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# Characterization of a Novel RNA Polymerase Mutant That Alters DksA Activity

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**The auxiliary factor DksA is a global transcription regulator and, with the help of ppGpp, controls the nutritional stress response in *Escherichia coli*. Although the consequences of its modulation of RNA polymerase (RNAP) are becoming better explained, it is still not fully understood how the two proteins interact. We employed a series of genetic suppressor selections to find residues in RNAP that alter its sensitivity to DksA. Our approach allowed us to identify and genetically characterize *in vivo* three single amino acid substitutions: β′ E677G, β V146F, and β G534D. We demonstrate that the mutation β′ E677G affects the activity of both DksA and its homolog, TraR, but does not affect the action of other secondary interactors, such as GreA or GreB. Our mutants provide insight into how different auxiliary transcription factors interact with RNAP and contribute to our understanding of how different stages of transcription are regulated through the secondary channel of RNAP *in vivo*.**

The vast majority of known transcription factors modulate gene activity by mediating the binding of RNA polymerase (RNAP) to promoters and other regulatory DNA sequences. Another class, the auxiliary transcription factors, interacts directly with the RNAP complex instead of binding DNA. Bacterial auxiliary transcription factors that bind to the secondary channel of RNAP share a set of common features: they are small proteins (up to about 150 amino acids) that consist of a globular head and a coiled-coil domain (1). Low-resolution cryo-electron microscopy data have revealed that the globular domain interacts with the surface of RNAP and the coiled-coil protrudes deep into the secondary channel of the RNAP complex (2–4). The secondary channel is an opening formed by the β and β′ subunits, through which free ribonucleoside triphosphates access the catalytic center of the enzyme. The common structure of this class of transcription factors allows them to reach the active site through the secondary channel and modulate the RNAP. Several such secondary channel interactors have been identified to date in bacteria: transcription elongation and fidelity factors GreA and GreB (5–9); two anti-Gre factors, Gfh1 (10, 11) and Rnk (12); and the transcription initiation and elongation factor DksA (13–16) and its analog, DksA2, from *Pseudomonas aeruginosa* (17), as well as its structural homolog, TraR, often found on conjugative plasmids (18).

A considerable amount of work has been done to comprehend the role of DksA in *Escherichia coli*. Recent evidence has prompted a shift in the understanding of its function: its role has evolved from a transcriptional cofactor to a major player regulating transcription at multiple levels. DksA was originally isolated as a suppressor of the mutant phenotype resulting from the loss of function of the chaperone DnaK (19). The involvement of DnaK in restarting stalled DNA polymerase suggests a probable role for DksA in replication (20). In fact, DksA was recently shown to play a role in prevention of the collision between transcription and replication machineries (14). DksA also ensures integrity of the genome by a not fully understood pathway implicating transcription and DNA repair mechanisms (21). Additionally, DksA is known to act as a potentiator of the small molecule ppGpp in response to nutritional stress (15, 22). ppGpp, which serves as a bacterial alarmone, is synthesized by RelA and SpoT in response to amino acid (RelA) (23), carbon (24), fatty acid (24), phosphate (22, 25, 26), and iron (SpoT) (27) starvation. Accumulation of ppGpp triggers the stringent response, which allows the cell to shift its transcriptional, translational, and replicational efforts toward survival in a nutrient-poor environment (25, 28, 29). DksA and ppGpp function synergistically with RNAP to change the promoter complex lifetime (30). The allosteric changes following RNAP-DksA binding destabilize the transcription initiation complex at rRNA promoters, which results in dissociation of RNAP from the DNA. Inhibition of rRNA synthesis during nutritional stress allows cells to conserve energy consumed by the production of ribosomes and diverts cellular RNAP to activate genes requiring a higher polymerase concentration, such as amino acid synthesis genes. Moreover, free RNAP is more accessible to alternative sigma factors that coordinate various stress responses (28). The absence of either ppGpp or DksA often results in very similar phenotypes, which usually relate to the inability to respond to nutritional stress. However, in some situations their effects are different and sometimes even opposing (31).

The nature of the interplay between DksA, ppGpp, and the RNAP complex has been of substantial interest (32, 33). Recent studies report that ppGpp binds to the interface between the β′ and ω subunits of RNAP, which could slow down its ratcheting motion, resulting in decreased lifetimes for certain promoter complexes (34–36). DksA structurally resembles the transcript cleavage factors GreA and GreB, but unlike the RNAP-GreA/B cocrystal structure, the RNAP-DksA cocrystal structure has not been determined at atomic resolution. On the basis of the structural similarity between DksA and GreA/B, it has been assumed that the interaction occurs on the rim and inner surface of the secondary channel (13). The active residue of DksA (aspartic acid

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74) is located at the tip of the coiled-coil domain, which would contact the trigger loop (TL) of RNAP. Such an interaction was recently tested using various DksA-GreA/B chimeras where the Gre factors could mimic DksA and initiate transcription when equipped with a DksA-like active tip (37). Additionally, DksA acts antagonistically to GreA in the regulation of rRNA promoters, which suggests that both molecules compete for the same surface on RNAP (38–41).

The most recent study by Lennon et al. provides extensive biochemical evidence of DksA being a secondary channel interactor (42). RNAP-DksA cross-linking and protein-protein footprinting allowed these authors to perform semiflexible simulated annealing, which resulted in an evidence-based prediction of the docking model. According to the proposed model, the head domain of DksA interacts with a coiled-coil domain on the surface of RNAP (rim helices [RHs]), with the coiled-coil tip of DksA entering the secondary channel and contacting the TL region of the complex.

Although it is clear that DksA is a major player in transcription in *E. coli*, the nature of its ability to interact with RNAP *in vivo* and compete with other secondary channel interactors remains to be fully understood. Our goal therefore was to use a genetic approach to identify the residues of the RNAP complex required for its interaction with DksA. Here we report a novel RNAP mutant that is insensitive to the presence of DksA and therefore whose phenotype resembles the phenotype of loss of *dksA*. Moreover, we describe a genetic suppressor of this phenotype, which restores the effect of DksA on RNAP. Our findings generally agree with the model of DksA docking into the secondary channel proposed by Lennon et al. (42) and enhance our knowledge about the finely tuned RNAP-DksA relationship in the regulation of transcription with other transcription factors acting through the secondary channel.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Strains are listed in Table S1 in the supplemental material, plasmids are listed in Table S2 in the supplemental material, and oligonucleotides are listed in Table S3 in the supplemental material. Unless otherwise stated, all strains used are derivatives of *E. coli* MG1655. Bacteria were grown in LB broth and on LB and M9-glucose (0.2%) agar plates. Media were supplemented with the following antibiotics: ampicillin (Amp; 50 μg/ml) or carbenicillin (CB; 50 μg/ml), rifampin (Rif; 50 μg/ml), tetracycline (Tet; 12.5 μg/ml), chloramphenicol (CM; 12.5 μg/ml), and kanamycin (Kan; 30 μg/ml). Casamino Acids (CAA; 0.3%) were added when necessary. IPTG (isopropyl-β-D-thiogalactopyranoside; 0.1 mM) was used as an inducer for overexpression of the respective constructs (see Table S2 in the supplemental material). Transduction with P1*vir* and other standard genetic methods were performed as described in reference 43. The promoter-*lacZ* fusions have been described earlier (22).

**Genetic manipulations.** Genes to be overexpressed were cloned into the pBA169 or pBR322 vector by standard cloning and transformation techniques (44). Gene replacements were made as reported previously (45). Specific mutations were transferred between different genetic backgrounds using P1 phage transduction (43).

**Localized mutagenesis.** The *rpoC* E677G mutation was obtained using a selection for the suppressors of overexpression of DksA L15F N88I. The Δ*btuB*3191::Tn*10* and Δ*yjaZ*::*kan* markers were transduced into MG1655, and then the entire Δ*btuB*3191::Tn*10*-*rpoBC*-Δ*yjaZ*::*kan* construct was moved into a Δ*mutL mutD5* background, which is a strong mutator. The mutator strain was grown in LB broth overnight at 37°C, transformed with pDksA L15F N88I, and plated on M9-glucose-CAA-Amp medium with IPTG for overexpression of DksA L15F N88I. Overexpression of DksA L15F N88I selects for mutants insensitive to its inhibiting effect on cell growth. The isolated colonies were cultured, and the Δ*btuB*3191::Tn*10*-*rpoBC*-Δ*yjaZ*::*kan* fragments were transduced into MG1655 for analysis.

**Spontaneous mutagenesis.** The *rpoB* V146F and *rpoB* G534D mutations were isolated as a result of spontaneous rifampin resistance. The *rpoC* E677G (Δ*dksA*) mutant was grown overnight at 37°C and plated on LB-rifampin medium. The resulting colonies were then tested for suppression of auxotrophy.

**Complementation assay.** Auxotrophic RNAP mutants were transformed with plasmids carrying either *rpoB* (pIA545) or *rpoC* (pRL662). The transformants were streaked on M9-glucose-ampicillin with IPTG (0.1 mM) plates to determine which gene complements the auxotrophy.

**Plating efficiency.** Serial dilutions of overnight cultures grown in LB medium were performed in 10 mM $MgSO_4$. Appropriate volumes of the dilutions of interest were then plated on M9-glucose and M9-glucose-CAA plates, both of which were supplemented with IPTG and antibiotics when appropriate. The plates were incubated for 3 days at 37°C, and colonies were counted. Percentages were obtained from the ratio of the number of colonies growing on M9-glucose plates to the number of colonies growing on M9-glucose-CAA plates.

**β-Galactosidase activity assay.** Overnight cultures were diluted 1/100 and grown with shaking at 32°C in LB medium supplemented with ampicillin and, when appropriate, IPTG (0.1 mM) to express plasmid-encoded DksA alleles. Aliquots (0.5 ml) were assayed as described earlier (43), except that samples were centrifuged to pellet cell debris before determination of the optical density at 420 nm ($OD_{420}$). The β-galactosidase activity was assessed for at least three independent experiments and is represented as $OD_{420}$/ml culture as a function of the $OD_{600}$ of the culture. Polynomial (third-order) regression lines were plotted using the Microsoft Excel program.

**Sequence determination.** All described mutations were identified by analysis of the DNA sequence of the *rpoBC* region. The oligonucleotides used were previously published (30). Δ*btuB3191*::Tn*10* is located 15.7 kb downstream of *rpoB*, but both markers—*tet* and *kan*—were used for selection. Δ*yjaZ*::*kan* is 213 bp downstream of *rpoC*. The two markers are 0.58 min away, which corresponds to a cotransduction frequency of ~40% (theoretical and observed). Cotransduction of Δ*btuB3191*::Tn*10* and Δ*yjaZ*::*kan* when selection was not available (in a Δ*dksA*::*tet* strain) was determined using PCR: oligonucleotides OC465 and OC466 were used to amplify the *tetC-tetD* junction of Tn*10*, and oligonucleotides OC467 and OC468 were used to amplify an internal fragment of *btuB* (see Table S3 in the supplemental material).

**Gene overexpression.** Genes overexpressed in this study were controlled under p*lac* or p*trc* promoters, and therefore, IPTG (0.1 mM) was used to induce the expression. GreA and GreB were overexpressed using the pBR322 vector under their native promoters, since the pBA169 backbone with a cloned *greA* or *greB* gene was not compatible with the β′ E677G background.

**Sensitivity to MMC.** Overnight cultures were diluted 1/100 and grown at 37°C in LB medium to an $OD_{600}$ of approximately 0.4. Five microliters of 1/10 serially diluted cultures was spotted on LB medium plates with or without 1 μg/ml mitomycin C (MMC). The plates were incubated overnight at 37°C.

**Isolation of DksA mutants.** pBA169 DksA was mutagenized as described previously (46). pDksA H61R was originally isolated with other super DksAs, but unlike those alleles, it was not published.

## RESULTS

**Isolation of an *rpoC* mutant insensitive to DksA.** The function of DksA with RNAP in transcription initiation *in vivo* depends on the presence of the alarmone ppGpp. This ppGpp dependence can be alleviated by introduction of point mutations in DksA. These amino acid substitutions have been shown to increase the affinity of DksA for RNAP (46). One such ppGpp-independent super

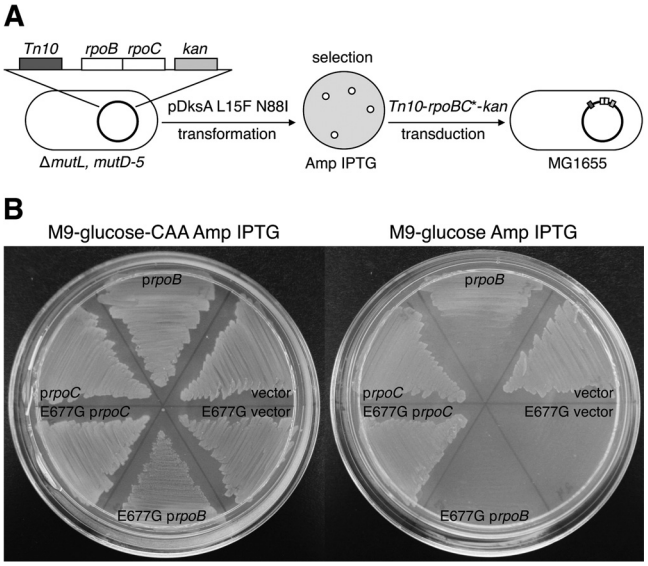


**FIG 1** Isolation and identification of a novel RNAP mutant. (A) Genetic selection for RNAP mutants insensitive to DksA. Flanked by two selectable markers (Tn*10* and *kan*), the *rpoBC* operon was mutagenized in a mutator background *in vivo*. The mutagenized strain was then transformed with a plasmid carrying a hyperactive allele of DksA L15F N88I and plated on medium containing IPTG for overexpression of DksA L15F N88I. Only mutants insensitive to its inhibitory effect on growth form colonies. The Tn*10-rpoBC*-kan* fragment (where the asterisk indicates a mutation) was then transduced into MG1655 for analysis. (B) Complementation test. The mutant obtained from the selection requires amino acids to grow. Overexpression of wild-type *rpoC* from a plasmid alleviates this phenotype, which indicates that a mutation in the chromosomal copy of *rpoC* causes auxotrophy. The top three sectors of each plate contain the wild-type strain, whereas the bottom three sectors contain the *rpoC* E677G mutant. Both strains carry the following plasmids: pIA545 carrying p*rpoB*, pRL662 carrying p*rpoC*, and pBA169 carrying the vector (see Table S2 in the supplemental material).

DksA carries two substitutions—L15F and N88I—and is so hyperactive that it efficiently shuts down rRNA synthesis when overexpressed, thereby completely inhibiting the growth of otherwise wild-type *E. coli* (46). We hypothesized that an RNAP mutant with decreased sensitivity or affinity to DksA would not only rescue the phenotype of DksA L15F N88I overexpression but also shed light on the mechanism of DksA-dependent RNAP modulation. To isolate such mutants, we used an *in vivo* localized mutagenesis approach (47) where the genes of interest—*rpoB* and *rpoC*, encoding β and β′ subunits of RNAP, respectively—were closely flanked by two selectable markers (Fig. 1A). The entire Δ*btuB3191*::Tn*10-rpoBC*-Δ*yjaZ*::*kan* construct was introduced into its native location in a strong DNA mutator background (Δ*mutL*, *mutD5*) by P1 transduction. The mutator strain containing the construct was transformed with pDksA L15F N88I and subject to overexpression. This selection allowed us to isolate colonies insensitive to the growth-inhibiting effects of pDksA L15F N88I. The Δ*btuB3191*::Tn*10-rpoBC*-p*yjaZ*::*kan* region was then transduced into MG1655 for analysis.

The absence of DksA causes auxotrophy (48), and a similar phenotype should be expected to result from the loss of RNAP's sensitivity to DksA. Indeed, the isolated mutation made strain MG1655 incapable of growth on minimal medium. Moreover, overexpression of wild-type *rpoC* relieved this phenotype, indicating that it was the chromosomal *rpoC* gene carrying a mutation that was responsible for making RNAP insensitive to DksA (Fig. 1B). Sequencing of the *rpoBC* operon of the mutant revealed a single amino acid substitution in the gene encoding the β′ subunit: E677G. As depicted below in Fig. 4, the mutation is located on the rim helices (RHs) on the surface of RNAP, which have been suggested to play a role in DksA binding (13, 42). Thus, glutamic acid residue 677 in the β′ subunit is critical for DksA's modulation of RNAP, and its replacement results in a phenotype that mimics the auxotrophic phenotype observed in the absence of DksA.

Super DksAs have an increased affinity for RNAP and therefore can alter transcription kinetics far more efficiently than wild-type DksA even in the absence of ppGpp (46). We tested whether these potent DksA mutations could overcome the growth defect of the β′ E677G mutation on minimal medium. The super DksA alleles L15F and N88I, as well as the double mutation DksA L15F N88I, were overexpressed, and plating efficiencies were determined by measuring the ratios of the numbers of CFU on minimal medium with and without amino acid supplement. Whereas wild-type DksA, as expected, did not rescue this phenotype due to the apparent DksA insensitivity conferred by the β′ E677G mutation, overexpression of the super DksAs allowed the nearly complete restoration of prototrophy (Table 1). An additional mutant with a new super DksA mutation, H61R, was also tested and showed full rescue of the phenotype (see Fig. 4 and Fig. S1 in the supplemental material). This suggests that the β′ E677G mutation can be overcome by the increased affinity of super DksAs for RNAP, allowing efficient transcriptional regulation. Therefore, either wild-type DksA does not bind to RNAP β′ E677G or the mutation could prevent a conformational change induced by DksA that is required for the effect of DksA on transcription.

The role of DksA in response to nutritional stress is to up- or downregulate certain promoters. One of the targets that becomes inhibited by DksA during the stringent response is the promoter

**TABLE 1** Super DksAs and the Gre factors rescue *rpoC* E677G auxotrophy[a]

| Strain | Relevant genotype | % CFU[b] |
|---|---|---|
| CH2624 | *rpoC* E677G/pBA169 | $(3.6 \pm 0.48) \times 10^{-3}$ |
| CH2625 | *rpoC* E677G/pDksA | $(7.7 \pm 3.7) \times 10^{-2}$ |
| CH2627 | *rpoC* E677G/pDksA N88I | $82.8 \pm 4.4$ |
| CH2628 | *rpoC* E677G/pDksA H61R | $64.8 \pm 5.6$ |
| CH2626 | *rpoC* E677G/pDksA L15F | $48.1 \pm 3.3$ |
| CH3650 | *rpoC* E677G/pDksA N88I L15F | $89.3 \pm 4.6$ |
| CH2459 | Δ*dksA*/pBR322 | $(7.3 \pm 0.45) \times 10^{-5}$ |
| CH2445 | Δ*dksA*/pGreB | $98.5 \pm 7.3$ |
| CH2444 | Δ*dksA*/pGreA | $68.1 \pm 7.2$ |
| CH965 | Δ*dksA*/pTraR | $103 \pm 6.5$ |
| CH2635 | *rpoC* E677G/pGreB | $89.4 \pm 2.0$ |
| CH2634 | *rpoC* E677G/pGreA | $102.3 \pm 4.2$ |
| CH2629 | *rpoC* E677G/pTraR | $8.1 \times 10^{-3}$ |

[a] A single E677G amino acid substitution in the β′ subunit phenotypically resembles the loss of *dksA* and results in auxotrophy, i.e., the inability to form colonies on minimal medium without amino acids due to the absence of the stringent response. The *rpoC* E677G mutant is suppressed by overexpression of super DksAs, i.e., by increasing the affinity of DksA for RNAP, but not by overexpression of the wild-type allele of DksA. Additionally, overexpression of GreA or GreB rescues the auxotrophy of both the *rpoC* E677G and Δ*dksA* strains. TraR, which acts similarly to super DksAs in a Δ*dksA* background, does not suppress the *rpoC* E677G mutation, indicating that RNAP is insensitive to this transcription factor. Full genotypes are available in Table S1 in the supplemental material.

[b] Number of CFU on M9-glucose-IPTG/number of CFU on M9-glucose-CAA-IPTG.

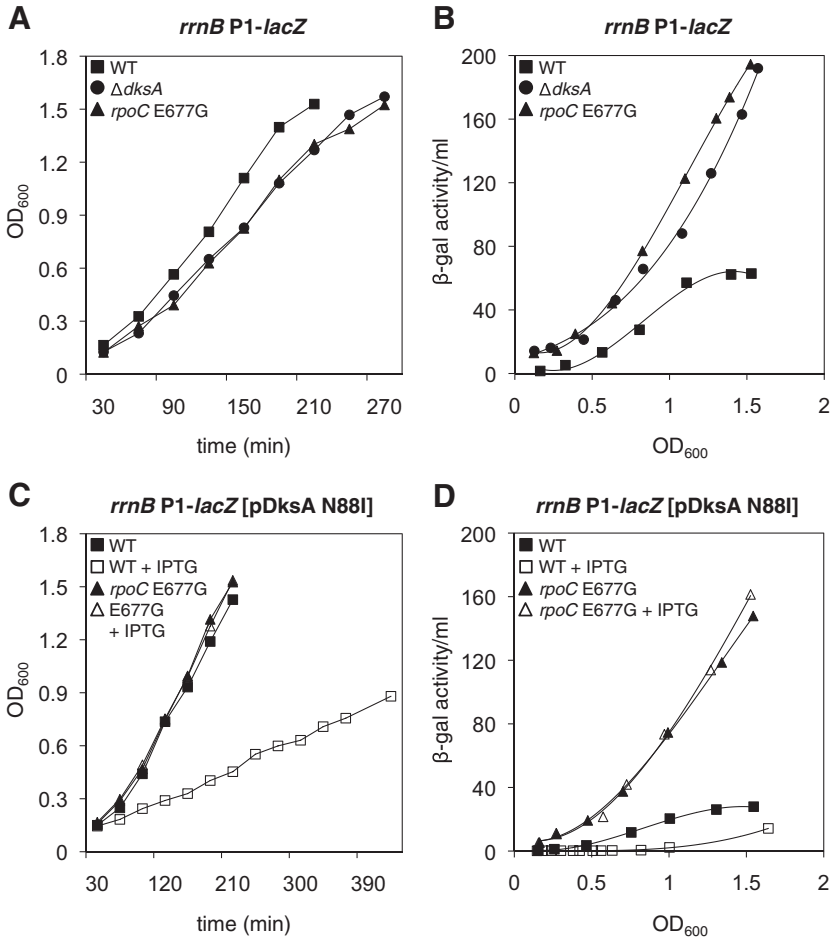


**FIG 2** β′ E677G prevents programmed regulation of *rrnB* P1 *in vivo*. The activity of *rrnB* P1-*lacZ* fusion was assessed. (A) The growth rates of the Δ*dksA* and β′ E677G mutants showed delays similar to the delay for wild-type *E. coli*. The following strains were used: CH958, which is the wild type (WT); CH980, which has the Δ*dksA* mutation; and CH3717, which has the *rpoC* E677G mutation. (B) Wild-type cells are capable of lowering the level of *rrnB* P1 expression when nutrients become scarce; the same promoter remains active in the Δ*dksA* and β′ E677G mutants. (C) Overexpression of the super DksA N88I allele severely affects the growth of wild-type *E. coli* by efficiently shutting down ribosomal genes expressed under the control of the *rrnB* P1 promoter. The growth rate of the β′ E677G mutant appeared to be undisturbed, suggesting a much smaller impact of the super DksA in this background. The wild-type strain was CH3767, and the strain with the *rpoC* E677G mutation was CH3769. (D) Low levels of DksA N88I due to the leakiness of the p*lac* promoter are sufficient to affect the activity of *rrnB* P1 in wild-type cells, whereas at high levels, it drastically inhibits the expression of the *rrnB* P1-*lacZ* fusion. β′ E677G cells are insensitive to growth inhibition caused by DksA N88I, suggesting that the mutated RNAP is much less affected by the presence of DksA than wild type. β-gal, β-galactosidase.

controlling a set of rRNA genes, *rrnB* P1 (49). We employed a previously described *rrnB* P1-*lacZ* fusion to monitor the activity of this promoter *in vivo* (22). Overnight LB cultures of wild-type, Δ*dksA*, and β′ E677G *E. coli* isolates were diluted 1/100 and grown until the $OD_{600}$ was 1.5. We observed a slight delay in the growth of both mutants (Fig. 2A). Moreover, the amount of β-galactosidase produced in the Δ*dksA* and β′ E677G strains increased exponentially during the course of the experiment, whereas the wild-type strain efficiently repressed the *rrnB* P1 promoter when the nutrients became limited and the cells entered into stationary phase (Fig. 2B). This indicates that the β′ E677G mutant is similar to strains lacking DksA or ppGpp, as they are incapable of responding to nutritional stress.

We also used the *rrnB* P1-*lacZ* construct to measure the sensitivity of wild-type RNAP and β′ E677G strains to transcription modulation by the super DksA L15F N88I allele. Overexpression of DksA L15F N88I drastically affects the growth rate of wild-type cells (Fig. 2C). This delay can be explained by a severe and unregulatable inhibition of the *rrnB* P1 promoter in wild type, which results in insufficient rRNAs to support robust growth. Overexpression of DksA N88I had no observable effect on the activity of *rrnB* P1, regardless of the β′ E677G mutation, a finding which is in contrast to the complete restoration of prototrophy (Fig. 2D). These data show either that wild-type DksA is not able to bind RNAP β′ E677G or, alternatively, that its binding does not affect mutant RNAP in the same way that it affects the wild-type complex.

**Effect of TraR, GreA, and GreB on the β′ E677G mutant.** In addition to various DksA alleles, we also overexpressed TraR, which regulates certain promoters in the same manner as DksA, but without the requirement of ppGpp (18). Overexpression of TraR is able to rescue the auxotrophy of Δ*dksA*. In contrast, we did not observe a full rescue of β′ E677G auxotrophy, suggesting that the single amino acid is critical for not only DksA-dependent transcription modulation but also TraR-dependent transcription modulation. Other secondary channel interactors have been

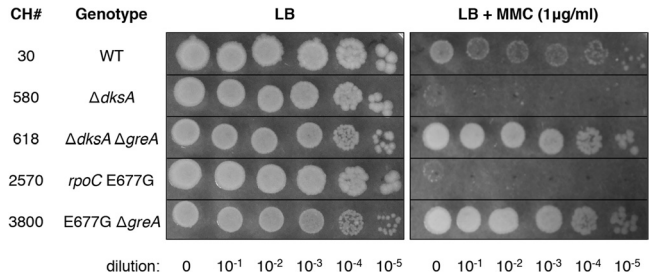


**FIG 3** The mitomycin C sensitivity of the β′ E677G mutant is alleviated by the absence of *greA*. Loss of DksA generates an *E. coli* strain particularly sensitive to the DNA cross-linking agent mitomycin C. Substitution of the single amino acid E677G in the β′ subunit of RNAP results in the same phenotype, suggesting a lack of DksA interaction. In both cases, subsequent deletion of GreA rescues the sensitivity, which indicates that the GreA transcription factor is capable of interaction with RNAP, despite the β′ E677G mutation. Therefore, the β′ E677G substitution specifically prevents DksA from modulating RNAP, but it does not affect RNAP's interaction with other auxiliary transcription factors, such as GreA. CH#, CH strain number.

shown to act to some extent like DksA (39, 40). GreB has been reported to decrease the activity of *rrnB* P1 *in vitro* and when overexpressed *in vivo* (39). We have not observed a similar phenomenon, which can be explained by the differences in the promoter strength used (see Fig. S2 in the supplemental material). We tested whether overexpression of GreA and GreB had any effect on the β′ E677G mutant and observed a nearly complete restoration of growth on amino acid-free medium when either GreA or GreB was ectopically expressed (Table 1). This result agrees with a recent report suggesting a certain level of functional redundancy between DksA and the Gre factors (40) and supports the *in vitro* results reported by Furman et al. showing that DksA and GreB interact with RNAP differently (41). Therefore, the E677G substitution in the β′ subunit of RNAP abolishes the functions of DksA and TraR, but it does not seem to affect RNAP-GreA/B interactions.

Apart from changing the affinity of RNAP for certain promoters and modulating transcription initiation, DksA has also been shown to play an important role in maintaining DNA integrity during DNA replication and transcription (14, 21). The study by Trautinger et al. indicated that DksA might be critical for the survival of cells suffering DNA damage as well as lesions caused by DNA cross-linking agents, such as mitomycin C (MMC) (21). We tested whether the sensitivity to MMC of the β′ E677G mutant is comparable to the loss of DksA and observed that DNA damage poses an extreme threat to the β′ E677G mutant similar to that to the Δ*dksA* strain (Fig. 3). Interestingly, the loss of GreA rescues the MMC sensitivity of the Δ*dksA* strain, and it also suppresses the MMC sensitivity of β′ E677G cells. Since the absence of GreA confers MMC resistance to both wild-type RNAP and the β′ E677G mutant RNAP, the interaction between RNAP and GreA is not likely affected by the substitution. Thus, these results also support the idea that the mutation harbored by the β′ subunit of RNAP is more DksA specific.

Additionally, these results agree with the recently proposed DksA docking model, which predicts that the C terminus of DksA (and, similarly, that of TraR) in certain conformations could extend along the RH on the surface of RNAP and reach the proximity of the glutamate residue, whereas the more globular C termini of GreA and GreB are located farther away (see Discussion).

**Isolation of RNAP suppressors of β′ E677G.** In order to further investigate the specificity of RNAP-DksA interactions, we proceeded to isolate Rif$^r$ suppressors of β′ E677G, since several rifampin resistance mutations have been shown to suppress the loss of DksA by reducing the stability of the RNAP-promoter complex, thereby mimicking the effects of DksA on complex stability observed *in vitro* (15, 30, 50–52). Rifampin is an RNAP inhibitor that binds the RNAP active site; numerous mutations in this region can confer resistance by preventing the drug from binding. We plated overnight cultures on LB-rifampin, selected resistant colonies, and tested whether they were capable of growth on minimal medium. This led to the isolation of a suppressor of β′ E677G. DNA sequencing revealed a single amino acid substitution in the *rpoB* gene encoding the β subunit of RNAP. β V146F suppresses β′ E677G and also abolishes the requirement of ppGpp, allowing growth on minimal medium in the absence of both RelA and SpoT (Table 2). Furthermore, the β′ E677G suppression by β V146F depends on the presence of DksA. This could be achieved either by strengthening the effect of DksA on RNAP, similar to the action of super DksAs, or by directly affecting the promoter complex lifetime. To test whether DksA-independent suppressors can also restore the β′ E677G phenotype, we repeated the selection for spontaneous rifampin resistance but this time in a β′ E677G Δ*dksA* background. DNA analysis of the *rpoBC* operon of a selected candidate identified a G534D substitution in *rpoB*. This single amino acid substitution is located near the active center of RNAP, in proximity to β V146F (Fig. 4). β G534D allows growth on minimal medium without amino acids in β′ E677G, Δ*dksA*, and Δ*relA* Δ*spoT* backgrounds, suggesting that the nature of the suppression is DksA and ppGpp independent, which could be explained by a strong, destabilizing influence on promoter complexes. By isolating spontaneous suppressors of β′ E677G, we have further characterized two previously reported Rif$^r$ mutations (53).

## DISCUSSION

Using a genetic selection, we have identified a novel RNAP mutation residing in the gene encoding the β′ subunit of the complex, *rpoC* E677G. Replacement of the acidic glutamate with glycine results in an inability of RNAP to respond to the auxiliary tran-

**TABLE 2** Suppression of β′ E677G by β V146F and β G534D[a]

| Strain | Relevant genotype | % CFU[b] |
|---|---|---|
| CH3532 | *rpoC* E677G | $(1.5 \pm 0.8) \times 10^{-5}$ |
| CH3178 | *rpoC* E677G Δ*dksA* | ND[c] |
| CH3539 | *rpoC* E677G Δ*relA* Δ*spoT* | $(3.34 \pm 1.43) \times 10^{-4}$ |
| CH3487 | *rpoC* E677G *rpoB* V146F | 91.1 ± 7.2 |
| CH3165 | *rpoC* E677G *rpoB* V146F Δ*dksA* | ND |
| CH3370 | *rpoC* E677G *rpoB* V146F Δ*relA* Δ*spoT* | 63.7 ± 12.0 |
| CH3511 | *rpoC* E677G *rpoB* G534D | 94.3 ± 5.8 |
| CH3332 | *rpoC* E677G *rpoB* G534D Δ*dksA* | 95.8 ± 5.4 |
| CH3372 | *rpoC* E677G *rpoB* G534D Δ*relA* Δ*spoT* | 102.9 ± 6.9 |

[a] Auxotrophy is measured as the percentage of cells forming colonies on medium without amino acids. The auxotrophy of the β′ E677G mutant is relieved by additional mutations in the β subunit of RNAP. The *rpoB* V146F mutation allows DksA to modulate RNAP β′ E677G even in the absence of ppGpp (Δ*relA* Δ*spoT*). Another single amino acid substitution—β G534D—generates RNAP independently of both DksA and ppGpp. Different effects of the mutation can be explained by different extents of RNAP-promoter complex destabilization. Full genotypes are available in Table S1 in the supplemental material.

[b] Number of CFU on M9-glucose/number of CFU on M9-glucose-CAA.

[c] ND, not detectable ($<10^{-5}$).

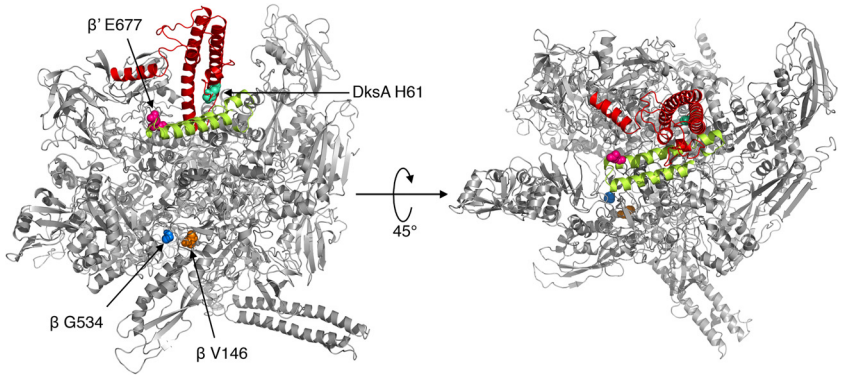


**FIG 4** β′ E677G is located at the tip of RH domain. The RNAP-DksA docking model proposed by Lennon et al. (42) shows the β′ subunit residue E677 (highlighted in pink) located at the tip of the rim helices (RHs; green). DksA (red) docks into the secondary channel. The H61 residue (cyan) that increases DksA's effect on RNAP is located near RHs (10 Å). Two suppressors of the β′ E677G mutation—β V146F (orange) and β G534D (blue)—are located approximately 40 Å from the active tip of DksA.

scription factor DksA. Our results show that the β′ E677G mutant is unable to shut down the *rrnB* P1 promoter during stationary-phase entry and is auxotrophic for amino acids, similar to the phenotype observed for the Δ*dksA* mutant. Thus, the lack of DksA-dependent modulation renders the mutant RNAP incapable of responding to nutritional stress. The Δ*dksA*-like phenotype of the β′ E677G mutant could also result from RNAP's insensitivity to ppGpp rather than DksA; however, TraR, a functional homolog of DksA, does not require ppGpp and is also unable to modulate the mutant RNAP. Even though β′ E677G is unable to respond to DksA or TraR, it does not appear to be impaired with respect to its interaction with GreA or GreB. These data agree with the recent report suggesting that different conformations of RNAP interact with different auxiliary transcription factors (41). Therefore, the β′ E677G mutation specifically abolishes the effect of DksA/TraR on RNAP. Since the super DksA mutants with higher affinity for RNAP compensate for the E677G mutation, it is possible that this substitution affects binding of DksA to RNAP. It is interesting to note that both super DksAs and TraR can bypass the ppGpp requirement for growth on minimum medium without amino acids but that only super DksAs can suppress the auxotrophy of the E677G mutant, suggesting that TraR may affect RNAP differently than the super DksAs.

Our discovery that a novel RNAP β′ E677G allele prevents RNAP modulation by DksA agrees with the recently proposed model of DksA binding to RNAP (42). β′ E677 is located at the tip of the RH domain, with which DksA is shown to interact. According to the published coordinates, the coiled-coil domain of DksA is responsible for the majority of the interaction with residues 648 to 900 of the β′ subunit encompassing the RH domain. The residues of DksA that increase its affinity for RNAP (L15F and N88I) as well as H61 are all located in the proximity of the RHs. β′ E677G is located approximately 15 Å from the C-terminal coil of DksA, which does not support the possibility of a direct interaction. However, as suggested by recent studies, auxiliary transcription factors may alter their position in the secondary channel as RNAP undergoes conformational changes between ratcheted and non-ratcheted states, yet the ratcheted state has not been confirmed for *E. coli* RNAP (11, 42). This could allow DksA to translocate along RHs and interact directly with β′ E677G. Alternatively, the mutation of a residue at the turn of RHs could destabilize the structure of the entire domain and result in altered DksA binding or a lack thereof. Therefore, the interaction requiring the glutamate tip of RHs may be either direct or indirect.

It has been a puzzle how RNAP can interact with various transcription factors competing for the secondary channel and be capable of selecting the right factor for each stage of gene expression. A recent study by Furman et al. explains how RNAP can react to the presence of DksA and GreB (41). These two secondary channel interactors bind different conformations of RNAP, and therefore, DksA is preferred during transcription initiation, whereas the Gre factors are favored during the elongation step (41). Nevertheless, it has been shown that in the absence of one of the secondary channel interactors, the others can take its place (39, 40). We tested how efficiently GreA and GreB can substitute for DksA when the β′ E677G mutation is present. DksA and the Gre factors share no sequence homology, but the overall structures of these proteins are very similar: they consist of a C-terminal head domain which interacts with the surface of RNAP and a helical hairpin (coiled-coil domain), at the end of which the active tip is located. Since the active residues of DksA and the Gre factors are located at the tip of the coiled-coil domain, the interaction of these transcription factors with RNAP should follow similar rules, where the head domain clashes with RHs and the coiled-coil protrudes deep into the secondary channel. Our data clearly demonstrate the interchangeability of DksA and the Gre factors when they are overexpressed. More importantly, β′ E677G, which is incapable of fruitful interaction with DksA, is a substrate for the Gre factors, which indicates that the single amino acid substitution at the turn of the RHs affects the action of DksA but not the Gre factors. The Gre factors have a globular head domain that is expected to interact with the RHs far away from β′ E677G (2); however, it has been shown *in vitro* that mutating β′ residues 672 and 673 located at the turn of the RHs drastically reduces the binding of RNAP to both DksA and GreB, presumably by disrupting the conformation of this domain (4). Our β′ E677G mutant differentiates DksA and the Gre factor interactions, as it specifically disrupts the RNAP-DksA interaction *in vivo*. Therefore, the β′ E677G mutant is a novel RNAP mutant that is insensitive to DksA but is functional for GreA/B activity.

Using the β′ E677G mutant, we isolated two suppressor mutations restoring its growth on minimal medium. Both muta-

tions—V146F and G534D—reside in the β subunit near the active center of RNAP. On the basis of the recently proposed DksA docking model, they are located approximately 40 Å from the tip of DksA and therefore are unlikely to affect the RNAP-DksA interaction directly (Fig. 4). β G534D has previously been reported to destabilize RNAP-promoter complexes and suppress Δ*dksA* and ppGpp$^0$ (Δ*relA* Δ*spoT*) strains. Other β-subunit substitutions located in the same region as V146F (Q148P and R151S) have also been documented to suppress the absence of ppGpp in a DksA-dependent manner, and one of them (Q148P) has been shown to destabilize promoter complexes *in vitro* (54, 55). Our observations of the β V146F phenotype suggest that this substitution has a very similar effect on transcription; i.e., it can suppress the absence of ppGpp but not that of DksA. This could suggest that either β V146F allosterically restores or potentiates the DksA-β′ E677G interaction or DksA binds to RNAP, regardless of the β′ E677G mutation, but its effect is so weak that it requires the promoter complex-destabilizing mutation at β V146F to function. Additional mutants suppressing the growth defect of E677G need to be isolated to further characterize the mode of action of DksA on RNAP, and biochemical affinity assays should provide an unequivocal answer to this question.

We show that β′ E677 specifically affects RNAP-DksA activity. The E-to-G substitution can abolish the modulating effect of DksA on RNAP either by preventing the interaction of RNAP directly or by distorting the RH domain on the surface of the β′ subunit of RNAP. Further *in vitro* characterization of these mutants will be extremely useful to the understanding of the details of the mechanism used by DksA to regulate transcription initiation/elongation. This work also serves to dissect the details of how different transcription factors compete for the same binding partner and how global RNAP regulation is achieved in a living cell.

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