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RESEARCH ARTICLE

DksA and ppGpp Regulate the σ^S Stress Response by Activating Promoters for the Small RNA DsrA and the Anti-Adapter Protein IraP

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ABSTRACT σ^S is an alternative sigma factor, encoded by the *rpoS* gene, that redirects cellular transcription to a large family of genes in response to stressful environmental signals. This so-called σ^S general stress response is necessary for survival in many bacterial species and is controlled by a complex, multifactorial pathway that regulates σ^S levels transcriptionally, translationally, and posttranslationally in *Escherichia coli*. It was shown previously that the transcription factor DksA and its cofactor, ppGpp, are among the many factors governing σ^S synthesis, thus playing an important role in activation of the σ^S stress response. However, the mechanisms responsible for the effects of DksA and ppGpp have not been elucidated fully. We describe here how DksA and ppGpp directly activate the promoters for the anti-adaptor protein IraP and the small regulatory RNA DsrA, thereby indirectly influencing σ^S levels. In addition, based on effects of $DksA_{N88I}$, a previously identified DksA variant with increased affinity for RNA polymerase (RNAP), we show that DksA can increase σ^S activity by another indirect mechanism. We propose that by reducing rRNA transcription, DksA and ppGpp increase the availability of core RNAP for binding to σ^S and also increase transcription from other promoters, including P*dsrA* and P*iraP*. By improving the translation and stabilization of σ^S, as well as the ability of other promoters to compete for RNAP, DksA and ppGpp contribute to the switch in the transcription program needed for stress adaptation.

IMPORTANCE Bacteria spend relatively little time in log phase outside the optimized environment found in a laboratory. They have evolved to make the most of alternating feast and famine conditions by seamlessly transitioning between rapid growth and stationary phase, a lower metabolic mode that is crucial for long-term survival. One of the key regulators of the switch in gene expression that characterizes stationary phase is the alternative sigma factor σ^S. Understanding the factors governing σ^S activity is central to unraveling the complexities of growth, adaptation to stress, and pathogenesis. Here, we describe three mechanisms by which the RNA polymerase binding factor DksA and the second messenger ppGpp regulate σ^S levels.

KEYWORDS DksA, *Escherichia coli*, ppGpp, regulation of gene expression, small RNA, starvation, stress response

In nature, bacteria are in constantly changing environments and are frequently assaulted by stressors. Accordingly, bacteria have evolved various cellular stress responses to survive and adapt to such unpredictable threats. One such adaptation is the ability to transition efficiently from exponential growth in rich nutrients to the stagnant, nutrient-exhausted state known as stationary phase. This transition into

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stationary phase is governed by the alternative sigma factor σ^S (RpoS) and is often referred to as the general stress response. Although σ^S induction is commonly associated with entry into stationary phase, it can also be triggered by several other environmental stresses, such as starvation for specific nutrients (e.g., carbon, nitrogen, or phosphate), oxidative stress, hyperosmotic shock, and changes in temperature or pH (reviewed in references 1 and 2). In response to these stressors, σ^S redirects RNA polymerase (RNAP) to transcribe a large family of genes known as the σ^S regulon that comprises approximately 10% of the *Escherichia coli* genome (3). These genes are responsible for a highly coordinated cellular response involving diverse functions, such as DNA repair, stress (ethanol, UV, or oxidation) protection, nutrient scavenging, and cellular maintenance (reviewed in reference 4).

σ^S has also been implicated in regulating the expression of virulence factors in some pathogens. For example, in *Salmonella* spp., σ^S is required for expression of both the plasmid-borne *spv* virulence genes and all five chromosomally encoded pathogenicity islands (5, 6). Further supporting the role of σ^S in virulence regulation, *rpoS* deletion mutants of *Salmonella enterica* serovar Typhimurium are attenuated in virulence and unable to cause infection in a murine model (7).

Because σ^S responds to multiple signals, the mechanisms governing its activity and expression are complex. *trans*-acting factors positively or negatively regulate virtually every step in σ^S gene expression, including *rpoS* transcription, mRNA transcript stability, translation of the *rpoS* mRNA, and σ^S protein stability. Transcription from the *rpoS* gene is initiated at an internal promoter within the upstream gene *nlpD* located 567 nucleotides upstream of the *rpoS* AUG start codon (8). This results in a long 5′ untranslated region (UTR) with a secondary structure that is the basis for posttranscriptional regulation of the *rpoS* transcript, because it creates an inhibitory stem-loop that obscures the ribosome binding site (9). It has been reported that the effects of this inhibitory stem-loop are alleviated by competitive binding of the DsrA (10, 11), RprA (12), and ArcZ small noncoding RNAs (13). These small RNAs (sRNAs) are assisted by the chaperone Hfq in binding to the 5′ UTR, revealing the translation start site and stabilizing the mRNA transcript (10, 14).

After translation and in the absence of stress signals, σ^S is recruited by the adaptor protein RssB, which delivers σ^S to the protease ClpXP for degradation (15, 16). Depending on the stress signal, the anti-adaptor protein IraP, IraD, or IraM binds and sequesters RssB, thereby preventing σ^S degradation and allowing its rapid accumulation (17). Together, this complex multifactorial regulation of σ^S levels allows an exquisitely fine-tuned cellular response for adaptation and survival in diverse, potentially harmful environments.

σ^S synthesis is also affected by the transcription factor DksA and the small signaling molecules guanosine tetraphosphate and pentaphosphate (abbreviated here as ppGpp) (18, 19). DksA is a transcription factor that binds in the secondary channel of RNAP, altering the kinetics of transcription initiation (20–22). As nutrients are depleted, ppGpp accumulates and, in conjunction with DksA, coordinates a global transcriptional response to alleviate the effects of the stress (23–25).

ppGpp and DksA directly and synergistically regulate large sets of promoters negatively (20) and positively (26). ppGpp binds to two sites on *E. coli* RNAP, site 1 (27), known to play a role in DNA break repair (28), and site 2 (29). The site 1 pocket is formed by the interaction of the β' and ω subunits, and the site 2 binding pocket is formed by the interaction of DksA and the β' rim helices. The requirement for both DksA and RNAP for formation of site 2 explains the DksA-ppGpp synergism (29). DksA and ppGpp are both required for full induction of σ^S in *S.* Typhimurium (30) and *E. coli* (19).

It was initially suggested that ppGpp regulates σ^S expression after transcription initiation, e.g., via effects on the *rpoS* mRNA (8), and subsequently, it was suggested that ppGpp helps induce σ^S at the translational level, likely in an indirect manner (19). Further clues resulted from studies on the anti-adaptor protein IraP and the small regulatory RNA DsrA. Cells deficient in ppGpp and DksA showed reduced expression of *iraP*, suggesting that the effects observed *in vivo* resulted from positive regulation of

iraP transcription by DksA/ppGpp (31). A DsrA and ppGpp deficiency also reduced *rpoS* expression, but it was concluded that DsrA and ppGpp acted through independent pathways (18). DksA and ppGpp together also directly stimulated transcription from the promoter for Hfq, the protein that serves as a chaperone for most small RNAs (32), suggesting another potential contribution of DksA/ppGpp to *rpoS* expression. Finally, part of the activation of *rpoS* expression by DksA/ppGpp could occur through other indirect mechanisms. For example, DksA/ppGpp inhibition of rRNA transcription could lead to an increase in the availability of RNAP for utilization by promoters that otherwise recruit RNAP poorly (33) and/or inhibition of rRNA transcription could allow alternative sigma factors to compete better with the primary sigma factor for available core RNAP.

As a result of this complexity in *rpoS* regulation, the complete mechanism by which ppGpp and DksA contribute to the σ^S stress response remains unclear. To help clarify their roles in *rpoS* expression, here, we took advantage of $DksA_{N88I}$, a variant with higher affinity for RNAP than wild-type (WT) DksA (34) that can enhance transcription of *rpoS* even in the absence of ppGpp *in vivo*. Our results provide support for a model in which DksA and ppGpp directly increase the expression of the DsrA small regulatory RNA and the anti-adaptor protein IraP, thus increasing σ^S levels. Furthermore, our results also suggest that DksA and ppGpp can increase *dsrA* and *iraP* expression indirectly by another mechanism, possibly by reducing rRNA transcription and thereby increasing RNAP availability.

RESULTS

$DksA_{N88I}$ activates the σ^S general stress response. To better understand the mechanism of DksA regulation of the σ^S stress response, we used the previously characterized variant $DksA_{N88I}$, also referred to as super DksA (34). The N88I substitution is in the coiled-coil domain of DksA that inserts into the secondary channel of RNAP, increasing DksA's affinity for RNAP, thereby increasing its effects on transcription initiation. A key feature of this variant is that it can inhibit rRNA transcription dramatically compared to WT DksA and allow growth even in a strain lacking ppGpp *in vivo* (34). Thus, utilization of this super DksA allele provided a means of studying the effects of DksA in the absence of confounding effects of starvation or of ppGpp binding to cellular targets other than RNAP.

We first validated that $DksA_{N88I}$, like WT DksA (29), activated the σ^S stress response *in vivo*. As an indicator of activation of a σ^S-dependent promoter and more broadly of the σ^S regulon in general (35), we examined expression of a P_{katE}-*lacZ* transcriptional fusion. Induction of plasmid-encoded $DksA_{N88I}$ strongly increased β-galactosidase (β-Gal) activity, and this increase was eliminated in a Δ*rpoS* mutant (Fig. 1A). Consistent with previous *in vivo* studies on P_{livJ}, a promoter activated by the stringent response (34), WT DksA overexpression was unable to induce P_{katE} in exponential phase (when ppGpp levels are very low) (Fig. 1A), whereas $DksA_{N88I}$ increased P_{katE} activity even in a Δ*relA* Δ*spoT* ($ppGpp^0$) background (Fig. 1B). As a result, $DksA_{N88I}$ induction resulted in accumulation of σ^S protein throughout exponential phase, allowing detection as early as an optical density at 600 nm (OD_{600}) of 0.2 (Fig. 1C). In summary, these results show that $DksA_{N88I}$ strongly induces the σ^S stress response, as shown previously for WT DksA (19), and that $DksA_{N88I}$ can do so even in log phase.

Activation of the σ^S stress response by $DksA_{N88I}$ is not completely dependent on IraP *in vivo*. Under stress conditions, the posttranslational regulatory factor IraP prevents σ^S degradation by ClpXP protease, leading to accumulation of σ^S (17). Previously, it was reported that P_{iraP}-*lacZ* activity was reduced in a Δ*dksA* strain (31), and this requirement for *dksA* for activation of P_{iraP} was direct, since it occurred *in vitro* with purified DksA/ppGpp (29). Expression of $DksA_{N88I}$, like that of WT DksA (29), activated a P_{iraP}-*lacZ* fusion (Fig. 2A). To address whether the effect of $DksA_{N88I}$ on IraP accounted for the entire requirement for $DksA_{N88I}$ to increase σ^S levels, we measured the effect of $DksA_{N88I}$ on the P_{katE}-*lacZ* transcriptional fusion in a Δ*iraP* mutant (Fig. 2B). $DksA_{N88I}$ overexpression induced P_{katE} expression throughout exponential phase in a Δ*iraP*

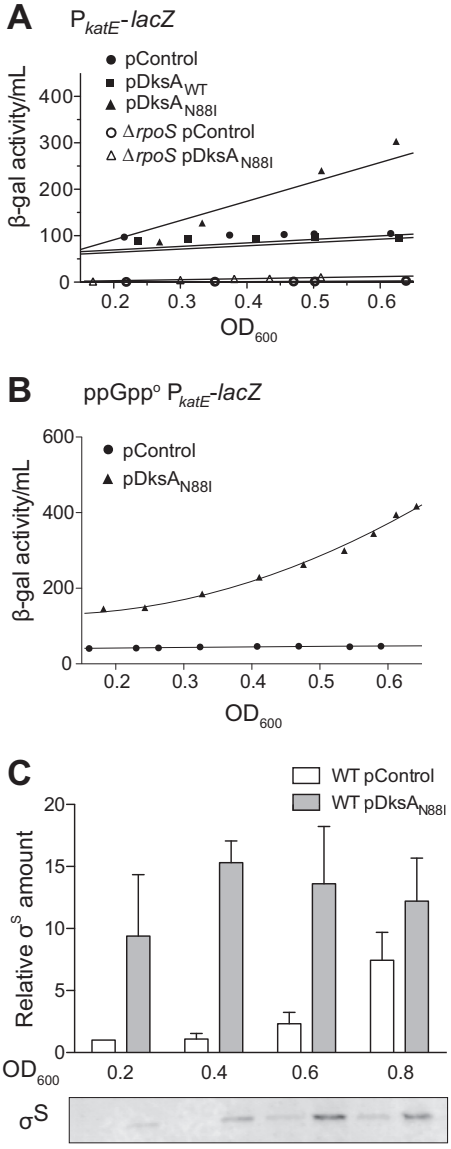

FIG 1 Super DksA ($DksA_{N88I}$) stimulates the σ^S general stress response and increases σ^S levels. (A) β-Gal activity was measured during exponential growth at 32°C in LB with 0.1 mM IPTG and was plotted against the OD_{600}. Shown are P_{katE}-*lacZ* transcriptional fusions in WT (solid symbols) or Δ*rpoS* (open symbols) strains. Strains contained a plasmid expressing $pDksA_{WT}$, $pDksA_{N88I}$, or the pControl plasmid. (B) Same P_{katE}-*lacZ* transcriptional fusions as in panel A but in a ppGpp° background (Δ*relA* Δ*spoT*). Representative experiments are shown. (C) Western blot analysis of σ^S protein levels in WT cells with or without $DksA_{N88I}$ induction at OD_{600} values of 0.2, 0.4, 0.6, and 0.8 relative to pControl protein levels at an OD_{600} of 0.2. Bands from a representative Western blot are shown below the bar graph the error bars represent the means and SD from three independent experiments ($n = 3$).

mutant, similar to the effect observed in a strain containing *iraP* (Fig. 1A and 2B). Thus, there must be a mechanism by which $DksA_{N88I}$ increases σ^S levels, in addition to its role in increasing expression of IraP.

$DksA_{N88I}$ specifically activates the expression of the small regulatory RNA DsrA *in vivo*. We next compared the effects of $DksA_{N88I}$ on σ^S levels in strains with two small RNAs previously implicated in regulation of σ^S, DsrA and RprA, deleted. Deletion of *rprA* only marginally reduced the effect of $DksA_{N88I}$ on σ^S levels, but deletion of *dsrA* (or both *iraP* and *dsrA*) virtually eliminated the effects of $DksA_{N88I}$ on σ^S levels (Fig. 3A). The effects of the double-deletion mutant were also examined at different times, from OD_{600} values of 0.2 to 0.8 (Fig. 3B). At each time point, the effects of $DksA_{N88I}$ were greatly diminished in the double mutant.

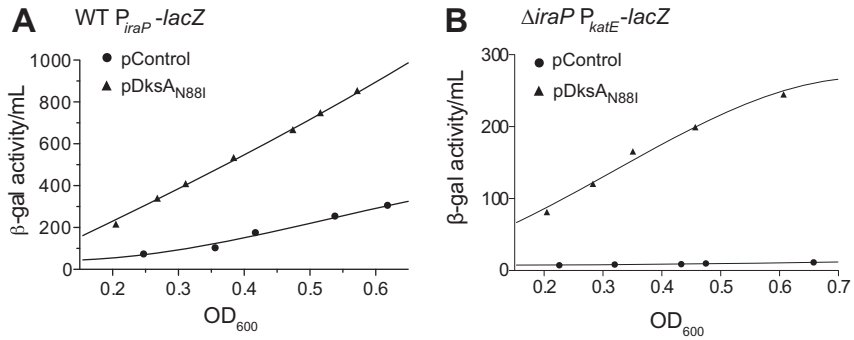

FIG 2 DksA stimulates expression of IraP *in vivo*. Shown is β-Gal activity during exponential growth at 32°C in LB with 0.1 mM IPTG. (A) P_{iraP}-*lacZ* transcriptional fusion with pControl or pDksA$_{N88I}$ in WT cells. (B) P_{katE}-*lacZ* transcriptional fusion in a Δ*iraP* mutant with pControl and DksA$_{N88I}$. The β-Gal data are representative of at least three independent experiments. The absolute activities in panels A and B should not be compared directly, since the fusions are to different promoters transcribed by different holoenzymes.

We next assessed the role of DsrA by measuring the effect of DksA$_{N88I}$ on an *rpoS-lacZ* translational fusion (36). There was a large increase in *rpoS* translation in exponential phase when DksA$_{N88I}$ was provided in *trans*, and this increase was largely eliminated in a Δ*dsrA* strain (Fig. 4A). Consistent with a role for factors in addition to *dsrA* in *rpoS* transcription, or even of posttranslational effects on the activity of the fusion protein, the residual activity of the translational fusion still increased slightly in the Δ*dsrA* pDksA$_{N88I}$ strain relative to the Δ*dsrA* pControl strain (Fig. 4A). However, the major conclusion was that DksA$_{N88I}$ greatly increased the expression of *rpoS* in a *dsrA*-dependent manner.

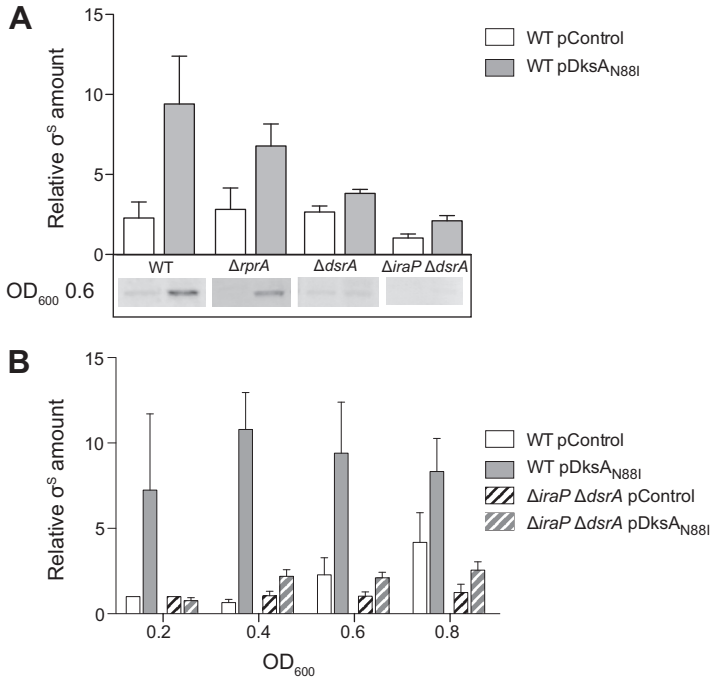

FIG 3 The effect of DksA$_{N88I}$ on σ^S levels is dependent on *dsrA*. Strains were grown to an OD$_{600}$ of 0.6 in LB at 32°C with 0.1 mM IPTG. Shown is Western blot analysis of σ^S protein levels. (A) Protein extracts were made from the indicated strains containing pControl or pDksA$_{N88I}$, and σ^S was normalized to the WT with pControl. A representative Western blot is shown below the graph. (B) Proteins were extracted from WT or Δ*iraP* Δ*dsrA* strains containing pControl or pDksA$_{N88I}$ as indicated. The bands were quantified and normalized relative to WT pControl (the error bars indicate means and SD; $n = 3$).

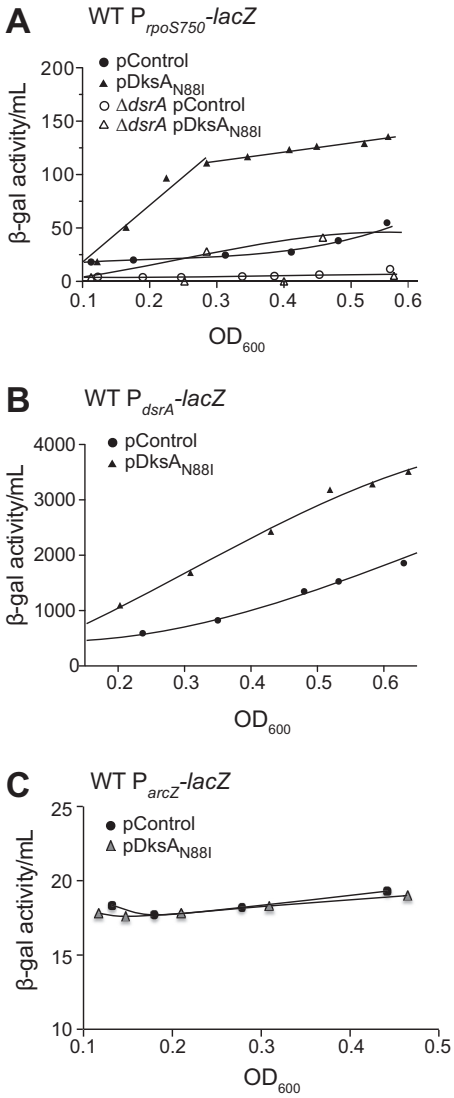

FIG 4 $DksA_{N88I}$ activates transcription of the small regulatory RNA DsrA *in vivo*. (A) β-Galactosidase activity from a $P_{rpoS750}$-*lacZ* translational fusion in the WT (solid symbols) or a Δ*dsrA* mutant (open symbols) containing either pControl or $pDksA_{N88I}$. (B) P_{dsrA}-*lacZ* β-Gal activity plotted against increasing OD_{600} in WT background. (C) P_{arcZ}-*lacZ* β-Gal activity plotted against increasing OD_{600} in WT background. The curves shown are representative of at least 3 independent experiments.

Since DksA (in conjunction with ppGpp) is a transcription activator, we tested whether it might exert its effect on *rpoS* expression by stimulating *dsrA* promoter activity *in vivo*. Consistent with this model, $DksA_{N88I}$ expression increased the activity of a P_{dsrA}-*lacZ* promoter fusion (Fig. 4B). This result was also consistent with previous observations implicating DksA in expression of the small RNA chaperone Hfq, and thus of small RNAs, in regulation of *rpoS* synthesis by DksA (32). In contrast, $DksA_{N88I}$ expression did not increase the activity of a P_{arcZ}-*lacZ* promoter fusion (Fig. 4C), suggesting that under the conditions tested, the small RNA ArcZ is not involved in the upregulation of *rpoS* expression by DksA. Taken together with the effects of $DksA_{N88I}$ on *iraP* expression shown in Fig. 2, our results support a role for DksA in both *dsrA* and *iraP* expression and thus in posttranscriptional regulation of *rpoS*.

Effects of $DksA_{N88I}$ on transcription from P_{iraP} and P_{dsrA} *in vitro*. To address whether the effects of WT DksA and $DksA_{N88I}$ on the *dsrA* and *iraP* promoters *in vivo* were direct, we measured transcription *in vitro* with purified DksA, ppGpp, and RNA

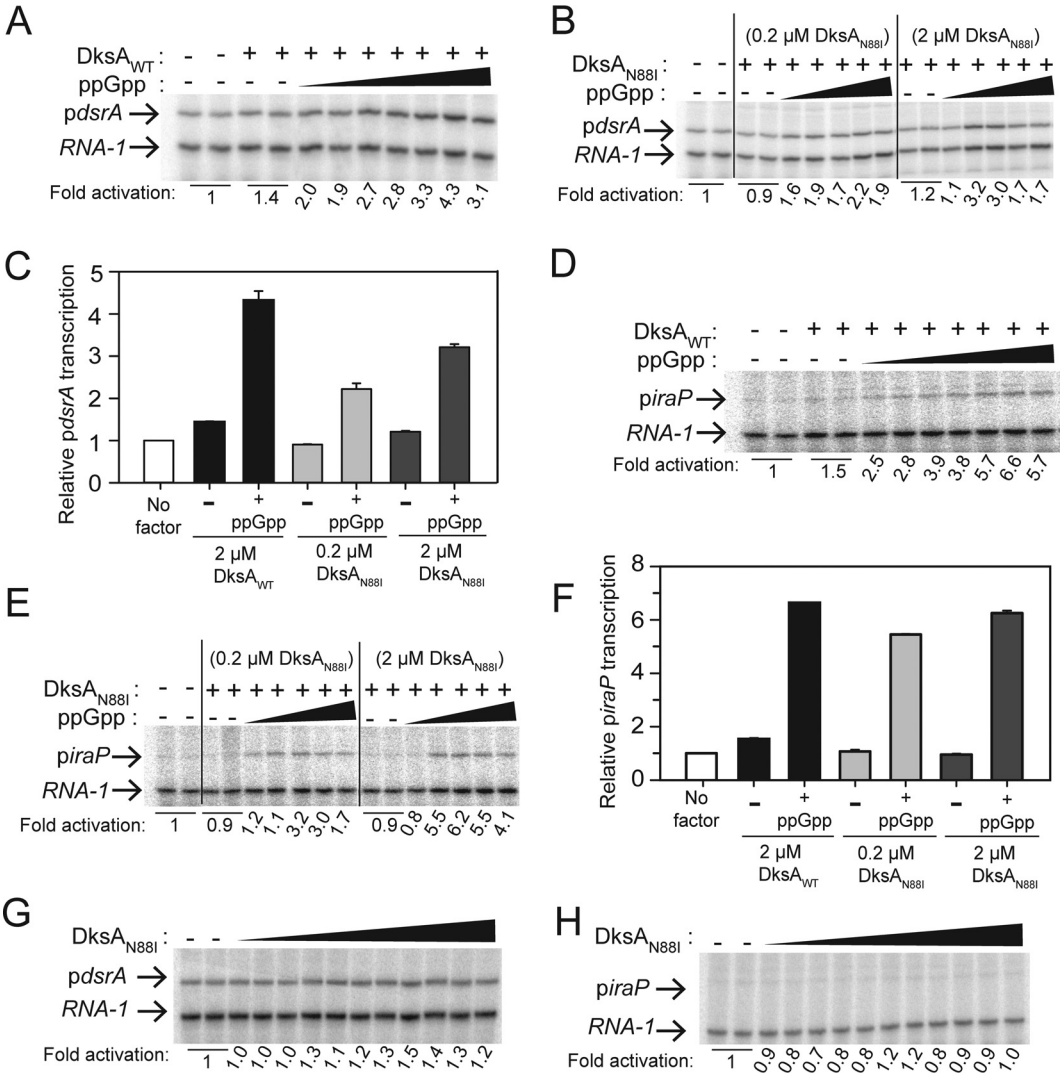

FIG 5 Stimulation of P_{iraP} and P_{dsrA} promoter activity by $DksA_{WT}$ or $DksA_{N88I}$ *in vitro* with or without ppGpp. (A) Representative gel image illustrating fold activation by DksA with or without ppGpp on the *dsrA* promoter. Transcription was normalized to that in the absence of any factor (lanes 1 and 2), and fold activation for each reaction is shown beneath each lane (average of 2 reactions). Multiple-round *in vitro* transcription was performed with 10 nM RNAP and 170 mM NaCl at room temperature. Lanes 1 and 2, buffer only; lanes 3 and 4, 2 μM WT DksA only; lanes 5 to 11, 2 μM WT DksA plus ppGpp. The wedge indicates increasing ppGpp, from 3 to 200 μM. Plasmid templates also contained the *RNA-1* promoter. Transcripts are indicated by arrows. (B) Representative gel image of transcription from the *dsrA* promoter as in panel A but with 0.2 μM or 2 μM $DksA_{N88I}$, as indicated. (C) Maximum activation observed from data sets illustrated in panels A and B plotted relative to transcription in the absence of either DksA or ppGpp. Shown is fold activation with 2 μM $DksA_{WT}$ alone or with $DksA_{WT}$ plus 100 μM ppGpp (black bars), 0.2 μM $DksA_{N88I}$ alone or 0.2 μM $DksA_{N88I}$ plus 25 μM ppGpp (light-gray bars), and 2 μM $DksA_{N88I}$ alone or 2 μM $DksA_{N88I}$ plus 6.25 μM ppGpp (dark-gray bars). The error bars indicate means and SD or range from at least two independent experiments. (D) Same as panel A except the template contained the *iraP* promoter. (E) Same as panel B except the template contained the *iraP* promoter. (F) Maximum activation of the *iraP* promoter from the data sets illustrated in panels D and E plotted relative to transcription in the absence of either DksA or ppGpp. Black bars, 2 μM $DksA_{WT}$ alone or $DksA_{WT}$ plus 100 μM ppGpp; light-gray bars, 0.2 μM $DksA_{N88I}$ alone or 0.2 μM $DksA_{N88I}$ plus 12.5 μM ppGpp; dark-gray bars, 2 μM $DksA_{N88I}$ alone or 2 μM $DksA_{N88I}$ plus 12.5 μM ppGpp. (G and H) Same as panels B and E except the $DksA_{N88I}$ concentration was varied from 4 nM to 4 μM and no ppGpp was included in the reaction mixture ($n = 3$).

polymerase (Fig. 5). In the presence of ppGpp, WT DksA stimulated transcription from the *dsrA* promoter as much as 4-fold *in vitro* (Fig. 5A and C) (37) and from the *iraP* promoter as much as 6-fold (Fig. 5D and F) (29). Like WT DksA, $DksA_{N88I}$ also stimulated transcription from these promoters *in vitro* in the presence of ppGpp (Fig. 5B, C, E, and F). However, as with WT DksA on other positively regulated promoters (34), the increased RNAP binding activity of $DksA_{N88I}$ did not relieve the requirement for ppGpp for activation of transcription *in vitro* (Fig. 5G and H).

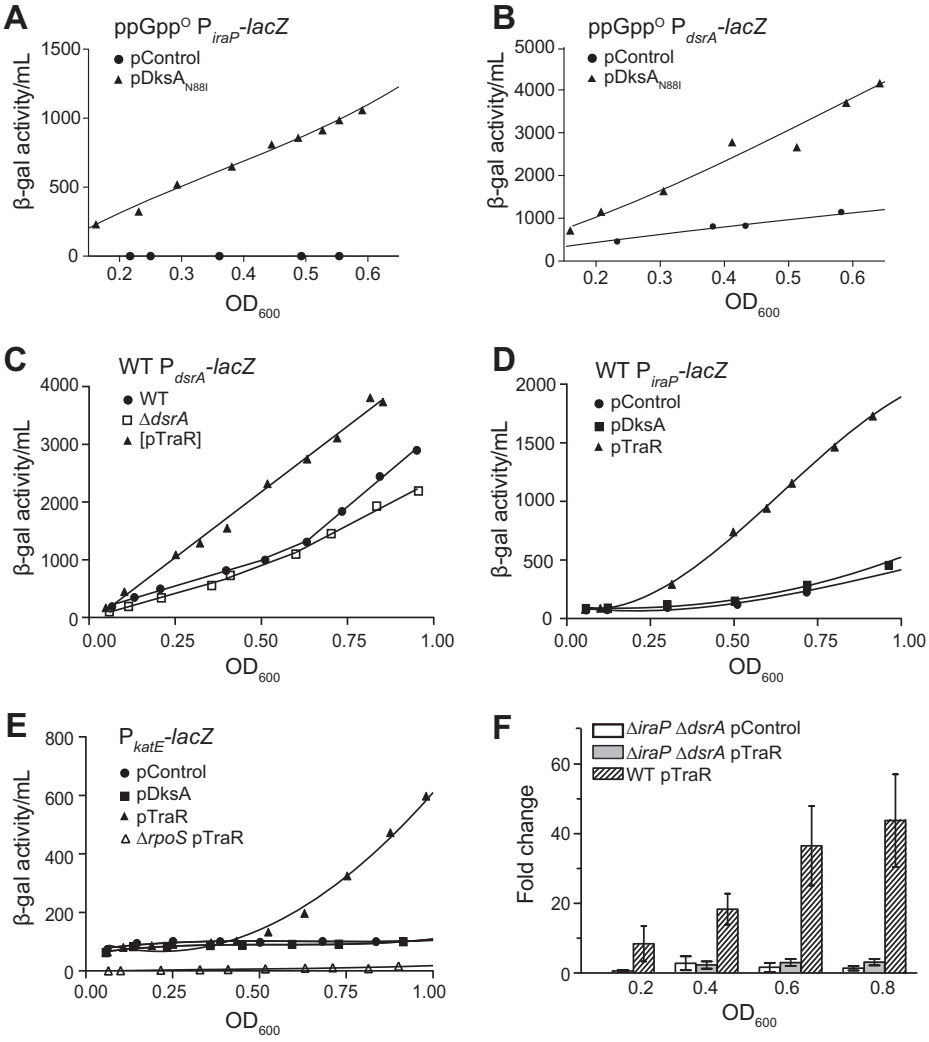

FIG 6 $DksA_{N88I}$ and TraR stimulate the *dsrA* and *iraP* promoters in the absence of ppGpp *in vivo*. Overnight cultures were diluted 1:100 in LB medium at 32°C with 0.1 mM IPTG to induce $DksA_{N88I}$ or TraR expression. (A to E) The activities of P_{iraP}-*lacZ*, P_{dsrA}-*lacZ*, or P_{katE}-*lacZ* fusions were monitored by measuring β-Gal activity during exponential growth at the optical densities shown on the *x* axes. The cells contained pControl, $pDksA_{N88I}$, or pTraR. Experiments were performed three times, and representative data are shown. The ppGpp° strain (Δ*relA* Δ*spoT*) is incapable of synthesizing ppGpp. It was shown previously that TraR can function without ppGpp *in vivo* (35). (A) Effect of $DksA_{N88I}$ on expression of the P_{iraP}-*lacZ* fusion. (B) Effect of $DksA_{N88I}$ on expression of the P_{dsrA}-*lacZ* fusion. (C) Effect of TraR on expression of the P_{dsrA}-*lacZ* fusion. (D) Effect of TraR on expression of the P_{iraP}-*lacZ* fusion. (E) Effect of DksA or TraR on expression of the P_{katE}-*lacZ* fusion in a WT or Δ*rpoS* background (open triangles). (F) Western blotting was performed, and σ^S levels were quantified at different times after TraR induction in a WT or Δ*dsrA* Δ*iraP* background as described in Materials and Methods. The error bars indicate SD.

$DksA_{N88I}$ and TraR activate transcription from P_{iraP} and P_{dsrA} better *in vivo* than *in vitro*. $DksA_{N88I}$ was identified originally because it allowed growth of a strain lacking ppGpp (ppGpp°; Δ*relA* Δ*spoT*) on media lacking amino acids. $DksA_{N88I}$ inhibited rRNA promoters and stimulated amino acid-biosynthetic promoters in a ppGpp° strain (34). As with the amino acid-biosynthetic promoters, we found that $DksA_{N88I}$ also stimulated the activities of the *iraP* and *dsrA* promoters in a ppGpp° strain (Fig. 6A and B). Furthermore, the activities of P_{iraP}-*lacZ* and P_{dsrA}-*lacZ* fusions after induction of $DksA_{N88I}$ in a ppGpp° strain background were approximately the same as in the WT background (compare the results in Fig. 6 with those in Fig. 2A and 4B). Thus, even though $DksA_{N88I}$ was unable to activate the *dsrA* and *iraP* promoters *in vitro* without ppGpp (Fig. 5G and H), it was able to activate these promoters strongly *in vivo* in the absence of ppGpp (Fig. 6A and B) and to turn on the σ^S stress response (Fig. 2).

We next examined the effect of TraR on *dsrA* and *iraP* expression as an independent means of evaluating the roles of DsrA and IraP in activating the σ^S regulon. TraR is an RNAP secondary channel binding factor that works without ppGpp but mimics the combined effects of DksA and ppGpp *in vivo* and *in vitro* (37, 38). As with $DksA_{N88I}$, expression of TraR *in vivo* increased expression of the P_{dsrA}-*lacZ* and P_{iraP}-*lacZ* fusions (Fig. 6C and D). Consistent with the model in which increased levels of DsrA and IraP contribute to increased levels of σ^S, TraR increased σ^S activity, as monitored by the activity of the σ^S-dependent P_{katE}-*lacZ* transcriptional fusion (Fig. 6E). Correspondingly, TraR increased the levels of σ^S protein, and these increased levels of σ^S were not observed in the Δ*dsrA* Δ*iraP* strain (Fig. 6F).

However, as observed with $DksA_{N88I}$ by itself, TraR had only a modest effect (~2- to 2.5-fold) on activating the *dsrA* and *iraP* promoters *in vitro*, smaller than the effects of DksA/ppGpp on the same promoters (Fig. 2; see Fig. S2 in reference 37). The discrepancy between the magnitude of the effects of TraR and $DksA_{N88I}$ on the *dsrA* and *iraP* promoters *in vitro* versus *in vivo* suggests that part of the effect of $DksA_{N88I}$ and TraR on these promoters *in vivo* might be indirect. We return to this observation in Discussion below.

DISCUSSION

ppGpp and DksA contribute in multiple ways to increased σ^S. DksA and ppGpp play crucial roles in the σ^S response by increasing σ^S holoenzyme activity (19). We propose that this occurs in at least three different ways. First, DksA and ppGpp together activate the *dsrA* promoter, thereby improving *rpoS* translation. Second, DksA and ppGpp together activate the *iraP* promoter, thereby improving σ^S stability. Third, we suggest that DksA and ppGpp can increase σ^S levels by an additional indirect mechanism: by reducing rRNA transcription, DksA and ppGpp increase the availability of RNAP for transcription from other promoters, including σ^{70}-dependent promoters, such as P_{iraP} and P_{dsrA}, and σ^S-dependent promoters, such as P_{katE}. We show all three of these contributions of DksA and ppGpp to the σ^S response schematically in Fig. 7.

WT DksA can partially inhibit rRNA transcription in the absence of ppGpp (20), but because $DksA_{N88I}$ increases the affinity of DksA for RNAP, it can inhibit rRNA transcription more strongly than WT DksA and bypass the requirement for ppGpp for inhibition. The mechanism of activation by DksA/ppGpp remains poorly understood, but ppGpp binding to the site at the interface of DksA and the β′ rim helices (site 2) is necessary and sufficient for activation (26, 29). How $DksA_{N88I}$ can inhibit rRNA promoters by itself, without ppGpp, but cannot activate promoters without ppGpp remains unclear. We suggest that $DksA_{N88I}$ can cause the conformational change in the promoter-RNAP complex that inhibits transcription but not the one that activates transcription.

The requirement for ppGpp for activation *in vitro* can be bypassed *in vivo*. We were able to uncover the third mechanism in the absence of confounding effects of ppGpp because rRNA transcription represents a large fraction of total transcription *in vivo* during log-phase growth and $DksA_{N88I}$ can inhibit rRNA transcription without ppGpp both *in vivo* and *in vitro*. We suggest that the inhibitory effects of $DksA_{N88I}$ and TraR increase the free RNAP concentration *in vivo*, and this allows transcription from a set of stress-related promoters that are otherwise too weak to be expressed.

We note that activation of weak promoters in this model is not limited to the σ^{70}-dependent promoters P_{iraP} and P_{dsrA} or to σ^S-dependent promoters like P_{katE}. Furthermore, effects on unknown factors or solution conditions (pH, salt, and template conformation) could also allow DksA to activate specific promoters in the absence of ppGpp. We note that effects of DksA independent of ppGpp or sRNAs have been reported previously *in vivo* (39, 40).

We have attempted to compare the contributions of direct activation (as observed with ppGpp/DksA *in vitro*) with the indirect activation proposed to occur on the same promoter in cells. Although we found they are roughly comparable, these are not ideal comparisons, at least in part because ppGpp concentrations are constantly changing

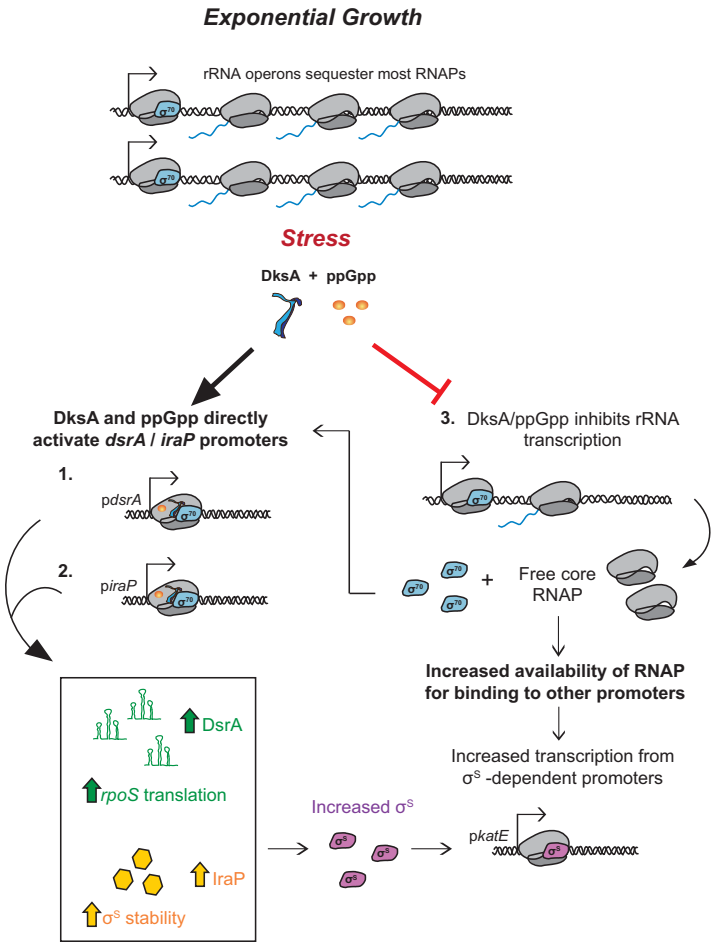

FIG 7 Model for stimulation of σ^S general stress response by DksA/ppGpp. During exponential growth, most RNAP is devoted to transcribing rRNA to accommodate the high demand for ribosomes for protein synthesis. (1 and 2) As nutrients are depleted, either from entry of cells into stationary phase or from starvation for specific nutrients, ppGpp is induced and binds to RNAP, along with DksA, to modulate RNAP activity and activate the *dsrA* (1) and *iraP* (2) promoters. (3) At the same time, potent inhibition of rRNA transcription initiation by DksA/ppGpp reduces the level of RNAP transcribing rRNA genes, making more core RNAP available for binding sigma and transcribing from the *dsrA* and *iraP* promoters. For brevity, only 4 RNAPs are shown on individual rRNA operons during exponential growth, but well over 100 RNAPs can occupy each of the 7 rRNA operons at high growth rates. Enhanced expression of *dsrA* and *iraP* increases *rpoS* translation and stabilizes σ^S protein. Thus, DksA/ppGpp upregulates σ^S expression by at least 3 different mechanisms.

and because the solution conditions *in vitro* are very different from those in cells. Therefore, we caution against placing too much emphasis on such comparisons.

Effects of DksA/ppGpp on stress responses involving different σ factors. DksA and ppGpp are best recognized for their central roles in the stringent response, largely through their effects on the holoenzyme containing the housekeeping sigma factor σ^{70}. We showed here that DksA and ppGpp also help activate the σ^S response indirectly by directly stimulating the *iraP* and *dsrA* promoters, which in turn results in an increase in *rpoS* synthesis/stability. DksA and ppGpp have also been shown to regulate the σ^E regulon directly, a stress response to extracytoplasmic and cell envelope stressors (39, 41). Finally, DksA and ppGpp have been implicated in controlling transcription dependent on σ^{54} (42, 43). Although some of the effects of DksA and ppGpp are indirect, it appears that they are truly global in scope, affecting regulons controlled by many or most of the cell's sigma factors, allowing the bacterium to survive diverse environmental challenges.

Prospects. Previous studies have linked ppGpp/DksA and sRNA expression to increased σ^S levels (18, 19), but our work describes the step-by-step chain of events from ppGpp

production to increased σ^S activity. A major component of this signal transduction pathway is the direct activation of a promoter for a small RNA, DsrA. It will be of interest to explore whether ppGpp/DksA regulation of additional sRNAs contributes to the breadth of the ppGpp/DksA regulon, the stringent response, and adaptation to stress.

MATERIALS AND METHODS

Media and bacterial growth. All strains were grown at 32°C with aeration in LB broth supplemented as required with the following: carbenicillin (50 μg ml^{-1}), kanamycin (30 μg ml^{-1}), chloramphenicol (12.5 μg ml^{-1}), and IPTG (isopropyl-β-D-thiogalactopyranoside) (0.1 mM). Plasmids were introduced using standard transformation techniques, and chromosomal alleles were moved into strains via P1 transduction (44). Further details on the methods for strain construction have been described previously (34, 38). All plasmids and strains with their genotypes are listed in Table S1 in the supplemental material.

β-Galactosidase activity assays. Overnight cultures were diluted 1:100 in LB containing carbenicillin, supplemented with 0.1 mM IPTG as needed for induction. Samples (0.5 ml) were taken approximately at every increase of the OD_{600} by 0.1 until early stationary phase and assayed for β-galactosidase (β-Gal) activity as described previously (44). The graphs are plotted as β-Gal activity per milliliter [$OD_{420} \times (2 \times 10^3)$/reaction time] versus OD_{600}, and a line of best fit was determined with GraphPad Prism. A representative sample is shown for at least three independent experiments.

Measurement of σ^S protein via Western blot analysis. Overnight cultures were diluted 1:100 in LB plus carbenicillin, with 0.1 mM IPTG as required for induction, taking 0.9-ml samples at various OD_{600} values (0.2, 0.4, 0.6, and 0.8), which were then added to 100 μl ice-cold 50% trichloroacetic acid (TCA) to precipitate protein. Samples were pelleted and resuspended at an OD_{600} of 0.25 in SDS sample buffer (14 μl β-mercaptoethanol, 1 ml 2× SDS buffer, 1 ml elution buffer [10 mM Tris-HCl, pH 8.0]). The same volume of cells from samples at an OD_{600} of 0.25 was loaded on all lanes and run on an SDS-12% PAGE gel, probed for σ^S with a polyclonal rabbit antibody (a gift from S. Wickner, NIH), and labeled with Alexa Fluor 647–goat anti-rabbit IgG (Invitrogen) as the secondary antibody. A Typhoon Tri scanner (GE) was used to visualize the bands under the following conditions: emission (Em) filter parameter of 670BP 30 Cy5; voltage sensitivity (PMT) of 300. The bands were then quantified using ImageQuant TL software and graphed for at least three experiments, with bands from one representative Western blot shown. To ensure proper loading, contrast was enhanced in ImageQuant TL to visualize the background banding patterns for consistency across wells.

***In vitro* transcription.** Multiple-round *in vitro* transcription was performed by standard procedures as previously described (20, 26). $DksA_{WT}$ and $DksA_{N88I}$ were purified as described previously (34). The plasmid templates contained the promoter P_{dsrA} (pRLG13072) or P_{iraP} (pRLG11350) and the transcription terminators *rrnB* T1 and T2 downstream from the promoter fragment cloning site. $DksA_{WT}$ and $DksA_{N88I}$ concentrations are shown in Fig. 5. The ppGpp concentrations were 3, 6.25, 12.5, 25, 50, 100, and 200 μM (indicated as wedges in Fig. 5). ppGpp was obtained from TriLink Inc. Transcription was quantified by phosphorimaging using ImageQuant 5.2 software, normalized to transcription in the absence of factors, and graphed for each condition. Transcription experiments were performed at least twice.

Statistics. Data were graphed using GraphPad Prism 5 software. The error bars indicate means and standard deviations (SD) for the indicated number of replicate experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00463-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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