes, which in *E. coli* is coordinated in large part by a homodimeric, DNA-bending protein known as Fis. Originally implicated in site-specific DNA recombination, Fis is now known to act in a wide range of processes (Finkel and Johnson, 1992; Nilsson *et al*, 1992; Xu and Johnson, 1995; Gonzalez-Gil *et al*, 1996). These include DNA supercoiling

(Schneider *et al*, 1997), expression of translation components (Nilsson *et al*, 1990; Ross *et al*, 1990; Zhang and Bremer, 1996), reinitiation of DNA replication after temperature upshift (Gille *et al*, 1991; Filutowicz *et al*, 1992), the cold shock response (Brandi *et al*, 1999), oxidative stress (Weinstein-Fischer *et al*, 2000) and the catabolism of sugars and nucleic acids (Gonzalez-Gil *et al*, 1996). Fis appears to operate by stabilizing local DNA architectures around the promoter regions of target genes, thereby stimulating or repressing transcription according to the gene in question (Schneider *et al*, 2001).

Fis expression varies dramatically during cell growth (Ball *et al*, 1992; Ninnemann *et al*, 1992; Azam *et al*, 1999). Thus, when stationary-phase cells are placed in a rich medium, Fis increases to a peak of > 50 000 molecules within 90 min (Ball *et al*, 1992; Azam *et al*, 1999). At this stage, it is the most abundant transcriptional modulator in *E. coli*, with sufficient molecules present to bind every 250 bp in the *E. coli* chromosome. Fis levels then decline precipitously, to the extent that it is undetectable in stationary-phase cells (< 100 molecules/cell). It should be noted, however, that *fis*<sup>-</sup> cells in the stationary phase still exhibit certain mutant phenotypes, suggesting that the protein plays a role even at this stage of growth (Xu and Johnson, 1995).

In keeping with its role as a key regulator of cellular processes, Fis expression is governed by multiple control mechanisms (Nasser *et al*, 2002; Mallik *et al*, 2004). Transcription of *fis* is strongly enhanced by increases in negative supercoiling and is also positively regulated by the DNA-bending protein IHF (Pratt *et al*, 1997; Schneider *et al*, 2000). In contrast, it is negatively autoregulated (Ninnemann *et al*, 1992). The *fis* gene is also regulated by the CRP transcription factor, whose activity is dependent on cAMP levels. In the absence of Fis, CRP enhances *fis* transcription, whereas the presence of Fis leads to synergistic repression (Nasser *et al*, 2001). Fis expression is also regulated by the growth rate. Promoter sequences between -36 and +6 are sufficient for this type of control and the presence of a GC-rich discriminator sequence, situated between the -10 region and the transcription start point, is required (Ninnemann *et al*, 1992). However, the precise roles of these elements remain unclear. Changes in the intracellular pools of nucleoside triphosphates, for example, due to different growth conditions, also affect *fis* transcription, although they appear mainly to influence the site of transcription initiation rather than the total amount of *fis* mRNA (Walker and Osuna, 2002). Fis has also been reported to be regulated by the stringent control system (Ninnemann *et al*, 1992; Mallik *et al*, 2004), which is activated by the nucleotide ppGpp in response to starvation (Wendrich *et al*, 2002). However, the pattern of expression and relative levels of *fis* mRNA are comparable in wild-type (WT) cells and cells lacking both ppGpp synthases, raising the possibility that some other mechanism is involved (Ball *et al*, 1992).

We report here that *fis* is also regulated at the translational level and that this process requires a new member of the

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elongation factor GTPase superfamily termed BipA or TypA. BipA was uncovered as a protein that is strongly upregulated when *Salmonella enterica* is exposed to the host defence protein BPI (Qi *et al*, 1995). However, null mutants of BipA are pleiotropic, with defects in key processes, including growth at low temperatures (Grant *et al*, 2001; Pfennig and Flower, 2001), flagella-mediated cell motility (Farris *et al*, 1998; Grant *et al*, 2003), the expression of the K5 capsule system (Rowe *et al*, 2000) and resistance to certain antimicrobial peptides (Qi *et al*, 1995; Barker *et al*, 2000). In the case of enteropathogenic *E. coli*, BipA also governs the expression of at least two gene clusters associated with virulence, known as the Locus of Enterocyte Effacement (LEE) and EspC pathogenicity islands (Grant *et al*, 2003). The wide-ranging nature of these processes highlights the global regulatory properties exhibited by BipA.

In this paper, we show that BipA interacts with the ribosome, at a site that overlaps the binding site for elongation factor G (EF-G), and that its GTPase activity is maximally stimulated by 70S ribosomes programmed with mRNA and aminoacylated tRNAs, but is inhibited at high GDP:GTP ratios. The results also show that *fis* mRNA is efficiently expressed only when BipA is present and indicate that a BipA-responsive element maps to the translation initiation region of the *fis* transcript. Finally, we show that the expression of BipA is itself regulated by the growth rate control systems. Taken together, these results indicate that BipA plays a critical function in coordinating cellular responses to nutritional upshift. Its role as a dedicated translation factor provides evidence for a fast-track mechanism to ensure the translation of a key mRNA transcript.

## Results

### ***BipA* interacts with 70S ribosomes—binding studies and effects of translational inhibitors**

BipA shares sequence identity with other members of the elongation factor superfamily, which is strongest in the amino-terminal third of the protein, corresponding to a guanine nucleotide-binding pocket, and decreases markedly towards the C-terminus. Accordingly, it was important to determine the functional significance of this similarity and in particular to determine if BipA interacts with ribosomes. Gel filtration was therefore used to examine the ability of BipA to associate with the 70S ribosome. In the absence of 70S ribosomes, BipA eluted from the column in fractions 12–17 (Figure 1A). In contrast, when ribosomes were present, BipA eluted in fractions 5–12, with the ribosomes eluting mainly in fractions 5–7. The extended elution profile of BipA in the presence of ribosomes may be due to transient interactions. Control experiments showed that a non-ribosome-binding protein (phosphofructokinase) did not coelute under the same conditions (data not shown).

All four of the G proteins involved in the major steps of bacterial protein synthesis share essentially the same binding site on the ribosome (Cameron *et al*, 2002). Given the sequence similarity between BipA and EF-G, it was of interest to determine if BipA also uses this site during its interaction with ribosomes. Addition of EF-G significantly reduced the coelution of BipA, with the bulk of the 70S ribosomes in fractions 5–7. Instead, EF-G eluted with the ribosomes, similarly displaying a tailing effect indicative of transient

binding (Figure 1B). The simplest interpretation of these results is that BipA and EF-G bind to the same site—or to a closely overlapping one—on the 70S ribosome and that EF-G is able to displace BipA from the ribosome.

To further define BipA interactions with the ribosome, we studied the effect of ribosome addition on the GTPase activity of the protein. BipA had a very low basal level of GTPase activity, but this was markedly stimulated when 70S ribosomes were present (Figure 1C). The  $K_M$  for the 70S-stimulated GTPase activity of BipA (42.6  $\mu\text{M}$ ) was approximately five-fold higher than that reported for EF-G in the presence of 70S ribosomes (Savelsbergh *et al*, 2000). GTP hydrolysis by BipA in the presence of ribosomes was also markedly slower than that of EF-G (Figure 1D).

We next compared the effect of GDP, and additionally translation inhibitors, on the GTPase activity of BipA. High GDP:GTP ratios had a considerable inhibitory effect on the 70S ribosome-stimulated GTPase activity of BipA (Figure 2A). Indeed, when the concentration of GDP equalled the concentration of GTP, the activity of BipA was reduced by approximately one-third. In contrast, the GTPase activity of EF-G was unaffected by the presence of GDP under the same conditions. The  $K_i$  for the binding of GDP (46.5  $\mu\text{M}$ ) was approximately twice the  $K_D$  value of BipA for GTP.

The translational inhibitor fusidic acid blocks the release of GDP from EF-G, thereby stalling recycling (Spahn and Prescott, 1996). While fusidic acid reduced the 70S ribosome-stimulated GTPase activity of EF-G almost to background levels, it had no discernible effect on the activity of BipA (Figure 2B). Thiostrepton inhibits the uncoupled GTPase activity of EF-G by binding strongly to the L11 region, thereby blocking binding of EF-G. The L11-rRNA region of the ribosome is thought to be part of the GTPase-associated region, which stimulates the GTPase activity of the translation GTPases (Wimberly *et al*, 1999). Similarly,  $\alpha$ -sarcin cleaves a phosphodiester bond in 23S rRNA in the sarcin/ricin loop region (A2654–A2665), which is also part of the GTPase-associated region, thereby blocking EF-G interactions (Correll *et al*, 1999). Figure 2C and D demonstrates that both thiostrepton and  $\alpha$ -sarcin markedly inhibited the 70S-stimulated GTPase activity of BipA. Taken together, these results provide further evidence for BipA interaction with ribosomes similar to that of EF-G, but also indicate that BipA differs in its response to high GDP:GTP ratios and to fusidic acid.

### **Components required for maximal GTPase activity of BipA**

Comparison of the GTPase activity of BipA in the presence of 70S ribosomes or purified 50S or 30S subunits, as well as with preparations of total ribosomal proteins or RNAs, showed that an intact ribosome was necessary for stimulation of GTP hydrolysis (Figure 3A). To determine if the fully programmed ribosome, that is, with occupied tRNA-binding sites and containing mRNA, influenced the GTPase activity of BipA, various ribosomal complexes were assembled and assayed for their effects on the GTPase activity of BipA (Figure 3B). Addition of polyU mRNA to 70S ribosomes did not affect BipA-mediated GTP hydrolysis relative to the stimulatory effect of 70S ribosomes alone (cf. complexes I and II). In contrast, GTP hydrolysis almost doubled when mRNA and tRNAs were present (complexes III and IV), although neither of these components stimulated the GTPase activity by

themselves. Collectively, these data show that maximal GTPase activity requires intact 70S ribosomes in their fully functional configuration.

**BipA modulates the expression of Fis and its downstream targets**

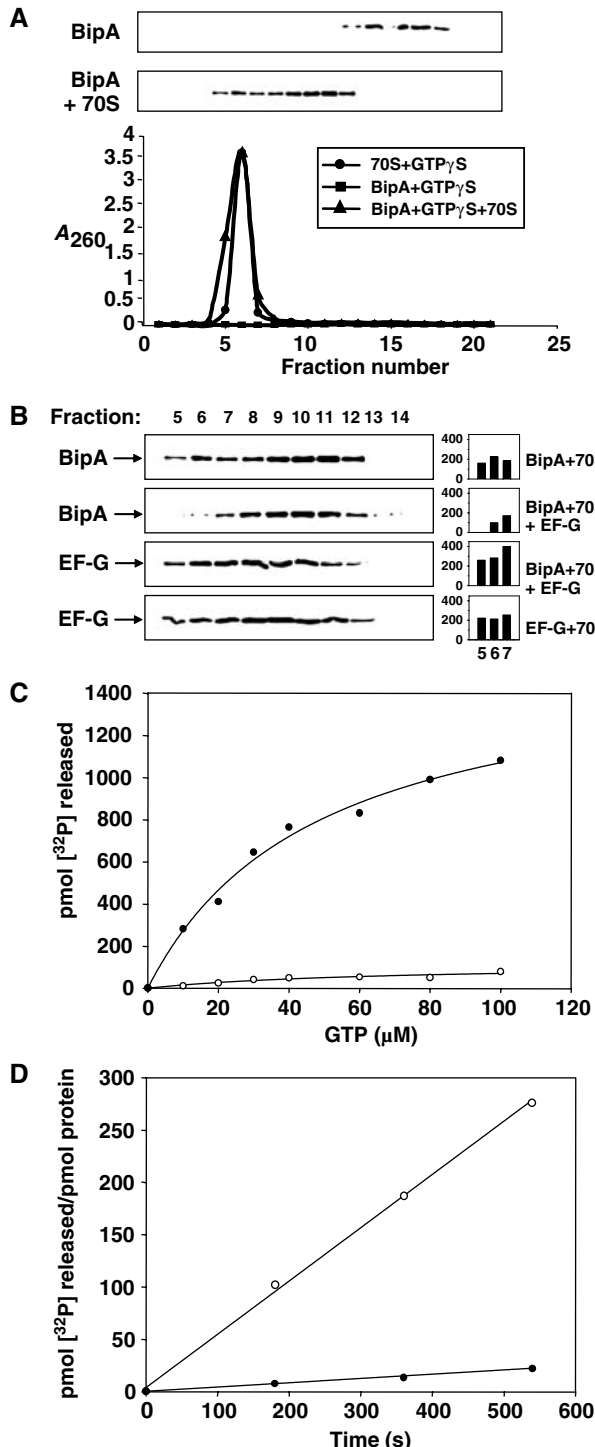
The above results, together with the pleiotropic nature of BipA mutants, suggested a model in which BipA regulates the expression of one or more global regulatory proteins. Previous work on the BipA-mediated control of the LEE cluster of virulence-associated genes in enteropathogenic

*E. coli* eliminated several candidate regulatory proteins as targets of BipA, including H-NS and IHF (Grant *et al*, 2003). However, the studies did not rule out potential BipA control of the Fis protein, which also regulates genes in the LEE cluster (Goldberg *et al*, 2001). To test this possibility, we compared Fis levels in WT and *bipA*<sup>-</sup> cells. While Fis expression could be readily detected in the former, it was barely discernible in the null mutant. However, introduction of a *bipA*<sup>+</sup> plasmid into the mutant restored Fis expression (Figure 4A).

We next examined the effect of constitutive production of BipA on the expression of Fis, using a strain in which *bipA* transcription is tightly regulated by L-arabinose (Grant *et al*, 2001). On BipA induction, *fis* expression remained high as cells progressed through the exponential phase of growth; the protein was still readily detectable 3 h after nutrient upshift in the cells expressing BipA, but was undetectable in cells in which BipA expression was repressed (Figure 4B). Both BipA and Fis are required for efficient expression of a range of virulence-associated proteins in enteropathogenic *E. coli*, including EspA and EspD (Goldberg *et al*, 2001; Grant *et al*, 2003). Ectopic expression of Fis, via the plasmid pTrcfis, restored the ability of a *bipA* mutant of enteropathogenic *E. coli* to synthesize EspA and EspD (Figure 4C). Together, these results indicate that BipA is required for Fis synthesis and hence modulates the expression of its downstream targets. They also indicate that the constitutive expression of BipA delays the growth phase-dependent decrease in Fis levels.

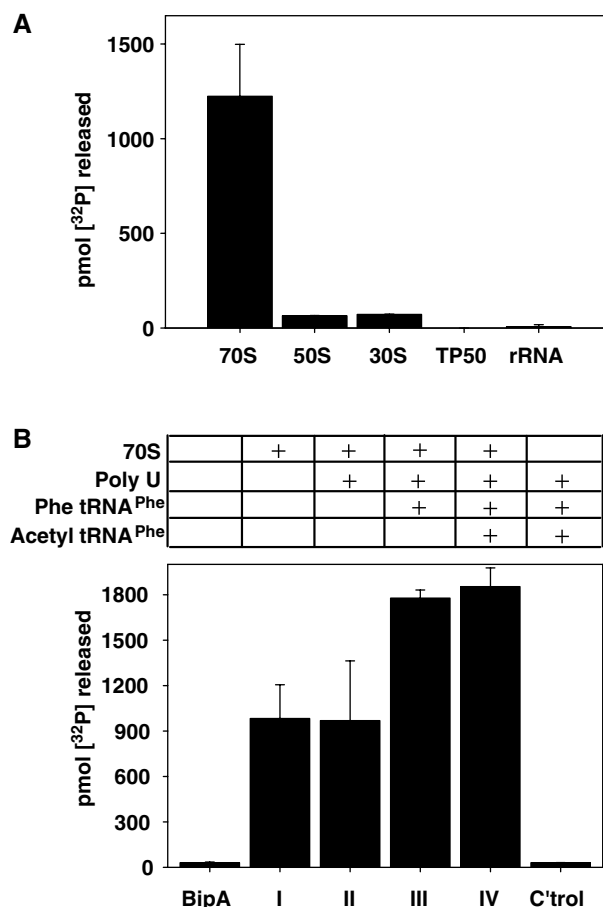
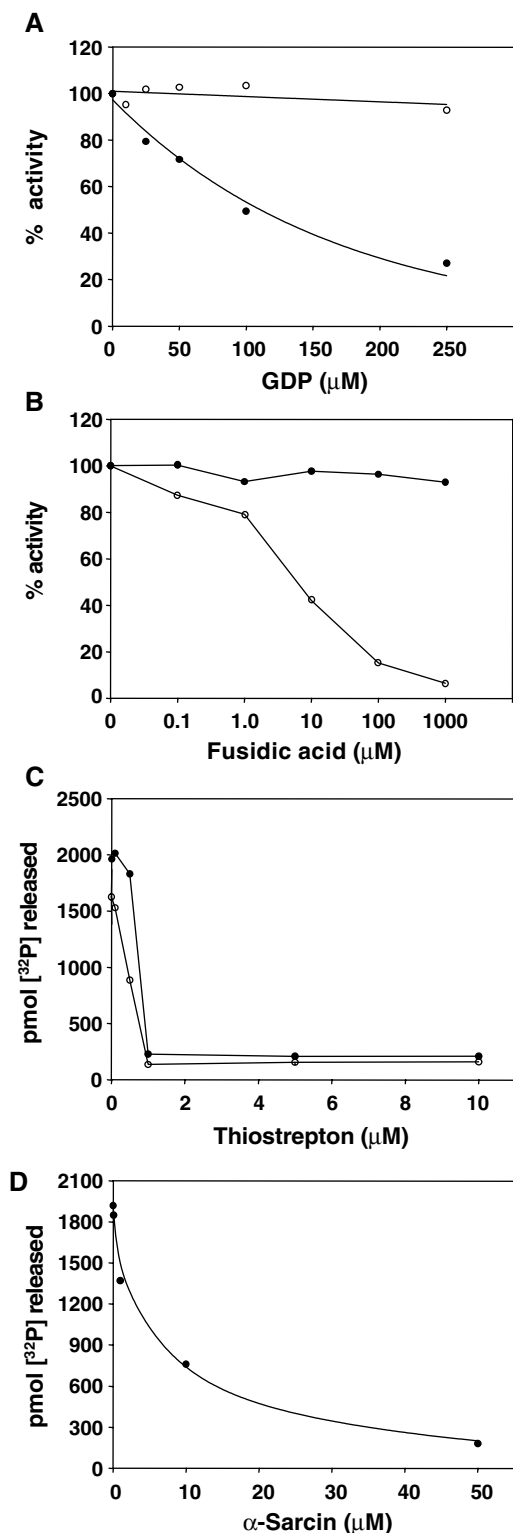
**Control of Fis expression by BipA occurs at the level of translation and requires the 5' UTR of fis mRNA**

The interaction of BipA with ribosomes and its sequence similarity to EF-G strongly suggest that it regulates the translation of Fis, rather than its transcription. To test this idea, we first compared the levels of *fis* transcription in the presence or absence of BipA, using a construct in which transcription of *lacZ* is controlled by the *fis* promoter region.



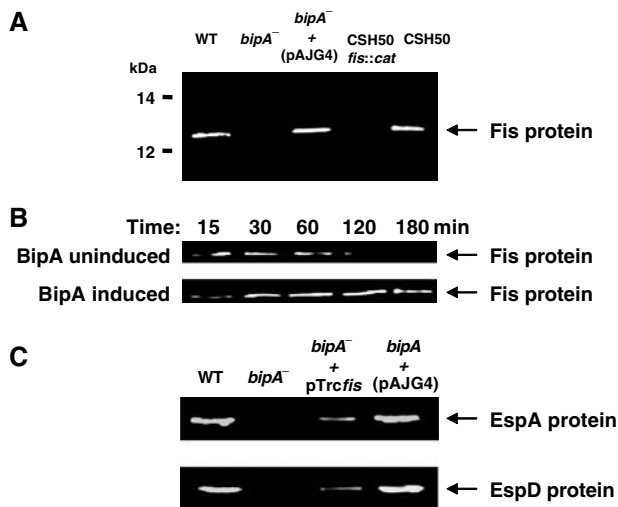
**Figure 1** Interaction of BipA with 70S ribosomes as shown by gel filtration (A, B) or stimulation of GTPase activity (C, D). Components, either singly or in combination, were passed down a Sephacryl S300 column and eluted fractions containing BipA and/or EF-G were detected following SDS-PAGE and immunoblotting. 70S ribosome-containing fractions were identified by absorbance at 260 nm. Panel A shows the elution profiles of BipA in the presence or absence of ribosomes, whereas panel B shows the elution profiles of BipA and EF-G, either singly or in combination, in the presence of 70S ribosomes. The amounts of BipA and EF-G in the ribosome-containing fractions (fractions 5–7), obtained by densitometry, are indicated in the histograms (intensities in arbitrary units). Panel C shows the effect of addition of 70S ribosomes on the GTPase activity of BipA. To obtain the 70S-stimulated GTPase activity, the basal level of GTP hydrolysis by BipA was subtracted from the total level of GTP hydrolysis in the presence of 70S ribosomes. ○, BipA basal activity; ●, BipA 70S-stimulated GTPase activity. Controls with 70S ribosomes and [<sup>32</sup>P]GTP alone, and additionally in the presence of a G77V mutant of BipA (Grant *et al*, 2003), showed minimal levels of GTP hydrolysis. Panel D shows the rates of GTP hydrolysis of BipA and EF-G. GTPase assays of BipA or EF-G ± 70S ribosomes were carried out as described in Materials and methods. ●, BipA; ○, EF-G. For the gel filtration assays, BipA, EF-G and 70S ribosomes were all present at 1.5 μM and GTPγS was included at 50 μM. For the GTPase assays, EF-G and 70S ribosomes were used at 0.2 μM while [BipA] was 10-fold higher.

WT and *bipA*<sup>-</sup> cells harbouring the construct had very similar levels of  $\beta$ -galactosidase activity during outgrowth from the stationary phase (measured by  $A_{600}$  values of cultures), suggesting that there were no major differences in transcription (Figure 5A). Direct measurement of the levels of *fis* transcripts in early exponential phase cells also indicated that BipA depletion had no significant effect on transcription (Figure 5B).



**Figure 3** Components required for maximal stimulation of the GTPase activity of BipA. In all cases, the components were pre-incubated for 15 min at 37°C prior to addition of BipA in the GTPase assays. (A) The intact 70S ribosome is required for stimulation of BipA GTPase activity. BipA (1  $\mu$ M) was preincubated with 70S ribosomes, 50S subunit, 30S subunit, TP50 (total protein content of the ribosome) or rRNA (total ribosomal RNA) (all at 0.2  $\mu$ M) prior to the addition of [ $\gamma$ -<sup>32</sup>P]GTP (50  $\mu$ M) and assay for GTP hydrolysis. (B) A programmed intact 70S ribosome is required for maximal stimulation of the GTPase activity of BipA. 70S ribosomes (0.2  $\mu$ M) were assembled into the following complexes before addition of BipA (1  $\mu$ M) and [ $\gamma$ -<sup>32</sup>P]GTP (50  $\mu$ M): complex I, 70S ribosomes; complex II, 70S ribosomes and polyU mRNA (1 mg/ml); complex III, 70S ribosomes, polyU mRNA (1 mg/ml) and Phe tRNA<sup>Phe</sup> (0.4  $\mu$ M); complex IV, 70S ribosomes, polyU mRNA (1 mg/ml) and acetyl tRNA<sup>Phe</sup> (0.2  $\mu$ M); control, polyU mRNA (1 mg/ml), Phe tRNA<sup>Phe</sup> (0.4  $\mu$ M) and acetyl tRNA<sup>Phe</sup> (0.2  $\mu$ M).

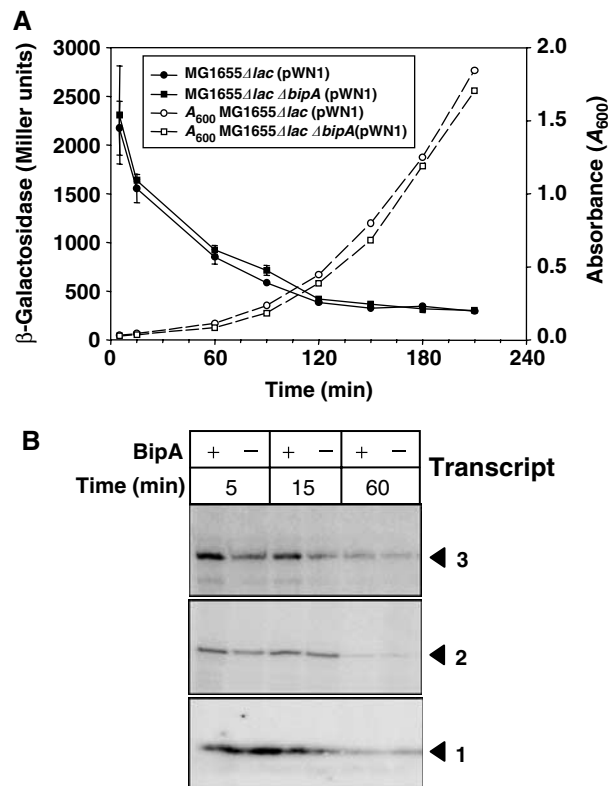
**Figure 2** Effect of inhibitors on the GTPase activity of BipA. (A) Effect of GDP:GTP ratio on the 70S-stimulated GTPase activities of BipA and EF-G. BipA or EF-G was incubated with 70S ribosomes and GDP (1–250  $\mu$ M). ●, BipA; ○, EF-G. The 100% values for BipA and EF-G were 1803.6 and 2376.3 pmol [<sup>32</sup>P] released, respectively. (B) Effect of fusidic acid on the 70S-stimulated GTPase activities of BipA and EF-G. BipA or EF-G was incubated  $\pm$  70S ribosomes and fusidic acid (1–1000  $\mu$ M). ●, BipA; ○, EF-G. The 100% values for BipA and EF-G were 1760.4 and 1833.9 pmol [<sup>32</sup>P] released, respectively. (C) Effect of thiostrepton on the 70S-stimulated GTPase activity of BipA. BipA or EF-G was incubated  $\pm$  70S ribosomes and thiostrepton (0–10  $\mu$ M). ●, BipA; ○, EF-G. (D) Effect of  $\alpha$ -sarcin on the 70S-stimulated GTPase activity of BipA. BipA was incubated  $\pm$  70S ribosomes and  $\alpha$ -sarcin (0–50  $\mu$ M). For the experiments described in (B–D), EF-G, TetO and 70S ribosomes were used at 0.2  $\mu$ M while [BipA] was 10-fold higher.



**Figure 4** Effect of BipA on the expression of Fis and its target genes. (A) Immunoblot of whole-cell extracts of WT, *bipA*, *fis* and trans-complemented mutants, probed with Fis-specific antibodies. In all cases, cells were harvested in the early exponential phase. (B) Effect of constitutive BipA expression on the level of Fis. MG1655Δ*bipA* (pAJG38) cells at  $A_{600} = 1$  were inoculated into LB medium supplemented with 0.1% L-arabinose or 0.1% D-glucose and samples were taken at the indicated times for immunoblotting with Fis-specific antibodies. (C) Ectopic expression of Fis in a *bipA* mutant of enteropathogenic *E. coli* restores the expression of the EspA and EspD virulence-associated proteins. A *bipA* null mutant of enteropathogenic *E. coli* (Grant *et al*, 2003), together with derivatives bearing *fis*<sup>+</sup> and *bipA*<sup>+</sup> plasmids and the parent strain, was harvested at the mid-exponential phase. Whole-cell extracts of the strains were then immunoblotted and probed for EspA and EspD expression.

The observation that *fis* mRNA transcripts are abundant in *bipA*<sup>-</sup> cells, even though an insignificant amount of Fis protein is present (cf. Figures 4, 5A and B), is consistent with a post-transcriptional regulatory mechanism. We therefore examined the *fis* mRNA sequence for unusual features that might be indicative of a BipA-response element. Strikingly, the Shine-Dalgarno (S-D) segment of the 5' untranslated region (UTR) displays an exceptionally high degree of complementarity with the 3' end of 16S rRNA. Ribosome-binding sites of *E. coli* mRNAs typically consist of 3–6 nucleotides that are complementary to the 3' end of 16S rRNA (Shultzaberger *et al*, 2001). In contrast, 12 of the 18 nucleotides immediately preceding the *fis* start codon were complementary to 16S rRNA (Figure 6A). The positioning of the anti-S-D segment of 16S rRNA relative to the start codon also differed between *fis* and other mRNA transcripts (cf. *fis* and *lacZ* S-D regions in Figure 6B).

To study the role of this extended translation initiation region in the BipA-mediated regulation of *fis*, the WT sequence (nucleotides –1 to –25) and mutant derivatives were fused to the structural gene for *lacZ* (Figure 6B). The levels of β-galactosidase expressed *in vivo* in the presence or absence of BipA were then compared, following induction of *lacZ* transcription. BipA significantly enhanced the expression of hybrid mRNA carrying the WT *fis* translation initiation region as well as that of the mutant derivatives specified by plasmids pJGP2, pJGP3 and pJGP5 (Figure 6C). In contrast, it had no significant effect when the native *lacZ* translation initiation



**Figure 5** Post-transcriptional regulation of Fis expression by BipA. (A) Measurement of *fis* transcription, via the *lac* fusion construct pWN1 (Nasser *et al*, 2002), during outgrowth from the stationary phase in the presence or absence of BipA. Filled and open symbols denote β-galactosidase and  $A_{600}$  measurements of cultures, respectively. The circles and square symbols correspond to MG1655 Δ*lac* (pWN1) and MG1655 Δ*lac* Δ*bipA* (pWN1), respectively. (B) Primer extension analysis of total RNA from MG1655 and MG1655Δ*bipA* using *fis*-specific primer 13' (Nasser *et al*, 2002). RNA was extracted from cells 5, 15 and 60 min after nutritional upshift. As previously reported by Nasser *et al* (2002), three transcripts were detected; however, recent studies suggest that they may originate from a single promoter (Mallik *et al*, 2004).

region (pJGP6) was present, indicating that the 5' UTR of *fis* mRNA contains a BipA-response element.

#### BipA expression is growth rate dependent

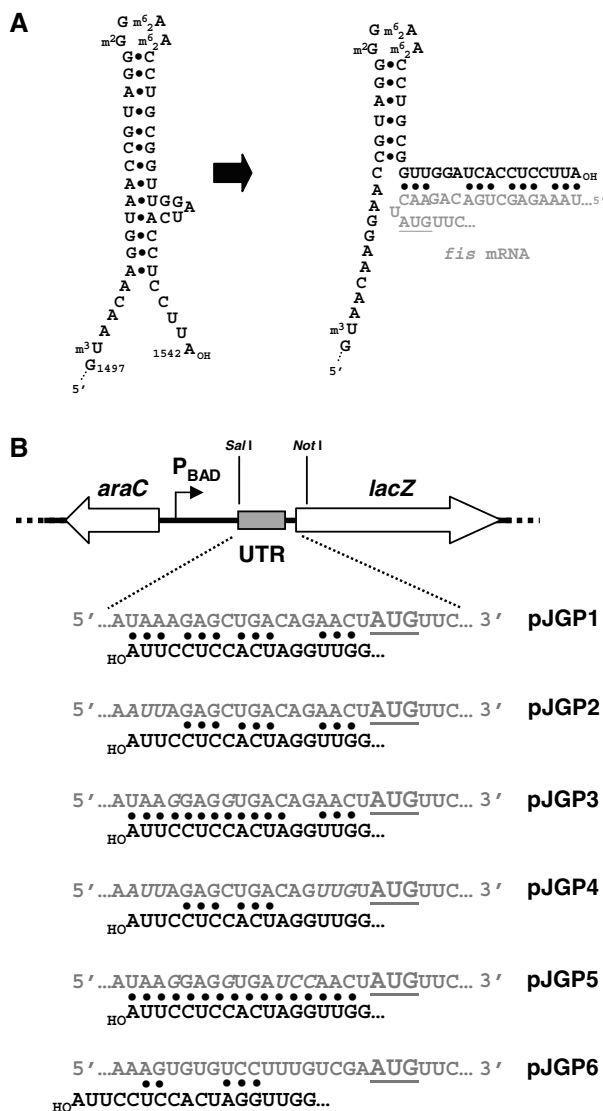
The shared sequence similarity of BipA and EF-G, its interaction with 70S ribosomes, binding competition with EF-G and inhibition of its GTPase activity by thiostrepton and α-sarcin collectively suggest that it shares the same (or overlapping) binding site on the ribosome as the four major G proteins involved in translation (Cameron *et al*, 2002). This raises questions about how BipA operates without disrupting normal ribosome function. One possibility is that BipA levels remain low relative to the number of ribosomes in a cell. Alternatively, BipA might be highly expressed but only at a particular stage in growth so as to minimize its potential interference with normal translation. To address these questions, we measured the level of BipA protein at different stages in growth. Changes in the abundance of BipA following nutrient upshift of MG1655 cells were monitored using the AQUA MS method. BipA expression was maximal in cells in the early exponential phase of growth (>18 000 mo-

lecules/cell) but decreased over 25-fold (<700 molecules/cell) as cells progressed into the stationary phase (Figure 7A).

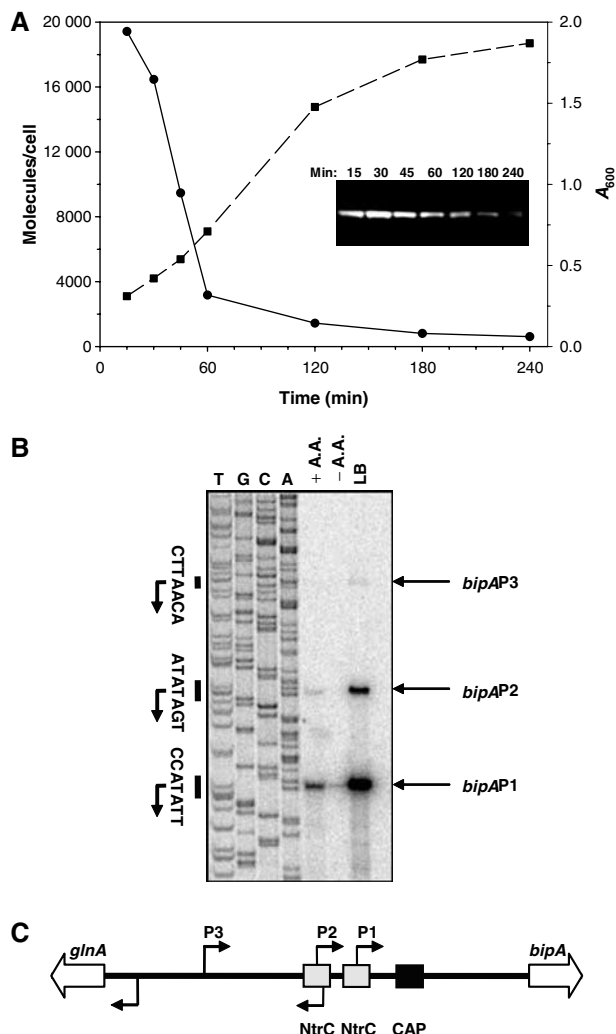
To determine if the growth rate-dependent expression of *BipA* occurred at the transcriptional or translational level, we mapped the promoter region of *bipA* and examined the transcript abundance under different growth conditions using primer extension assays. As shown in Figure 7B, three transcripts were detected, with 5' ends at positions -160, -189 and -236 relative to the first nucleotide of the *bipA*

start codon. *bipA* transcription was highest in rich media (LB) and lowest in a minimal medium (M9 glucose medium) lacking amino acids. In each case, however, the -160 transcript was the most abundant. Subcloning of the relevant regions upstream of each putative transcription start site into a promoter probe plasmid indicated that three bona fide promoters, designated P1, P2 and P3, were present (Figures 7C and 8). Interestingly, a previously mapped binding site for CRP is located between the promoters and the beginning of the structural gene for *bipA*, suggesting that the gene is negatively controlled by this global regulator. Additionally, the P1 and P2 promoters overlap binding sites for the NtrC transcription factor, suggesting that *bipA* expression may be under the control of the  $\sigma^{54}$  system, in addition to growth rate control.

The reporter gene fusions were used to study the growth rate regulation of *bipA* in further detail. A *lac* fusion to the entire *bipA* promoter region showed that transcription was proportional to the richness of the growth medium (Figure 8A). Additionally, the net level of *bipA* transcription was maximal approximately 60 min after nutrient upshift, that is, just after cells entered the exponential phase of growth. Thereafter, transcription declined progressively. Analysis of *lac* fusions to the P1, P2 and P3 promoters of *bipA* indicated that all are growth rate-regulated. However, the maximal level of transcription for each occurred earlier and depended on the growth medium used (Figure 8B-D). Thus, transcription of P1 (most proximal to the *bipA* start codon) was maximal at 30 min after inoculation and increased progressively when cells were cultivated in 0.1  $\times$  LB, 0.5  $\times$  LB and 1  $\times$  LB, respectively. However, a further increase in the richness of the medium (2  $\times$  YT) resulted in an overall decrease in transcription. Promoter P2 showed the same trend, although transcription peaked earlier and the decrease in transcription in 2  $\times$  YT medium was less marked. In contrast, the P3 promoter only displayed growth rate-dependent activity with 2  $\times$  YT medium and with a peak approximately 30 min after nutrient upshift. Collectively, these results indicate that *BipA* expression is growth rate-dependent and, like *Fis* expression, peaks in



**Figure 6** The 5' UTR of *fis* is required for translational control by *BipA*. (A) Extended complementarity between the translation initiation region of *fis* mRNA and the 3' end of 16S rRNA. The predicted stem-loop structures at the 3' end of 16S rRNA before and after interaction with the 5' UTR of *fis* mRNA are shown. The *fis* start codon is underlined. (B) Construction of plasmids carrying the *lacZ* ORF fused to translation initiation region of WT *fis* (pJGP1), mutant derivatives (pJGP2, pJGP3, pJGP4 and pJGP5) or *lacZ* (pJGP6). Only the relevant region of the plasmids is shown, together with the sequences of the inserted translation initiation regions and the 3' end of 16S rRNA. In each case, transcription of *lacZ* is driven by the L-arabinose-inducible promoter  $P_{BAD}$ . The extent of complementarity with the 3' end of 16S rRNA is indicated (●) and the *lacZ* start codon is underlined. (C) Effect of *BipA* on the translation of *lacZ* mRNAs carrying the translation initiation regions of *fis* and its mutant derivatives, or of *lacZ*. MG1655 $\Delta$ *lac* and MG1655 $\Delta$ *lacZ* $\Delta$ *bipA* cells carrying the pJGP series of plasmids were grown in LB + ampicillin medium to  $A_{600} = 1$ , prior to dilution into fresh LB medium containing 0.1% arabinose to induce transcription.  $\beta$ -Galactosidase activity was measured 60 min after induction. Comparison of the levels of the corresponding *lacZ* mRNA transcripts by RT-PCR under the same conditions showed that they varied less than 1.5-fold. The black and grey shading denote the presence or absence of *BipA*, respectively.



**Figure 7** Effect of growth rate on the expression of BipA. (A) AQUA analysis of protein extracts from MG1655 to measure the levels of BipA at different stages after nutrient upshift; see Materials and methods for details. ●, BipA molecules/cell; ■, cell growth ( $A_{600}$  values). The inset shows a BipA-specific immunoblot of equivalent samples taken at the same time points. Equal amounts of proteins were immunoblotted. (B) Determination of *bipA* transcription start sites and effect of growth medium on *bipA* transcription. Cells were grown to mid-log phase in M9 glucose minimal medium in the presence (+ A.A.) or absence (-A.A.) of added amino acids, or in LB medium. Primer extension analyses were carried out on equal amounts of total RNA using the Bip358 primer, which was also used in sequencing reactions with a *bipA*<sup>+</sup> plasmid template to generate size markers. (C) Distribution of promoters in the intergenic region separating *glnA* from *bipA*. The positions of the transcriptional start points and the *glnA* and *bipA* ORFs are indicated by thin and open arrows, respectively. The relative positions of NtrC- and CRP-binding sites are also shown.

early exponential phase. In addition, the individual promoters are also sensitive to the growth rate but are tuned to respond to growth media of different degrees of richness.

## Discussion

Subcellular complexes frequently have multiple recognition modules to allow interactions with different types of target. For example, prokaryotic RNA polymerases possess interchangeable sigma factors that recognize different classes of

promoter. In contrast, ribosomes of a given cellular compartment have generally been assumed to be identical and to be competent to translate all available mRNAs. The results presented here indicate that, contrary to this view, the translation of the transcriptional modulator Fis only takes place when a specific ribosome-binding G protein, BipA, is present. In the absence of BipA, *fis* mRNA is abundant but yields negligible amounts of the protein (Figures 4 and 5). This results in many mutant phenotypes, for example, impaired DNA supercoiling, hypermotility and defective type III secretion. When BipA is present, however, Fis accumulates to high levels (> 50 000 copies/cell at the end of the lag phase), thereby altering the expression of many genes. This in turn allows *E. coli* to reconfigure its metabolism to benefit from nutrient-rich conditions as well as to express a number of virulence-associated genes.

### The mechanism of action of BipA and its relationship with normal translation process

How does BipA bring about the expression of Fis? Its interaction with 70S ribosomes, shared sequence identity with EF-G, binding competition of EF-G with BipA and inhibition of its GTPase activity by thiostrepton and  $\alpha$ -sarcin strongly suggest that it operates at the same site on 70S ribosomes as EF-G. BipA also appears to function during translation initiation, as the region in *fis* mRNA that confers responsiveness to BipA maps to an extended S-D sequence with substantial complementarity to the anti-S-D domain of 16S rRNA. The extensive complementarity (Figure 6A) suggests that 70S ribosomes may be locked on to the translation initiation region of *fis* transcripts in the absence of BipA and hence are unable to proceed further in the translation process. Since EF-G induces a ratchet-like movement of the subunits during translocation (Frank and Agrawal, 2000), BipA might also induce conformational changes, although it cannot induce translocation of the tRNA<sub>2</sub>mRNA complex on the ribosome. The role of BipA may therefore be to destabilize the interaction between the 3' end of 16S rRNA and the 5' UTR of *fis* mRNA, thereby allowing translation to continue.

### Biological rationale for BipA-mediated regulation of Fis

The results reported here suggest at least three possible explanations for BipA-mediated control of Fis expression. First, BipA-associated ribosomes may allow the continued synthesis of Fis under conditions where the general rate of translation is very high. The induction of Fis expression occurs just before cells enter the early exponential phase of growth and signals a major increase in gene transcription. The large number of mRNAs available for translation may therefore temporarily compete with *fis* mRNA for ribosomes, potentially decreasing expression of the latter at a critical time. The presence of a subpopulation of ribosomes that interact with BipA to translate *fis* mRNA may thus provide a 'fast-track' route to ensure the continued expression of this crucial transcription modulator.

A second explanation is that preassembly of the 30S subunit (or a 70S ribosome) onto the 5' end of *fis* mRNA may accelerate Fis expression in response to altered environmental conditions. Models for prokaryotic translation initiation and start site selection suggest that, while the association of 30S subunits with pseudo-S-D sequences may be transient in the presence of initiation factors and initiator tRNAs,

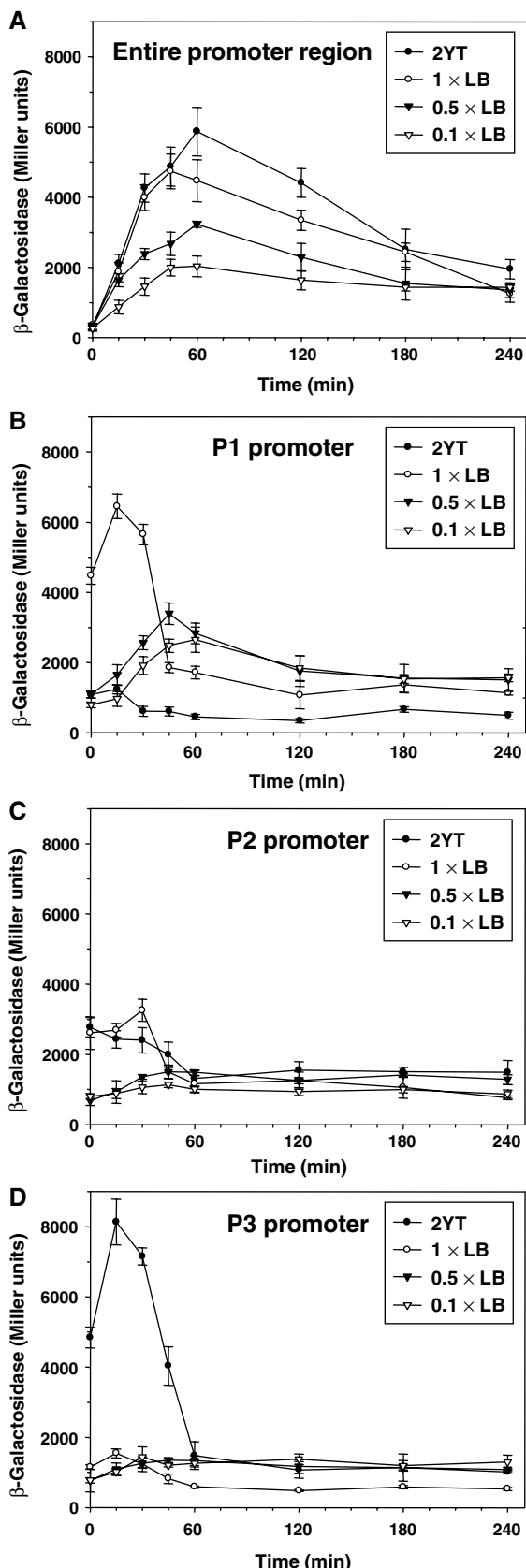
complexes of 30S subunits with bona fide S-D sequences are stable (Gualerzi and Pon, 1990; Mawn *et al*, 2002). The presence of 30S subunits preassembled on untranslated

*fis* mRNAs may therefore serve as a further 'fast-track' contingency measure to allow a rapid response to nutrient upshift.

The third possibility is that BipA acts as a sensor of intracellular [GTP]:[GDP] status and only allows the translation of *fis* mRNA when the ratio is high. Translation is a significant source of GTP consumption and it is thus important that the intracellular GTP pool is large enough to cope with the increased protein synthesis that occurs following Fis expression. The inappropriate expression of Fis is also deleterious to cells. For example, *Salmonella* has decreased viability when Fis is expressed in stationary-phase cells (Osuna *et al*, 1995). BipA may thus serve as a regulatory checkpoint to ensure that Fis is only expressed under appropriate environmental conditions. In support of this idea, we found that the GTPase activity of BipA is inhibited by high GDP:GTP ratios ( $K_i = 46.5 \mu\text{M}$ ), whereas EF-G showed negligible effects under similar conditions (Figure 3C). Clearly, the above explanations are nonexclusive; any or all may account for the BipA-mediated regulation of Fis expression.

The results described here also raise the question as to how BipA functions without disrupting the translation of other proteins. In particular, if BipA and the four major G proteins involved in protein synthesis share essentially the same binding site, how are their activities coordinated? One possibility is that BipA only binds with high affinity to ribosomes specifically associated with *fis* mRNA transcripts and at an early stage in the translation cycle. If BipA operates after the assembly of the 70S initiation complex, it is also possible that the position of fMet-tRNA, or at a later stage peptidyl-tRNA, in the ribosome and the presence of the nascent peptide control its binding and GTPase activity (Zavialov and Ehrenberg, 2003). It is also noteworthy that the GTPase activity of EF-G is some 10-fold higher than that of BipA. The expression of BipA appears to be carefully regulated so as to coincide with the transcription of the *fis* gene. The transcription of *bipA* is driven by multiple tandem promoters that are under growth rate control. Although each promoter responds to a different level of nutrient, the net result is an expression profile that closely resembles that of the *fis* gene (Figures 7A and 8). Thus, BipA may also avoid disrupting general protein synthesis by only being expressed under conditions that are likely to favour *fis* expression.

In addition to regulating Fis, BipA may also govern the expression of certain other mRNAs in *E. coli*. For example, the ectopic expression of *fis*, using a heterologous promoter and S-D sequence, does not restore the ability of *E. coli bipA* mutants to grow at low temperatures, even though many of the other mutant phenotypes associated with BipA can be corrected in this way (see e.g. Figure 4C). Similarly, the phylogenetic distribution of BipA is wide (see <http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac=IPR006298>), whereas Fis appears to be restricted to enteric bacteria.



**Figure 8** Effect of growth medium on transcription from the entire *glnA-bipA* intergenic region (A) or from the individual *bipA* promoters P1, P2 and P3 (B–D). Individual BipA promoters, and additionally the entire *glnA-bipA* intergenic region, were inserted upstream of the promoterless *lacZ* gene in pRS415 and introduced into *E. coli* MC1000. The expression of *lacZ* from the promoters was then monitored as a function of  $\beta$ -galactosidase activity, following nutritional upshift.

Intriguingly, several eukaryotes have putative GTP-binding proteins with a high degree of sequence identity with BipA. For example, the protein At2g31060 from the plant *Arabidopsis thaliana* has 43% sequence identity and 61% sequence similarity over 618 residues. Such observations raise the possibility that the use of dedicated ribosome-binding G proteins to regulate specific mRNA transcripts may be more widespread.

## Materials and methods

### Bacterial strains and growth conditions

*E. coli* K-12 MG1655 (ATCC number 47076) and its  $\Delta$ bipA derivative (formerly known as  $\Delta$ yihK) have been described (Guyer *et al*, 1981; Arigoni *et al*, 1998), as have CSH50, CSH50fis::kan, E2348/69 and E2348/69  $\Delta$ bipA::cat (Koch *et al*, 1988; Grant *et al*, 2003).  $\Delta$ lac derivatives of MG1655 and MG1655  $\Delta$ bipA were constructed as described previously (Grant *et al*, 2003). Bacteria were grown aerobically at 37°C in Luria-Bertani (LB) medium, diluted 2- or 10-fold where necessary, or in 2 × YT or M9 minimal glucose medium. Where necessary, antibiotics were used at the following final concentrations: ampicillin, 100 µg/ml; kanamycin, 25 µg/ml. For growth measurements, an overnight culture was diluted 1:6 into fresh, prewarmed medium and grown to an absorbance at 600 nm ( $A_{600}$ ) of 1.0. The cells were then diluted 6- or 100-fold, as appropriate, into fresh, prewarmed media and samples were taken at timed intervals for various assays.

### Plasmids

The bipA<sup>+</sup> plasmids pAJG4 and pAJG38 have been described (Grant *et al*, 2003), as have pTrcfis, pTM918, pWN1 and the lacZ reporter plasmids pRS414 and pRS415 (Simons *et al*, 1987; Schneider *et al*, 1997; Nasser *et al*, 2002). Derivatives of pRS415 in which the three individual bipA promoters or the entire *glnA-bipA* intergenic region were transcriptionally fused to lacZ were made by cloning appropriate PCR fragments between the EcoRI and BamHI sites. The plasmids carrying bipA promoters P1–P3 were designated pDJS73.7, pDJS70.33 and pBip116, respectively, while the construct carrying all three bipA promoters was designated pWP1. A plasmid in which the region encoding the fis S-D sequence was fused to the lacZ ORF and transcribed from the arabinose-inducible p<sub>BAD</sub> promoter was constructed by using PCR to amplify the lacZ ORF from pRS414 and to simultaneously add the fis S-D region (forward primer: 5'-aaaaaaaaagtcgacacagaataaagagctgacagaactATGtgcgaacaacgcccgcgctgttttaacaacgtcgactgg-3'; reverse primer: cccccccctgcagattattttgacaccagacc-3'; restriction sites underlined; start codon in capitals). The resulting fragment was trimmed with Sall and PstI and inserted into similarly cut pAJG38 (which carries the P<sub>BAD</sub> promoter), thereby generating pJGP1. Derivatives of pJGP1, carrying the regions encoding the WT lacZ S-D or mutant versions of the fis S-D, were constructed by replacing a SalI–NotI fragment with appropriate synthetic dsDNA fragments.

### Preparation of ribosomes, BipA and EF-G

BipA, carrying an N-terminal hexahistidiny tag, and EF-G similarly tagged at its C-terminus were purified as described (Owens *et al*, 2001; Connell *et al*, 2003). Ribosomes and associated components were prepared by the methods described by Blaha *et al* (2000). (For brevity, the recombinant (His)<sub>6</sub>BipA and EF-G(His)<sub>6</sub> proteins are referred to as BipA and EF-G in this paper.)

### Gel filtration assay

The assay used was adapted from that of Blaha *et al* (2000). BipA, EF-G and 70S ribosomes were all present at 1.5 µM. GTP $\gamma$ S was included, where appropriate, at a concentration of 50 µM. Reactions were started by incubation at 37°C for 5 min, whereupon 100 µl samples were applied to a pre-equilibrated S300 cDNA column (Pharmacia). A 2 ml portion of binding buffer (20 mM HEPES, 6 mM MgCl<sub>2</sub>, 150 mM NH<sub>4</sub>Cl, 2 mM spermidine, 0.05 mM spermine, pH 7.6) was added to the columns and 20 × 100 µl fractions were collected by gravity flow (Blaha *et al*, 2000). In all, 10 µl samples were removed from each fraction, diluted to 1 ml and their absorbance at 260 nm was recorded to detect the presence of 70S ribosomes. The fractions were also analysed by SDS–PAGE and

subsequently immunoblotted with antibodies specific for either (His)<sub>6</sub>BipA or EF-G(His)<sub>6</sub>.

### GTPase and fluorescence assays

The GTPase assay, which was adapted from the method of R Adlung (PhD thesis, 1994), has been described previously (Owens *et al*, 2001). Unless otherwise stated, 70S ribosomes, and EF-G were used at 0.2 µM, while BipA was included at 2 µM. GTPase reactions were started by adding [ $\gamma$ -<sup>32</sup>P]GTP (50 µM) and were carried out at 37°C for 15 min. The results were expressed in terms of pmol <sup>32</sup>P released per reaction or as percentages.  $K_d$  values of BipA for GTP and GDP were measured by fluorescence spectroscopy. The displacement of 1-anilino-8-naphthalene sulphonic acid (100 µM) from the nucleotide-binding site was measured upon addition of guanosine nucleotides (0–500 µM). The dissociation constants and number of binding sites were calculated using the Klotz plot equation.

### AQUA analysis

BipA levels were quantified using the AQUA method (Gerber *et al*, 2003). MG1655 cells were grown in 2 × YT broth to  $A_{600}$  = 1, diluted six-fold into fresh 2 × YT broth and grown aerobically at 37°C. Samples (35 ml) were taken at timed intervals and cell numbers were estimated by both  $A_{600}$  and haemocytometer measurements. Each sample was pelleted (12 000 g, 5 min, 4°C), resuspended in 2 × final sample buffer (4% SDS, 20% glycerol, 200 mM DTT, 120 mM Tris–HCl, pH 6.8, 0.002% bromophenol blue) and incubated at 100°C for 3 min. The resulting cell extracts were fractionated by SDS–PAGE and the regions containing the BipA protein were excised. After digestion *in situ* with excess trypsin in the presence of 500 fmol of a [<sup>13</sup>C] reference peptide (\*LDYVIPSR, where \* indicates the <sup>13</sup>C-labelled residue) corresponding to residues 440–446 of BipA, peptides were extracted as previously described (Adams *et al*, 1999). Peptides were analysed by LC–MS/MS using a Dionex PepMap C18, 3 µm, 100 Å (15 cm × 75 µm, i.d.) column coupled to a Q-TOF Global Ultima (Waters). Doubly charged parent ions ( $m/z$ : 481.17 and 484.17) were fragmented to generate  $\gamma$  ions of 534.27. Using reconstructed ion chromatograms, the ratios of the areas under each peak were used to calculate the absolute amount of BipA in each sample. The precise amount of added [<sup>13</sup>C] reference peptide was determined by amino-acid analysis.

### Primer extension and RT–PCR analyses

Extraction of total RNA and primer extension analyses were carried out as previously described (Grant *et al*, 2003). Analysis of the BipA transcripts used the primer Bip358 (5'-CTTGCCTCAGGCATTA GAAATAGCGCG-3'). Quantitative RT–PCR of cDNA was carried out using an SYBR Green PCR Master Mix (Eurogentec) in conjunction with primers specific for the lacZ coding sequence (5'-GACGTCTCGTTGCTGCATAA-3' and 5'-CAGCAGCAGACCATT TTTCAA-3') and gapA (5'-CATCTCAGAACATCATCCCGTCT-3' and 5'-GCCATACCAGTCAGTTTGCCATT-3'). PCR products were quantified using an Opticon 2 detection system (MJ Research) with the following cycling parameters: 95°C, 15 s; 60°C, 1 min; 72°C for 15 s. A melt curve was performed after each run to ensure the absence of nonspecific PCR products using the following parameters: 95°C, 1 min; 55°C for 1 min; ramp from 55 to 95°C (0.1°C/s). The number of cycles required for fluorescence, resulting from synthesis of PCR product, to become significantly higher than background fluorescence was then calculated and normalized to the corresponding data for gapA.

### Other procedures

Immunoblotting and  $\beta$ -galactosidase assays were carried out as described (Schneider *et al*, 1997; Grant *et al*, 2003).

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