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Growth Phase and (p)ppGpp Control of IraD, a Regulator of RpoS Stability, in *Escherichia coli*[▽]

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The antiadaptor protein IraD inhibits the proteolysis of the alternative sigma factor, RpoS, which promotes the synthesis of >100 genes during the general stress response and during stationary phase. Our previous results showed that IraD determines RpoS steady-state levels during exponential growth and mediates its stabilization after DNA damage. In this study, we show by promoter fusions that *iraD* was upregulated during the transition from exponential growth to stationary phase. The levels of RpoS likewise rose during this transition in a partially IraD-dependent manner. The expression of *iraD* was under the control of ppGpp. The expression of *iraD* required RelA and SpoT (p)ppGpp synthetase activities and was dramatically induced by a "stringent" allele of RNA polymerase, culminating in elevated levels of RpoS. Surprisingly, DksA, normally required for transcriptional effects of the stringent response, repressed *iraD* expression, suggesting that DksA can exert regulatory effects independent of and opposing those of (p)ppGpp. Northern blot analysis and 5′ rapid amplification of cDNA ends revealed two transcripts for *iraD* in wild-type strains; the smaller was regulated positively by RelA during growth; the larger transcript was induced specifically upon transition to stationary phase and was RelA SpoT dependent. A reporter fusion to the distal promoter indicated that it accounts for growth-phase regulation and DNA damage inducibility. DNA damage inducibility occurred in strains unable to synthesize (p)ppGpp, indicating an additional mode of regulation. Our results suggest that the induction of RpoS during transition to stationary phase and by (p)ppGpp occurs at least partially through IraD.

Bacteria such as *Escherichia coli* control gene expression in response to environmental signals. One component of this control is the use of alternative sigma factors that change promoter recognition by RNA polymerase and therefore the repertoire of expressed genes. The transcription factor σ^S, or RpoS, controls the synthesis of more than 100 genes, many of which are induced during the stationary phase of growth (15, 16, 24, 33, 45). RpoS levels respond sensitively to growth rate (42) and σ^S accumulates during the transition from proliferative growth to quiescence (17, 26, 41). Even during exponential growth, σ^S controls gene expression in response to a wide variety of environmental insults, comprising the so-called "general stress response" (reviewed in references 19, 21, and 27).

RpoS is regulated at every conceivable level: transcriptional, translational, protein stability, and activity (reviewed in references 20 and 23), and the integration of signals that impinge on RpoS is complex. Posttranslational control appears to underlie much of the regulation of σ^S in response to stress during growth. During cell proliferation, σ^S is synthesized but rapidly degraded, with a half-life of 2 to 3 min, but RpoS becomes stabilized upon entry into stationary phase (26, 40). The ClpXP protease degrades RpoS but does not directly recognize the protein; degradation requires the adaptor molecule RssB (5, 30, 31, 38, 47), which binds RpoS directly (48). RssB levels are limiting in the cell (39) and can be titrated by increased RpoS synthesis. RssB is a two-component response regulator protein, but the consequence of phosphorylation of the aspartate in the predicted receiver domain (8) on RpoS stability is negligible (36).

In the presence of stress such as starvation or DNA damage, RpoS levels are induced by the synthesis of specific small antiadaptor molecules that bind RssB and prevent its action as an adaptor for RpoS proteolysis. The first of these to be discovered, IraP, is induced by phosphate starvation (11); IraD, the subject of the present study, is induced by DNA damage (9, 29); IraM is induced by magnesium starvation (9). Accumulation of any of these proteins leads to RpoS stabilization in vivo and protection from proteolysis in vitro (9, 11). The antiadaptor mechanism therefore allows the induction of situation-specific factors that can rapidly and transiently affect RpoS levels.

The induction of the σ^S regulon has been correlated with a second signal, the production of (p)ppGpp during the stringent response (reviewed in references 13 and 37). Levels of ppGpp (the more predominant of the two signaling molecules) and RpoS rise dramatically at the late exponential phase of growth (13, 17, 26, 41). (p)ppGpp is synthesized by two enzymes: RelA and SpoT (46). SpoT has dual functions and possesses the only known ppGpp hydrolase activity, regulated apart from its synthetase function (22, 32).

The best-studied (p)ppGpp response is the RelA-dependent stringent response to amino acid starvation mediated through effects of ppGpp on RNA polymerase activity. In the presence of uncharged tRNA, RelA is activated to synthesize (p)ppGpp

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(7). RNA polymerase bound by ppGpp has altered properties with respect to open complex formation, resulting in down-regulation of stable RNA synthesis and upregulation of certain biosynthetic operons. At rRNA promoters such as the well-studied *rrnB* P1 promoter, ppGpp disfavors initiation by preventing stable open complex formation (3, 4). Upregulation of biosynthetic promoters may be indirect, by reallocation of RNAP newly freed from stable RNA synthesis, but ppGpp can also directly activate transcription in vitro, in the presence of the small transcription factor DksA (35). In vivo, DksA is required for the full effect of (p)ppGpp on both negatively and positively regulated promoters (34, 35). However, DksA has effects on phenotypes such as adhesion, motility and chemotaxis in *relA spoT* double mutant strains [so-called "(p)ppGpp^0" strains] unable to synthesize (p)ppGpp (1, 28), suggesting that DksA can function independently of (p)ppGpp. Microarray and fusion experiments indicated that DksA and (p)ppGpp have opposing effects on a subset of genes primarily involved in fimbriae regulation, chemotaxis and motility (1, 2).

The connection between the stringent response and RpoS is complex. (p)ppGpp has been implicated in transcriptional (25), translational (12), and posttranslational (10) control of RpoS. Perhaps by a combination of these mechanisms, RpoS accumulates during the transition from exponential growth to stationary phase, and this is greatly diminished in (p)ppGpp^0 strains (17). Artificial induction of high levels of (p)ppGpp leads to elevated RpoS in the cell (12, 17). At least one level of control impinges on the antiadaptor mechanism of RpoS regulation: induction of *iraP* during phosphate starvation and stabilization of RpoS requires ppGpp synthesis capacity (10).

In the present study we examine the effects of the stringent response on the IraD antiadaptor. We identified *iraD* in a mutant screen for sensitivity to a variety of DNA damaging agents (29). In the presence of DNA damage such as oxidative stress or the replication inhibitor, azidothymidine (AZT), SOS-independent induction of *iraD* leads to RpoS stabilization (29). Like IraP, IraD directly binds RssB and prevents proteolysis of RpoS by ClpXP (9). Unlike IraP, which has effects only on RpoS after starvation, IraD appears to promote higher steady-state levels of RpoS, even in the absence of stress (29). We show here that *iraD* is growth phase regulated and that (p)ppGpp accumulation is necessary for full *iraD* induction during the transition to stationary phase. In strains containing "stringent" alleles of RNA polymerase that mimic the transcriptional effects after (p)ppGpp accumulation (49), *iraD* is constitutively induced. In such strains, RpoS levels are also induced, in a partially IraD-dependent manner, suggesting that IraD is a critical regulator of RpoS under conditions when ppGpp accumulates, such as during the stringent response and during transition to stationary phase.

MATERIALS AND METHODS

Strains, growth conditions. All strains used in the present study are listed in Table 1 and are isogenic with MG1655 (F$^-$ *rph-1*) (6). Cultures were grown at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics kanamycin (20 μg/ml), chloramphenicol (15 μg/ml), ampicillin (100 μg/ml), and/or tetracycline (10 μg/ml). Strains were constructed by P1 transductions and phage lysates grown in LCG (LB medium supplemented with 1% glucose and 2 mM calcium chloride). Strain 13119 [*btuB*::Tn*10 spoT*(E219Q)] was constructed with P1 donor CF11608 (provided by D. Jin [18]) crossed with MG1655, selecting tetracycline resistance; the presence of the *spoT* allele was confirmed by PCR, followed by sequence analysis. *relA*Δ::FRT and *dksA*Δ::FRT *cat* were both constructed by PCR amplification of the *cat* gene from pKD4 with *Taq* DNA polymerase, using primers carrying 40-nucleotide (nt) homology to the open reading frame (ORF) of each gene as described previously (14). After purification and DpnI digestion of the PCR fragment, it was transformed into MG1655 harboring recombination facilitator plasmid pKD46, selecting chloramphenicol resistance. pKD46 was subsequently cured by growth at 42°C.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Isogenic strains		
MG1655	Wild-type *E. coli* K-12, *rph-1*	
STL8198	Δ*relA*::FRT	This study
STL8246	Δ*dksA*::FRT *cat*	This study
CF1693	*relA*::*kan spoT207*::*cat*	46
STL11655[a]	*rpoB3370*	49
STL13119	*spoT*(*E319Q*)	This study
Plasmids		
iraD::*luxCDABE*		29
P1-$iraD_{600/375}$::*luxCDABE*		This study
P2-$iraD_{262/1}$::*luxCDABE*		This study

[a] Strain provided by D. Jin, denoted previously (49) as "MG1655 *rpoB3370*."

Plasmid constructions. Plasmid *iraD*::*luxCDABE* (29) was constructed as previously described. Plasmids P1-$iraD_{600/375}$::*luxCDABE* and P2-$iraD_{262/1}$::*luxCDABE* were constructed from the primer pairs 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CGT AAA CAA ATG ACA TGC ATG TTT CT-3′/5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC AAT TGA TAA CAT ATC AAA TAC ATC AAA AAT-3′ and 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CCT ATT CGG AAT ATT CAG AAT ATA CTA GCG G-3′/5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TTT GCG CAC TCC TGA CGT TTA GCA A-3′, respectively, as previously described (29).

Transcriptional start site (TSS) mapping. The 5′ ends of *iraD* mRNA were mapped by using 5′ RACE (rapid amplification of cDNA ends) as previously described (43) with the following modifications. cDNA was prepared by incubation of 2 μg of total RNA (isolated as described below) with 200 U of Moloney murine leukemia virus reverse transcriptase (NEB), 2 mM deoxynucleoside triphosphate, 40 μM *iraD* ORF specific primer (5′-TTA GCT GAC ATT CTC CAG CGT CGC ACT GCG-3′), and 1× Moloney murine leukemia virus RT buffer at 42°C for 1 h, followed by treatment with 10 μg of RNase A (Sigma)/ml and 5 U of RNase H (NEB) at 37°C for 30 min. cDNA was purified by using a QIAquick PCR purification kit (Qiagen). After ligation of cDNA with an anchor oligonucleotide (5′-TTT AGT GAG GGT TAA TAA GCG GCC GCG TCG TGA CTG GGA GCG C-3′), touchdown PCR was performed by using 1 U of Phusion polymerase (Finnzyme), 12 ng of purified anchored-cDNA, 0.5 μM anchor, and *iraD* ORF specific primers (5′-CTC CCA GTC ACG ACG CGG CCG C-3′ and 5′-CAA ACG TTA GCG GTT CAT CG-3′). The PCR cycling conditions were as follows: 98°C for 3 min (1 cycle); 98°C for 30 s to 72°C (at 1°C/cycle) for 1 min (15 cycles); 72°C for 1.5 min (1 cycle); 98°C for 30 s, 58°C for 1 min, and 72°C for 1.5 min (20 cycles); 72°C for 5 min (1 cycle); followed finally by holding at 4°C. PCR products were resolved on 1.5% agarose gels and bands were excised by using a QIAEX II gel extraction kit (Qiagen) and sequenced using the *iraD* ORF and anchor-specific primers (5′-TTC TTT CAA ATT AAC CTG CAA CGC C-3′ and 5′-TAA AAA GAG TGA GGA GAT CGC-3′, respectively).

RpoS levels and stability experiments. RpoS steady-state levels during growth were determined by Western blot analysis (29) from cultures harvested at the indicated phase of growth. The stability of RpoS was determined after the addition of chloramphenicol (200 μg/ml) to exponential-phase LB cultures harvested at the time points indicated. Protein was precipitated after a 30-min incubation on ice in 20% ice-cold trichloroacetic acid (TCA), and the pellet was resuspended in sodium dodecyl sulfate buffer. RpoS Western blots were performed as described previously (29).

Lux reporter assays. Luciferase reporter assays were performed as described previously (29, 44). For expression measurements throughout growth, cultures were inoculated and grown overnight at 37°C in LB medium containing only

ampicillin (100 μg/ml). After a 14-h incubation period, 25 ml of the same medium in the absence of antibiotics was inoculated with cells to an optical density at 600 nm (OD_{600}) of 0.05. Time points (OD_{600} and counts per minute) were determined every 20 min after a 90-min initial incubation. All cultures were grown with aeration throughout the time course, and time points were taken at 37°C without the introduction of variability in temperature. In AZT induction experiments, cultures were grown as described above and treated with 1 μg of AZT/ml for 40 min before measurements were taken. For *rpoB** experiments, time points were taken every 30 min after an initial 90-min incubation in 40 ml of LB medium. Luminescence from strains containing the reporter vector (with no inserted promoter region) was measured and did not significantly contribute to the reported data.

RNA purification and Northern blot analysis. Cells were grown in LB as described above, and samples for exponential phase were taken at an optical density of 0.7 (± 0.1) and for stationary phase at 1.6 (± 0.1). RNA was isolated by using the RiboPure -Bacteria kit from Ambion according to the manufacturer's instructions. Concentrations of RNA samples were determined by measuring the OD_{260}. Analysis of *iraD* mRNA by Northern blots was performed with 7.5 μg of total RNA per sample, loaded equally on a 1% agarose-formaldehyde denaturing gel, and ran in MOPS buffer. Samples were transferred onto a positively charged membrane (Amersham Hybond N+), cross-linked by using UV, and transcripts were detected by using a specific, 400-nt DNA probe complementary to *iraD*'s ORF in ExpressHyb Hybridization Solution (Clontech). RNA integrity and equal loading of each sample lane was monitored via methylene blue staining of the membranes prior to hybridization. The *iraD* specific probe was constructed from the PCR product generated by a forward primer (5′-CAA TCA CTT CAG GCT GTT TTA CC-3′) and a reverse primer (5′-TTA GCT GAC ATT CTC CAG CGT CGC ACT GCG-3′). The PCR product was labeled according to manufacturer's instructions using ATP-αP^{32} and the Prime-It II Random Primer Labeling Kit (Stratagene). The sizes of the apparent *iraD* transcripts were estimated by using RNA Century-Plus Markers (Ambion). The graphs below the Northern blots show quantification of each transcript in that condition relative to 16S rRNA as quantitated by phosphorImage analysis (GE Storm 860, with ImageQuant TL software), in the strain indicated. The standard deviation in the quantifications (shown with error bars) was determined by the product of the number of pixels and standard deviation of the pixel intensity as calculated by Adobe Photoshop.

RESULTS

Two TSSs for *iraD*. To define the promoters responsible for *iraD* expression, we performed 5′ RACE followed by sequence analysis of the resulting PCR product, both during exponential growth and during stationary phase (Fig. 1A). Sequence analysis of DNA generated from cells in exponential and/or stationary phase revealed two mRNA start sites; one at −417 (referred to as P1) and a second at −137 (referred to as P2), relative to the ATG start codon of the *iraD* ORF (Fig. 1A). A reasonable match to −10 and −35 consensus promoter sequences were found immediately upstream of both start sites (Fig. 1B). Interestingly, the ~800-nt transcript generated from the distal promoter (P1) was present during entry into stationary phase but not in exponential phase, whereas the ~500-nt transcript generated from the proximal promoter (P2) is present at both growth phases. There is no known protein coding region in this upstream region of *iraD*; the largest predicted ORF could encode a 47-amino-acid peptide.

***iraD* expression is growth phase dependent.** We examined *iraD* expression indirectly through fusion of 600 nt of its upstream intergenic region, including both promoter regions, to a luciferase operon reporter (29, 44) (*iraD*::*luxCDABE*) in a simple growth experiment. As apparent in Fig. 1C, *iraD* was expressed very highly in late exponential phase of growth, as determined from the optical density of the culture over time, and maintained at high levels during stationary phase. Expression reached a minimum as cells entered the middle exponential phase of growth.

The two *iraD* promoter regions show differences in their expression pattern. To dissect the contribution of each promoter site to the expression pattern of the full-length promoter fusion construct, we constructed two new luciferase reporters containing either the distal P1 or the proximal P2 TSSs. In a growth experiment, we found that the reporter fused to the region containing the distal P1 (spanning −600 to −375 relative to the start of the ORF) behaved similarly to the full-length promoter construct with a strong induction during the transition to stationary phase, although the expression levels were somewhat higher in exponential growth than the full-length promoter fusion. The reporter construct containing P2 (spanning −262 to −1 relative to the start of ORF) did not behave like the full-length fusion reporter, and showed expression levels that were low and steady (Fig. 1C), equivalent to that for the full-length fusion at its lowest point. These results suggest that the full-length promoter fusion construct may report the sum of both transcripts during exponential growth, with P1 predominating during the late stages of growth.

RpoS levels during normal growth are determined by IraD. Previously, we showed that IraD is involved in regulating RpoS stability in response to DNA damage (29). Based on the expression pattern of *iraD*, here we sought to determine whether IraD is involved in regulating RpoS levels in the late logarithmic phase of growth. Western blot analysis showed that RpoS levels begin to rise during the transition from exponential growth to stationary phase (Fig. 1D) as has been reported previously (12, 17). In mutants deficient in IraD, steady-state levels of RpoS were reduced by ca. 60% at mid to late exponential phase during growth, suggesting that IraD is an important factor promoting accumulation of RpoS as cells begin to enter stationary phase. Although at a much lower level, RpoS still accumulates in a similar time frame as in a wild-type background in the *iraD* mutant, indicating there are yet other factors influencing the timing of its accumulation.

***iraD* expression is positively influenced by both RelA and SpoT.** Because (p)ppGpp levels rise dramatically as cells enter the late exponential phase of growth, we examined the impact of the known (p)ppGpp synthetases on the expression of *iraD*. Using the *iraD*::*lux* reporter constructs, we looked at the expression profile of *iraD* in either a *relA* deletion mutant or in *spoT*(*E319Q*), a mutant specifically defective for (p)ppGpp synthetase activity (18). (We were unable to look at a *spoT*-null mutant since the ppGpp hydrolase activity of the protein is essential for survival for strains that can produce (p)ppGpp). We found that the expression of the gene was reduced in both the *relA* and *spoT*(*E319Q*) mutants strains throughout fast growth in the mid-exponential phase (Fig. 2A), although induction in late exponential phase was still evident. After overnight growth into the stationary phase, both mutants seemed to reach wild-type levels of *iraD* expression, suggesting that either *iraD* expression is independent of (p)ppGpp altogether during this time or that one of the two enzymes can compensate for the absence of the other (Table 2). Supporting the latter notion, a *relA spoT* double-knockout mutant resulted in even lower levels of expression from the *iraD*::*luxCDABE* reporter compared to that of either single knockout in early exponential phase (Fig. 2B) and in stationary phase, culminating in fivefold

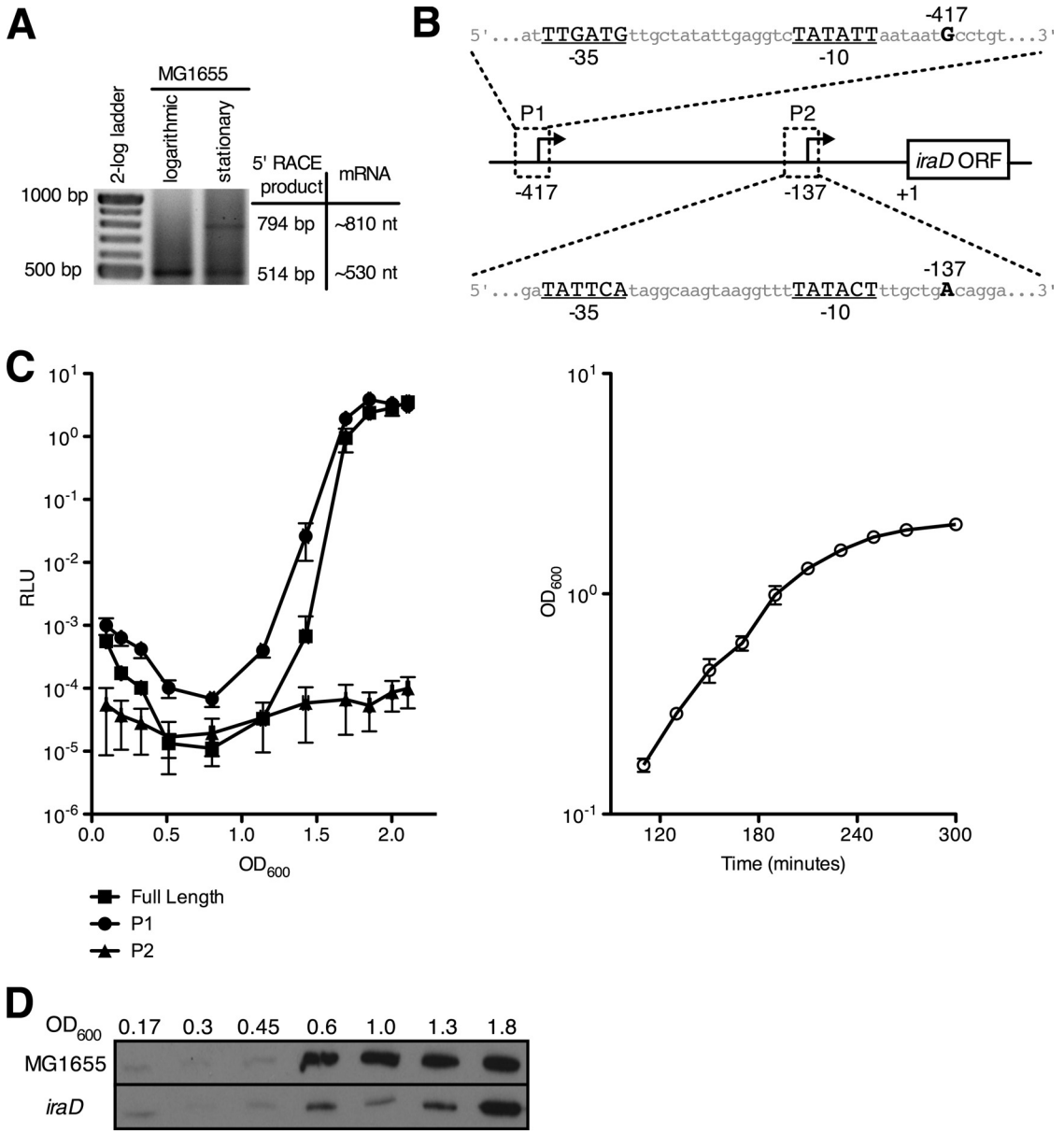

FIG. 1. Mapping of the *iraD* TSSs and correlation of *iraD* expression with growth phase and RpoS levels. (A) Agarose gel of 5′ RACE PCR products generated from MG1655 cells in either logarithmic or stationary phase as indicated. Sequencing of the PCR products revealed two products of 794 and 514 bp, predicting transcript lengths of ~800 and 500 nt, respectively. Neither transcript is present in Δ*iraD* cells (data not shown). (B) Map of the two TSSs identified in the *iraD* 5′ upstream region based on the sequencing data after the 5′ RACE shown in panel A. TSSs are indicated with their positions relative to the 5′ end of the *iraD* ORF, and putative −10 and −35 elements are indicated for each. The distal promoter is labeled as P1, and the proximal promoter is labeled as P2. (C) *iraD*::*luxCDABE* expression for the full-length promoter, the distal promoter P1, and the proximal promoter P2 throughout growth. Full-length reporter is a fusion of positions −600 to −1, the P1 reporter is a fusion of positions −600 to −375, and the P2 reporter is a fusion of positions −262 to −1 to luciferase (numbers are relative to the start of ORF-ATG). Each data point is an average of six independent determinations. The variability is shown with error bars in both graphs. RLU, relative luminescence units (bioluminescence counts per minute, normalized to the OD_{600}). The right panel shows the growth curve of wild-type cells in the experimental conditions used in the present study. The ODs are shown for time points of 20 min, starting at 90 min after the inoculation of each culture. (D) Steady-state RpoS levels in MG1655 and Δ*iraD* strains throughout growth. Samples were taken at ODs indicated, and TCA-precipitated as described in the methods section.

lower levels than in wild-type strains and single *relA* or *spoT*(*E319E*) mutants (Table 2). A fusion of the *recA* promoter to the same luciferase construct did not show any difference in expression in these (p)ppGpp-deficient backgrounds, suggesting that this is a specific effect on *iraD* and not the technique or the reporter fusion (data not shown). These results suggest that ppGpp production is one factor required for full induction of *iraD* expression during the transition to stationary phase of growth. We note, however, that in the absence of (p)ppGpp production, *iraD* induction does indeed occur as cells enter

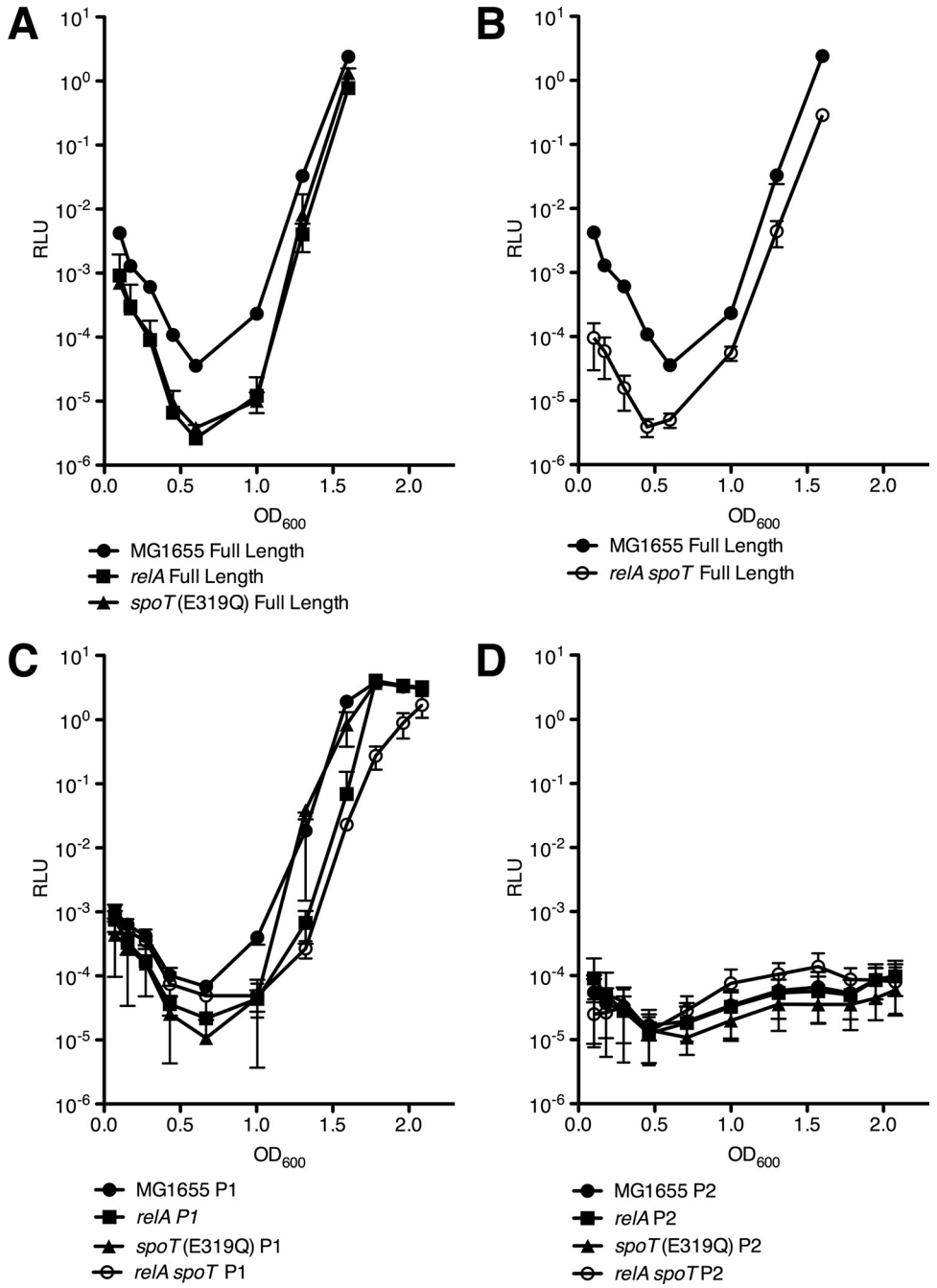

FIG. 2. *iraD*::*luxCDABE* expression in strains affected for (p)ppGpp synthesis. Each data point is an average of four independent determinations. The variability is shown with error bars in both graphs. RLU, relative luminescence units (bioluminescence counts per minute, normalized to the OD_{600}). Wild-type, *spoT*(*E319Q*), and *relA* on the graph represent expression in the MG1655, *spoT*(*E319Q*), and *relA*Δ::FRT strains, respectively, and *relA spoT* represents expression in the Δ*relA* Δ*spoT* double mutant. (A and B) Luciferase expression of the full-length *iraD*::*luxCDABE* fusion construct throughout growth. (C) Expression of a luciferase construct, fused to the −600 to −375 region of the *iraD* promoter containing the P1 start site at −417, throughout growth. (D) Expression of a luciferase construct, fused to the −262 to −1 region of the *iraD* upstream region containing the P2 start site at −137, throughout growth.

stationary phase, indicating an additional mode of growth regulation. This is quite similar to that observed for RpoS itself: in (p)ppGpp^0 strains, RpoS accumulation is delayed and modestly reduced, but not abolished, during the transition to stationary phase (12, 17).

To determine stringent regulation at each of the two promoter regions of *iraD*, we introduced the reporter constructs containing either the P1 or the P2 promoter regions into the various mutant backgrounds, and assayed their expression throughout growth. The luciferase reporter constructs showed that the distal segment of the *iraD* upstream region containing the P1 start site was positively regulated by (p)ppGpp, and

TABLE 2. Late-stationary-phase expression of *iraD*::*luxDCABE* in strains affecting the stringent response

Strain genotype	RLU	SD[a]
Wild type	2.8	0.52
Δ*relA*	2.6	0.086
spoT(*E319Q*)	2.4	0.17
Δ*relA* Δ*spoT*	0.52	0.078
Δ*dksA*	2.5	0.024

[a] Based on at least three independent determinations.

showed lower expression in the late logarithmic phase of growth in the *relA* mutant and in the *relA spoT* double-mutant backgrounds (Fig. 2C). In the *spoT*(*E319Q*) mutant, expression from the reporter construct spanning P1 was somewhat lower in late logarithmic phase (OD_{600} = 1.0) but not at later times ($OD_{600} \geq 1.5$) (Fig. 2C). In contrast, the reporter construct spanning P2 showed no significant change when it was introduced into the *relA* or *spoT* mutant backgrounds (Fig. 2D).

Both *iraD* transcripts are positively influenced by (p)ppGpp, in a growth phase dependent manner. We examined the (p)ppGpp regulation of *iraD* expression by Northern blot analysis. We were able to detect two transcripts, corresponding in length to those derived from promoter regions P1 and P2 through the *iraD* coding region. The longer transcript, presumably from distal promoter P1, was only detected in stationary phase cells. In agreement with the luciferase reporter assays, Northern blot analysis of the *iraD* transcripts showed positive regulation by (p)ppGpp. However, in contrast to the reporter assays, the Northern blot data revealed potential regulation by (p)ppGpp on the proximal promoter P2, in addition to the distal promoter P1. In exponential phase, levels of the 500-nt transcript were reduced dramatically by a mutation in *relA*, but less so by the *spoT*(*E319Q*) allele (Fig. 3); levels in the *relA spoT* double mutant were somewhat lower than *relA* alone. However, in stationary phase, the 500-nt transcript was not diminished, even in the *relA spoT* background. The larger stationary phase-specific transcript was virtually absent in the *relA spoT* double mutant (Fig. 3), although not significantly diminished by either *relA* or *spoT*(*E319Q*) alone.

RNA levels in Northern blot analysis confirmed the regulation of *iraD* transcripts by (p)ppGpp as apparent from the luciferase reporter assays, but indicated some discrepancies between the two that indicate a potentially more complex pattern of regulation. In particular, high levels of the smaller transcript did not correspond with high expression with the luciferase fusion. Whereas both small and large transcripts were almost equally abundant in stationary phase, expression from the fusion constructs appeared to be driven exclusively by the distal P1 promoter during stationary phase. The discrepancy between the two techniques may be due to postinitiation regulation of the transcripts, reflected in the reporter data but not at the level of *iraD* mRNA; alternatively, the reporter operon may be more sensitive to initiation or elongation effects specific to P2. Interestingly, each of the two transcripts had differential regulation with growth phase and responded to regulators of (p)ppGpp levels, although in different ways.

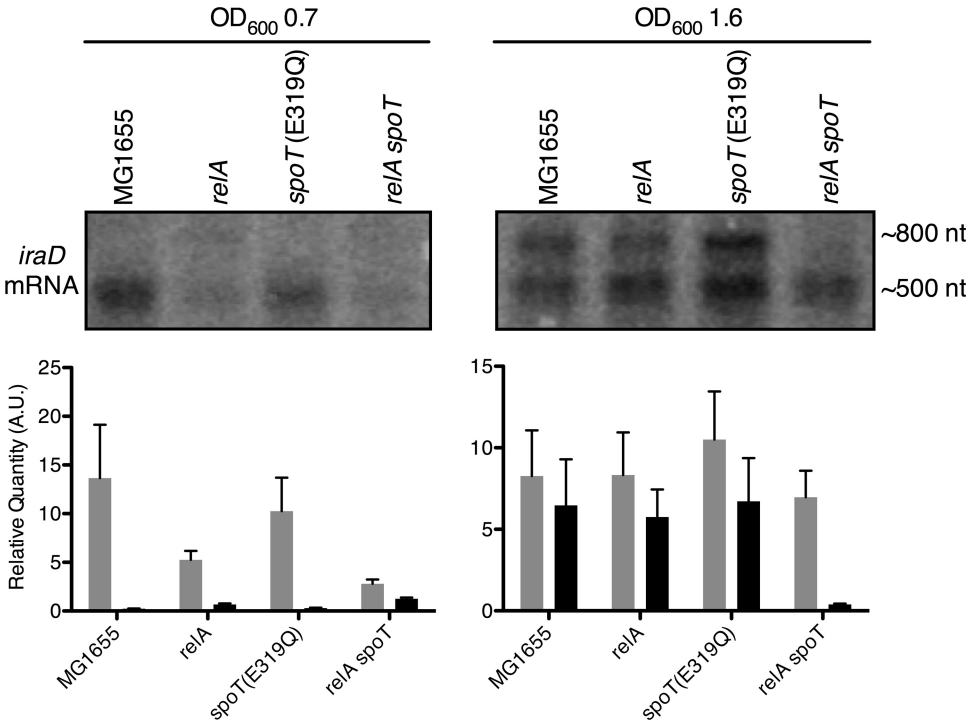

FIG. 3. Northern blot analysis of *iraD* transcripts in the MG1655, *spoT*(*E319Q*), *relA*Δ::FRT, and *relA*Δ *spoT*Δ strains. Samples were prepared from cells grown to either an OD of 0.7 (±0.1) or 1.6 (±0.1) as indicated. The graphs below the Northern blots show quantification of each transcript in that condition relative to 16S rRNA, in the strain indicated. Gray bars represent the smaller transcript of ~500 nt, and the black bars represent the larger transcript of 800 nt.

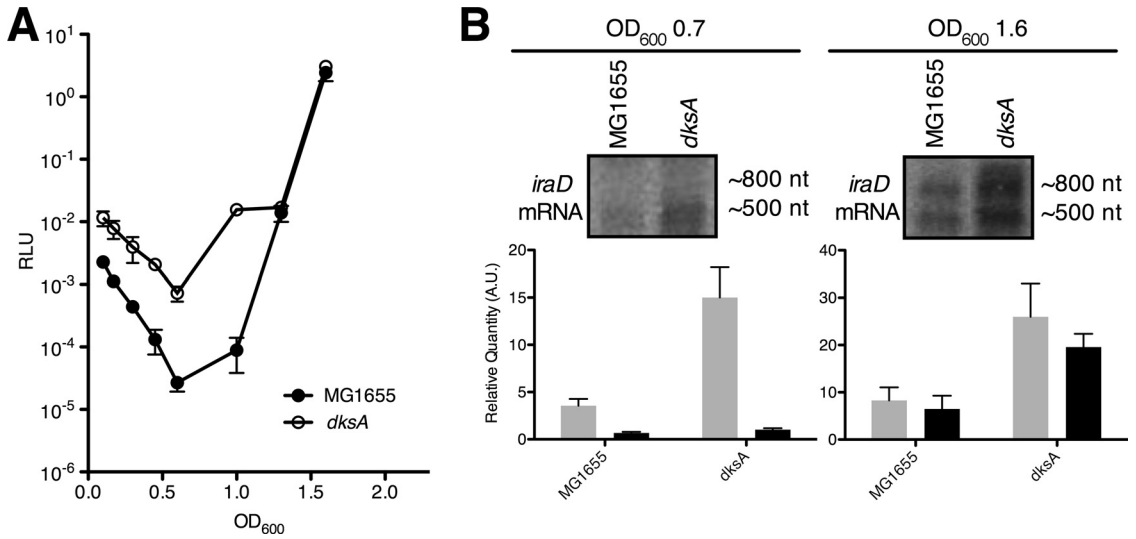

FIG. 4. Expression of the full-length IraD promoter fusion (*iraD*::*luxCDABE*) in strains lacking DksA. Each data point is an average of four independent determinations. The variability is shown with error bars in both graphs. RLU, relative luminescence units (bioluminescence counts per minute, normalized to the OD_{600}). (A) Wild-type and *dksA* strains on graph represent data from *iraD*::*luxCDABE* expression in the MG1655 and Δ*dksA*::FRT *cat* strains, respectively. (B) Northern blot analysis of *iraD* transcripts in the MG1655 and Δ*dksA*::FRT *cat* strains. Samples were prepared from cell grown to either an OD of 0.7 (±0.1) or 1.6 (±0.1) as indicated. The graphs below the Northern blots show quantification of each transcript in that condition relative to 16S rRNA loading control, in the strain indicated. Gray bars represent the smaller transcript of 500 nt, and the black bars represent the larger transcript of 800 nt.

DksA is a negative regulator of *iraD* transcription. DksA is a small protein that binds RNA polymerase and alters open complex formation on (p)ppGpp-regulated genes (34, 35). In vivo, DksA is required for the induction or repression of genes during the stringent response to amino acid starvation. We investigated a possible role for DksA in regulation of *iraD* by first examining expression of the full-length fusion of the upstream region (*iraD*::*luxCDABE*) in a *dksA*-null mutant. Surprisingly, the expression of *iraD*::*luxCDABE* was enhanced between 10 to 100-fold, depending on the growth phase of the cells, suggesting that DksA acts as a repressor of *iraD* expression (Fig. 4A). Overexpression of DksA from a high copy plasmid (data not shown), led to lower levels of *iraD*::*lux CDABE* expression, even in *relA spoT* strains, confirming a negative role of DksA on IraD expression.

Examination of *iraD* mRNA by Northern blots confirmed a repressive effect of DksA. In exponential phase, the 500-nt transcript was in greater abundance in the *dksA* mutant strain (Fig. 4D). In stationary phase, the level of the larger 800-nt transcript was likewise higher in the *dksA* mutant.

A stringent RNA polymerase highly induces *iraD* expression. To confirm the effect of the stringent response on *iraD*, we used a mutated version of RNA polymerase that mimics the "stringent" state (49). Interactions between stringent promoters and RNA polymerase are intrinsically unstable, and certain point mutations in the beta subunit of RNA polymerase (RpoB) core transcription complex exacerbate these interactions, thereby mimicking RNA polymerase bound to ppGpp during the stringent response (49). These "stringent" *rpoB* alleles reduce initiation at negatively regulated stringent promoters, such as those for rRNA, and increase expression of positively regulated stringent promoters. Therefore, if *iraD* is regulated by ppGpp, we would expect to see higher levels of expression from the *iraD*::*lux* reporter in any one of these stringent *rpoB* backgrounds.

We obtained one such allele, *rpoB3370*(*T563P*) (referred to below as *rpoB**), and looked at *iraD*'s expression in that strain background. As expected, *iraD*::*lux CDABE* was highly induced, by as much as 4 orders of magnitude, throughout the early, mid, and late exponential growth phases in the *rpoB** background relative to wild-type strains, supporting the model that *iraD* is stringently regulated (Fig. 5A). Similar induction by *rpoB** was seen for fusions carrying either P1 or P2 promoter regions (Fig. 5D and E), indicating that both promoters are probably regulated by the stringent response.

Induction of IraD by a stringent RNAP has consequences on RpoS levels. IraD appears a determinant of RpoS levels and stability (29) during normal exponential growth and, as shown here, during the transition to the stationary phase. To determine how much of the stringent response effect on RpoS was mediated through *iraD*, we examined RpoS steady-state levels and stability by Western blot analysis. Stability could be specifically ascertained by the addition of chloramphenicol to block new synthesis, followed by Western blotting with RpoS antibody. The effect of the *rpoB** mutation should increase and/or stabilize RpoS levels through IraD, IraP, and perhaps other antiadaptors (9, 11, 29).

As expected, there were higher levels of RpoS in the *rpoB** strain compared to the control wild-type strain (Fig. 5B and C). The elevated levels of RpoS protein detected in this strain were *iraD* dependent; this was apparent even when chloramphenicol was added to prevent new RpoS synthesis, supporting the fact that IraD effects on RpoS are posttranslational. A residual amount of RpoS was stabilized in *rpoB** strains compared to wild-type, even after the deletion of *iraD*, probably

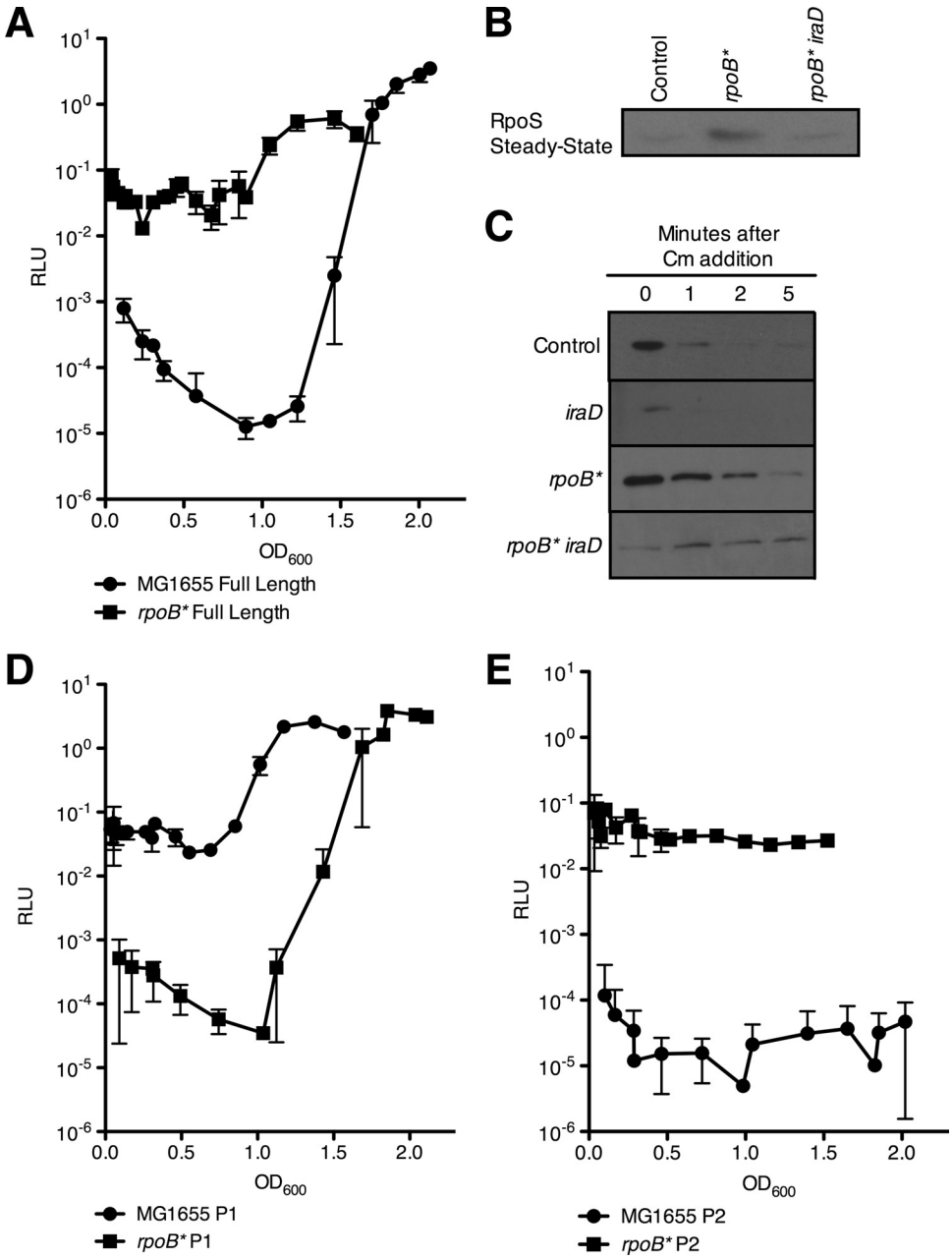

FIG. 5. Effects of a stringent *rpoB* allele on *iraD* transcription and RpoS levels. (A) *iraD*::*luxCDABE* expression from a −600 to −1 fusion construct in a "stringent" RNA polymerase mutant. Each data point is an average of three independent determinations. Wild-type and *rpoB** represent *iraD*::*luxCDABE* expression in the MG1655 and the *rpoB** strains, respectively. The variability is shown with error bars in both graphs. RLU, relative luminescence units (bioluminescence counts per minute, normalized to the OD_{600}). (B) Steady-state RpoS levels in MG1655 (Ctrl), *rpoB**, and *rpoB* iraD* double-mutant strains at a growth phase corresponding to an OD_{600} of 0.3. (C) RpoS stability in the same strains at the same growth stage, following chloramphenicol treatment to block new protein synthesis. Levels of RpoS are shown at times indicated after chloramphenicol treatment. (D and E) Luciferase expression from promoter fusions to P1 (−600 to −375) (D) or P2 (−262 to −1) of the *iraD* upstream region (E). The data represent averages of at least three determinations.

through the action of other stringently regulated antiadaptors such as IraP (10).

DNA damage induction of *iraD* is not dependent on the stringent response and is mediated by the distal promoter. We showed previously that *iraD* is highly induced after DNA damage by AZT or oxidative stress and that this induction is independent of the SOS response (29). To determine a role for (p)ppGpp in this induction, we assayed the *iraD*::*luxCDABE* reporter in the absence of (p)ppGpp or *dksA*. After AZT treatment, *iraD*::*luxCDABE* was induced to wild-type levels in $(p)ppGpp^0$ strains, implying that (p)ppGpp is not necessary for the induction of *iraD* after DNA damage (Fig. 6A). In the *dksA*-null strain, the induction after AZT treatment was higher than that of the wild-type, probably due to the derepression of

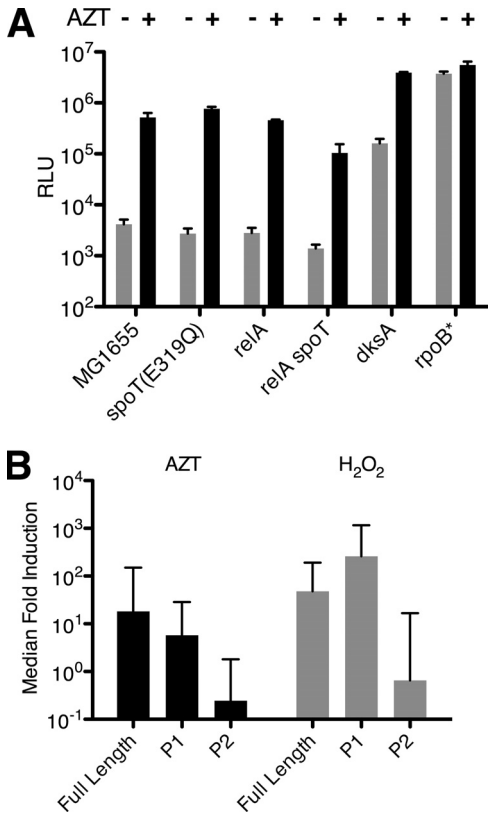

FIG. 6. Expression of various *iraD*::*luxCDABE* reporters in response to DNA damage. (A) Gray bars show expression in response to H_2O (Ctrl), and black bars show expression in response to 1 μg of AZT/ml for at least two isolates. RLU, relative luminescence units (bioluminescence counts per minute, normalized to the OD_{600}). (B) *iraD*::*luxCDABE* expression for the 600-bp promoter fusion (P1 and P2), the distal promoter P1, and the proximal promoter P2 after treatment with either AZT or peroxide as indicated, normalized to untreated controls. Each data point represents the median value derived from data from at least eight isolates.

the promoter in these backgrounds as we observed earlier. We did not observe any induction in the *rpoB** strain background, probably because the gene is maximally induced even before AZT treatment (Fig. 5A).

Since we observed differences in the growth-phase regulation of the two transcripts of *iraD*, we wondered whether the DNA damage induction was also different between the two promoters. To address this using the luciferase fusion constructs, we looked at induction by AZT and peroxide of either the distal or the proximal promoter regions compared to the wild type. Interestingly, the distal promoter fusion containing the P1 start site showed strong induction with AZT treatment as well as with peroxide treatment, whereas the proximal promoter fusion containing the P2 start site did not (Fig. 6B). This suggests that, similar to growth-phase regulation, the regulation of *iraD* expression in response to DNA damage may be predominantly at the distal promoter. The biological readout of *iraD* expression, accumulation of RpoS after DNA damage and during the transition to stationary phase, therefore appears to correlate with expression from the distal promoter. The role of the proximal promoter, if any, to *iraD* expression remains to be determined.

DISCUSSION

Expression of *iraD* is (p)ppGpp dependent. Expression of *iraD*, as detected by a luciferase fusion, varies dramatically during the growth of cultures and is induced as cells begin to starve and enter the stationary phase of growth in rich medium. Similar to another antiadaptor, IraP, IraD expression seems to be regulated by (p)ppGpp, the signaling molecule of the stringent response, produced by either RelA and SpoT. In RNA polymerase mutants that mimic the stringent transcriptional state even in the absence of ppGpp production (49), *iraD* expression as detected by luciferase fusions was dramatically elevated, particularly in the exponential phase of growth when its levels increased by more than 4 orders of magnitude. In this *rpoB** mutant, growth phase effects on *iraD* expression were minimized, with expression high throughout. This suggests that (p)ppGpp has the potential to modulate IraD not only during the transition to stationary phase, when its levels begin to accumulate, but also very sensitively in the mid-exponential phase of growth, when (p)ppGpp production is presumably low or transient in nature. This regulation by (p)ppGpp may be either direct or indirect via other regulatory factors, although we note that *iraD* putative promoter P1 has a AT-rich "discriminator" region downstream of the −10 sequence, a finding consistent with direct positive regulation by ppGpp.

Levels of ppGpp (which predominates over pppGpp) increase in response to a number of cellular stresses, the best studied of which is amino acid starvation, and naturally increase during late stages of exponential growth when nutrients begin to be depleted (reviewed in references 13 and 37). RelA, a ribosome-associated factor, is responsible for (p)ppGpp synthesis during translational stalling that accompanies amino acid starvation; SpoT has been associated with ppGpp synthesis associated with energy stress, such as carbon starvation.

As detected by promoter fusions to the luciferase operon, *iraD* expression was dependent on (p)ppGpp production during all phases of growth, with a reduction of >10-fold at the mid-exponential phase of growth and ~5-fold in the stationary phase. Surprisingly, the low level of *iraD* expression seen during exponential phase was reduced by both the loss of RelA and the ppGpp synthetase activity of SpoT (specifically affected by the SpoT E319Q mutation), indicating that both enzymes are required for maximal IraD expression in this growth phase. This is a puzzling result, since SpoT and RelA act independently and respond to different stimuli; this result may be explained by upregulation of SpoT (p)ppGpp hydrolase activity in the *spoT*(*E319Q*) mutant, causing a decrease in RelA-dependent (p)ppGpp synthesis in this mutant. In contrast, high levels of expression of *iraD* as cells were in transition to stationary phase were dependent on (p)ppGpp production by either RelA or SpoT, with a strong reduction seen only in the double mutant strain. Nutrient depletion in rich medium, such as that used in the present study, is likely to involve a complex combination of factors, with contributions to (p)ppGpp production by both RelA and SpoT.

Although both IraP and IraD antiadaptors appear to be regulated by the stringent response, there is a notable differ-

ence. The effect of ppGpp on IraP appear to be restricted to an integrated signal with phosphate starvation (10). In contrast, the effects of the stringent response on *iraD* appeared to be independent of its induction through DNA damage.

Furthermore, our results show that IraD is a determinant of RpoS levels during the transition to stationary phase, when high levels of (p)ppGpp begin to accumulate. An allele of *rpoB*, encoding the beta subunit of RNA polymerase, that causes the enzyme to display stringent transcription characteristics (49) constitutively caused a dramatic increase in *iraD* expression and elevated levels of RpoS levels, partially dependent on IraD. In the absence of IraD, RpoB* mutants had lower levels of RpoS, which were nonetheless stable, suggesting that other antiadaptors, including IraP, may substitute for IraD but with limited capacity relative to IraD.

DksA inhibits IraD expression. DksA, a small transcription factor involved in the stringent response, acted primarily as a negative regulator of *iraD* through mid-exponential growth, and these effects were diminished in stationary phase. This effect could be direct or indirect.

Surprisingly, we found that, unlike many stringently regulated genes, the regulatory effects of ppGpp and DksA on *iraD* are opposing: (p)ppGpp is a positive regulator, whereas DksA is a negative regulator of the gene. The opposing and independent relationship between DksA and (p)ppGpp in *iraD*'s regulation is similar to that found in a previous study wherein (p)ppGpp promoted and DksA antagonized cell adhesion, presumably a consequence of effects on *fimB* expression (2), as cultures entered stationary phase (28). DksA and (p)ppGpp also have opposing effects on a number of genes, primarily involved in chemotaxis and motility (1). The *iraD* gene was not found among this set but may have been missed because of growth condition differences or due to difficulty in the detection of the *iraD* transcript. The mechanism of this antagonistic and (p)ppGpp-independent effect by DksA on some genes appears to be competition with the GreA/B elongation factors (1, 2).

IraD transcripts. Northern blot analysis confirmed features revealed by the gene fusion studies and presented new complexities. We detected two primary transcripts in wild-type strains: an ~500-nt RNA in exponential phase and an 800-nt transcript present in the stationary phase of growth. We mapped the start sites corresponding to these transcripts by sequence analysis and 5′ RACE at positions −137 and −417 relative to the start codon of *iraD*, which are juxtaposed to identifiable promoter sequences, P2 and P1, respectively. Analyses of these regions fused to luciferase are consistent with these as the two promoters for *iraD*, with the distal promoter induced strongly during stationary phase.

There are some discrepancies between the full-length fusion reporter and RNA analysis. One difference is that the luciferase fusion exhibited an increase of many orders of magnitude in expression in late exponential phase, whereas the Northern analysis indicates a modest increase in the total RNA levels, with a dramatic increase only in the larger transcript. Reporter fusions suggested that only the distal promoter, P1, contributed to late-exponential-phase induction of *iraD*. This suggests that the larger 800-nt transcript emanating from P1 has properties that improve the elongation, stability, or translatability of the mRNA as reflected in luciferase production. This property of the larger transcript is also apparent in the partial fusion reporter containing the distal promoter P1 compared to the proximal promoter P2.

A need for posttranscriptional activation of the P2 transcript could explain a number of the observed discrepancies between the reporter data and Northern blots. This includes reduction of transcript levels by RelA, but with no reduction in expression detected by the P2 luciferase reporter construct. An additional mode of regulation of *iraD* in the exponential phase could promote a rapid, sensitive, and potentially transient activation during this growth phase.

Our results suggest that the growth-phase regulation of *iraD* is mediated by a combination of (p)ppGpp-dependent positive regulation and DksA-dependent negative regulation and possibly other factors. Even in (p)ppGpp^0 strains, *iraD* expression, as detected by the reporter fusions, was growth phase dependent, indicating potentially an additional mode of growth regulation. Both P1 and P2 transcripts were positively regulated by (p)ppGpp, although the small transcript became (p)ppGpp independent during the stationary phase. The small transcript required RelA predominantly during exponential growth, whereas the larger requires either RelA or SpoT during the transition to stationary phase. This suggests that (p)ppGpp production is primarily RelA dependent in growing cells but switches to more balanced production by both RelA and SpoT in the late exponential phase. Both transcripts were repressed by DksA, with higher levels in *dksA* mutants. Again, as with (p)ppGpp, the smaller transcript became resistant to DksA effects during the stationary phase, indicating a switch in its regulation.

Connection between stringent response and RpoS through IraD. We observed IraD-dependent stabilization of RpoS during normal growth and in stringent-mimic RpoB* cells. The accumulation of RpoS as cells enter the stationary phase was primarily dependent on IraD. A previous report (12) did not detect stabilization of RpoS during accumulation of (p)ppGpp, although we note that this latter experiment was performed with different growth conditions (minimal versus rich medium growth), and the reported RpoS half-life was much longer than what we observed (5 min versus <1 min). RpoS accumulation with (p)ppGpp may involve a number of influences, including both increases in translational efficiency, as suggested in the previous study, and protein stability, as suggested by our study. The only known biochemical function of IraD is to bind the adaptor RssB that targets RpoS for proteolysis by ClpXP (9); however, it is also conceivable that IraD either directly or indirectly affects other factors that influence RpoS levels.

Both RpoS and (p)ppGpp lead to major changes in gene expression in response to cellular stress and different growth conditions. These two systems may be connected through the regulation of RpoS at many levels, including transcriptional, translational, and protein stability. Our results raise the possibility that (p)ppGpp may regulate RpoS, at least in part, through effects on expression of the stabilizing factor, IraD.

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REFERENCES

1. **Aberg, A., J. Fernández-Vázquez, J. D. Cabrer-Panes, A. Sánchez, and C. Balsalobre.** 2009. Similar and divergent effects of ppGpp and DksA deficiencies on transcription in *Escherichia coli*. J. Bacteriol. **191:**3226–3236.
2. **Aberg, A., V. Shingler, and C. Balsalobre.** 2008. Regulation of the *fimB* promoter: a case of differential regulation by ppGpp and DksA in vivo. Mol. Microbiol. **67:**1223–1241.
3. **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.
4. **Barker, M. M., and R. L. Gourse.** 2001. Regulation of rRNA transcription correlates with nucleoside triphosphate sensing. J. Bacteriol. **183:**6315–6323.
5. **Bearson, S. M., W. H. Benjamin, Jr., W. E. Swords, and J. W. Foster.** 1996. Acid shock induction of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. J. Bacteriol. **178:**2572–2579.
6. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
7. **Block, R., and A. W. Haseltine.** 1975. Purification and properties of stringent factor. J. Biol. Chem. **250:**1212–1217.
8. **Bouche, S., E. Klauck, D. Fischer, M. Lucassen, K. Jung, and R. Hengge-Aronis.** 1998. Regulation of RssB-dependent proteolysis in *Escherichia coli*: a role for acetyl phosphate in a response regulator-controlled process. Mol. Microbiol. **27:**787–795.
9. **Bougdour, A., C. Cunning, P. J. Baptiste, T. Elliott, and S. Gottesman.** 2008. Multiple pathways for regulation of σ^S (RpoS) stability in *Escherichia coli* via the action of multiple anti-adaptors. Mol. Microbiol. **68:**298–313.
10. **Bougdour, A., and S. Gottesman.** 2007. ppGpp regulation of RpoS degradation via anti-adaptor protein IraP. Proc. Natl. Acad. Sci. USA **104:**12896–12901.
11. **Bougdour, A., S. Wickner, and S. Gottesman.** 2006. Modulating RssB activity: IraP, a novel regulator of σ^S stability in *Escherichia coli*. Genes Dev. **20:**884–897.
12. **Brown, L., D. Gentry, T. Elliott, and M. Cashel.** 2002. DksA affects ppGpp induction of RpoS at a translational level. J. Bacteriol. **184:**4455–4465.
13. **Cashel, M., D. R. Gentry, V. H. 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*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
14. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
15. **Dong, T., M. G. Kirchhof, and H. E. Schellhorn.** 2008. RpoS regulation of gene expression during exponential growth of *Escherichia coli* K12. Mol. Genet. Genomics **279:**267–277.
16. **Dong, T., and H. E. Schellhorn.** 2009. Control of RpoS in global gene expression of *Escherichia coli* in minimal media. Mol. Genet. Genomics **281:**19–33.
17. **Gentry, D. R., V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel.** 1993. Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. J. Bacteriol. **175:**7982–7989.
18. **Harinarayanan, R., H. Murphy, and M. Cashel.** 2008. Synthetic growth phenotypes of *Escherichia coli* lacking ppGpp and transketolase A (*tktA*) are due to ppGpp-mediated transcriptional regulation of *tktB*. Mol. Microbiol. **69:**882–894.
19. **Hengge-Aronis, R.** 1996. Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. Mol. Microbiol. **21:**887–893.
20. **Hengge-Aronis, R.** 2002. Recent insights into the general stress response regulatory network in *Escherichia coli*. J. Mol. Microbiol. Biotechnol. **4:**341–346.
21. **Hengge-Aronis, R.** 2002. Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. Microbiol. Mol. Biol. Rev. **66:**373–395.
22. **Hernandez, V. J., and H. Bremer.** 1991. *Escherichia coli* ppGpp synthetase II activity requires *spoT*. J. Biol. Chem. **266:**5991–5999.
23. **Klauck, E., A. Typas, and R. Hengge.** 2007. The sigmaS subunit of RNA polymerase as a signal integrator and network master regulator in the general stress response in *Escherichia coli*. Sci. Prog. **90:**103–127.
24. **Lacour, S., and P. Landini.** 2004. SigmaS-dependent gene expression at the onset of stationary phase in *Escherichia coli*: function of σ^S-dependent genes and identification of their promoter sequences. J. Bacteriol. **186:**7186–7195.
25. **Lange, R., D. Fischer, and R. Hengge-Aronis.** 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli*. J. Bacteriol. **177:**4676–4680.
26. **Lange, R., and R. Hengge-Aronis.** 1994. The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. Genes Dev. **8:**1600–1612.
27. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor sigma S (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
28. **Magnusson, L. U., B. Gummesson, P. Joksimovic, A. Farewell, and T. Nystrom.** 2007. Identical, independent, and opposing roles of ppGpp and DksA in *Escherichia coli*. J. Bacteriol. **189:**5193–5202.
29. **Merrikh, H., A. E. Ferrazzoli, A. Bougdour, A. Olivier-Mason, and S. T. Lovett.** 2009. A DNA damage response in *Escherichia coli* involving the alternative sigma factor, RpoS. Proc. Natl. Acad. Sci. USA **106:**611–616.
30. **Moreno, M., J. P. Audia, S. M. Bearson, C. Webb, and J. W. Foster.** 2000. Regulation of sigma S degradation in *Salmonella enterica* var. typhimurium: in vivo interactions between sigma S, the response regulator MviA(RssB) and ClpX. J. Mol. Microbiol. Biotechnol. **2:**245–254.
31. **Muffler, A., D. Fischer, S. Altuvia, G. Storz, and R. Hengge-Aronis.** 1996. The response regulator RssB controls stability of the σ^S subunit of RNA polymerase in *Escherichia coli*. EMBO J. **15:**1333–1339.
32. **Murray, K. D., and H. Bremer.** 1996. Control of *spoT*-dependent ppGpp synthesis and degradation in *Escherichia coli*. J. Mol. Biol. **259:**41–57.
33. **Patten, C. L., M. G. Kirchhof, M. R. Schertzberg, R. A. Morton, and H. E. Schellhorn.** 2004. Microarray analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. Mol. Genet. Genomics **272:**580–591.
34. **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.
35. **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.
36. **Peterson, C. N., N. Ruiz, and T. J. Silhavy.** 2004. RpoS proteolysis is regulated by a mechanism that does not require the SprE (RssB) response regulator phosphorylation site. J. Bacteriol. **186:**7403–7410.
37. **Potrykus, K., and M. Cashel.** 2008. (p)ppGpp: still magical? Annu. Rev. Microbiol. **62:**35–51.
38. **Pratt, L. A., and T. J. Silhavy.** 1996. The response regulator SprE controls the stability of RpoS. Proc. Natl. Acad. Sci. USA **93:**2488–2492.
39. **Pruteanu, M., and R. Hengge-Aronis.** 2002. The cellular level of the recognition factor RssB is rate-limiting for σ^S proteolysis: implications for RssB regulation and signal transduction in σ^S turnover in *Escherichia coli*. Mol. Microbiol. **45:**1701–1713.
40. **Schweder, T., K. H. Lee, O. Lomovskaya, and A. Matin.** 1996. Regulation of *Escherichia coli* starvation sigma factor (σ^S) by ClpXP protease. J. Bacteriol. **178:**470–476.
41. **Tanaka, K., Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi.** 1993. Heterogeneity of the principal sigma factor in *Escherichia coli*: the *rpoS* gene product, sigma 38, is a second principal sigma factor of RNA polymerase in stationary-phase *Escherichia coli*. Proc. Natl. Acad. Sci. USA **90:**3511–3515.
42. **Teich, A., S. Meyer, H. Y. Lin, L. Andersson, S. Enfors, and P. Neubauer.** 1999. Growth rate related concentration changes of the starvation response regulators σ^S and ppGpp in glucose-limited fed-batch and continuous cultures of *Escherichia coli*. Biotechnol. Prog. **15:**123–129.
43. **Troutt, A. B., M. G. McHeyzer-Williams, B. Pulendran, and G. J. Nossal.** 1992. Ligation-anchored PCR: a simple amplification technique with single-sided specificity. Proc. Natl. Acad. Sci. USA **89:**9823–9825.
44. **Van Dyk, T. K., Y. Wei, M. K. Hanafey, M. Dolan, M. J. Reeve, J. A. Rafalski, L. B. Rothman-Denes, and R. A. LaRossa.** 2001. A genomic approach to gene fusion technology. Proc. Natl. Acad. Sci. USA **98:**2555–2560.
45. **Weber, H., T. Polen, J. Heuveling, V. F. Wendisch, and R. Hengge.** 2005. Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and sigma factor selectivity. J. Bacteriol. **187:**1591–1603.
46. **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.
47. **Zhou, Y., and S. Gottesman.** 1998. Regulation of proteolysis of the stationary-phase sigma factor RpoS. J. Bacteriol. **180:**1154–1158.
48. **Zhou, Y., S. Gottesman, J. R. Hoskins, M. R. Maurizi, and S. Wickner.** 2001. The RssB response regulator directly targets σ^S for degradation by ClpXP. Genes Dev. **15:**627–637.
49. **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.