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DksA Is Required for Growth Phase-Dependent Regulation, Growth Rate-Dependent Control, and Stringent Control of *fis* Expression in *Escherichia coli*

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DksA is a critical transcription factor in *Escherichia coli* that binds to RNA polymerase and potentiates control of rRNA promoters and certain amino acid promoters. Given the kinetic similarities between rRNA promoters and the *fis* promoter (P*fis*), we investigated the possibility that DksA might also control transcription from P*fis*. We show that the absence of *dksA* extends transcription from P*fis* well into the late logarithmic and stationary growth phases, demonstrating the importance of DksA for growth phase-dependent regulation of *fis*. We also show that transcription from P*fis* increases with steady-state growth rate and that *dksA* is absolutely required for this regulation. In addition, both DksA and ppGpp are required for inhibition of P*fis* promoter activity following amino acid starvation, and these factors act directly and synergistically to negatively control P*fis* transcription in vitro. DksA decreases the half-life of the intrinsically short-lived *fis* promoter-RNA polymerase complex and increases its sensitivity to the concentration of CTP, the predominant initiating nucleotide triphosphate for this promoter. This work extends our understanding of the multiple factors controlling *fis* expression and demonstrates the generality of the DksA requirement for regulation of kinetically similar promoters.

Fis is the most abundant nucleoid-associated protein during logarithmic growth phase in *Escherichia coli* growing in rich medium (2). Besides stimulating specialized site-specific DNA inversion events (hence its name: factor for inversion stimulation) and λ phage DNA recombination (17), Fis acts as a transcription factor to stimulate rRNA and various tRNA promoters (35, 48). In addition, a growing number of genes are reported to be regulated positively or negatively by Fis (13, 15, 21, 22, 24, 29, 61, 63), including several genes that play roles in pathogenesis in various bacterial species (16, 20, 52, 60). In cases where positive control by Fis has been examined in detail (e.g., at the *rrnB* P1 and *pro*P promoters), activation requires a direct interaction between Fis and the RNA polymerase (RNAP) α subunit C-terminal domain (1, 30).

Regulation by Fis results from large changes in its intracellular levels, which are triggered by changes in the nutritional environment or the growth phase (3, 36, 37, 55). A dramatic burst in both *fis* mRNA and protein levels is observed when cells in stationary phase are outgrown in rich medium. *fis* mRNA and protein levels peak during early logarithmic growth phase, decrease soon thereafter, and become nearly undetectable as cells enter the stationary phase, an expression pattern referred to as growth phase-dependent regulation (GPDR). GPDR is controlled by changes in *fis* transcription initiation and not by changes in the decay rates of the *fis* mRNA (47). Additionally, it was reported recently that translational control of *fis* expression is exerted by BipA, a ribosome-associated protein (40).

A single promoter (P*fis*) is responsible for transcribing the *fis* operon in *E. coli* (28). P*fis* is negatively autoregulated by Fis binding at two sites centered at −44 and +25 relative to the transcription start site (3, 37, 47) and positively controlled by integration host factor (IHF) binding at a single site centered at −94 (47). P*fis* is also negatively controlled during the stringent response by ppGpp (28, 37, 57). However, GPDR of P*fis* is relatively normal in strains lacking ppGpp (3). Both the stringent response and GPDR require only the core promoter region from about −38 to +5, the binding site for RNAP holoenzyme (37, 47), and these regulatory properties are strictly conserved in other bacterial species (8, 28, 39). Additionally, because maximal Fis levels increase with the quality of the growth medium (3, 36), it has been suggested that P*fis* activity might increase with steady-state growth rate, and this in turn might contribute to the growth rate-dependent control of some Fis-activated promoters (6, 35, 46). However, growth rate-dependent control of P*fis* itself has not been demonstrated directly.

P*fis* is among a small number of *E. coli* promoters that initiate transcription predominantly with CTP (23, 27), a feature that is strongly conserved in P*fis* in other bacteria (28, 59). Mutations in the transcription initiation region that caused a switch in CTP to ATP or GTP as the predominant initiating nucleoside triphosphate (iNTP) profoundly altered GPDR of *fis* (57). As in the case of the *rrnB* P1 and P2 promoters (18, 33, 43), complexes formed by the P*fis* promoter with RNAP are unusually short-lived, hypersensitive to salts, and strongly dependent on high concentrations of the iNTP for maximal transcription (58).

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TABLE 1. Strains used in this work

Strain	Description	Reference or source
VH1000 (RLG3499)	MG1655 *pyrE*$^+$ *lacI lacZ*	18
RO1250	VH1000 Δ*dksA*	This work
RO1261	VH1000 P*fis*::*lacZ*	This work
RO1265	RO1250 P*fis*::*lacZ*	This work
RO1275	VH1000/pBR322	This work
RO1276	RO1250/pBR322	This work
RO1279	MG1655 Δ*relA* Δ*spoT*/pBR322	This work
RLG7062	VH1000 Δ*dksA rrnB* P1[-61/+1]::*lacZ*	41

Recently, it was found that the small RNAP-associated protein DksA is a critical component of the transcription initiation complex that is required for negative regulation of rRNA promoters (41, 44) and positive regulation of several amino acid promoters in vitro and in vivo (42). DksA destabilizes intrinsically unstable rRNA promoter complexes, enhances their sensitivity to ppGpp and the iNTP concentration, and is essential for their growth rate-dependent control, growth phase-dependent control, and stringent control (41).

Considering the kinetic and regulatory similarities between rRNA promoters and P*fis*, we investigated the possibility that DksA might also target P*fis* for transcriptional regulation. Here we demonstrate that DksA greatly affects growth phase-dependent control, growth rate-dependent control, and stringent control of P*fis*. DksA acts on the P*fis* promoter directly in vitro, decreasing the lifetime of its complex with RNAP, enhancing its iNTP sensitivity, and, together with ppGpp, negatively regulating *fis* transcription. These results expand our understanding of *fis* regulation and demonstrate the generality of the role of DksA in facilitating regulation of promoters sharing similar kinetic characteristics.

MATERIALS AND METHODS

Chemicals, enzymes, and growth media. Chemicals were purchased from Sigma-Aldrich, Fisher Scientific, Life Technologies Inc. (Gibco BRL), Pharmacia, or VWR Scientific. Nucleotides and radioisotopes were from Amersham Biosciences Corp. or Tri-Link BioTechnologies. Oligonucleotides were synthesized at the Center for Comparative Functional Genomics, State University of New York at Albany. Enzymes were from New England BioLabs, Inc., Promega Corp., or Roche Molecular Biochemicals. RNA polymerase ($E\sigma^{70}$) purified by standard procedures (10) was a gift from R. Landick (University of Wisconsin—Madison) or purchased from Epicentre (Madison, WI). N-terminally hexahistidine-tagged DksA was purified by affinity chromatography and functioned indistinguishably from native DksA in vivo and in vitro (41).

Bacterial culture media were from Difco Laboratories. Cultures were grown at 31°C in Luria-Bertani (LB) medium or M9 minimal medium (50) supplemented with 0.4% carbon source (glucose or glycerol), 0.4% Casamino Acids, or 40 μg/ml methionine, aspartic acid, and threonine, as indicated for each experiment. To select for drug resistance, 100 μg of ampicillin per ml or 12 μg of tetracycline per ml was added to the growth medium as appropriate for each strain.

Strains and plasmids. Relevant strains used in this work are listed in Table 1. RO1250 was made by P1 transduction of the *dksA*::*tet* mutation from RLG7062 (41) into VH1000 following procedures described previously (31). Transductants were selected on LB agar containing tetracycline, and the presence of the *dksA*::*tet* construct was verified by PCR using the oligonucleotides 5′-dGCTAT CCGGAAAAGCATCTGC and 5′-dCTGTGGTAAACGTGATGGAAC, which anneal at 54°C within regions upstream and downstream of *dksA*, respectively, to give distinct DNA fragments for the *dksA*::*tet* mutation and the wild-type gene. PCR was conducted using *Taq* polymerase (Roche Molecular Biochemicals), as described by the manufacturer. Strains (RO1261 and RO1265) carrying prophage-containing promoter-*lacZ* fusions (P*fis* DNA from −373 to +83 with respect to the transcription start site) were made by infection of wild-type (WT) or *dksA* mutant strains with phage from previously existing lysogens or by P1 transduction (with the same result). This DNA region contains a single promoter (P*fis*) responsible for transcribing the *fis* operon (3, 28, 47). pRO362 is a pKK223-3-based plasmid that contains the P*fis* region from −166 to +83 cloned within EcoRI and BamHI restriction sites. This replaces the *tac* promoter in pKK223-3 and positions the P*fis* start site about 340 bp upstream of the *rrnB* T_1 rho-independent terminator (28).

RNA extraction and primer extension assays. Primer extension assays were conducted to examine effects of *dksA* on P*fis* GPDR in RO1275 and RO1276. The relative *fis* mRNA levels measured at 0 min of growth were from total RNA prepared from cultures grown in LB medium for 18 h. Subsequent measurements were from cultures diluted in LB to an optical density at 600 nm (OD_{600}) of 0.06 and grown at 31°C with shaking. At intervals, cells were harvested for total RNA preparation using the hot acid-phenol method of extraction (11). Primer extension reactions were performed as described previously (57, 59) using 10 μg total cellular RNA and 2 pmol of ^{32}P-end-labeled oRO109 oligonucleotide (5′-dGC TGATATTGTCCGATG), which anneals to the *fis* mRNA region from +54 to +38, relative to the transcription initiation site at +1C. A second oligonucleotide oRO133 (5′-dGGTGAGCAAAAACAGGAAGG) anneals to the β-lactamase (*bla*) promoter region from +76 to +57 on pBR322 present in these strains and was used as a control. The resulting products were separated on 8 M urea–8% polyacrylamide gels, and the relative signal intensities were quantified by phosphorimaging. The relative intensities measured for the P*fis* transcripts were adjusted by those of the *bla* transcripts in each data set to correct for loading errors and were then normalized to the amount of cells (based on OD_{600} readings) that yielded the RNA signals in each reaction. The proximity of the primer-annealing site to the *fis* promoter allows the primer extension assay to accurately measure changes in transcription initiation.

Starvation induction. Saturated cultures of RO1275, RO1276, and RO1279 were diluted in LB to an OD_{600} of 0.06 and grown at 31°C with shaking for 30 min to induce *fis* expression. At this time half the culture was transferred to another flask containing sufficient serine hydroxamate (SH) to generate a final concentration of 1 mg/ml and allowed to continue shaking at 31°C. The non-treated (control) culture received an equivalent volume of sterile distilled water and was allowed to continue shaking at 31°C. At various times after addition of SH or water (see Fig. 3), a sample from each culture was harvested and used to prepare total cellular RNA. Primer extension reactions were performed with 10 μg total RNA and oligonucleotides oRO109 and oRO133 as described above.

β-Galactosidase assays. For determining the effect of *dksA* on growth phase-dependent regulation, saturated cultures of RO1261 and RO1265 were diluted in LB medium to an OD_{600} of 0.06 and grown at 31°C. Culture samples were removed at various times during growth for β-galactosidase assays, which were performed as described elsewhere (31). For determining the effect of *dksA* on growth rate-dependent control of P*fis*, the same strains were diluted in the experimentally specified growth medium and allowed to grow at 31°C. Culture samples were removed during early logarithmic growth phase, when *fis* expression is high (OD_{600} from 0.08 to 0.15, depending on the growth medium) and assayed for β-galactosidase activity. Results are given as averages from three independent assays.

In vitro transcription. Multiple round in vitro transcription assays were performed using supercoiled plasmid template pRO362 containing P*fis* with the promoter endpoints −168 and +83 with respect to the transcription start site (28). Reactants (0.5 nM plasmid and specified concentrations of ppGpp and DksA) were incubated at 30°C for 10 min in 40 mM Tris · HCl (pH 7.9), 150 mM KCl, 10 mM NaCl, 5% glycerol, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 μg/ml bovine serum albumin (BSA), 200 μM ATP, CTP and GTP, 10 μM UTP, and [α-^{32}P]UTP (2.5 μCi). Transcription was initiated by addition of 5 nM RNAP and terminated after 10 min by addition of an equal volume of formamide loading buffer, and the products were electrophoresed on 7 M urea–6% polyacrylamide gels and visualized and quantified by phosphorimaging.

CTP concentration dependence of transcription. To evaluate the CTP dependence on transcription, multiple-round in vitro transcription assays were performed using buffer conditions similar to those described above, except that 200 mM KCl, 40 μM ATP and GTP, 4 μM UTP, 2.5 μCi [α-^{32}P]UTP, and various concentrations of CTP (see Fig. 6) were used. Reactions were initiated by addition of RNAP and allowed to proceed for 30 min at 22°C, and the products were analyzed as described above. As a control, ATP dependence was similarly examined but with the NTP concentrations adjusted to 40 μM GTP and CTP, 4 μM UTP, 2.5 μCi [α-^{32}P]UTP, and the concentrations of ATP indicated in Fig. 6. To evaluate the effect of DksA on the CTP dependence, multiple-round in vitro transcription assays were performed in the absence or presence of 6 μM DksA.

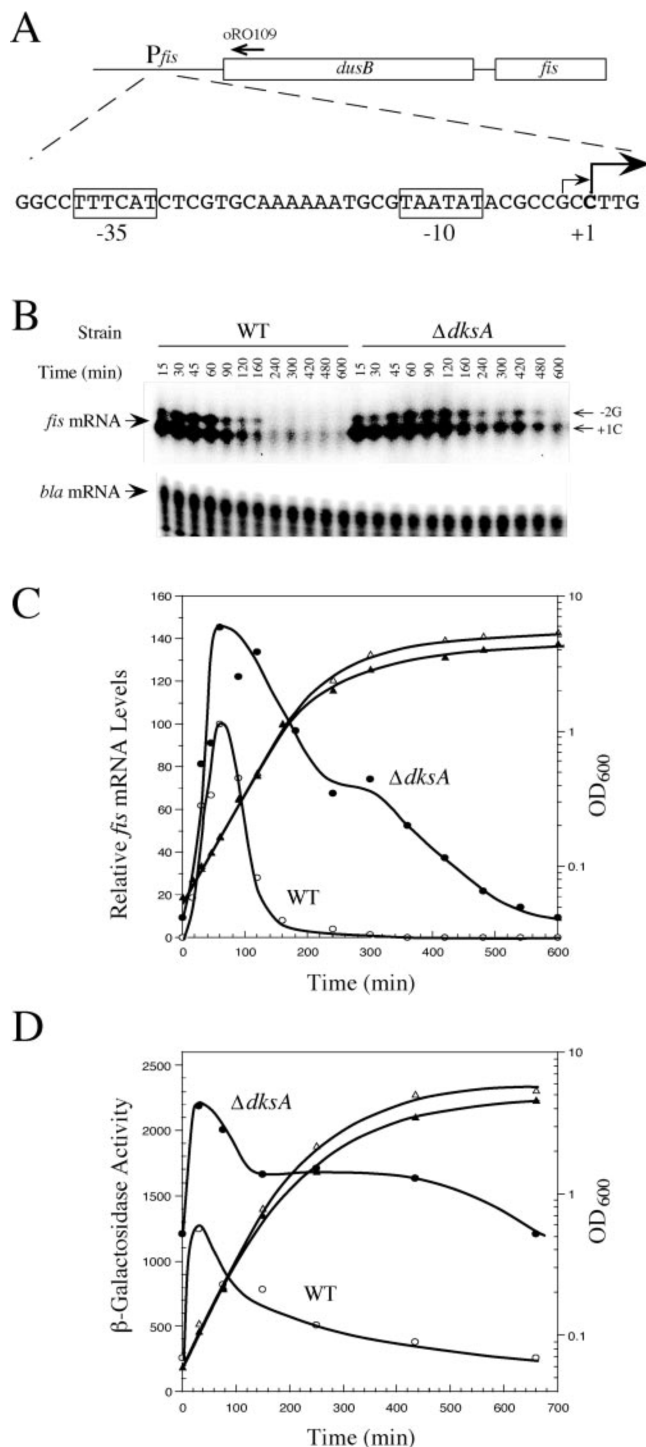

FIG. 1. Effect of *dksA* on growth phase-dependent regulation of P*fis*. (A) Schematic representation of the *fis* operon, consisting of two genes (*dusB* and *fis*) that are transcribed by a single promoter (P*fis*). The approximate annealing region of the oligonucleotide oRO109 used in the primer extension assays is represented with an arrow. Beneath is the sequence of the minimal P*fis* promoter. The operationally defined −10 and −35 promoter sequences (57) are boxed, and the major and minor transcription start sites are indicated with a large and small arrow, respectively. (B) Effect of DksA on GPDR of P*fis*. Primer extension assays were performed to detect P*fis* transcripts synthesized from the chromosome using total RNA obtained from RO1275 (WT) or RO1276 (Δ*dksA*) at various times of outgrowth from stationary phase in LB medium. The signals corresponding to the two *fis* mRNA start sites (+1C and −2G) and for the *bla* mRNA produced in these strains are shown with arrows. (C) Effect of *dksA* on the *fis* mRNA expression pattern. Primer extension results similar to those shown in panel B were quantified by phosphorimaging, normalized to the amount of cells used (as determined by OD_{600} readings) in each reaction, and plotted relative to the maximum value in RO1275, which was assigned a value of 100. The results from two experiments were averaged, with standard deviations being within 30% or less of the values. Relative *fis* mRNA levels are shown for RO1275 (WT for *dksA*) (○) and RO1276 (Δ*dksA*) (●); growth curves based on OD_{600} measurements are shown for WT (△) and Δ*dksA* (▲) strains. (D) Effect of *dksA* on β-galactosidase activity from a P*fis-lacZ* fusion during growth in LB medium. Saturated cultures of RO1261 (WT) (○, △) and RO1265 (Δ*dksA*) (●, ▲) were diluted to an OD_{600} of 0.06 in LB and grown at 31°C. β-Galactosidase assays (○, ●) were performed at the indicated times during growth. Results are averages from three independent cultures; standard deviations were within 14% of the average values. Growth curves for both strains (△, ▲) are shown.

Promoter complex half-life assays. Complex lifetime was determined using a transcription-based assay essentially as described previously (5). pRO362 (0.5 nM) and RNAP (5 nM) were incubated in the absence or presence of 2 μM DksA for 10 min at 30°C in 40 mM Tris · HCl (pH 7.9), 100 mM KCl, 10 mM NaCl, 5% glycerol, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 μg/μl BSA. At time zero, heparin at a final concentration of 10 μg/ml was added as a competitor, and at different times 10-μl aliquots were added to tubes containing 1.25 μl NTPs (final concentrations, 200 μM ATP, CTP, and GTP, 10 μM UTP, and 2.5 μCi [α-^{32}P]UTP). Reactions were stopped after 10 min, and the products were analyzed as described above. We note that at 175 mM KCl, the half-life of the competitor-resistant complex was too short (<15 s) to quantify (58).

RESULTS

DksA alters growth phase-dependent regulation of *fis*. We used primer extension assays to measure the relative levels of chromosomally derived *fis* transcripts in strains with and without *dksA* (Fig. 1A, B, and C). As reported previously (58), *fis* mRNA levels in strains grown in LB medium were maximal during early logarithmic phase (15 to 60 min after dilution of stationary-phase cells), decreased during mid-log phase, and then became undetectable as cells entered stationary phase. In the Δ*dksA* mutant, *fis* mRNA levels increased during early log phase, reaching peak levels that were about 1.5-fold higher than in the *dksA* wild-type strain (WT). These levels then decreased more slowly during mid-log and stationary phases, exceeding those in the wild-type strain even 4 h after entry into stationary phase. Similar results were observed when P*fis* was present on a multicopy plasmid in a Δ*dksA* strain (not shown). In contrast, the β-lactamase (*bla*) transcript showed minimal variation with growth phase and was unaffected by *dksA*. Thus, *dksA* is required for inhibition of *fis* transcription in mid-log and late log growth and in stationary phase.

We confirmed that *dksA* alters GPDR of the *fis* promoter by comparing expression from a chromosomal P*fis-lacZ* fusion in WT versus Δ*dksA* strains (Fig. 1D). β-Galactosidase activity peaked during early logarithmic growth in the WT strain and then decreased during mid-logarithmic and late logarithmic growth. Consistent with the primer extension results described above, P*fis* activity was higher in the Δ*dksA* than in the WT strain during all phases of growth, and this increase was most prominent during late log phase and in stationary phase. The enzymatic activity observed in the WT strain during late log

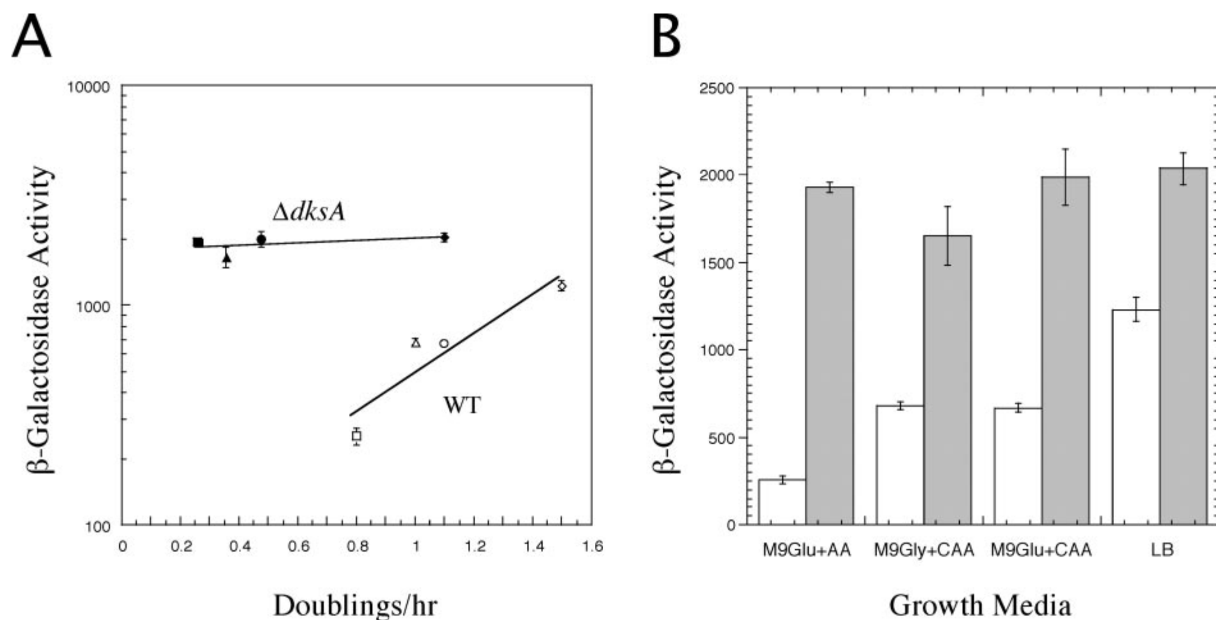

FIG. 2. Effect of *dksA* on growth rate-dependent control of P*fis*. (A) Transcription activity from P*fis* was monitored by β-galactosidase assays in strains RO1261 (WT for *dksA*; open symbols) and RO1265 (Δ*dksA*; filled symbols) grown in different media at 31°C to generate different growth rates. Growth media used were (□, ■) M9 salts, 0.4% glucose, and 40 μg/ml each of methionine, aspartic acid, and threonine, (△, ▲) M9 salts, 0.4% glycerol, and 0.4% Casamino Acids, (○, ●) M9 salts, 0.4% glucose, and 0.4% Casamino Acids, and (◇, ◆) LB. Results are averages of three independent cultures, with standard deviations indicated by error bars. (B) The results from panel A are displayed in a bar graph to compare the effects of *dksA* on P*fis* transcription in each growth medium used. Open bars, RO1261; filled bars, RO1265.

and stationary phases is likely attributable to β-galactosidase that accumulated earlier in growth, since β-galactosidase is stable and P*fis*-*lacZ* mRNA hybrid transcripts were not detected in late log or stationary phase (37) (data not shown). These results indicate that DksA acts negatively on P*fis* and is required for normal GPDR of this promoter.

DksA is required for growth rate-dependent regulation of P*fis*. To determine if P*fis* transcription is controlled by the growth rate, we measured P*fis* activity using promoter-*lacZ* fusions in media of varying nutritional quality during steady-state growth, when *fis* is maximally expressed (Fig. 2). β-Galactosidase activity increased >4-fold (255 to 1,230 units), with an ~2-fold increase in growth rate (0.8 to 1.5 doublings/h), demonstrating that P*fis* transcription is subject to growth rate-dependent control. In contrast, P*fis* activity was higher and changed little or not at all with growth rate in the Δ*dksA* mutant (Fig. 2A). There was a 5.5-fold difference in P*fis* activity in the two strains grown in M9 glucose medium supplemented with glucose, methionine, aspartic acid, and threonine and a 1.6-fold difference in LB medium (Fig. 2B). These results demonstrate that DksA is essential for limiting P*fis* promoter activity, especially at low steady-state growth rates.

DksA greatly amplifies stringent control of P*fis*. To determine if DksA affected stringent control of P*fis* in vivo, we compared relative transcript levels from P*fis* in a WT, Δ*dksA*, and Δ*relA* Δ*spoT* strain that is unable to synthesize ppGpp (62), before and after starvation for serine (Fig. 3). *fis* transcription decreased ~10-fold by 15 min after starvation (induced by addition of serine hydroxamate) in the WT strain. In contrast, *fis* transcription decreased <2-fold in the Δ*dksA* and not at all in the Δ*relA* Δ*spoT* strain. As expected, there was no significant effect of starvation on transcripts from the *bla* promoter, confirming that the effects of DksA and ppGpp are promoter specific. Taken together, our results indicate that although ppGpp may be able to inhibit *fis* expression slightly in the absence of DksA (41), both DksA and ppGpp are required for normal stringent control of P*fis*.

DksA directly inhibits transcription from P*fis*, amplifies inhibition by ppGpp, and decreases the lifetime of the P*fis* complex with RNAP. To determine whether the effects of ppGpp and DksA observed on P*fis* in vivo were direct, transcription from the promoter was analyzed in vitro in the presence or absence of purified DksA and/or ppGpp (Fig. 4). Whereas either DksA or ppGpp alone reduced P*fis* activity ~2-fold, the two factors together reduced transcription ~10-fold. Thus, as observed for rRNA promoters (41), DksA and ppGpp inhibit P*fis* directly and synergistically.

It was previously shown that the P*fis* complex with RNAP is very short-lived, too short to measured accurately in buffers containing relatively high salt concentrations; e.g., the half-life of the complex was <15 s in a buffer containing 175 mM KCl (58). We took advantage of the fact that the complex is longer-lived at lower salt concentrations to measure effects of DksA on the half-life of the P*fis* complex with RNAP. Figure 5 demonstrates that DksA further decreases the lifetime of the complex directly, ~3-fold (from ~14 min to ~4.6 min) in a buffer containing 100 mM KCl. These results are consistent with the model that DksA inhibits transcription, in part, by further destabilizing intrinsically unstable promoter complexes.

DksA increases the sensitivity of P*fis* to CTP. Previous studies indicated that high concentrations of CTP stabilize the

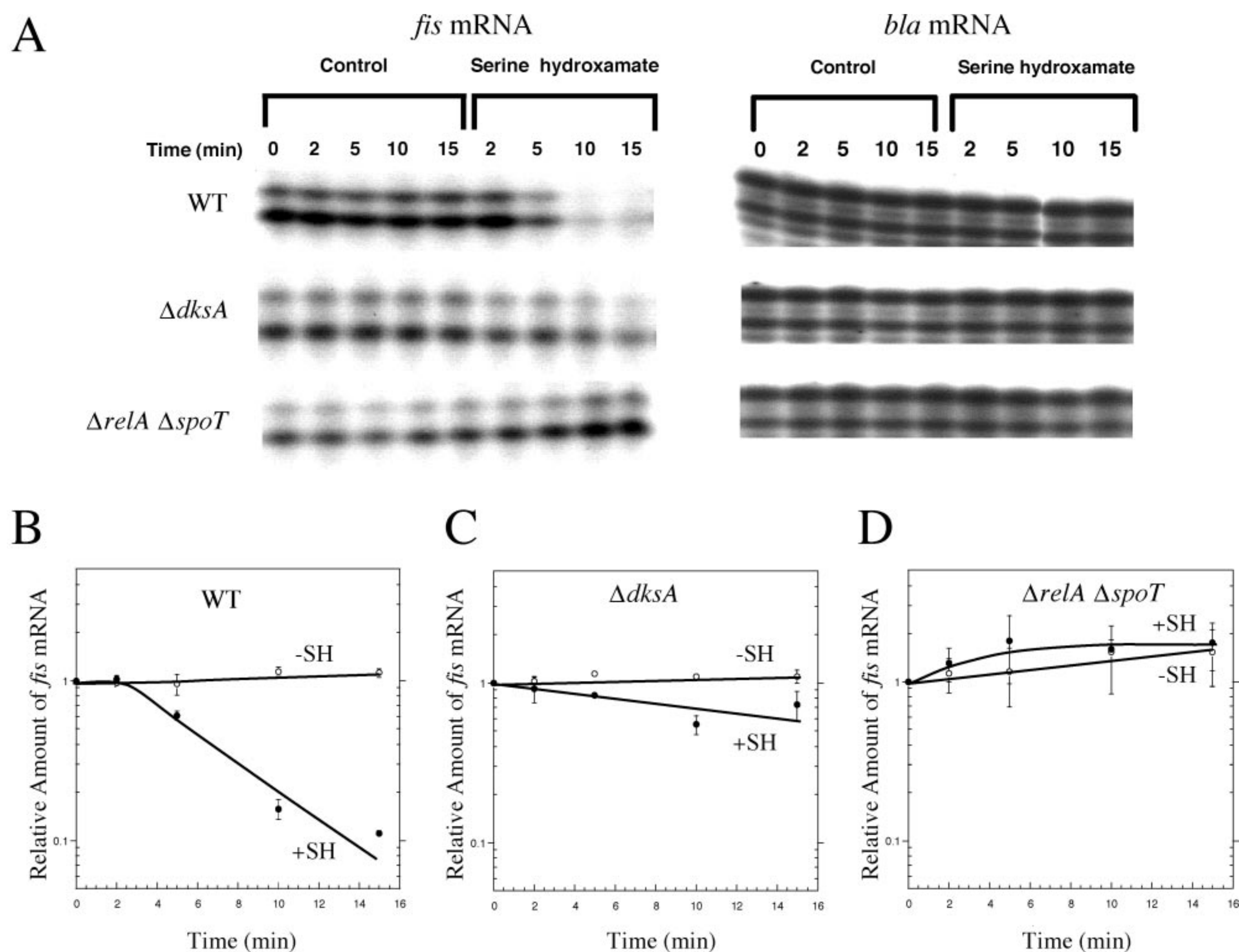

FIG. 3. Effect of *dksA* on stringent control of P*fis*. (A) Primer extension assays of *fis* mRNA under starved and nonstarved conditions. At the indicated times after SH addition, samples of the SH-treated and untreated (control) cell cultures were harvested and used to prepare total RNA. Primer extension reactions were performed to detect both the *fis* and *bla* mRNA signals. The two P*fis* transcript signals correspond to those initiating at +1C and −2G; two prominent transcript signals were also detected for the *bla* transcript. Strains used in this experiment were RO1275 (WT for *dksA*), RO1276 (Δ*dksA*), and RO1279 (Δ*relA* Δ*spoT*). Duplicate reactions similar to those represented in panel A for (B) *dksA* WT (WT), (C) Δ*dksA*, and (D) Δ*relA* Δ*spoT* strains were quantified by phosphorimaging and averaged and are shown relative to the values at time zero in each set, which were assigned a value of 1.0. Error bars indicate standard deviations. Open circles, relative *fis* transcript levels in the control cultures (-SH); filled circles, relative *fis* transcript levels in the serine hydroxamate-treated cultures (+SH).

short-lived P*fis* complex with RNAP to increase transcription >20-fold (58), suggesting that transcription from P*fis* is highly sensitive to the concentration of CTP. To verify the P*fis* dependence on high CTP concentrations, we performed multiple-round transcription from P*fis* with increasing concentrations of CTP or ATP while the remaining NTP concentrations were kept constant (Fig. 6). We found that half-maximal P*fis* promoter activity required ~280 μM CTP and maximal transcription required ~900 μM CTP. In contrast, half-maximal promoter activity required only ~5 μM ATP and maximal activity required only ~100 μM ATP. Thus, P*fis* transcription in vitro is highly sensitive to the concentration of the iNTP, CTP, but not to that of ATP.

To determine if DksA affected the sensitivity of P*fis* to the iNTP, we performed transcription in the presence of 6 μM DksA with various concentrations of CTP while keeping the other three NTP concentrations constant. The results show that DksA further increased the relative K_{NTP} for CTP by about 185 μM (from ~280 μM to ~465 μM) and caused the maximal transcription activity to require >1,500 μM CTP (Fig. 6). We emphasize that the absolute K_{NTP} values are dependent on the solution conditions, and it is not valid to compare the apparent K_{NTP} values obtained under different conditions. Nevertheless, our results demonstrate that not only does DksA amplify the sensitivity of P*fis* to ppGpp, but also it increases the concentration of the initiating NTP required for transcription.

DISCUSSION

In a Δ*dksA* strain, we found that the *fis* mRNA levels are higher than in a WT strain, persisting through mid-logarithmic and late logarithmic growth and several hours into stationary phase and that this results from an increase in P*fis* promoter activity. Steady-state P*fis* activity increases with growth rate,

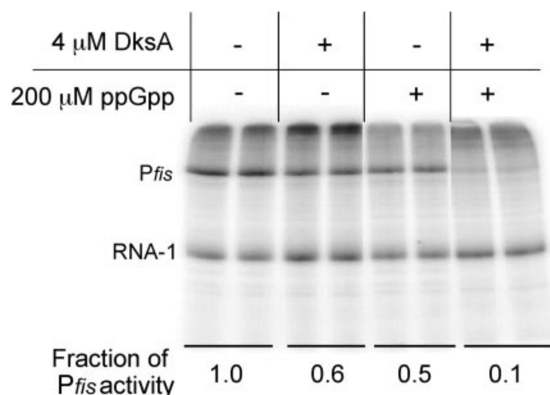

FIG. 4. Effect of ppGpp and DksA on P*fis* transcription in vitro. Duplicate reactions of multiple round in vitro transcription assays were performed with pRO362 in the absence or presence of 4 μM purified DksA and 200 μM ppGpp, as indicated. Transcripts from P*fis* and the RNA-1 promoter (derived from the plasmid) are indicated. The P*fis* signals from two independent reactions were averaged and are shown as a fraction of the P*fis* activity in the absence of DksA and ppGpp.

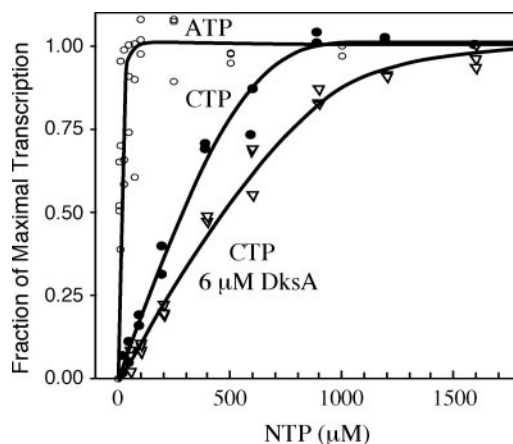

FIG. 6. Effect of DksA on promoter sensitivity to CTP. Multiple-round in vitro transcription assays were performed with supercoiled pRO362 in transcription buffer containing 200 mM KCl and various concentrations of ATP (○) or CTP (●, ▽), in the absence (○, ●) or presence (▽) of 6 μM DksA, as described in Materials and Methods. *fis* transcripts were electrophoretically separated and quantified by phosphorimaging and are shown relative to the maximal levels obtained at the highest NTP concentrations used.

and this is entirely dependent on *dksA*. Stringent control of *fis* is nearly abolished in a Δ*dksA* strain. Furthermore, we show that DksA acts directly on P*fis* promoter complexes in vitro, reducing the lifetime of the competitor-resistant complex with RNAP. DksA elevates the concentration of the iNTP (CTP) required for efficient transcription from P*fis* and strongly amplifies the inhibitory effect of ppGpp on P*fis*. Our results are consistent with the model that DksA inhibits P*fis* activity, in part, by reducing the half-life of P*fis* promoter complexes. In conjunction with changes in the concentrations of ppGpp and CTP, DksA constrains *fis* expression primarily to early log phase at high growth rates, and it inhibits expression at low growth rates or following amino acid starvation. We conclude that DksA is crucial for regulation of *fis* expression in *E. coli*.

Blastn and Blastx searches indicate that *fis* homologues are present throughout the γ and β subdivisions of proteobacteria, which include the *Enterobacteriales*, *Pasteurellaceae*, *Pseudomonadaceae*, *Vibrionaceae*, *Xanthomonadaceae*, *Burkholderiaceae*, and *Neisseriaceae*, among others (not shown). In every one of these organisms, a *dksA* homologue is also evident. Additionally, *fis* core promoter sequences and transcription initiation of P*fis* with CTP, as well as GPDR and stringent control of *fis*, are strongly conserved among enteric bacteria (3, 28, 37, 39). Therefore, we suggest that DksA-dependent control of *fis* expression is likely to be a feature conserved in these bacteria as well.

The effects of DksA on P*fis* transcription are strikingly similar to those observed on *rrnB* P1 (41). This can be attributed to the overall similarities in the kinetic properties of these promoters. The parallel regulation of these promoters is consistent with the role of Fis in stimulating rRNA promoter activity (9, 48), and this is apparently accomplished by linking their synthesis rates to the same regulatory molecules: DksA, ppGpp, and NTPs.

DksA does not bind to DNA but instead binds directly to RNAP to mediate its effect (41). The structural similarities among DksA (44, 56) and the transcription elongation factors GreA and GreB (26, 38, 53, 54) suggested a model whereby the coiled coil of DksA inserts deep into the RNAP secondary channel with its tip approaching the active site (44). There are strong correlations between the effects of promoter mutations on the lifetimes of *rrnB* P1 promoter complexes (18, 33) and P*fis* promoter complexes (P. Mallik and R. Osuna, unpublished results), the sensitivity of these complexes to small molecule regulators, and their control in vivo, suggesting that a short-lived complex with RNAP is a prerequisite for regulation by DksA. However, the precise mechanism(s) by which DksA and

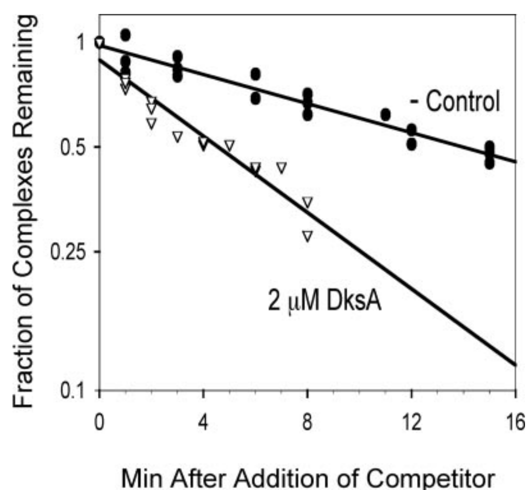

FIG. 5. Effect of DksA on the stability of RNAP complexes with P*fis*. P*fis* complexes with RNAP were preformed with supercoiled pRO362 in the presence (▽) or absence (●) of 2 μM DksA in transcription buffer containing 100 mM KCl as described in Materials and Methods. The complexes were challenged with heparin and assayed for single-round transcription at various times thereafter. Electrophoretically separated transcripts were quantified by phosphorimaging and are shown relative to the amount present prior to the heparin addition.

ppGpp ultimately affect the kinetics of promoter complexes remains to be determined.

Relative DksA levels change only slightly in different phases of growth and with changes in steady-state growth rate (41; S. T. Rutherford and R. L. Gourse, unpublished results). Thus, rapid changes in the intracellular concentrations of ppGpp and iNTPs specify when rRNA promoter regulation occurs and which of these small molecules is responsible (34). The rapid increase in ppGpp levels that occurs during the stringent response (12) effectively inhibits *fis* expression. The role of DksA in amplifying negative control by ppGpp likely explains the DksA requirement for stringent control of P*fis*. In contrast, CTP pools rapidly increase during outgrowth from stationary phase (33), fluctuate during logarithmic growth, and substantially decrease as cells enter the stationary phase, all in a pattern that qualitatively correlates with the pattern of *fis* expression (58). We suggest that the DksA requirement for limiting *fis* expression to the logarithmic growth phase may derive from its role in decreasing the lifetime of P*fis* complexes and increasing the sensitivity to the CTP concentration (58).

Since ppGpp functions negatively and its concentrations increase during outgrowth from stationary phase, ppGpp cannot be responsible for the increase in *fis* expression during this time (34). In contrast, there is a transient increase in ppGpp levels during entry into stationary phase (34) as well as a decrease in CTP levels (34, 58). Thus, it is possible that the changes in both ppGpp and CTP levels contribute to the shutdown of *fis* expression at this time. However, since normal GPDR of *fis* was observed in a Δ*relA* Δ*spoT* strain (3), apparently other mechanisms can compensate for the role of ppGpp in this process.

Although ppGpp concentrations correlate inversely with control of rRNA and *fis* promoter activities, whereas NTP pools remain relatively unchanged at different steady-state growth rates (43, 45, 49, 51, 64), growth rate-dependent regulation of *rrnB* P1 and P2 is maintained in Δ*relA* and Δ*relA* Δ*spoT* strains (5, 7, 19, 32). DksA, however, is essential for growth rate-dependent regulation of both rRNA and *fis* expression. Thus, other factors are apparently able to compensate for the loss of ppGpp and confer DksA-dependent regulation on rRNA promoters (and possibly on P*fis*) when the cell is provided with enough time to adjust its macromolecular synthesis rates.

In addition to the mechanisms that control *fis* transcription, it has been reported that there is also control of *fis* translation by a mechanism requiring BipA (40). Levels of BipA vary with the growth phase and with the nutritional quality of the growth medium in a manner that correlates with variations in Fis levels. Thus, control of Fis synthesis may also involve BipA, directly or indirectly. Contributions from multiple mechanisms is a common feature for regulation of genes associated with synthesis of the translation machinery (12, 14, 25, 43).

DksA has emerged as an essential component of three mechanisms that involve negative regulation of *fis* and stable RNA promoters: stringent, growth phase-dependent, and growth rate-dependent control. Furthermore, its direct effects are not limited to negative control of promoters sharing similar kinetic properties; in conjunction with ppGpp, DksA also plays a role in direct positive control of several amino acid promoters (42). DksA thus has global effects in regulating gene expression, some of which are direct and some of which are indirect (resulting from its direct effects on stable RNA expression) (4, 65). Since Fis acts as both an activator and a repressor of numerous genes, it can be expected that some of the indirect effects of DksA on global gene expression will be mediated through its effects on *fis* expression.

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REFERENCES

1. **Aiyar, S. E., S. M. McLeod, W. Ross, C. A. Hirvonen, M. S. Thomas, R. C. Johnson, and R. L. Gourse.** 2002. Architecture of Fis-activated transcription complexes at the *Escherichia coli rrnB* P1 and *rrnE* P1 promoters. J. Mol. Biol. **316:**501–516.
2. **Ali Azam, T., A. Iwata, A. Nishimura, S. Ueda, and A. Ishihama.** 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. J. Bacteriol. **181:**6361–6370.
3. **Ball, C. A., R. Osuna, K. C. Ferguson, and R. C. Johnson.** 1992. Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. J. Bacteriol. **174:**8043–8056.
4. **Barker, M. M., T. Gaal, and R. L. Gourse.** 2001. Mechanism of regulation of transcription initiation by ppGpp. II. Models for positive control based on properties of RNAP mutants and competition for RNAP. J. Mol. Biol. **305:**689–702.
5. **Barker, M. M., T. Gaal, C. A. Josaitis, and R. L. Gourse.** 2001. Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro. J. Mol. Biol. **305:**673–688.
6. **Bartlett, M. S., T. Gaal, W. Ross, and R. L. Gourse.** 2000. Regulation of rRNA transcription is remarkably robust: FIS compensates for altered nucleoside triphosphate sensing by mutant RNA polymerases at *Escherichia coli rrn* P1 promoters. J. Bacteriol. **182:**1969–1977.
7. **Bartlett, M. S., and R. L. Gourse.** 1994. Growth rate-dependent control of the *rrnB* P1 core promoter in *Escherichia coli*. J. Bacteriol. **176:**5560–5564.
8. **Beach, M. B., and R. Osuna.** 1998. Identification and characterization of the *fis* operon in enteric bacteria. J. Bacteriol. **180:**5932–5946.
9. **Bokal, A. J., W. Ross, T. Gaal, R. C. Johnson, and R. L. Gourse.** 1997. Molecular anatomy of a transcription activation patch: FIS-RNA polymerase interactions at the *Escherichia coli rrnB* P1 promoter. EMBO J. **16:**154–162.
10. **Burgess, R. R., and J. J. Jendrisak.** 1975. A procedure for the rapid, large-scale purification of Escherichia coli DNA-dependent RNA polymerase involving Polymin P precipitation and DNA-cellulose chromatography. Biochemistry **14:**4634–4638.
11. **Case, C. C., S. M. Roels, J. E. Gonzalez, E. L. Simons, and R. W. Simons.** 1988. Analysis of the promoters and transcripts involved in IS*10* anti-sense RNA control. Gene **72:**219–236.
12. **Cashel, M., D. R. Gentry, V. J. Hernandez, and D. Vinella.** 1996. The stringent response, p. 1458–1496. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
13. **Claret, L., and J. Rouviere-Yaniv.** 1996. Regulation of HU alpha and HU beta by CRP and FIS in *Escherichia coli*. J. Mol. Biol. **263:**126–139.
14. **Condon, C., C. Squires, and C. L. Squires.** 1995. Control of rRNA transcription in *Escherichia coli*. Microbiol. Rev. **59:**623–645.
15. **Falconi, M., A. Brandi, A. La Teana, C. O. Gualerzi, and C. L. Pon.** 1996. Antagonistic involvement of FIS and H-NS proteins in the transcriptional control of *hns* expression. Mol. Microbiol. **19:**965–975.
16. **Falconi, M., G. Prosseda, M. Giangrossi, E. Beghetto, and B. Colonna.** 2001. Involvement of FIS in the H-NS-mediated regulation of *virF* gene of *Shigella* and enteroinvasive *Escherichia coli*. Mol. Microbiol. **42:**439–452.
17. **Finkel, S. E., and R. C. Johnson.** 1992. The Fis protein: it's not just for DNA inversion anymore. Mol. Microbiol. **6:**3257–3265.
18. **Gaal, T., M. S. Bartlett, W. Ross, C. L. Turnbough, Jr., and R. L. Gourse.** 1997. Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science **278:**2092–2097.
19. **Gaal, T., and R. L. Gourse.** 1990. Guanosine 3′-diphosphate 5′-diphosphate is not required for growth rate-dependent control of rRNA synthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**5533–5537.
20. **Goldberg, M. D., M. Johnson, J. C. Hinton, and P. H. Williams.** 2001. Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli*. Mol. Microbiol. **41:**549–559.
21. **Gonzalez-Gil, G., P. Bringmann, and R. Kahmann.** 1996. FIS is a regulator of metabolism in *Escherichia coli*. Mol. Microbiol. **22:**21–29.

22. **Green, J., M. F. Anjum, and J. R. Guest.** 1996. The *ndh*-binding protein (Nbp) regulates the *ndh* gene of *Escherichia coli* in response to growth phase and is identical to Fis. Mol. Microbiol. **20:**1043–1055.
23. **Hershberg, R., G. Bejerano, A. Santos-Zavaleta, and H. Margalit.** 2001. PromEC: an updated database of *Escherichia coli* mRNA promoters with experimentally identified transcriptional start sites. Nucleic Acids Res. **29:** 277.
24. **Jacobson, B. A., and J. A. Fuchs.** 1998. Multiple cis-acting sites positively regulate *Escherichia coli nrd* expression. Mol. Microbiol. **28:**1315–1322.
25. **Keener, J., and M. Nomura.** 1996. Regulation of ribosome biosynthesis, p. 1417–1431. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
26. **Laptenko, O., J. Lee, I. Lomakin, and S. Borukhov.** 2003. Transcript cleavage factors GreA and GreB act as transient catalytic components of RNA polymerase. EMBO J. **22:**6322–6334.
27. **Lisser, S., and H. Margalit.** 1993. Compilation of *E. coli* mRNA promoter sequences. Nucleic Acids Res. **21:**1507–1516.
28. **Mallik, P., T. S. Pratt, M. B. Beach, M. D. Bradley, J. Undamatla, and R. Osuna.** 2004. Growth phase-dependent regulation and stringent control of *fis* are conserved processes in enteric bacteria and involve a single promoter (*fis* P) in *Escherichia coli*. J. Bacteriol. **186:**122–135.
29. **Martin, R. G., and J. L. Rosner.** 1997. Fis, an accessorial factor for transcriptional activation of the *mar* (multiple antibiotic resistance) promoter of *Escherichia coli* in the presence of the activator MarA, SoxS, or Rob. J. Bacteriol. **179:**7410–7419.
30. **McLeod, S. M., S. E. Aiyar, R. L. Gourse, and R. C. Johnson.** 2002. The C-terminal domains of the RNA polymerase alpha subunits: contact site with Fis and localization during co-activation with CRP at the *Escherichia coli proP* P2 promoter. J. Mol. Biol. **316:**517–529.
31. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Murray, H. D., J. A. Appleman, and R. L. Gourse.** 2003. Regulation of the *Escherichia coli rrnB* P2 promoter. J. Bacteriol. **185:**28–34.
33. **Murray, H. D., and R. L. Gourse.** 2004. Unique roles of the *rrn* P2 rRNA promoters in *Escherichia coli*. Mol. Microbiol. **52:**1375–1387.
34. **Murray, H. D., D. A. Schneider, and R. L. Gourse.** 2003. Control of rRNA expression by small molecules is dynamic and nonredundant. Mol. Cell **12:**125–134.
35. **Nilsson, L., and V. Emilsson.** 1994. Factor for inversion stimulation-dependent growth rate regulation of individual tRNA species in *Escherichia coli*. J. Biol. Chem. **269:**9460–9465.
36. **Nilsson, L., H. Verbeek, E. Vijgenboom, C. van Drunen, A. Vanet, and L. Bosch.** 1992. FIS-dependent trans activation of stable RNA operons of *Escherichia coli* under various growth conditions. J. Bacteriol. **174:**921–929.
37. **Ninnemann, O., C. Koch, and R. Kahmann.** 1992. The *E. coli fis* promoter is subject to stringent control and autoregulation. EMBO J. **11:**1075–1083.
38. **Opalka, N., M. Chlenov, P. Chacon, W. J. Rice, W. Wriggers, and S. A. Darst.** 2003. Structure and function of the transcription elongation factor GreB bound to bacterial RNA polymerase. Cell **114:**335–345.
39. **Osuna, R., D. Lienau, K. T. Hughes, and R. C. Johnson.** 1995. Sequence, regulation, and functions of *fis* in *Salmonella typhimurium*. J. Bacteriol. **177:**2021–2032.
40. **Owens, R. M., G. Pritchard, P. Skipp, M. Hodey, S. R. Connell, K. H. Nierhaus, and C. D. O'Connor.** 2004. A dedicated translation factor controls the synthesis of the global regulator Fis. EMBO J. **23:**3375–3385.
41. **Paul, B. J., M. M. Barker, W. Ross, D. A. Schneider, C. Webb, J. W. Foster, and R. L. Gourse.** 2004. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell **118:**311–322.
42. **Paul, B. J., M. B. Berkmen, and R. L. Gourse.** 2005. DksA potentiates direct activation of amino acid promoters by ppGpp. Proc. Natl. Acad. Sci. USA **102:**7823–7828.
43. **Paul, B. J., W. Ross, T. Gaal, and R. L. Gourse.** 2004. rRNA transcription in *Escherichia coli*. Annu. Rev. Genet. **38:**749–770.
44. **Perederina, A., V. Svetlov, M. N. Vassylyeva, T. H. Tahirov, S. Yokoyama, I. Artsimovitch, and D. G. Vassylyev.** 2004. Regulation through the secondary channel—structural framework for ppGpp-DksA synergism during transcription. Cell **118:**297–309.
45. **Petersen, C., and L. B. Moller.** 2000. Invariance of the nucleoside triphosphate pools of *Escherichia col*i with growth rate. J. Biol. Chem. **275:**3931–3935.
46. **Pokholok, D. K., M. Redlak, C. L. Turnbough, Jr., S. Dylla, and W. M. Holmes.** 1999. Multiple mechanisms are used for growth rate and stringent control of *leuV* transcriptional initiation in *Escherichia coli*. J. Bacteriol. **181:**5771–5782.
47. **Pratt, T. S., T. Steiner, L. S. Feldman, K. A. Walker, and R. Osuna.** 1997. Deletion analysis of the *fis* promoter region in *Escherichia coli*: antagonistic effects of integration host factor and Fis. J. Bacteriol. **179:**6367–6377.
48. **Ross, W., J. F. Thompson, J. T. Newlands, and R. L. Gourse.** 1990. *E. coli* Fis protein activates ribosomal RNA transcription in vitro and in vivo. EMBO J. **9:**3733–3742.
49. **Ryals, J., R. Little, and H. Bremer.** 1982. Control of rRNA and tRNA syntheses in *Escherichia coli* by guanosine tetraphosphate. J. Bacteriol. **151:** 1261–1268.
50. **Sambrook, J. E., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
51. **Schneider, D. A., and R. L. Gourse.** 2004. Relationship between growth rate and ATP concentration in *Escherichia coli*: a bioassay for available cellular ATP. J. Biol. Chem. **279:**8262–8268.
52. **Sheikh, J., S. Hicks, M. Dall'Agnol, A. D. Phillips, and J. P. Nataro.** 2001. Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. Mol. Microbiol. **41:**983–997.
53. **Sosunova, E., V. Sosunov, M. Kozlov, V. Nikiforov, A. Goldfarb, and A. Mustaev.** 2003. Donation of catalytic residues to RNA polymerase active center by transcription factor Gre. Proc. Natl. Acad. Sci. USA **100:**15469–15474.
54. **Stebbins, C. E., S. Borukhov, M. Orlova, A. Polyakov, A. Goldfarb, and S. A. Darst.** 1995. Crystal structure of the GreA transcript cleavage factor from *Escherichia coli*. Nature **373:**636–640.
55. **Thompson, J. F., L. Moitoso de Vargas, C. Koch, R. Kahmann, and A. Landy.** 1987. Cellular factors couple recombination with growth phase: characterization of a new component in the lambda site-specific recombination pathway. Cell **50:**901–908.
56. **Vassylyeva, M. N., A. A. Perederina, V. Svetlov, S. Yokoyama, I. Artsimovitch, and D. G. Vassylyev.** 2004. Cloning, expression, purification, crystallization and initial crystallographic analysis of transcription factor DksA from *Escherichia coli*. Acta Crystallogr. D **60:**1611–1613.
57. **Walker, K. A., C. L. Atkins, and R. Osuna.** 1999. Functional determinants of the *Escherichia coli fis* promoter: roles of −35, −10, and transcription initiation regions in the response to stringent control and growth phase-dependent regulation. J. Bacteriol. **181:**1269–1280.
58. **Walker, K. A., P. Mallik, T. S. Pratt, and R. Osuna.** 2004. The *Escherichia coli fis* promoter is regulated by changes in the levels of its transcription initiation nucleotide CTP. J. Biol. Chem. **279:**50818–50828.
59. **Walker, K. A., and R. Osuna.** 2002. Factors affecting start site selection at the *Escherichia coli fis* promoter. J. Bacteriol. **184:**4783–4791.
60. **Wilson, R. L., S. J. Libby, A. M. Freet, J. D. Boddicker, T. F. Fahlen, and B. D. Jones.** 2001. Fis, a DNA nucleoid-associated protein, is involved in *Salmonella typhimurium* SPI-1 invasion gene expression. Mol. Microbiol. **39:**79–88.
61. **Wu, H., K. L. Tyson, J. A. Cole, and S. J. Busby.** 1998. Regulation of transcription initiation at the *Escherichia coli nir* operon promoter: a new mechanism to account for co-dependence on two transcription factors. Mol. Microbiol. **27:**493–505.
62. **Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel.** 1991. Residual guanosine 3′,5′-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem. **266:**5980–5990.
63. **Xu, J., and R. C. Johnson.** 1995. Identification of genes negatively regulated by Fis: Fis and RpoS comodulate growth-phase-dependent gene expression in *Escherichia coli*. J. Bacteriol. **177:**938–947.
64. **Zhang, X., P. Dennis, M. Ehrenberg, and H. Bremer.** 2002. Kinetic properties of *rrn* promoters in *Escherichia coli*. Biochimie **84:**981–996.
65. **Zhou, Y. N., and D. J. Jin.** 1998. The rpoB mutants destabilizing initiation complexes at stringently controlled promoters behave like "stringent" RNA polymerases in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **95:**2908–2913.