RESEARCH ARTICLE

A *Rhodobacter sphaeroides* Protein Mechanistically Similar to *Escherichia coli* DksA Regulates Photosynthetic Growth

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ABSTRACT **DksA is a global regulatory protein that, together with the alarmone ppGpp, is required for the "stringent response" to nutrient starvation in the gammaproteobacterium *Escherichia coli* and for more moderate shifts between growth conditions. DksA modulates the expression of hundreds of genes, directly or indirectly. Mutants lacking a DksA homolog exhibit pleiotropic phenotypes in other gammaproteobacteria as well. Here we analyzed the DksA homolog RSP2654 in the more distantly related *Rhodobacter sphaeroides*, an alphaproteobacterium. RSP2654 is 42% identical and similar in length to *E. coli* DksA but lacks the Zn finger motif of the *E. coli* DksA globular domain. Deletion of the RSP2654 gene results in defects in photosynthetic growth, impaired utilization of amino acids, and an increase in fatty acid content. RSP2654 complements the growth and regulatory defects of an *E. coli* strain lacking the *dksA* gene and modulates transcription *in vitro* with *E. coli* RNA polymerase (RNAP) similarly to *E. coli* DksA. RSP2654 reduces RNAP-promoter complex stability *in vitro* with RNAPs from *E. coli* or *R. sphaeroides*, alone and synergistically with ppGpp, suggesting that even though it has limited sequence identity to *E. coli* DksA ($DksA_{Ec}$), it functions in a mechanistically similar manner. We therefore designate the RSP2654 protein $DksA_{Rsp}$. Our work suggests that $DksA_{Rsp}$ has distinct and important physiological roles in alphaproteobacteria and will be useful for understanding structure-function relationships in DksA and the mechanism of synergy between DksA and ppGpp.**

IMPORTANCE **The role of DksA has been analyzed primarily in the gammaproteobacteria, in which it is best understood for its role in control of the synthesis of the translation apparatus and amino acid biosynthesis. Our work suggests that DksA plays distinct and important physiological roles in alphaproteobacteria, including the control of photosynthesis in *Rhodobacter sphaeroides*. The study of $DksA_{Rsp}$, should be useful for understanding structure-function relationships in the protein, including those that play a role in the little-understood synergy between DksA and ppGpp.**

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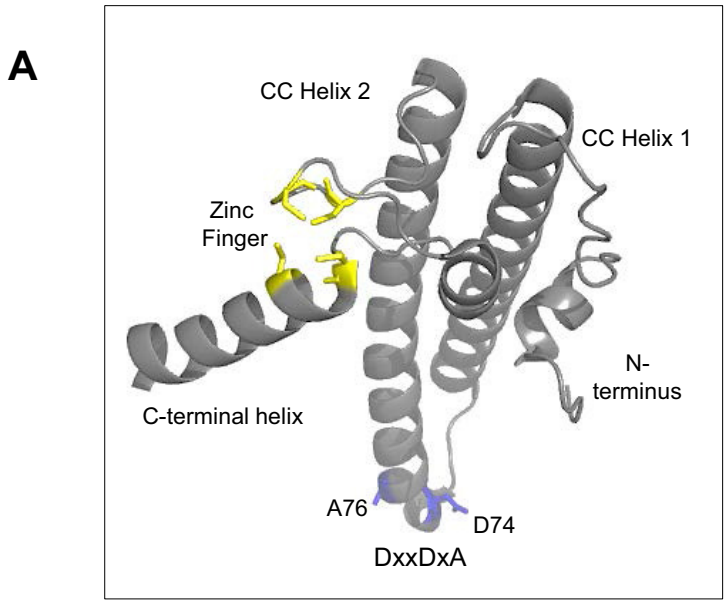
In *Escherichia coli*, the 151-residue regulatory protein DksA, together with the modified guanine nucleotides ppGpp and pppGpp (together referred to here as ppGpp), regulates transcription in response to various nutritional conditions and cellular stresses (e.g., the stringent response) (1–4). Pleiotropic effects of deletion of *dksA*-like genes have been found in a number of species, including effects on functions required for pathogenesis (e.g., in *Vibrio cholerae* [5], *Pseudomonas aeruginosa* [6], *Shigella flexneri* [7], *Campylobacter jejuni* [8], and *Legionella pneumophila* [9]). However, direct demonstrations of effects on transcription *in vitro* have been carried out with DksA only from three closely related gammaproteobacteria, *E. coli* (10), *P. aeruginosa* (11), and *S. flexneri* (7).

DksA has been studied most extensively in *E. coli*, where it inhibits transcription from a large number of promoters both *in vivo* and *in vitro*, including those for synthesis of ribosomal RNAs, ribosomal proteins, fatty acids, the flagellar cascade master regulator FlhDC, the transcription activator Fis, and the promoter for the *dksA* gene itself (2, 10, 12–16). DksA also activates transcription from a different set of promoters *in vivo* and *in vitro*, including those for some amino acid biosynthesis genes (17). Unlike most transcriptional regulators, *E. coli* DksA ($DksA_{Ec}$) does not interact with promoter DNA. Its promoter specificity derives from the fact that different promoters are rate limited at different steps in the kinetic mechanism. DksA binds to RNA polymerase (RNAP) in all promoter complexes tested to date and alters the rates of a specific step(s) in the pathway to open complex formation, but it affects transcriptional output only from promoters rate limited at that step(s) (2, 10, 18–20). $DksA_{Ec}$ is also associated with elongating RNAP *in vivo*, reduces transcription-replication conflicts, and affects DNA repair (21–23).

$DksA_{Ec}$ has three major structural features, a coiled-coiled domain with a DxxDxA motif in the loop at its tip (residues 35 to 109), a globular Cys4 zinc finger domain (residues 7 to 33 and 110 to 134), and a C-terminal α-helix (residues 135 to 151) (Fig. 1A) (18, 24). The coiled-coil domain binds in the RNAP secondary



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                                  N-terminus                                  CC Helix 1
                                             10        20        30        40        50        60        70
Eco_DksA          -----MQEGQN-RKTSSLSILAIAGVEPYQEKPGEEYMNEAQLAHFRRILEAWRNQLRDEVDRTVTHMQDEAANFP
Rsp 2654          MTVNHISEPAGLQQRAAAMKAEIFLPEDYRPAENEPFMNERQLEYFRRKLLNWKQELLDQSAETIEGLQESGRNVP
Pae DksA1         -----MS---T-KAKQQSSQQMTRGFEPYQETKGEEYMSERMRAHFTAILNKWKQELMEEVDRTVHHMQDEAANFP
Pae DksA2         ----------------------MTEQELLAQPDAAYMDEAQQDFFRDLLLRQRQELQARIEGEFGELRDLE-RPS
Rsp 0166          ---------------------------------------- MIDIARRKSQLEALADLGARLEGIEAELDSHNSR-

                                  80        90        100       110       120       130       140       150
Eco_DksA          DPVDRAAQEEEFSLELRNRDRERKLIKKIEKTLKKVEDEDFGYCESCGVEIGIRRLEARPTADLCIDCKTLAEIREKQMAG-
Rsp 2654          DIADRASEETDRALELRTRDRQRKLVAKIDAALRRIEAGEYGYCEVTGEPISLKRLDARPIATMTLEAQERHERRERVHRDE
Pae DksA1         DPADRASQEEEFSLELRARDRERKLIKKIDETLQLIEDEEYGWCDSCGVEIGIRRLEARPTATLCIDCKTLAEIREKQLGS-
Pae DksA2         DEADLASREEQRQWQLRLLEREKKLLDKIDEALERLARGDYGWCQETGEPIGLRRLLLRPTATLCIECAKERQEKRERHVRHN
Rsp 0166          DWEELATERETEEVLESMGNSGQQEIRAIMAALARIEADEYGYCMKCGAPIGDARLDVLPYTPFCRNCAG------------

                                  DxxDxA              CC Helix 2                    Zinc Finger     C-terminal helix
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FIG 1 (A) Structural features of *E. coli* DksA (adapted from reference 18; PDB 1TJL). The N-terminal region, coiled-coil (CC) helices 1 and 2, zinc finger-containing globular domain, and C-terminal helix are indicated. Coiled-coil tip residues D74 and A76 (blue, within the DxxDxA motif [24, 25]) and zinc finger residues C114, C117, C135 and C138 (yellow) are shown in stick form. (B) Alignment (ClustalW) of *E. coli* DksA (Eco DksA) with *R. sphaeroides* RSP2654 and RSP0166 and *P. aeruginosa* DksA1 and DksA2. Identical or conservatively substituted residues are shown in red. Locations of structural features shown in panel A are indicated. Cysteine residues are underlined.

channel, with tip residues (D74 and A76; part of the DxxDxA motif) positioned near the trigger loop of the enzyme, whereas the Zn finger-containing globular domain has been modeled to interact with the rim of the secondary channel (18, 20, 24, 25). The DxxDxA motif and the Zn-finger motif are essential for $DksA_{Ec}$ function *in vitro* and *in vivo* (10, 25, 26), and effects of deleting residues from the C-terminal α-helix suggest a role for this feature as well (24).

$DksA_{Ec}$ is in the DksA/TraR family, whose members have been defined by protein sequence conservation and annotated in the genomes of many bacterial species. Some bacterial species encode more than one member of the DksA/TraR family. For example, *P. aeruginosa* encodes two members with lengths similar to that of $DksA_{Ec}$, one containing and one lacking a Zn finger motif, DksA1 and DksA2, respectively, and both function similarly to $DksA_{Ec}$ *in vitro* and *in vivo* (11). Although *P. aeruginosa* DksA2 lacks a Zn finger, an X-ray structure showed that it contains a globular fold very similar to that of $DksA_{Ec}$ (26). Expression of *P. aeruginosa*

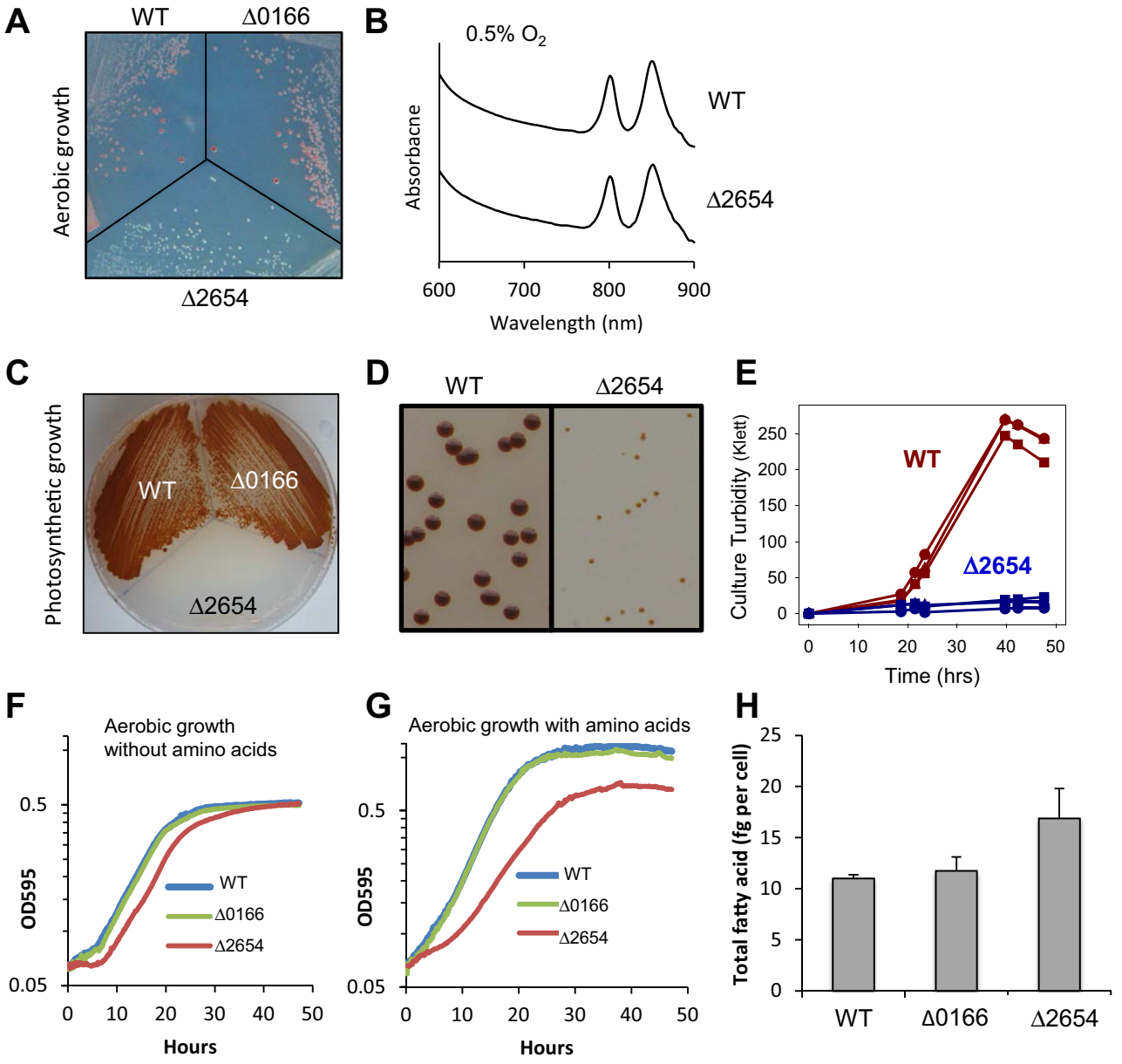


FIG 2 Phenotype of *R. sphaeroides* strains with deletions of RSP2654 (Δ2654) and RSP0166 (Δ0166). (A) Colony pigmentation for wild-type (WT) and mutant strains grown aerobically on SIS agar. (B) Absorbance spectra of wild-type and Δ2654 liquid cultures bubbled with 0.5% O_2 in the dark show the presence of photosynthetic pigment-protein complexes in both strains. Spectra were obtained from intact cells, normalized to equal absorbance at 680 nm, and staggered for presentation on one vertical axis. (C) Photosynthetic growth of wild-type and mutant strains streaked on SIS agar plates and grown anaerobically in the light. (D) Colony morphology of wild-type and Δ2654 cells grown photosynthetically for 7 days in the light. (E) Photosynthetic growth of triplicate liquid cultures of wild-type (red) or Δ2654 (blue) strains. (F and G) Growth curves of wild-type and mutant strains. Cells were grown aerobically in 96-well plates in either SIS medium lacking all amino acids (F) or SIS medium supplemented with 0.4% Casamino Acids (G), and the generation times (in hours) were calculated from three independent experiments, each containing at least 4 biological replicates. One representative curve for each strain is shown. The generation times (average ± standard deviation) in SIS medium lacking all amino acids were as follows: WT, 5.7 ± 0.5 h; Δ0166, 5.8 ± 0.6 h; Δ2654, 6.5 ± 0.7 h. The generation times in SIS medium supplemented with 0.4% Casamino Acids were as follows: WT, 4.4 ± 0.1 h; Δ0166, 4.3 ± 0.1 h; Δ2654, 6.8 ± 0.5 h. (H) Fatty acid content of wild-type and mutant strains grown aerobically in liquid cultures bubbled with 30% O_2. The 1.5-fold increase in fatty acid content of Δ2654 relative to that of the wild type is statistically significant ($P < 0.005$). Fatty acids were analyzed by GC-MS and normalized to CFU; each value represents the average for three independent experiments ± standard deviation.

DksA2 is regulated by the zinc-responsive transcriptional regulator Zur (11), suggesting that it plays a role under zinc-limiting conditions.

DksA-like proteins from other species have not been characterized *in vitro* for effects on transcription, and it is not clear which other DksA/TraR family members exhibit $DksA_{Ec}$-like function.

Analysis of predicted proteins in representatives of approximately 570 bacterial genera has indicated various degrees of conservation of the DxxDxA and Zn finger motifs, including some with both motifs and others with only a DxxDxA-like or only a Zn finger motif (24). Based on this bioinformatic analysis, in conjunction with the *in vitro* analysis of DksAs from *E. coli* and

P. aeruginosa, it was suggested that an intact DxxDxA motif is an indicator that the protein functions like DksA, whereas a Zn finger in the globular domain is not essential for DksA function (26). Other sequence and/or structural features important for function potentially could be revealed by *in vitro* analysis of DksA-like proteins from more distantly related species.

In this work, we investigated the functions of two gene products annotated as DksA/TraR family members (RSP2654 and RSP0166) in *Rhodobacter sphaeroides*, an energetically and metabolically diverse alphaproteobacterium capable of growth by anaerobic photosynthesis or by aerobic or anaerobic respiration (27). Aerobically grown cells lack the machinery for photosynthesis and morphologically resemble other Gram-negative bacteria. However, under low-oxygen or anaerobic conditions, *R. sphaeroides* synthesizes light-harvesting and electron transfer components of the photosynthetic apparatus and inserts them into specialized invaginations of the cytoplasmic membrane called intracytoplasmic membranes (28).

R. sphaeroides transcriptional networks affecting growth and the roles of different RNAP holoenzymes under different conditions have been examined (27–32), but the function(s) of its *dksA*-like gene(s) is not known. Here we have shown that an *R. sphaeroides* strain deleted for the RSP2654 gene is severely impaired in anaerobic photosynthetic growth, while aerobically grown cells display altered amino acid utilization properties and increased fatty acid content. In contrast, we did not detect a phenotype for an *R. sphaeroides* strain deleted for the RSP1066 gene. RSP2654 (but not RSP0166) also complemented an *E. coli dksA* null mutant, and like $DksA_{Ec}$, it inhibited transcription *in vitro*, reduced promoter open complex stability, and functioned synergistically with ppGpp on RNAP from either *E. coli* or *R. sphaeroides*. We suggest that RSP2654 is a functional homologue of $DksA_{Ec}$ and that it has functions in this alphaproteobacterium other than or in addition to those identified in *E. coli* and other analyzed gammaproteobacteria.

RESULTS

***R. sphaeroides* RSP2654 encodes a DksA-like protein that is required for normal photosynthetic growth.** Annotation of the *R. sphaeroides* 2.4.1 genome sequence identified two open reading frames, RSP2654 and RSP0166, encoding DksA-like proteins (33). RSP2654 is expressed both anaerobically and aerobically, whereas RSP0166 is expressed at low levels aerobically and is induced to higher levels under anaerobic conditions (31, 34). RSP2654 encodes a 158-amino-acid protein that is 42% identical to $DksA_{Ec}$ and contains the conserved DxxDxA motif but only one of the four cysteines in its globular domain that would correspond to the Zn finger motif in $DksA_{Ec}$ (Fig. 1B). RSP0166 encodes a 105-amino-acid protein that is 35% identical to $DksA_{Ec}$, contains the cysteine ligands for the Zn finger motif, and has an aspartate-to-glutamate substitution in the DxxDxA motif (Fig. 1B).

To test the function of these two DksA-like proteins in *R. sphaeroides*, we constructed mutant strains with markerless deletions of either open reading frame, strains Δ2654 and Δ0166. Wild-type cells form red-pigmented colonies when grown on agar plates aerobically in the dark, because as oxygen becomes limiting in the colony, cells induce expression of components of the photosynthetic apparatus, including bacteriochlorophyll and carotenoid pigments (Fig. 2A) (35). The Δ0166 mutant formed red-pigmented colonies like those of wild-type *R. sphaeroides*, but the Δ2654 mutant formed much paler colonies, indicating that it was defective in pigment production under these conditions (Fig. 2A).

To test whether assembly of photosynthetic pigment-protein complexes was impaired under the more strictly anaerobic conditions in which they are normally inserted into the intracytoplasmic membrane (but not used for photosynthesis), we grew wild-type and Δ2654 cells under lower oxygen tension (0.5%) in liquid cultures in the dark. Wild-type and Δ2654 cultures both appeared red pigmented (data not shown), and the visible spectra of intact cells for both strains were nearly identical, with characteristic peaks at 800 and 850 nm (from the B800-850 light-harvesting pigment-protein complex) and a characteristic shoulder at 875 nm (from the B875 light-harvesting pigment protein complex), suggesting that there were equal amounts of these complexes in both strains (Fig. 2B). When we measured the total amount of bacteriochlorophyll present in acetone-soluble extracts of these cultures using the method described by Cohen-Bazire et al. (36), we found equivalent levels of this photosynthetic pigment in Δ2654 and wild-type cells (data not shown). Thus, Δ2654 was able to make pigments, assemble pigment-protein complexes, and insert them into membranes as did wild-type cells in low-oxygen liquid cultures.

Together these observations suggest that there is a partial defect in the oxygen induction of pigment. This defect is apparent in aerobically grown colonies, but it is not apparent under lower oxygen tensions (0.5%) in liquid cultures (Fig. 2A). Normal synthesis of bacteriochlorophyll and assembly of light-harvesting complexes occurs at this low oxygen tension.

We tested the ability of the photosynthetic apparatus to function by assessing growth anaerobically in the light, either on plates or in liquid culture. Wild-type and Δ0166 cells grew normally when streaked on a photosynthetic plate, but Δ2654 exhibited a marked growth defect (Fig. 2C). When plated for single colonies, the Δ2654 strain plated with 93 to 99% efficiency under photosynthetic growth conditions and was pigmented, but it formed much smaller colonies than the wild type, even when incubated photosynthetically for 7 days (Fig. 2D and data not shown). In liquid culture, the wild-type strain grew well after an initial lag period, reaching maximum density after approximately 40 h. In contrast, Δ2654 showed no growth during this time (Fig. 2E). Upon further incubation, apparent suppressors of the Δ2654 defect grew, appearing at different times in independent cultures (data not shown). Thus, loss of RSP2654 results in a severe decrease, albeit not a complete loss, of photosynthetic growth.

We therefore suggest that the loss of RSP2654 results in two defects. First, a partial defect in the oxygen-sensing mechanism that induces pigment production occurs during aerobic colony formation, but induction and assembly of normal levels of photosynthetic pigment complexes occur under anaerobic conditions in liquid culture. Second, the loss of RSP2654 causes a defect in photosynthetic growth that is downstream of assembly of the light-harvesting pigment-protein complexes.

***R. sphaeroides* RSP2654 is necessary for utilization of exogenous amino acids.** In *E. coli*, $DksA_{Ec}$ acts with ppGpp to activate a subset of the promoters needed for amino acid biosynthesis and transport (17), and cells deleted for *dksA* are unable to grow on media lacking amino acids (10, 19). Therefore, we asked whether deletion of the RSP2654 or RSP0166 gene resulted in a similar phenotype. *R. sphaeroides* typically is grown in a defined medium (SIS) that contains low concentrations of aspartic acid and glu-

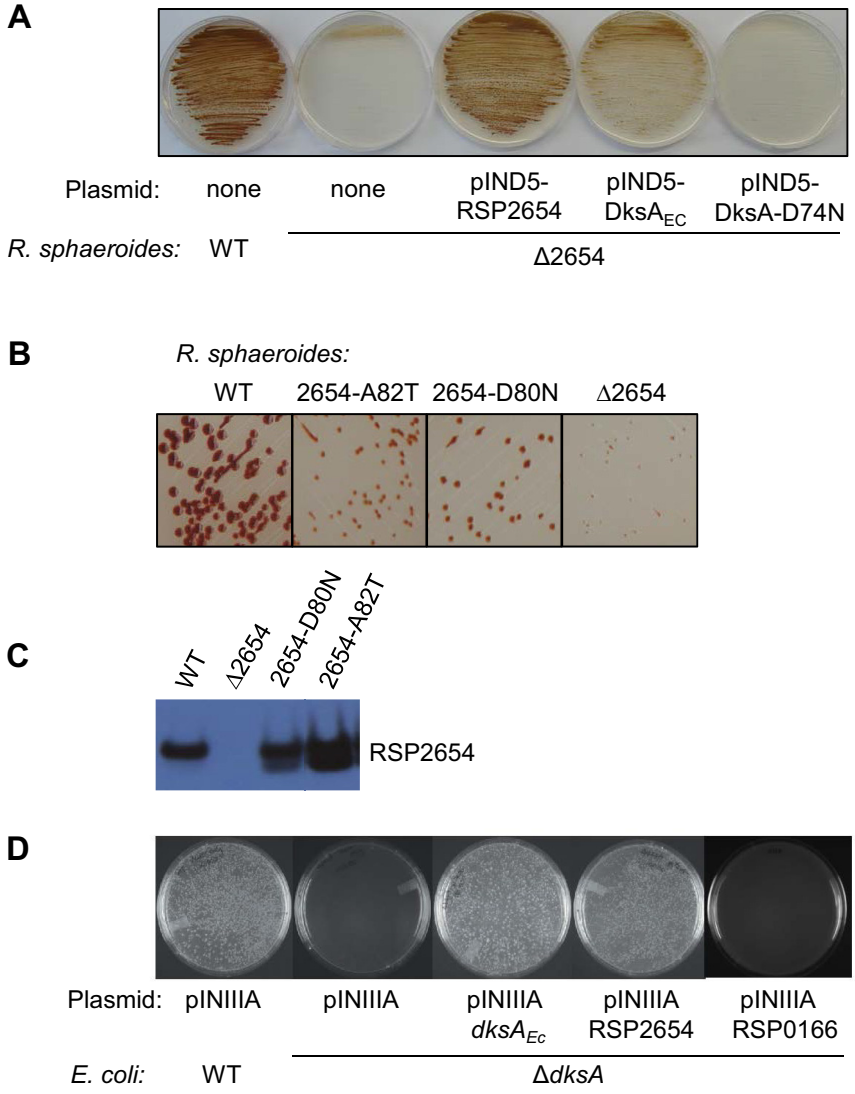


FIG 3 (A) Photosynthetic growth of *R. sphaeroides* Δ2654 is rescued by expression of plasmid-encoded RSP2654 or DksA$_{Ec}$. *R. sphaeroides* wild type, Δ2654, and Δ2654 derivatives carrying the indicated plasmids were streaked onto SIS agar plates containing 100 mM IPTG to induce gene expression and incubated anaerobically in the light. (B) Strains encoding substitutions in the putative coiled-coil tip residues D80 and A82 of RSP2654 show reduced colony size when grown photosynthetically. *R. sphaeroides* wild-type, 2654-D80N, 2654-A82T, and Δ2654 were streaked on SIS agar plates and grown anaerobically in the light. (C) Western blot analysis showing the levels of wild-type or mutant RSP2654 protein present in the strains shown in panel B. Equal amounts of total protein were loaded in each lane. (D) The growth defect of *E. coli* Δ*dksA* cells on minimal agar without amino acids is rescued by expression of plasmid-encoded DksA$_{Ec}$ or RSP2654 but not by RSP0166. DksA$_{Ec}$, RSP2654, and RSP0166 were expressed constitutively from the pINIIIA vector.

tamic acid (37), but for the following experiments, we used a modified SIS medium without any amino acids. The *R. sphaeroides* wild-type, Δ2654, and Δ0166 strains all grew aerobically with similar growth rates and to the same optical density in the medium lacking amino acids, although the Δ2654 mutant exhibited an extended lag before exponential growth and a small but statistically significant increase in doubling time (6.5 h for Δ2654 versus 5.7 h for the wild-type strain; $P = 0.001$) (Fig. 2F). In contrast, no significant difference in the aerobic growth rate was observed between Δ0166 and wild-type cells (Fig. 2F). The extents and rates of aerobic growth were similar for the three strains in SIS medium with or without aspartic acid and glutamic acid (data not shown).

In contrast to the similar growth rates (generation times) of the three strains in medium lacking amino acids described above, the wild-type and Δ0166 strains were able to utilize exogenous amino acids to increase their growth rate and biomass, whereas Δ2654 was not (Fig. 2G). The generation time of Δ2654 was virtually unaffected by addition of Casamino Acids to the SIS medium (6.8 h versus 6.5 h; Fig. 2F and G), whereas addition of Casamino Acids decreased the generation times of the wild-type and Δ0166 strains by 25% (4.3 versus 5.7 h; Fig. 2F and G). Thus, the difference in generation times between the wild-type and Δ2654 strains was more pronounced in the presence than in the absence of amino acids (6.8 versus 4.3 h, respectively, in Casamino Acids, compared to 6.5 versus 5.7 h without amino acids) (Fig. 2F). Δ2654 also grew to a lower optical density (optical density at 595 nm [OD_{595}] of 0.7) than the wild-type or Δ0166 strain (OD_{595}, 1.1) in the presence of Casamino Acids (Fig. 2G). These results suggest that RSP2654 may have a role in transport or utilization of one or more amino acids in *R. sphaeroides*.

Deletion of *R. sphaeroides* RSP2654 leads to an apparent increase in cellular fatty acid content. Because DksA$_{EC}$ inhibits some promoters for fatty acid biosynthesis genes (16), we also asked if the fatty acid content of the *R. sphaeroides* Δ2654 and Δ0166 mutants was altered. Total lipids were extracted from wild-type and mutant cells grown aerobically at 30% O_2, reacted to form fatty acid methyl esters, and analyzed quantitatively by gas chromatography-mass spectrometry (GC-MS). We found that the fatty acid content of Δ2654 cells was increased approximately 1.5-fold per CFU relative to that of wild-type or Δ1066 cells (Fig. 2H) ($P < 0.005$), consistent with a negative effect of RSP2654 on fatty acid accumulation. The relative amounts of the major fatty acid species were the same among the three strains (data not shown) and were consistent with the fatty acid composition observed in previous studies of *R. sphaeroides* (38). The Δ2654 mutant cells did not form chains or display other obvious differences in cell morphology when observed by phase-contrast microscopy (data not shown), suggesting that the apparent increase in fatty acid content per CFU did not result from cell division defects that reduced the number of CFU.

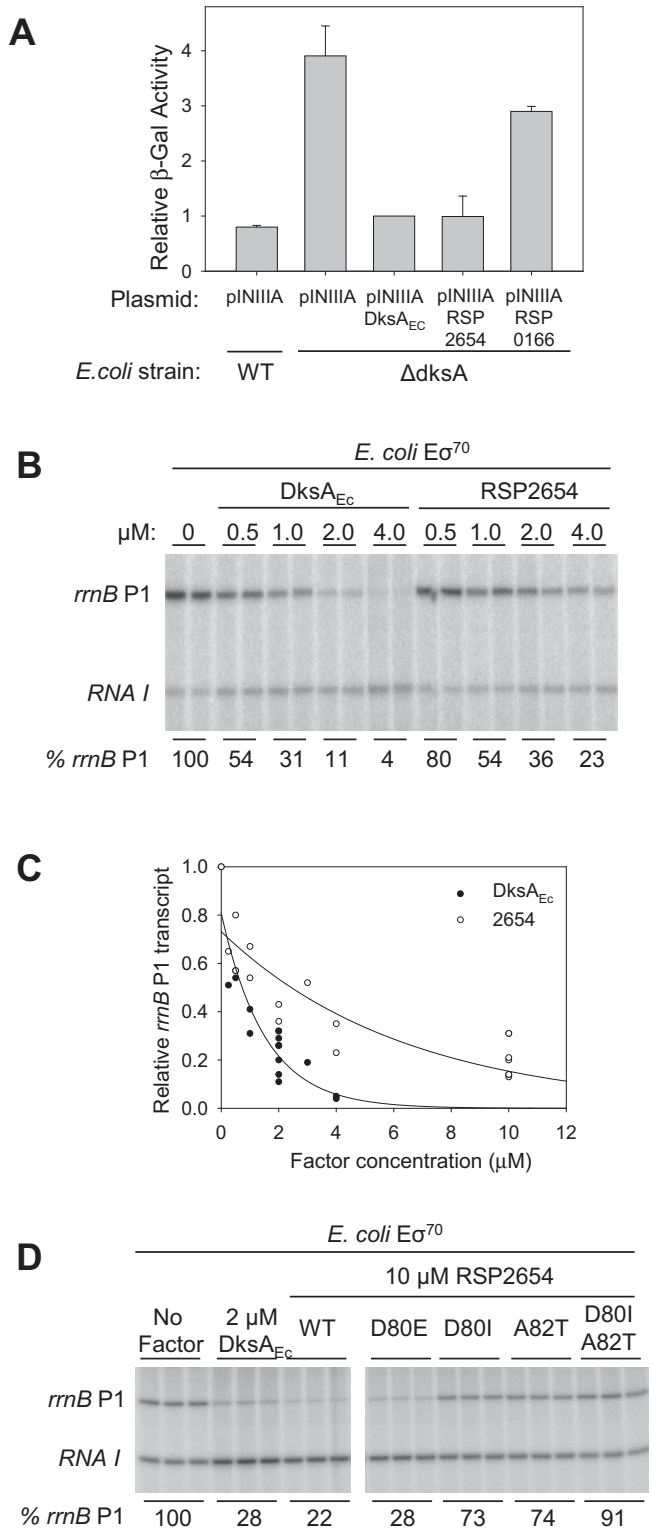


FIG 4 *R. sphaeroides* RSP2654 inhibits transcription from the *E. coli rrnB* P1 promoter *in vivo* and *in vitro*. (A) β-Galactosidase activity expressed in *E. coli* from a chromosomal *rrnB* P1-*lacZ* fusion was determined in a wild-type strain carrying the pINIIIA plasmid vector or in a Δ*dksA* strain carrying the pINIIIA1 vector or pINIIIA1 expressing *E. coli* DksA, RSP2654, or RSP0166. Activities were normalized to that of the Δ*dksA* strain carrying the pINIIIA1 DksA$_{Ec}$ plasmid. *rrnB* P1 promoter activity was elevated ~3- to 4-fold in the Δ*dksA* strain and was restored to wild-type levels by plasmid-encoded DksA$_{Ec}$ or

(Continued)

The photosynthetic growth defect of *R. sphaeroides* Δ2654 is complemented by plasmid-encoded RSP2654 or by E. coli DksA. Photosynthetic growth of *R. sphaeroides* Δ2654 was rescued by complementation with a plasmid expressing RSP2654 from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter (Fig. 3A), strongly suggesting that loss of the RSP2654 protein rather than potential polar effects from deletion of the RSP2654 gene was responsible for the defect. Ectopic expression of DksA$_{Ec}$ from an IPTG-inducible promoter also complemented the photosynthetic growth defect of Δ2654, although not as well as expression of RSP2654. Ectopic expression of the DksA$_{Ec}$ D74N mutant protein (which greatly reduces the activity of DksA$_{Ec}$ *in vitro* and *in vivo* in *E. coli* [25]) failed to complement *R. sphaeroides* Δ2654 for photosynthetic growth (Fig. 3A). These results suggest that some or all of the defects associated with the loss of RSP2654 in *R. sphaeroides* can be restored by DksA$_{Ec}$ and that the coiled-coil tip critical for function of DksA$_{Ec}$ in *E. coli* is also critical for its ability to compensate for the loss of RSP2654 in *R. sphaeroides*. The lack of full complementation of the photosynthetic growth phenotype by DksA$_{Ec}$ could reflect different levels of expression of DksA$_{Ec}$ and RSP2654 and/or sequence-dependent differences in the functions of the two proteins.

RSP2654 residues D80 and A82 correspond to the functionally critical DksA$_{Ec}$ residues D74 and A76 in the conserved DxxDxA motif (Fig. 1). Single amino acid substitutions at D80 and A82 were constructed in the chromosomal copy of the RSP2654 gene by markerless homologous recombination (see Materials and Methods). The resulting strains (2654-D80N and 2654-A82T) made smaller colonies than wild-type *R. sphaeroides* when grown anaerobically in the light, although not as small as those of the Δ2654 mutant (Fig. 3B). Western blotting showed that the levels of the RSP2654 mutant proteins were at least as high as that of wild-type RSP2654 *in vivo* (Fig. 3C), indicating that decreased protein stability was not responsible for the altered photosynthetic growth phenotype of cells containing the variant RSP2654 proteins. These results indicate that the conserved DxxDxA motif in RSP2654 is important for its function in *R. sphaeroides* photosynthetic growth.

***R. sphaeroides* RSP2654 complements *E. coli* Δ*dksA* cells for growth in the absence of amino acids and inhibits rRNA promoter activity *in vivo*.** The above results (Fig. 3A and B) suggested that DksA$_{Ec}$ functions similarly to RSP2654 in *R. sphaeroides*. We next asked whether the *R. sphaeroides* protein RSP2654 or RSP0166 could complement the growth defect of *E. coli* Δ*dksA*

Figure Legend Continued

RSP2654 but not by RSP0166. (B) Products of single-round *in vitro* transcription from the *E. coli rrnB* P1 and RNA-I promoters by *E. coli* RNAP Eσ^{70} in the absence of added factor or with the indicated concentrations of DksA$_{Ec}$ or RSP2654 (0.5 to 4 μM), purified as described in Materials and Methods. Transcripts were resolved on 5.5% acrylamide–7 M urea gels. Average transcript levels from duplicate reactions relative to that with no added factor (100%) are shown below the gel lanes. (C) Quantification of *in vitro* transcription data from three replicates of the experiment shown in panel B. (D) *In vitro* transcription as for panel B of *rrnB* P1 with *E. coli* RNAP Eσ^{70} in the absence of added factor or with the indicated concentrations of DksA$_{Ec}$, RSP2654, or RSP2654 variants containing substitutions for residues analogous to the *E. coli* DksA coiled-coil tip residues (RSP2654-D80E, -D80I, -A82T, or -D80I/A82T), purified as described in Materials and Methods. Average transcript levels from triplicate reactions relative to that with no added factor are indicated below the gel lanes.

cells grown on minimal medium. Wild-type *E. coli* cells or Δ*dksA* cells expressing plasmid-encoded $DksA_{Ec}$ grew on minimal medium lacking amino acids, whereas Δ*dksA* cells did not, consistent with previous observations (Fig. 3D) (10, 25). Plasmid-encoded RSP2654 restored the ability of Δ*dksA* cells to grow without amino acids, suggesting that RSP2654 functions in *E. coli* similarly to $DksA_{Ec}$. In contrast, plasmid-encoded RSP0166 did not restore growth for the *E. coli* Δ*dksA* strain in the absence of amino acids, indicating that it lacks activities associated with DksA in this host as well (Fig. 3D).

To test the functional similarity of RSP2654 and $DksA_{Ec}$ further, we compared their effects on rRNA promoter-specific transcription in *E. coli* using an *rrnB* P1-*lacZ* fusion as a reporter (Fig. 4A). In log-phase growth, *rrnB* P1 activity was elevated 3- to 4-fold in the Δ*dksA* strain compared to that in wild-type cells, consistent with findings of our previous studies (10, 25, 39). When either $DksA_{Ec}$ or RSP2654 was expressed ectopically in Δ*dksA* cells, *rrnB* P1 promoter activity was restored to the level in wild-type cells (Fig. 4A), whereas RSP0166 affected *rrnB* P1 activity only very slightly if at all, consistent with its inability to complement plating of Δ*dksA* cells in the absence of amino acids (Fig. 3D). Without an RSP0166-specific antibody, we could not eliminate the possibility that low RSP0166 levels were responsible for the absence of its effects in *E. coli*. However, since we also did not detect phenotypes of the Δ1066 mutant in *R. sphaeroides*, we focused on RSP2654 in the studies described below.

***R. sphaeroides* RSP2654 specifically reduces *E. coli rrnB* P1 activity *in vitro*.** We tested whether the effect of RSP2654 on rRNA promoter activity *in vivo* resulted from direct interactions with RNAP at the promoter, as observed previously for $DksA_{Ec}$ (10). Single-round *in vitro* transcription assays with the *E. coli rrnB* P1 promoter and *E. coli* RNAP showed that $DksA_{Ec}$ and RSP2654 each inhibited *rrnB* P1 transcription in a concentration-dependent manner. Neither protein inhibited transcription from the RNA-I promoter (from the plasmid origin-of-replication region) (Fig. 4B and D), indicating the effects were promoter specific. The 50% inhibitory concentration (IC_{50}) for inhibition by RSP2654 was approximately 3- to 4-fold higher than that for $DksA_{Ec}$ (approximately 1 μM for $DksA_{Ec}$ and 3 to 4 μM for RSP2654) (Fig. 4C). This slightly higher IC_{50} for RSP2654 than for $DksA_{Ec}$ could reflect either the divergence of the protein sequences or differences in the specific activities of the two preparations.

We also tested the *in vitro* activities of variants of RSP2654 with substitutions in residues that correspond to the functionally important $DksA_{Ec}$ tip positions D74 and A76 (RSP2654 residues D80 and A82). Wild-type RSP2654 reduced *rrnB* P1 transcript levels by 70 to 75% relative to the control lacking RSP2654. In contrast, *rrnB* P1 activity was reduced by only 10 to 25% by comparable concentrations of the RSP2654 proteins containing D80I, A82T, or the double substitution D80I plus A82T (Fig. 4D), consistent with the inability of other substitutions in the coiled-coil tip (D80N and A82T) to restore normal photosynthetic growth in *R. sphaeroides* (Fig. 3B). In contrast to the loss-of-function phenotype observed previously for a $DksA_{Ec}$ D74E variant (25), RSP2654-D80E retained the ability to inhibit transcription from *rrnB* P1 (Fig. 4D). Thus, RSP2654 appears to have a less-strict requirement for aspartate at this position.

RSP2654 functions synergistically with ppGpp *in vitro*. $DksA_{Ec}$ functions together with the regulatory nucleotide ppGpp

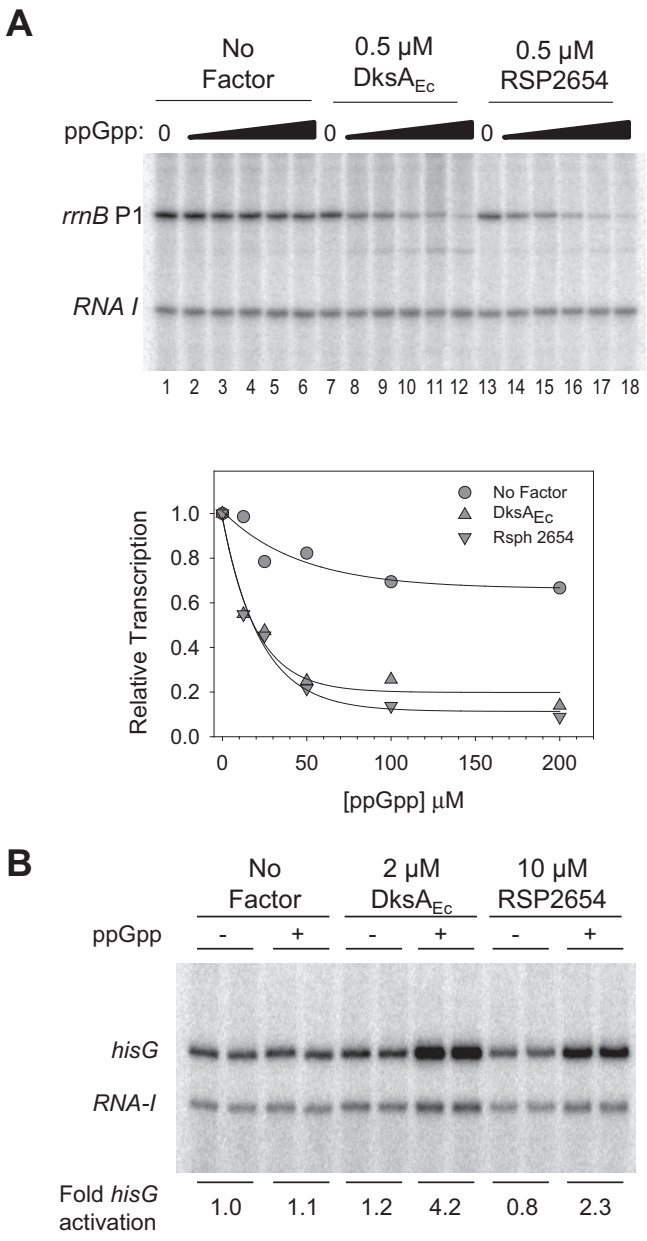
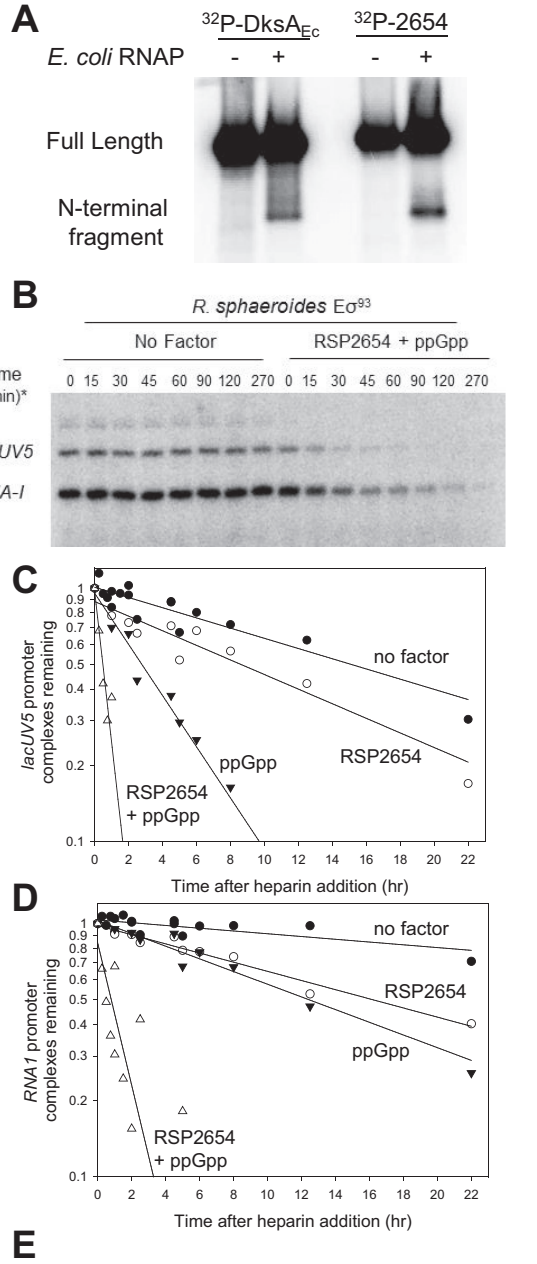


FIG 5 RSP2654 potentiates the negative (A) or positive (B) effects of ppGpp on *in vitro* transcription of *E. coli* promoters. (A) Products of single-round *in vitro* transcription of the *E. coli rrnB* P1 promoter either in the absence of $DksA_{Ec}$ or RSP2654 (No Factor; lanes 1 to 6), with 0.5 μM $DksA_{Ec}$ (lanes 7 to 12), or with 0.5 μM RSP2654 (lanes 13 to 18). Samples either lacked ppGpp (lanes 1, 7, and 13) or contained ppGpp at 12.5 μM (lanes 2, 8, and 14), 25 μM (lanes 3, 9, and 15), 50 μM (lanes 4, 10, and 16), 100 μM (lanes 5, 11, and 17), or 200 μM (lanes 6, 12, and 18). In the absence of ppGpp, transcription was reduced by $DksA_{Ec}$ to 67% (lane 7) or by RSP2654 to 52% (lane 13) relative to transcription observed in the absence of both the factor and ppGpp (lane 1). The observed inhibition as a function of ppGpp concentration is quantified and graphed below the gel image. Values were normalized to the level of transcription observed in the absence of ppGpp for each condition (i.e., relative to the transcription observed without factor in lane 1 or with $DksA_{Ec}$ or RSP2654 alone in lane 7 or 13, respectively). (B) Products of multiple-round *in vitro* transcription of the *E. coli hisG* promoter with *E. coli* RNAP in the presence of $DksA_{Ec}$ (2 μM) or RSP2654 (10 μM) with or without 100 μM ppGpp. Average transcription from duplicate reactions carried out in the presence of ppGpp relative to that in the absence of ppGpp for each factor is shown below the gel lanes.



E

Factor	*lacUV5* Promoter t½ (hrs)	*lacUV5* Promoter Fold Reduction	*RNA-1* Promoter t½ (hrs)	*RNA-1* Promoter Fold Reduction
none	16	-	50	-
RSP2654	9	<2	17	3
ppGpp	4	4	13	4
RSP2654 + ppGpp	0.33	48	1	50

FIG 6 The mechanism of effects of RSP2654 on transcription is similar to that of $DksA_{Ec}$. (A) RSP2654 binds in the secondary channel of *E. coli* RNAP. Cleavage of *E. coli* DksA or RSP2654, ^{32}P-labeled at an N-terminal HMK site, by hydroxyl radicals generated from Fe^{2+} in the active site of *E. coli* RNAP. Full-length DksA or RSP2654 and its N-terminal cleavage product are shown on a phosphorimage of the SDS gel. Cleavage of $DksA_{Ec}$ occurs at or near coiled-coil tip residue 73 (19). A representative gel is shown in panel A, but

(Continued)

to inhibit or activate transcription in a promoter-specific fashion (10, 17). Transcription from *rrnB* P1 is inhibited by $DksA_{Ec}$ alone and by ppGpp alone (10, 39–41), but the magnitude of the inhibition is greatly amplified when both ppGpp and $DksA_{Ec}$ are present (10). Under the conditions used in the experiment shown in Fig. 5A, transcription from *rrnB* P1 was reduced by ppGpp alone by ≤2-fold (compare lane 1 with lanes 2 to 6, 7, or 13), whereas $DksA_{Ec}$ and ppGpp together (lanes 8 to 12) or RSP2654 and ppGpp together (lanes 14 to 18) reduced transcription by ~10-fold (see the gel image in Fig. 5A; quantitation is in the accompanying graph). Thus, RSP2654 and $DksA_{Ec}$ each contain determinants needed for synergistic regulation of transcription with ppGpp.

In contrast to promoter inhibition, neither $DksA_{Ec}$ nor ppGpp alone can activate transcription of amino acid biosynthesis promoters; activation requires both $DksA_{Ec}$ and ppGpp (17). Consistent with those results, we found that transcription from the *hisG* promoter by *E. coli* RNAP was increased 4.2-fold by $DksA_{Ec}$ and ppGpp together but not by either ppGpp or $DksA_{Ec}$ alone (17). Similarly, RSP2654 and ppGpp together increased transcription, whereas either factor alone did not (Fig. 5B). However, the magnitude of the increase (2.3-fold) was not quite as large as that with $DksA_{Ec}$ and ppGpp together.

RSP2654 and $DksA_{Ec}$ affect RNAP by similar mechanisms. $DksA_{Ec}$ alters transcription by *E. coli* RNAP by binding in the secondary channel of the enzyme and shifting the equilibrium between closed and open promoter complexes in the dissociation direction (10, 19). Therefore, as an indicator of its mechanism of action, we tested whether RSP2654 binds in the RNAP secondary channel and reduces open complex stability.

Binding of DksA in the RNAP secondary channel has been detected previously from its cleavage by hydroxyl radicals generated by Fe^{2+} bound at the active site of RNAP followed by SDS-PAGE (18, 20, 25, 42). In this assay, cleavage of $DksA_{Ec}$ occurs in the coiled-coil tip region at or near residue 73, indicating that this functionally critical region of the protein is located at the base of the RNAP secondary channel within ~10 Å of the active site (42). ^{32}P-labeled N-terminal cleavage products of a similar size were generated from $DksA_{Ec}$ or RSP2654 in an RNAP-dependent reac-

Figure Legend Continued

similar results were obtained in multiple experiments. (B) RSP2654 and ppGpp directly reduce the lifetime of *R. sphaeroides* RNAP-Eσ^{93} complexes formed with the *lacUV5* or RNA-I promoters. Eσ^{93} RNAP-promoter complexes were preformed in the absence of ribonucleoside triphosphates (rNTPs) and the presence or absence of RSP2654 (4 μM) and ppGpp (333 μM), as indicated. Aliquots were sampled at the indicated times after addition of heparin as a competitor for free RNAP, and promoter complexes remaining were determined by transcription in the presence of added rNTPs. Transcripts were resolved on 5.5% acrylamide–7 M Urea gels. A representative gel is shown. (C) Decay of Eσ^{93}-*lacUV5* promoter complexes in the absence of factors or in the presence of RSP2654 (4 μM), ppGpp (333 μM), or both RSP2654 (4 μM) and ppGpp (333 μM), determined as for the experiment shown in panel B. Transcript levels were quantified, and the complexes remaining at each time point after competitor addition were determined as a fraction of the complexes present at time zero. (D) Decay of Eσ^{93} RNA-I promoter complexes determined as for panel C. (E) The half-life (in hours) of *R. sphaeroides* RNAP Eσ93-*lacUV5* and RNA-I promoter complexes in the presence or absence of RSP2654 and ppGpp was determined from the plots in panels C and D. Fold reduction in half-life indicates the ratio of the half-life in the presence of the factor(s) to that in the absence of any added factor. The values in panel E derive from the graphs in panels C and D.

tion (Fig. 6A). These results suggest that RSP2654 residues D80 and A82 are likely positioned similarly to $DksA_{Ec}$ D74 and A76, near the *E. coli* RNAP active site, consistent with their requirement for inhibition of *E. coli rrnB* P1 transcription (Fig. 4D).

$DksA_{Ec}$ and ppGpp directly reduce the lifetime of all *E. coli* Eσ^{70} RNAP-promoter DNA complexes that have been examined, and they inhibit transcription from the subset of promoters that form intrinsically unstable complexes with RNAP (2, 10, 43). We analyzed the effect of RSP2654, ppGpp, and both factors together on complexes formed by the major *R. sphaeroides* RNAP holoenzyme (Eσ^{93}) (29), using a promoter-RNAP half-life assay (43) and two *E. coli* promoters that were shown previously to be recognized and transcribed by *R. sphaeroides* Eσ^{93}, *lacUV5* and the plasmid-encoded promoter RNA-I (29). *R. sphaeroides* RNAP was prebound to promoter DNA, and the fraction of complexes remaining at times after addition of a competitor to sequester free RNAP was measured by transcription (25, 43). Separately, RSP2654 or ppGpp each decreased the lifetimes of the promoter-complexes a few fold, but when combined, RSP2654 and ppGpp decreased Eσ^{93} complex stability dramatically (~50-fold reduction in complex lifetime) (Fig. 6B to E). Taken together with the evidence that RSP2654 binds in the secondary channel of RNAP (Fig. 6A), these results suggest that the phenotypes of the *R. sphaeroides* Δ2654 mutant likely reflect direct interactions of RSP2654 and ppGpp with promoter complexes and alteration of transcription by a mechanism similar to that of $DksA_{Ec}$ and ppGpp. We therefore designate RSP2654 $DksA_{Rsp}$.

Interestingly, $DksA_{Ec}$ and $DksA_{Rsp}$ reduced *E. coli* holoenzyme (Eσ^{70}) complex lifetimes similarly, but ppGpp and $DksA_{Rsp}$ together had a larger effect on the lifetime of the *R. sphaeroides* RNAP complex than was observed previously for $DksA_{Ec}$ and ppGpp on the *E. coli* RNAP complex (Fig. 6) (10, 25). Further investigation of the interactions of ppGpp-$DksA_{Rsp}$ with *R. sphaeroides* RNAP may provide opportunities to unravel the mechanism of the DksA-ppGpp synergism.

DISCUSSION

Comparison of proteins that function like DksA provides information about DksA structure-function. Bacterial proteins annotated as members of the DksA/TraR protein family vary in length but share sequence similarity to one or more domains of *E. coli* DksA (24). Although some of these proteins may function like $DksA_{Ec}$ to regulate transcription, others may lack critical sequence features and may act in different, as yet uncharacterized capacities. Although DksA-like proteins in several species have been implicated genetically in regulation of transcription, only a small set of these proteins, all from gammaproteobacteria, have been shown to function as transcription regulators in a purified system *in vitro* (7, 10, 11, 24, 26). We report here that RSP2654, one of two proteins annotated as DksA in the alphaproteobacterium *R. sphaeroides*, is functionally and mechanistically similar *to E. coli* DksA. Our inability to identify evidence for DksA-like function for the second *R. sphaeroides* protein, RSP0166, reinforces the need for corroboration of function of proteins annotated as members of the DksA/TraR family solely from bioinformatic criteria.

R. sphaeroides $DksA_{Rsp}$ shares several important properties with $DksA_{Ec}$, including inhibition of transcription by *E. coli* RNAP both *in vivo* and *in vitro*, direct activation of transcription of some promoters in the presence of ppGpp, and reduction of the lifetime of promoter complexes formed with either *E. coli* RNAP or *R. sphaeroides* RNAP, either alone or synergistically with ppGpp. RSP2654 residues analogous to the coiled-coil tip residues of $DksA_{Ec}$ were required for function (RSP2654 D80 and A82), and as for $DksA_{Ec}$, these residues are within ~10 Å of the RNAP active site (Fig. 6A). The location of RSP2654 when bound to RNAP and the mechanism of its effect on transcription initiation thus appear similar to those described for *E. coli* DksA (10, 17, 20, 25).

Although models for protein-protein interactions in the RNAP-$DksA_{Ec}$ complex have been proposed (18, 20, 24) and coiled-coil tip residues critical for regulation of transcription but not for RNAP binding are known, the interacting surfaces of the two proteins as well as other residues important for binding and function remain to be identified. There is no crystal structure for a DksA-RNAP complex. Analysis of conserved residues among divergent DksA proteins with conserved function, like $DksA_{Rsp}$, can provide critical structure-function information.

The four DksA proteins that have been characterized *in vitro*, $DksA_{Ec}$, $DksA_{Rsp}$, and *P. aeruginosa* DksA1 and DksA2 (Fig. 1B), have high sequence identity/similarity in their C termini, including the coiled-coil tip (the DxxDxA motif; $DksA_{Ec}$ residues 71 to 76), the second helix of the coiled coil (residues 77 to 109), the C-terminal portion of the globular domain (residues 110 to 134), and the C-terminal helix (residues 135 to 151) (Fig. 1A). Residues corresponding to $DksA_{Ec}$ 86 to 151 are 52% identical or contain conservative substitutions in these four proteins, and models for binding of $DksA_{Ec}$ to RNAP suggest that a surface in the distal half of the second helix of the coiled coil and the adjacent surface of the globular domain are likely to interact with the RNAP secondary channel rim (20, 24). Consistent with this model, specific substitutions in the RNAP secondary channel rim decrease $DksA_{Ec}$ function (24, 44, 45), but the interacting residues in $DksA_{Ec}$ have not yet been identified. A substitution for residue N88 in $DksA_{Ec}$ (the "super DksA" substitution N88I) confers increased RNAP binding and activity (20, 46), but N88 is not conserved in $DksA_{Rsp}$ or *P. aeruginosa* DksA1 or DksA2 (Fig. 1). Other DksA residues involved in binding to RNAP remain to be identified.

Many annotated DksA/TraR family members contain a Cys4-zinc finger motif in the globular domain, whereas others share sequence similarity with this domain but lack the ligands to bind zinc (11, 24, 26). DksA2 from *P. aeruginosa* has only 2 of the 4 cysteines found in $DksA_{Ec}$. Like DksA2, $DksA_{Rsp}$ also lacks the Cys4 motif, but it has only 1 of the 4 cysteines (corresponding to $DksA_{Ec}$ C114). The identities of the two cysteine substitutions in DksA2 are the same in $DksA_{Rsp}$ (threonine for $DksA_{Ec}$ C117 and alanine for $DksA_{Ec}$ C138). The third substitution for a cysteine in $DksA_{Rsp}$ is a threonine (corresponding to $DksA_{Ec}$ C135). Despite the absence of a zinc finger, there is considerable overall sequence conservation of this region between $DksA_{Rsp}$ and DksA2 (64% identity in the region corresponding to the zinc finger and C-terminal helix regions of $DksA_{Ec}$, residues 109 to 151) (Fig. 1B), suggesting that $DksA_{Rsp}$, like DksA2, has a structure similar to that of $DksA_{Ec}$.

In contrast to the high degree of evolutionary conservation in the distal half of the four *in vitro*-characterized DksA proteins, the N-terminal regions (corresponding to $DksA_{Ec}$ 1 to 70) are variable in length and less well conserved (13% identity) (Fig. 1). Consistent with this lack of conservation, removal of residues 1 to 18 of $DksA_{Ec}$ did not impair function but in contrast was reported to increase DksA activity (24). Additionally, the 73-amino-acid TraR protein, which lacks sequence corresponding to the entire

N-terminal half of $DksA_{Ec}$, retains some functions of DksA *in vivo* and *in vitro* (47). Although extensive differences in the N-terminal regions of these proteins suggest that this area is not essential for DksA function, they could reflect as yet unknown roles unique to the individual species. Conservation of amino acid sequences from $DksA_{Rsp}$ and the three other DksA proteins previously characterized *in vitro* thereby suggests regions responsible for DksA binding to RNAP, activity, and synergism with ppGpp.

The Gre factors also interact with RNA polymerase in the secondary channel (24, 39, 44, 48). Despite sharing a major structural feature with DksA, a long coiled-coil domain with conserved acidic residues in the tip loop required for function (18), elsewhere these factors lack recognizable sequence similarity to the DksA/TraR family and carry out functions distinct from that of DksA. Gre factors are not required for regulation of transcription initiation *in vivo* (39), but rather they increase transcription elongation by facilitating transcript cleavage in backtracked or arrested elongation complexes, thereby realigning the RNA 3′ end with the RNAP active site. In contrast, DksA does not carry out RNA cleavage (18). It has been suggested that the Gre and DksA factors recognize distinct conformations of the RNAP active site region, thereby targeting different functional states of the enzyme (24).

Pleiotropic effects of the *R. sphaeroides* mutant lacking $DksA_{Rsp}$. Our phenotypic analyses indicate that $DksA_{Rsp}$ mediates some functions in *R. sphaeroides* that are related to those affected by DksA in *E. coli*, including roles in fatty acid biosynthesis and amino acid utilization (Fig. 2), and others that pertain to the photosynthetic lifestyle distinct to *R. sphaeroides* (Fig. 2 and 3). Although identification of the *R. sphaeroides* promoters regulated by $DksA_{Rsp}$ will be the subject of further studies, the similar properties of $DksA_{Ec}$ and $DksA_{Rsp}$ suggest that ppGpp will also be found to participate in their regulation.

$DksA_{Ec}$ and ppGpp often function together, consistent with the similar (albeit not identical) expression profiles of *E. coli* strains lacking either factor (4). ppGpp has long been recognized as a signal mediating the stringent response, a broadly conserved bacterial stress response to starvation and stress conditions that regulates critical growth processes like the biosynthesis of the translational apparatus and amino acids (2, 3). $DksA_{Ec}$ is a required cofactor for many of the direct transcription responses to the changes in ppGpp concentration, both positive and negative, that accompany changes in growth conditions for *E. coli* (2, 10, 13–15, 17). Consistent with a model for joint regulation by these factors in *R. sphaeroides*, $DksA_{Rsp}$ functioned synergistically with ppGpp to alter *R. sphaeroides* RNAP-promoter complexes *in vitro* (Fig. 6), and ppGpp concentrations in *R. sphaeroides* are known to vary in response to light intensity (49, 50). Further supporting the joint regulation model, gene expression profiling of mutants lacking a *dksA*-like gene or ppGpp synthesis gene in another alphaproteobacterium, *Sinorhizobium meliloti*, indicated that a large number of genes are affected by both factors (51).

In *R. sphaeroides*, deletion of RSP2654 resulted in increased fatty acid content, but further studies will be needed to determine whether the effects of $DksA_{Rsp}$ on fatty acid content in *R. sphaeroides* derive from direct effects on promoters in fatty acid synthesis pathways. Deletion of the RSP2654 gene also altered utilization of amino acids by *R. sphaeroides*, although it did not result in the amino acid auxotrophy observed for *E. coli* cells lacking the *dksA* gene (19). Wild-type *R. sphaeroides* and *R. sphaeroides* Δ2654 cells grew at similar rates in the absence of added amino acids, but wild-type *R. sphaeroides* cells utilized exogenous amino acids to increase the growth rate and total biomass, whereas Δ2654 cells did not (Fig. 2F and G). Together, these observations suggest that $DksA_{Rsp}$ is not required for amino acid biosynthesis *per se*, but it plays a role in uptake of amino acids, their incorporation into proteins, or in some related pathway(s).

These findings are consistent with reports that, unlike the case for *E. coli*, starvation for (single) amino acids does not induce ppGpp synthesis in *R. sphaeroides* and in other alphaproteobacteria (52–54). Amino acid biosynthesis is not dependent on DksA/ppGpp in these species. *Sinorhizobium meliloti*, *Rhizobium etli*, and *Caulobacter crescentus* strains lacking either their DksA homologs or ppGpp grow on minimal medium (51, 55, 56). Similar to our observation that *R. sphaeroides* without RSP2654 is defective in utilizing exogenous amino acids, *R. etli* lacking ppGpp is impaired in utilization of exogenous amino acids as its sole nitrogen source (55). Thus, DksA/ppGpp may affect amino acid metabolism somewhat differently in alphaproteobacteria compared to the case for gammaproteobacteria, perhaps regulating uptake and/or catabolism but not biosynthesis of one or more amino acids.

Photosynthetic growth defect of *R. sphaeroides* lacking $DksA_{Rsp}$. Although *R. sphaeroides* lacking $DksA_{Rsp}$ (Δ2654) synthesized and assembled normal levels of light-harvesting pigment-protein complexes when grown anaerobically, it was severely impaired in anaerobic photosynthetic growth, and the reduced colony pigmentation of aerobically grown mutant colonies suggests a direct or indirect role for $DksA_{Rsp}$ in the global response of this bacterium to O_2 limitation (Fig. 2). Supporting the model that DksA/ppGpp plays a role in the response to O_2 availability, it was reported previously that a ppGpp null strain in the closely related species *Rhodobacter capsulatus* displayed a similar reduced pigmentation phenotype in response to O_2 limitation (although this mutant remained able to grow by photosynthesis) (57).

Because *R. sphaeroides* Δ2654 cells produced normal amounts of light-harvesting complexes when grown at reduced O_2 levels (0.5%) (Fig. 2), we suggest that the photosynthetic defect of the Δ2654 mutant results from events after the harvesting of light energy, rather than from a deficit in photosynthetic pigment production or an inability to assemble functional photosynthetic pigment-protein complexes (58–60). Events after the harvesting of light energy could include the funneling of photons into the reaction center complex, transportation of electrons to generate a proton gradient, or utilization of the proton gradient to generate ATP (28, 35). For example, a defect in photosynthetic growth was observed previously for an *R. sphaeroides* mutant deleted for the cytochrome c_2 electron carrier (61). $DksA_{Rsp}$ together with ppGpp may directly or indirectly affect the expression of additional genes required for photosynthesis, a role suggested for ppGpp in *R. capsulatus* but not yet fully characterized for any photosynthetic bacterium (57).

In many bacterial species, levels of ppGpp are determined by activation of a member(s) of the RelA protein family in response to a variety of nutrient limitation and/or other stress conditions, with specific inducing conditions and targets of ppGpp varying among different species, reflecting the particular ecological niche of the organism (3, 9, 53, 54). The lifestyle of *R. sphaeroides* is adapted to low-O_2 environments, where it can transition between aerobic respiration and anaerobic photosynthesis, with O_2 and light energy serving as "nutrients" that govern this lifestyle choice.

Loss of $DksA_{Rsp}$ altered the response to O_2 limitation, as reflected in the colony pigmentation phenotype described above. Previous studies have shown that ppGpp levels in *R. sphaeroides* increase rapidly upon a downshift in light intensity under anaerobic conditions (49, 50). These observations are consistent with a role for $DksA_{Rsp}$ and ppGpp in regulating some components of the photosynthetic apparatus in response to oxygen and light limitation, the environmental factors that regulate this lifestyle (28). Future work will identify target promoters of $DksA_{Rsp}$/ppGpp and examine how these factors function together with the known regulators of photosynthesis genes, including the transcriptional repressor PspR, the anti-PspR factor AppA, and the global transcriptional regulator PrrA or FnrL (31, 62, 63).

RSP0166. The *R. sphaeroides* RSP0166 gene was also annotated as a DksA family member (33), but *R. sphaeroides* cells lacking the RSP0166 gene displayed no obvious growth phenotypes even under anaerobic conditions, where expression of this gene was quite high (31, 34). Expression of RSP0166 in *E. coli*, unlike that of RSP2654, did not complement a Δ*dksA* mutant. Sequence conservation of RSP0166 and $DksA_{Ec}$ occurs mainly in the zinc finger region of the $DksA_{Ec}$ globular domain (Fig. 1). Although RSP0166 contains four appropriately spaced cysteine residues suggestive of a metal-binding domain, there is lower overall sequence identity/similarity to the other characterized DksA proteins in this region, and it does not contain sequence corresponding to most of the C-terminal helix. RSP0166 contains glutamate in place of $DksA_{Ec}$ D74 in the critical DxxDxA motif (a substitution that strongly reduces $DksA_{Ec}$ function [25]), although we note that the $DksA_{Rsp}$ D80E variant functions with *E. coli* RNAP, suggesting that the local context of this region can be important for DksA function.

The nuclear magnetic resonance (NMR) structure of another short DksA family member, a 112-amino-acid protein of unknown function from the alphaproteobacterium *Agrobacterium tumefaciens* strain C58 with 49% identity to RSP0166 (Atu0905; PDB 2KQ9; MMDB ID 78099; Northeast Structural Genomics Consortium), shows some similarity to that of $DksA_{Ec}$ (and *P. aeruginosa* DksA2) in the regions of sequence conservation but differs in the N terminus and in the length of the coiled coil. Although it contains the DxxDxA motif essential for $DksA_{Ec}$ function, this motif is located in a long, unstructured loop which would likely prevent it from accessing the active site region at the base of the secondary channel. These differences suggest either that RSP0166 interacts with RNAP differently from $DksA_{Ec}$, that refolding of the long unstructured loop could occur upon interaction with RNAP, or that it is not an RNAP binding protein.

Taken together, our data suggest that RSP0166 does not function like DksA in *R. sphaeroides*, and consistent with this hypothesis, it has also been annotated as a member of a different protein family (dimethylmenaquinone methyltransferase; NCBI reference sequence WP_002720312). Proteins of unknown function in other bacterial species have also been annotated as DksA/TraR family members (e.g., *P. aeruginosa* contains 3 proteins annotated as DksA/TraR family members in addition to the two longer ones that function like $DksA_{Ec}$; see above and see reference 11).

Although our results with RSP0166 suggest that caution should be used in drawing conclusions about the function of proteins with amino acid similarity to DksA only in the zinc finger region, we point out that some members of this class could still play a regulatory role in transcription. For example, *E. coli* Rnk has sequence similarity to the C-terminal domain of Gre factors but has a shorter coiled coil. Rnk has no effect on RNAP itself but competes with Gre factors for binding to RNAP and thus may regulate Gre factor function (64).

Summary. In conclusion, our data provide information useful for structure-function analysis of the DksA family and suggest that DksARsp is critical for photosynthesis. Finally, our investigations of RSP0166 illustrate the limitations of annotation of *dksA*-like genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are described in Table S1 in the supplemental material. *E. coli* strains were grown at 37°C in Luria-Bertani medium or in M9 minimal medium where appropriate (see below). *R. sphaeroides* 2.4.1 strains were grown at 30°C in a succinate-based minimal medium (SIS) (37) unless otherwise noted. When necessary, media were supplemented with kanamycin (25 μg/ml for *R. sphaeroides* or 20 or 50 μg/ml for *E. coli*) or ampicillin (100 μg/ml). For growth of *R. sphaeroides* in liquid, 500-ml cultures were bubbled with 69% N_2, 30% O_2, and 1% CO_2 for aerobic growth and 98.5% N_2, 0.5% O_2, and 1% CO_2 for low-O_2 growth. For photosynthetic growth, screw-cap tubes of liquid culture or sealed canisters containing agar plates and a GasPak EZ anaerobe container system packet (BD Biosciences) were incubated at room temperature in front of an incandescent light with a light intensity of 10 W/m^2 measured through a red glass filter.

For aerobic growth curves, *R. sphaeroides* strains were grown in SIS medium lacking aspartic acid and glutamic acid or in SIS medium supplemented with 0.4% Casamino Acids and 0.004% tryptophan. Two-hundred-microliter cultures were incubated at 30°C in clear 96-well plates in an Infinite F500 plate reader (Tecan, Männedorf, Switzerland) with shaking at 33.2 rpm orbitally. Absorbance was measured every ~10 min at 595 nm after 10 s of linear shaking.

Construction of *R. sphaeroides* mutants. Deletion of RSP2654 or RSP0166 was carried out to create strains Δ2654 and Δ0166 using the nonreplicable integration vector pK18mobsacB, which allows marker-free deletion by two-step homologous recombination (65). For each gene, ~2.3-kb fragments were amplified from genomic DNA of *R. sphaeroides* containing the open reading frame (ORF) flanked by 0.8 to 1.0 kb of sequence on each side with primers containing XbaI and EcoRI (RSP2654) or HindIII (RSP0166). These PCR products were inserted into pK18mobsacB to create plasmids pKC09 and pKCL07. The entire coding region of RSP2654 or RSP0166 was deleted from the respective plasmids by performing PCR with primers facing outward from each end of the ORF and ligation of the resulting fragment with T4 DNA ligase (Promega, Madison, WI) to create pKCL08 and pKCL10. These plasmids were mated into *R. sphaeroides* from *E. coli* S17-1. Single crossovers were selected by kanamycin resistance, and double crossovers by loss of sucrose sensitivity.

To create strains 2654-D80N and 2654-A82T, site-specific mutagenesis was performed using pK18mobsacB-derived plasmids. Two-step PCR mutagenesis was performed to create 2.3-kb genomic fragments containing RSP2654 with internal mutations. One base pair change in each PCR product resulted in a codon change of the desired amino acid substitution (D80N or A82T), and a second base pair change mutated an EarI restriction site. These PCR products were inserted into the XbaI and EcoRI sites of pK18mobsacB to make plasmids pKCL11 and pKCL12. The plasmids were mobilized into *R. sphaeroides* and selected as described above. The resulting sucrose-resistant strains were screened for a copy of RSP2654 containing the 2 mutated nucleotides by PCR of the gene and digestion with EarI. Strains containing a copy of RSP2654 that was not digested by EarI were sequenced for verification of the codon change.

Spectroscopy. To assess photosynthetic pigment-protein complex levels, aliquots of exponential-phase cell culture were assayed by visible spectroscopy on an Olis DW-2/2000 spectrophotometer. To normalize for cell density, all spectra were scaled to an absorbance of 1 at 680 nm.

Fatty acid analysis. *R. sphaeroides* strains were grown aerobically in liquid culture to an optical density at 600 nm (OD_{600}) of ~0.4 to 0.6.

Ten-milliliter samples were centrifuged at 1,000 × *g* and resuspended in 2.5 ml water, and 5 μl of 10-mg/ml pentadecanoic acid was added as an internal standard. Total lipids were extracted with chloroform-methanol and reacted to form fatty acid methyl esters (66). Gas chromatography-mass spectrometry (GC-MS) analysis was performed using a model 7890 Agilent GC instrument (Agilent Technologies, Santa Clara, CA) with a 30-m by 0.25-mm DB-5 capillary column (Agilent) and a model 5975 mass spectrometer. Quantification was performed using ChemStation software (Agilent) by comparison of integrated single ion peaks (74 for saturated fatty acids and 55 for monounsaturated fatty acids) with calibration curves of fatty acid methyl ester standards. Cell plating experiments with each strain were used to make a standard curve of OD_{600} versus CFU to normalize fatty acid content per cell.

Construction of plasmids for expression of RSP2654 or *E. coli* DksA in *R. sphaeroides*. The coding sequence for RSP2654 was PCR amplified from genomic DNA and inserted into the NdeI and HindIII sites of pIND5 downstream of the IPTG-inducible promoter. The coding sequences for $DksA_{Ec}$ and DksA-D74N were PCR amplified from plasmids pRLG6333 and pRLG8873 (17) and inserted into the NdeI and BglII sites of pIND5, adding a hexahistidine tag onto the C terminus of the expressed protein.

Western blot analysis. Exponentially growing cultures were harvested, resuspended in urea buffer (8 M urea, 100 mM NaH_2PO4, and 10 mM Tris [pH 8.0]) supplemented with 50 μM phenylmethylsulfonyl fluoride and then heated at 95°C for 10 min. Samples were centrifuged to remove debris, and the total protein concentration of the samples was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), following the manufacturer's protocol. Western blotting was performed as previously described (67), using a rabbit polyclonal antibody raised against His_6-HMK (heart muscle kinase)-RSP2654 produced in *E. coli* (Harlan Laboratories, Madison, WI). Detection was performed with Pierce enhanced chemiluminescence (ECL) Western blotting substrate (Pierce, Rockford, IL).

Construction of plasmids for expression of RSP2654 and RSP0166 in *E. coli*. *R. sphaeroides* RSP0166 and RSP2654 DNA fragments were synthesized (GeneArt) using codons optimized for expression in *E. coli* and cloned into the pINIIIA vector at the XbaI and HindIII sites and into the pET33 vector at the NheI and HindIII sites. Constructs expressed from the pET33 vector also contained vector-encoded N-terminal His_6 and HMK tags. Mutagenesis of the RSP2654 gene was performed using a QuikChange Lightning multisite-directed mutagenesis kit (Stratagene) by standard procedures using oligonucleotides purchased from IDT DNA.

***E. coli* growth without amino acids.** Wild-type *E. coli* cells were transformed with the pINIIIA vector, and Δ*dksA E. coli* cells were transformed with the pINIIIA vector or with pINIIIA constitutively expressing one of the following: *E. coli* DksA, *R. sphaeroides* RSP2654, or *R. sphaeroides* RSP0166.

Strains were grown overnight on LB agar with ampicillin and then harvested from the plates and washed in M9 minimal medium, and serial dilutions were plated on M9 minimal agar plates with 0.4% glucose and ampicillin (with no amino acids). Plates were incubated at 30°C for 2 days.

β-Galactosidase assay. RLG5950 (Wild-type *E. coli* containing the *rrnB* P1 promoter, −61 to +1, fused to a *lacZ* reporter) (19) was transformed with pINIIIA, and RLG7238 (Δ*dksA*::*tet E. coli* containing an *rrnB* P1 promoter, −61 to +1, fused to a *lacZ* reporter) was transformed with pINIIIA or pINIIIA constitutively expressing either *E. coli dksA*, *R. sphaeroides* RSP2654, or *R. sphaeroides* RSP0166. Cells were grown in M9 medium containing 0.2% glycerol, 0.2% Casamino Acids, and 100 μg/ml ampicillin to an optical density of ~0.4 at 600 nm (~4 generations) for log-phase measurements. Cells were chilled on ice for ~20 min and sonicated, and β-galactosidase activity was measured by standard procedures as described elsewhere (43).

Protein purification. His_6-HMK-$DksA_{Ec}$ and His_6-HMK-$DksA_{Rsp}$ were purified by Ni^{2+} affinity chromatography using conditions previously described for His_6-$DksA_{Ec}$ (10). Native *E. coli* RNAP holoenzyme ($E\sigma^{70}$) was purified as described elsewhere (68). Native *R. sphaeroides* core RNAP was purified as described previously (30), except that heparin resin was substituted for DNA cellulose. For the heparin purification step, partially pure RNAP in TGE (10 mM Tris-HCl, 0.1 mM EDTA, and 5% glycerol) plus 200 mM NaCl was bound to heparin resin equilibrated in the same buffer and washed with 1 column volume of TGE plus 1 vol of 300, 400, 500, 600, or 700 mM NaCl. RNAP eluted during the 500 to 700 mM NaCl wash steps and was subsequently concentrated into storage buffer (20 mM Tris-HCl [pH 7.9], 100 mM NaCl, 0.1 mM dithiothreitol [DTT], 0.1 mM EDTA, and 50% glycerol). His_6-σ^{93} was purified from the soluble fraction using Ni^{2+} affinity chromatography. Briefly, cells were resuspended in buffer A (40 mM Tris-HCl [pH 7.9] and 10 mM imidazole) plus 300 mM NaCl, lysed via sonication, and centrifuged, and the cleared lysate was passed over Ni-nitrilotriacetic acid (NTA) resin (Qiagen) equilibrated with buffer A plus 300 mM NaCl. The column was subsequently washed with buffer A plus 600 mM NaCl and buffer A plus 900 mM NaCl, eluted with buffer A plus 900 mM NaCl with 300 mM imidazole, and finally dialyzed for storage against 20 mM Tris-HCl (pH 7.9), 200 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA, and 50% glycerol.

***In vitro* transcription.** Supercoiled template DNA (150 ng) containing the *rrnB* P1 and RNA-I promoters (pRLG1616) or *hisG* and RNA-I promoters (pRLG4413) was incubated with His_6-HMK-DksA, His_6-HMK-2654 (wild type or variant), or no factor (storage buffer) in transcription buffer (20 mM Tris-HCl [pH 7.9], 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/ml BSA) at room temperature (~20°C) for 10 min. Additionally, transcription buffer contained either 50 mM NaCl (single round) or 165 mM NaCl (multiple round). ppGpp (TriLink Biotechnologies) was present at the needed concentrations. *E. coli* RNAP ($E\sigma^{70}$) was added to a final concentration of 10 nM, and nucleoside triphosphates (NTPs) were added at a final concentration of 500 μM ATP, 200 μM GTP, 200 μM CTP, 10 μM UTP, and 1.0 μCi of [α-^{32}P]UTP. For single-round reactions, template DNA, RNAP, and factors were preincubated and transcription was then initiated by the simultaneous addition of rNTPs and heparin (100 μg/ml). For multiple-round transcription, reactions were initiated by the addition of RNAP. Reactions were allowed to proceed for 10 min at room temperature (~20°C) or 30°C and halted by the addition of 2× stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Transcripts were separated using 5.5% polyacrylamide–7 M urea gels. RNA was quantified using phosporimaging and ImageQuant software. The presence of the His_6-HMK sequence at the N terminus of $DksA_{Ec}$ was previously shown not to affect its function (42).

Fe^{2+}-mediated cleavage assay of DksA homologs. His_6-HMK-$DksA_{Ec}$ or *R. sphaeroides* His_6-HMK-$DksA_{Rsp}$ was ^{32}P labeled as described previously (42). Excess [γ-^{32}P]ATP was removed, and proteins were exchanged into cleavage buffer (20 mM NaCl and 20 mM HEPES [pH 7.9]) using G-50 size exclusion spin columns (GE Healthcare). *E. coli* core RNAP was also exchanged into cleavage buffer. Core RNAP (1.8 μM) was incubated at 30°C for 10 min with ~20 nM ^{32}P-labeled His_6-HMK-$DksA_{Ec}$ or His_6-HMK-$DksA_{Rsp}$ in a 10-μl reaction mixture. Hydroxyl radicals were generated from the active site of RNAP by the concurrent addition of 1 μl 100 mM DTT and 1 μl 500 μM $(NH4)_2$-$Fe(SO_4)_2$. Reaction mixtures were incubated at 30°C for 10 min and stopped by the addition of an equal volume of 2× lithium dodecyl sulfate (LDS) (Invitrogen). Reactions were electrophoresed using 4 to 12% NuPAGE gels with morpholineethanesulfonic acid (MES) buffer (Invitrogen). ^{32}P-labeled products were visualized by phosphorimaging.

RNAP-promoter complex lifetime assay. Promoter complex lifetime with *R. sphaeroides* $E\sigma^{93}$ was determined by measuring transcription from a supercoiled plasmid template at different times following heparin addition. One hundred fifty nanograms DNA (pRLG3422) was incubated with ~10 nM RNAP in transcription buffer (200 mM NaCl, 20 mM Tris-HCl [pH 7.9], 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA) at room temperature (~20°C) for 10 min. His_6-HMK-$DksA_{Rsp}$ (4 μM), 333 μM ppGpp, His_6-HMK-$DksA_{Rsp}$ (4 μM) and 333 μM ppGpp, or no factor (storage

buffer) was then added, followed by heparin to a final concentration of 100 μg/ml. Transcription was initiated by NTP addition at the indicated times following heparin addition. Reactions were allowed to proceed for 10 min and then stopped, and transcripts were quantified as described above (see "*In vitro* transcription").

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01105-14/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.

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