Similar and Divergent Effects of ppGpp and DksA Deficiencies on Transcription in Escherichia coli

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The concerted action of ppGpp and DksA in transcription has been widely documented. In disparity with this model, phenotypic studies showed that ppGpp and DksA might also have independent and opposing roles in gene expression in Escherichia coli. In this study we used a transcriptomic approach to compare the global transcriptional patterns of gene expression in strains deficient in ppGpp (ppGpp<sup>0</sup>) and/or DksA (ΔdksA<sup>0</sup>). Approximately 6 and 7% of all genes were significantly affected by more than twofold in ppGpp- and DksA-deficient strains, respectively, increasing to 13% of all genes in the ppGpp<sup>0</sup> ΔdksA<sup>0</sup> strain. Although the data indicate that most of the affected genes were coexpressively or conegatively regulated by ppGpp and DksA, some genes that were independently and/or differentially regulated by the two factors were found. The large functional group of chemotaxis and flagellum synthesis genes were notably differentially affected, with all genes being upregulated in the DksA-deficient strain but 60% of them being downregulated in the ppGpp-deficient strain. Reversely, mutations in the antipausing Gre factors suppress the upregulation observed in the DksA-deficient strain, emphasizing the importance of the secondary channel of the RNA polymerase for regulation and fine-tuning of gene expression in E. coli.

Guanosine tetraphosphate and guanosine pentaphosphate, collectively called ppGpp, are modified nucleotides that act as intracellular transducing signals in most gram-negative and gram-positive bacteria and in the chloroplasts of plant cells. In 1969, Cashel and Gallant established that the intracellular level of ppGpp was induced in response to nutrient starvation (47). In 1969, Cashel and Gallant established that the intracellular level of ppGpp was induced in response to nutrient starvation. The hallmark of the stringent response is the downregulation of rRNA and tRNA synthesis that occurs upon response (7). The hallmark of the stringent response is the downregulation of rRNA and tRNA synthesis that occurs upon response (7).

ppGpp is produced very rapidly from GTP (or GDP) and ATP in response to any stress condition that will result in attenuation of growth (5, 9, 28). In E. coli, the synthesis of ppGpp is mediated by the enzymes RelA and SpoT (8, 52). The RelA protein synthesizes ppGpp in response to amino acid starvation by recognizing and binding to stalled ribosomes, which have an uncharged tRNA bound in their A site (8, 50). The SpoT protein has ppGpp synthetase and hydrolase activities, and its synthetase activation mechanism is not well understood (16, 50, 52). In many bacteria, a single RelA-SpoT homologue protein that has both synthetase and hydrolase activities is present (33).

A few years ago it was reported that DksA, a 17-kDa RNA polymerase (RNAP) binding protein, potentiates ppGpp-mediated repression of the rRNA promoter and ppGpp-mediated stimulation of some promoters of amino acid biosynthesis operons (34, 35). These landmark findings encouraged the hypothesis that DksA acts as a cofactor for the ppGpp-dependent effects on transcription (reviewed in reference 19). DksA has an overall structural similarity to the transcription elongation factor GreA, with a prominent coiled-coil domain in the N terminus and a Zn finger motif in the C terminus (36, 44, 49). It has been suggested that the protrusion of the DksA N terminus into the secondary channel of the RNAP would stabilize the interaction between RNAP and ppGpp (36). It has been proposed that the synergistic effect of ppGpp and DksA on transcription occurs by decreasing the energy required for open complex formation and also by reducing open complex stability, which will result in either repression or stimulation of transcription, depending on the kinetic properties of a given promoter (35).

The proposed model of concerted action of ppGpp/DksA to regulate transcription would imply that deficiency of either of the two factors would cause similar phenotypic consequences, as has been shown for several genes (19, 34, 35). However, differential phenotypes of DksA-deficient (ΔdksA<sup>0</sup>) and ppGpp-deficient (ppGpp<sup>0</sup>) mutant strains in several cellular processes, such as adhesion and motility, indicated that apparent discrepancies with the postulated concerted model exist (2, 20, 29, 38). For example, it was shown that the expression of the type 1 fimbriae was significantly downregulated in ppGpp<sup>0</sup> strains, while it was notably upregulated in DksA-deficient strains (1, 2). In contrast to these in vivo discrepancies, in vitro studies demonstrated that both ppGpp and DksA stimulate transcription from the fimB promoter that is responsible for the differences observed in vivo. It was postulated that the discrepancy...
between the in vivo and in vitro findings was, at least in part, a consequence of the increased availability of the secondary channel of the RNAP to interact with the antisuppressing Gre factors in the DksA-deficient strain (2).

In this work, comparative studies of the effects of ppGpp and DksA on the global pattern of gene expression in E. coli were performed using a transcriptome approach. Our results let us conclude that deficiency in either ppGpp and/or DksA causes major alterations in the general profile of gene expression. Interestingly, when the transcriptional profiles obtained were compared, the deduced roles of ppGpp and DksA in the expression of specific genes were much more diverse than initially expected. Hence, although many genes are conegatively or copositively affected, there are also genes that are differentially affected by ppGpp and DksA. Studies of the expression of genes involved in flagellum biosynthesis and chemotaxis indicate that the increased RNAP secondary-channel occupancy by the Gre factors in the absence of DksA might be responsible for some of the differential phenotypes detected in the DksA-deficient strains. These results also suggest that competition for the occupancy of the secondary channel of the RNAP may play a key role in transcriptional regulation.

### TABLE I. Bacterial strains used in this study

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<th>Strain</th>
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<td>AAG1</td>
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<td>PRG16 ΔdksA::Cm′ ΔdksA::Tc′</td>
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* Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline.

### MATERIALS AND METHODS

#### Bacterial strains and growth conditions.

The bacterial strains used are listed in Table 1. Strains were grown in LB medium (3) at 37°C with vigorous shaking (200 rpm), unless otherwise stated. When necessary, antibiotics were used at the following concentrations: chloramphenicol, 15 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 12.5 μg/ml. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) in a Shimadzu UVmini 1240 spectrophotometer, where 0.5 unit of OD_{600} corresponds to mid-log-phase growth and approximately 5 × 10^{10} bacteria/ml. Given the propensity of ppGpp- and DksA-deficient strains to accumulate suppressor mutations within spoBC and spoD loci, routine controls for the auxotrophy phenotype of the ppGpp- and DksA-deficient strains were performed using minimal M9-salt plates (41) supplemented with 0.4% (wt/ vol) glucose and 10 μM thiamine. In addition, the fidelity of the spoBDC genes in the mutant strains was confirmed as wild type (WT) by DNA sequencing as previously described (2).

### Genomic techniques.

Basic molecular genetic manipulations were performed essentially as described previously (41). DNA sequencing was performed using the DYEnamic ET terminator cycle sequencing kit according to the manufacturer’s protocol (GE Healthcare). Gene disruption of the relA allele (corresponding to amino acids 6 to 745) and the spoT allele (corresponding to amino acids 7 to 699) was carried out by allelic exchange using the suicide plasmid pK3 as previously described (27, 31). The in vitro transcription-translation of the ppGpp and DksA-deficient strains were performed using minimal M9-salt plates (41) supplemented with 0.4% (wt/vol) glucose and 10 μM thiamine. In addition, the fidelity of the spoBDC genes in the mutant strains was confirmed as wild type (WT) by DNA sequencing as previously described (2).

### Microarray analysis.

Total RNA was isolated from two independent cultures of MG1655, CF1693 (ppGpp−), AAG95 (ΔdksA), and AAG98 (ppGpp− ΔdksA) grown to the beginning of stationary phase (OD_{600} of 1.5), and cDNA was synthesized as described previously (2). Four comparisons were made for each combination pair, i.e., WT/ppGpp− mutant, WT/ΔdksA mutant, and WT/ppGpp− ΔdksA mutant, using the two different total RNA isolations and two different Cy5 labeling. The labeled cDNAs were mixed in hybridization buffer, hybridized to microarray slides (E. coli K-12 V2 array, MWG; Ocimum Biosolutions), scanned, and analyzed as previously described (2). The microarray slides contain 4,288 open reading frames (ORFs) of E. coli K-12 covered by 4,239 50-mer oligonucleotides. Forty-seven of the oligonucleotides are replicated, and the slide also contains 48 control oligonucleotides (Arabidopsis ORFs), giving a total of 4,688 spots on each microarray slide. The microarray chip covers most of the E. coli genome, but it does not include any of the noncoding RNAs (rRNA and tRNA).

### Statistical analysis of the microarray data.

Scanned images were processed using the software Genepix 6 (Axon Inc.), and raw expression values obtained from GPR files were processed as described previously (15). Data quality was tested by MA plots of mean intensities to illustrate existing biases and the need for normalization and by correlation plots to check the quality of technical and biological replicates. Nonspecific filtering of spots which had been flagged as “bad” by the image analysis program was used. Background signal which might be considered due to nonspecific hybridization was removed using the method of Kooperberg et al. (23). Data were normalized using a print-tip loess algorithm (33).

The selection of differentially expressed genes between combination pairs was based on a linear model analysis with empirical Bayes moderation of the variance estimates following the methodology developed by Smyth (43). P values were adjusted to obtain strong control over the false discovery rate as previously described (39). Genes were selected as being “statistically significantly differentially expressed” by using volcano plots; these had a significant adjusted P value and showed a fold change of at least 2 or −2. The analysis of biological significance was based on an enrichment analysis as described previously (22).

All the statistical analysis, were done using the free statistical language R and the libraries developed for microarray data analysis by the Bioconductor Project (http://bioconductor.org).

### Gene distribution in functional groups.

Functional classification of the ORFs present in the E. coli K-12 V2 array was performed according to the classification made at The Comprehensive Microbial Resource at the J. Craig Venter Institute (http://cmr.jcvi.org/cgi-bin/CMR/CMRHomePage.cgi). The ORF names in each functional group can be found at http://cmr.jcvi.org/tgi scripts/CMR /shared/GetNumAndPercentGenesInARole.cgi. The total number of ORFs in each functional group was compared to the number of ORFs affected in each mutant strain (compared to the WT strain), with differentiation between the ORFs that were up- or downregulated. The ORFs in the two hypothetical groups (groups 10 and 11) were combined into one functional group, as were unclassified (groups 21 and 22).

### RT-PCR assay.

Reverse transcription-PCR (RT-PCR) was performed using Ready-to-Go RT-PCR beads (GE Healthcare) and the primer pairs shown in Table S1 in the supplemental material, following the manufacturer indications. Saturation curves with increasing amounts of total RNA were made to determine...
empirically the amount of total RNA used for detection of each specific transcript. The total RNA used was isolated as described for the microarray analysis.

Chemosmotaxis assay. The chemotaxis response was analyzed on TB plates (1% tryptone, 0.5% NaCl) containing 0.25% agar and supplemented with 2 mM final concentrations of L-serine, maltose, glucose, or L-valine. Overnight bacterial cultures in LB at 37°C were spotted (5 μl) on the centers of the plates and incubated 12 h at 30°C. The experiments were repeated twice with four plates of each strain in each experiment. The colony diameter was measured and plotted, and standard errors were calculated.

β-Galactosidase assay. β-Galactosidase activity measurements were performed as described by Miller (32). Data are mean values from duplicate determinations in at least three independent experiments plotted with standard errors.

Western blot analysis. Protein levels were determined from crude extracts of soluble and/or total proteins after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transfer to polyvinylidene difluoride membrane (PolyScreen) (23a), and Western blot analysis using polyclonal rabbit anti-FliC and anti-FliA antibodies (polyclonal rabbit anti-FliC and anti-FliA; NEONET, anti-FliC, anti-FliA (50a), and anti-H-NS (21a). Antibody-decorated protein bands were detected with chemiluminescence reagents (GE Healthcare) and quantified using the Fluor-S scanner and Quantity One software (Bio-Rad). To be able to visualize the FliC and FliA proteins in the WT strain MG1655, highly (∼40-fold) concentrated protein extracts of soluble proteins were used.

RESULTS

Experimental layout of the transcriptional analysis. The intriguing debate on the role of ppGpp and DksA in transcription (2, 19, 29, 35, 38) led us to compare the global gene expression pattern of the E. coli WT strain MG1655 to that of the extensively studied ppGpp-deficient strain (CF1693) (ppGpp0), a ΔdksA mutant strain (AAG95), and a ppGpp0 ΔdksA mutant strain (AAG98). The bacterial cultures were grown in LB medium at 37°C to the beginning of stationary phase (OD600 of 1.5), and total RNA was extracted. To test the response to deficiency of ppGpp and/or DksA on the general transcriptional pattern, E. coli K-12 V2 arrays (MWG, Oci-num Biosolutions) were used.

In Fig. 1A, the experimental layout used in the microarray experiment is shown. Every mutant RNA sample (ppGpp0, ΔdksA, and ppGpp0 ΔdksA) was hybridized to WT RNA. For each combination pair, i.e., WT/ppGpp0, WT/ΔdksA, and WT/ppGpp0 ΔdksA, hybridizations using the two possible dye assignments (dye swaps) were performed using the same RNA preparations (technical replicates). Moreover, each combination was replicated twice using different RNA preparations (biological replicates). Therefore, the data shown are the results of four replicates for each strain combination. Quality control of the raw data obtained shows, according to current standards (15), a very high statistical quality of the experimental data as indicated by MA plots of the data after normalization (Fig. 1B). A tightly centered distribution, symmetrical around zero, was detected. High reproducibility was observed between technical or biological replicates, which ranged from 0.7 to 0.8 for technical replicates and from 0.82 to 0.93 for biological replicates.

To confirm and validate the transcriptomic results obtained, RT-PCR was used to analyze the mRNA levels of 10 repre-
sentative genes in the different strain backgrounds (Fig. 1C). The genes selected were hns, flu, ompT, flhD, fliC, fadL, tnaA, uspA, fliA, and fimA. The difference in the levels of mRNAs for the 10 selected genes in the different strains backgrounds was similarly detected by using either the transcriptome approach or RT-PCR methodologies. Further validation experiments with some of the selected genes were performed. The expression of genes involved in flagellum synthesis (fliC and fliC) and the expression of ompT, which encodes an outer membrane protease, were also studied by using Western blot analysis (see Fig. 6 and see Fig. S1B in the supplemental material). The fold difference in the expression of FliC, FliA, and OmpT in the ppGpp0, ΔdksA, and ppGppΔdksA strains compared to the WT was similar to what was observed at the transcriptional level. The expression of tnaA, which encodes the tryptophanase (TnaA) (12), was severely downregulated in the ppGpp-deficient strain compared to the WT (27-fold; see Table S2 in the supplemental material), consistent with earlier results (54). When tnaA expresion was monitored using a chromosomal transcriptional tnaA::lacZ fusion under the same experimental conditions as in the microarray study, a 22-fold downregulation was observed in the ppGpp0 strain. The expression of tnaA::lacZ in the ΔdksA and the ppGppΔdksA strains was equally consistent with the results obtained by microarray analysis (see Fig. S1A and Table S2 in the supplemental material). Moreover, the results obtained from the microarray data were consistent with previous studies of the transcriptional expres

Effect of ppGpp and DksA deficiencies on the gene expression pattern of E. coli K-12 strain MG1655. An initial comparison of the genes that were up- or downregulated more than twofold (P < 0.0001) in the different mutant backgrounds was performed using a Venn diagram (Fig. 2A). The number of genes affected in the ppGpp0, ΔdksA, and ppGppΔdksA strains compared to the WT were 265, 311, and 556, respectively, representing 6, 7, and 13% of the total number of ORFs represented in the DNA microarray. To obtain an overview of the effects of the single and double mutations on the biology of the bacterial cell, the ORFs with expression altered more than twofold were distributed into different functional groups and presented as percentages of the total number of ORFs of each functional group that were up- or downregulated in the different mutant strains compared to the WT (Fig. 2B). In all three strains, the ORFs involved in transcription; purine, pyrimidine, and protein synthesis; protein fate; and cell envelope biogenesis were mostly upregulated (i.e., repressed by ppGpp and DksA), and the ORFs involved in energy metabolism, transport/binding, and amino acid biosynthesis were mostly downregulated (i.e., stimulated by ppGpp and DksA). By looking at the data as the percentage of the total number of ORFs affected in the different mutant backgrounds (Fig. 2B), the predominantly affected functional categories, in ppGpp- and/or DksA-deficient strains, were found to be energy metabolism and transport/binding proteins, in agreement with the crucial role described for the ppGpp/DksA regulatory system in the adaptation of growing cells to severe nutritional stresses. In almost all the functional categories, higher numbers of genes were found to be altered in the double mutant than in the single mutants, as exemplified by the categories of transcription, protein synthesis, energy metabolism, and central intermediary metabolism (Fig. 2B). Consistent with the concerted model proposed, most of the genes affected in the double mutant were also affected in the single mutants, although to a lesser extent in most cases. Hence, looking at the genes that were strongly (more than sixfold) affected by ppGpp and DksA, 60% of the genes affected in the double mutant were slightly altered in the single mutant strains (Fig. 3C). It is remarkable that of those genes affected more than sixfold in the double mutant (62 genes), almost one-third of them (19) were affected in the ppGpp0 strain but not in the ΔdksA strain, while only 2 genes were affected in the ΔdksA strain but not in the ppGpp0 strain.

Effect of ppGpp and/or DksA deficiencies on the general pattern of transcription stimulation and repression. Next, all ORFs altered more than twofold were classified into ORFs whose transcription was either stimulated or repressed in the different genetic backgrounds (Fig. 3). This classification shows that some ORFs were differentially affected (i.e., were upregulated in one mutant background but downregulated in another mutant background). These differentially affected ORFs were excluded in the comparison shown in Fig. 3 and are discussed in following sections.

The analyses of the ORFs that were up- or downregulated show that in the double mutant, 30% more genes were downregulated than upregulated (293 versus 203) (Fig. 3A). When only ORFs that were up- or downregulated more than sixfold were considered, the relative difference in numbers was even higher (49 versus 14) (Fig. 3B). These results highlight a noteworthy role of ppGpp/DksA in stimulation of gene expression. The distributions of ORFs that were up- or downregulated in the ppGpp-deficient strain are similar (114 versus 106 and 6 versus 12 [genes altered by >2-fold or >6-fold, respectively]). On the other hand, in the single DksA-deficient strain, the number of genes upregulated was higher than the number of genes downregulated (159 versus 81 and 20 versus 9 [genes altered by >2-fold or >6-fold, respectively]). The difference was even greater when looking at the ORFs that were upregulated or downregulated only in the DksA-deficient strains (86 + 54 versus 41 + 6, respectively), suggesting that DksA might independently play a more crucial role in the repression of gene expression than in its stimulation. However, previous results indicate that stimulation of gene expression in a DksA-deficient strain may not necessarily be due to a direct repressive effect of DksA on transcription (2). It might also be consequence of an increased association of other factors (Gre factors) with the RNAP occurring in the absence of DksA.

The array data imply that not all genes are regulated by ppGpp and DksA in a concerted manner. Although our results partially support the proposed concerted model of ppGpp/DksA action on transcription, some data also indicate clear inconsistency with that model. For example, while 60% of the genes affected more than sixfold in a strain deficient in both factors were also altered in the single mutant strains (as would be expected from the concerted model), 40% were not (Fig. 3C). Moreover, an elevated number of genes were affected by single ppGpp or DksA deficiencies but not by deficiency of both regulatory factors, strongly suggesting that the concerted model is not valid for all genes (Fig. 2 and 3). Further-
more, ppGpp and DksA deficiencies cause differential effects on the expression of some ORFs. It is noteworthy that several ORFs of the cellular process functional group respond differentially to deficiency in either ppGpp or DksA (Fig. 2B). In the ppGpp-deficient strain, most of the genes in this functional category were downregulated, indicating that ppGpp stimulates their expression. Conversely, in the DksA-deficient strain, most of the genes were upregulated, indicating that DksA represses their expression. Extraordinarily, 77% of the genes (57 genes of 74) in this functional category affected by ppGpp were differentially regulated by DksA. Most of those genes (51 genes) are involved in chemotaxis and motility. The other six genes are katG, cypA, cspF, cspD, betB, and b1503. A similar pattern of regulation was also found for other gene determinants belonging to other functional categories, such as the fim genes (see Table S2 in the supplemental material), represented in the cell envelope group, that were previously reported to have such a pattern of expression (2).

In Fig. 4 the ORFs differentially affected in at least two of the mutant backgrounds (>2-fold compared to the WT) are grouped depending on their pattern of expression in the different mutant strains. Interestingly, most of the genes that were differentially regulated were stimulated by ppGpp (40 genes) (Fig. 4B), whereas only 6 genes (Fig. 4A) were repressed. Among them, in addition to the previously described fim genes, many ORFs coding for the flagellum and chemotaxis systems were found (Fig. 4C). Our data suggest that depending on promoter-specific properties, the requirements for ppGpp and DksA might be dissimilar and the mechanisms of repression and stimulation might be diverse.
Effect of ppGpp and/or DksA deficiencies on flagellum bio-
synthesis and chemotaxis operons. The transcriptional pattern
of the 54 ORFs coding for flagellum biosynthesis and chemotaxis
is shown in Fig. 5A. These genes are distributed in 18 operons
and are grouped into early, middle, and late operon
classes depending on their temporal expression (Fig. 5A) (10,
30). All the flagellum- and chemotaxis-encoding ORFs were
induced in the DksA-deficient strain compared to the WT
strain, whereas 60% of them (32 of the 54 ORFs) were down-
regulated in the ppGpp-deficient strain (Fig. 5A). Interest-
ingly, the expression of several of those ORFs was not signif-
cantly altered in the double mutant strain. The extent of the
effects caused by the deficiency in either DksA or ppGpp varies
depending on the operon class. In the DksA-deficient strain,
expression was slightly induced (2.5-fold) for the early operon
(flhDC), modestly induced (between 4- and 10-fold) for the
middle operons, and greatly induced for the late operons, i.e.,
fliC (36-fold), motB (20-fold), and tar (23-fold) (Fig. 5A). In
the ppGpp-deficient strain, the early and middle operons were
slightly repressed (1.4- and 2.9-fold on average, respectively),
while most of the late operons were unaffected (Fig. 5A).

The ppGpp/DksA-mediated control of flagellum and che-
motaxis genes was assessed by phenotypic characterization
of the chemotactic movement in the different strains (WT,
ppGpp0, ΔdksA, and ppGpp0 ΔdksA strains) using TB plates
with either different chemottractants (L-serine, maltose, or
glucose) or a chemorepellent (L-valine) (Fig. 5B). The WT
strain showed modest spreading and a weak response to the
different chemoattractants. Nevertheless, the ppGpp-deficient
strain showed even less motility, corroborating our transcrip-
strains independently of the presence or absence of ppGpp (Fig. 6B) (since the amount of FliC in MG1655 was very low, different amounts of each protein extract were loaded for quantification purposes). As a control, the protein level of H-NS (a major nucleoid-associated protein) was determined using the same protein extracts.

The increased expression of flagellum-related genes is abolished in the absence of the Gre factors. Repression and induction of gene expression caused by deficiency in ppGpp or DksA, respectively, has been previously described for the genes of the fim determinant, coding for the type 1 fimbriae (1, 2). In that case, it was shown that the upregulation of gene expression in a DksA-deficient strain in vivo did not imply that DksA has an active repressing effect on transcription. In fact, it was shown that the stimulation of the fim determinant in the DksA-deficient strain was suppressed by mutation of the genes coding for the Gre factors. Those results suggest that in the absence of DksA, the availability of the secondary channel of the RNAP to interact with GreA and GreB might increase, to account for the upregulation observed (2). To determine whether the increased expression profile of flagellum genes in the DksA-deficient strain might be explained by a similar mechanism, the effects of gre mutations on the expression of FliC were analyzed (Fig. 7). The expression of FliC (a major subunit of the flagella) in the DksA-deficient background was greatly reduced in the presence of a greA mutation (Fig. 7A), in agreement with the mechanism proposed to underlie DksA-mediated repression of fimB transcription. The results obtained when the greA allele was added in the dksA background indicate that, under the conditions used, GreA seems to play a relevant role in the control of fliC. An interesting question is whether the interplay between the DksA and Gre factors affects fliC expression either at transcription initiation or during transcription elongation. To discriminate among those possibilities in vivo, early (+70, first codon of the fliC coding sequence) and late (+1210) chromosomal lacZ fusions within the fliC gene were constructed. Expression from those fusions in the WT, ΔdksA, and ΔdksA ΔgreA strains was determined (Fig. 7B). Interestingly, for the early fliC fusion, no important response to DksA deficiency was observed. However, a very important increase in expression was detected in the absence of DksA when the late fliC fusion was used. Remarkably, this increased expression dropped to WT levels when GreA was eliminated. These results suggest that fliC expression is importantly regulated at the elongation level in a GreA-dependent manner. Moreover, the results with the early fusion do not let us rule out that the interplay between DksA and the Gre factors might also contribute to the regulation at the transcription initiation level. Remarkably, the expression of FliC was severely reduced in a ΔgreA ΔgreB strain even in the presence of DksA, suggesting that the expression of FliC is strictly dependent on the presence of the anti-pausing Gre factors (Fig. 7A). To elucidate whether the Gre factors directly or indirectly affect transcription of the fliC gene will require further studies.

**DISCUSSION**

The ppGpp mediator of the stringent response induces major changes in the pattern of gene expression, with consequential effects on different cellular processes (reviewed in refer-
A common experimental approach to establish whether a genetic system is under ppGpp control is to compare expression in a WT strain and its ppGpp-deficient counterpart in the beginning of stationary phase (1, 2, 17, 26). In this report, we describe comparative microarray analysis of the global transcriptional patterns of a widely studied ppGpp-deficient E. coli strain (ppGpp0, CF1693) and its otherwise WT counterpart (MG1655) grown in LB medium at 37°C to the beginning of stationary phase (OD600 of 1.5). The discovery that DksA can potentiate the effects of ppGpp on transcription stimulation and repression (2, 29, 35, 38) led us to also compare the global gene expression patterns of a ΔdksA strain and a ppGpp0 ΔdksA strain.

Of the total number of protein-encoding ORFs screened (all genes of E. coli excluding those for rRNA and tRNA), we found that 6% were up- or downregulated more than twofold in the ppGpp-deficient strain compared to the WT. Our results are reminiscent of microarray studies of the effects of other global regulators in E. coli, such as H-NS (21) and Fis (4), where a similar percentage of genes (~5%) were found to be affected. The number of genes affected when comparing the DksA-deficient strain to the WT was similar to the number affected in the ppGpp-deficient strain (approximately 7%). In the strain deficient in both ppGpp and DksA, the number rose to 13% of the ORFs. In accordance with previous reports describing the general effect of ppGpp and DksA on bacterial physiology, the cellular functions mostly affected in the strains deficient in those factors were metabolism, transport, translation, and DNA/RNA metabolism (8, 13, 37). In a recent report by Traxler et al. (48), the gene expression profiles of WT and ppGpp-deficient strains after induction of the stringent response were compared. Although the experimental setup and the growth conditions used were different in the two approaches, we found in the ppGpp-deficient strain (see Table S2 in the supplemental material) altered expression of genes involved in the control of important cellular processes (protein synthesis, transcription, replication, fatty acid biosynthesis, stress response, etc.) that were likewise detected by Traxler et al. (48), corroborating the important role of ppGpp in the control of diverse cellular processes in the bacterial cells. Interestingly, in comparison with our results, a higher number of genes showed altered expression in the ppGpp-deficient strain, which presum-
ably might be explained by the fact that the expression profiles were compared after inducing the stringent response by amino acid starvation (48).

Based on expression studies performed with the rrnB and amino acid biosynthetic operons (34, 35), a model of concerted coregulation by ppGpp and DksA was proposed, which entails that mutants deficient in either ppGpp or DksA would possess similar features independently of whether the ppGpp/DksA-mediated regulation was direct or indirect. However, phenotypic studies indicated that ppGpp and DksA can have identical but also independent and opposing roles in gene expression in *E. coli* (2, 20, 29, 38). Our transcriptional data support the phenotypic diversity previously described. (i) Most of the ORFs affected in either ppGpp- or DksA-deficient strains were also affected in the strain lacking both factors. Hence, many of the 346 ORFs affected in the ppGpp0/H9004 dksA strain are also affected in the ppGpp0 and the dksA strains, but by less than twofold (Fig. 2A). (ii) Interestingly, a notable number of genes whose expression is altered in either the ppGpp-deficient (89 + 58) or DksA-deficient (124 + 58) strain, representing 55 and 58% of all genes affected in the ppGpp- and DksA-deficient strains, respectively, did not show altered expression in the ppGpp/DksA-deficient strain. Moreover, 58 of those genes were affected in both the ppGpp- and DksA-deficient strains (Fig. 2A). (iii) Many genes exhibited opposite effects on transcription by ppGpp and DksA (Fig. 4). Thus, our data indicate that the mechanisms of regulation by ppGpp and DksA are most likely more diverse than initially suggested, and not all effects observed can be explained by the concerted coregulation. Elucidation of the presumably different modes of action of these two regulatory factors will require detailed studies of genetic determinants of regulation of the individual systems.

An extensive group of genes that encode flagellum synthesis and chemotaxis were downregulated in ppGpp-deficient strains while being upregulated in the DksA-deficient counterpart. A similar expression pattern was previously de-

![FIG. 6. ppGpp and DksA deficiencies differentially affect flagellum production. (A) Electron micrographs of bacterial cultures of the WT (MG1655), ppGpp0 (CF1693), ΔdksA (AAG95), and ppGpp0 ΔdksA (YVF14) strains, grown under the same conditions as for Fig. 1. Bar, 5 μm. (B) Effect of ppGpp and DksA deficiencies on expression of the major flagellum subunit FlIC and the sigma subunit FlIA. Determinations of the protein levels of FlIC, FlIA, and H-NS (control) were performed by Western blot analysis of protein extracts from the WT (MG1655), ppGpp0 (CF1693), ΔdksA (AAG95), and ppGpp0 ΔdksA (AAG98) strains cultured as for Fig. 1. For FlIC and FlIA immunodetection, 100 μg of protein extracts was used for the WT and ppGpp0 strains, while 5 μg of the ΔdksA extract and 10 μg of the ppGpp0 ΔdksA extract were used. For H-NS detection, 25 μg of each extract was used. The values shown below the gels represent the relative amount of protein found in the mutant strains compared to the WT strain (set as 1), after adjusting for the amount of extract used.](image)

![FIG. 7. GreA and GreB regulate expression of the major flagellum subunit FlIC. (A) FlIC immunodetection in protein extracts (100 μg) from the WT (MG1655), ppGpp0 (CF1693), ΔdksA (AAG95), ppGpp0 ΔdksA (AAG98), ΔgreA ΔgreB (AAG107), and ΔdksA ΔgreA (AAG101) strains cultured as for Fig. 1. Detection after 30 s and 5 min of exposure is shown. (B) Expression from two chromosomal fliC::lacZ transcriptional fusions. lacZ fusions within the fliC gene at positions +70 (left panel) and +1210 (right panel) were constructed, and their expression in the WT (PRG13 and PRG16), ΔdksA (PRG14 and PRG17), and ΔdksA ΔgreA (PRG15 and PRG18) strains was monitored. Cultures were grown in LB medium to an OD_{600} of 1.5 at 37°C. Relative average values and standard deviations from three independent experiments are shown. For each fliC::lacZ fusion, the value in Miller units in the WT was set as 1, which corresponds to 60 and 106 Miller units for PRG13 and PRG16, respectively.](image)
scribed for the type 1 fimbriae-encoding genes (1, 2, 29); however, it was demonstrated that in vitro, both ppGpp and DksA stimulate the transcription from the responsible promoter, namely, that for \textit{fimB} (2). A model was suggested in which the upregulation of \textit{fimB} detected in the DksA-deficient strain in vivo was a consequence of the increased vacancy of the secondary channel of the RNAP, due to the absence of DksA, that could promote the binding of other regulators such as the GreA/B antipausing factors. Here we showed evidence that the transcriptional stimulation of flagellum- and chemotaxis-related genes observed in DksA-deficient strains was suppressed when the Gre factors were removed (Fig. 7). These results suggest that when analyzing the in vivo DksA-mediated effects on gene expression, the passive consequence of vacancy of the secondary channel of the RNAP should be considered. GreA-regulated genes in \textit{E. coli} have been identified using a microarray approach comparing the expression profiles of a \textit{greA} \textit{greB} strain, a \textit{greA} \textit{greB} strain, and a strain overexpressing GreA from a plasmid in a \textit{greA} \textit{greB} background (46). Although the growth conditions used were different from those in our study (30°C and mid-log-phase cultures as opposed to 37°C and late mid-log-phase cultures), 39 of the 311 genes with altered expression in the DksA-deficient strain were found to be regulated by GreA, supporting the hypothesis that some of the phenotypes found in a DksA-deficient strain might be related to the interplay between DksA and the Gre factors. The influence of the Gre factors on the expression of ppGpp/ DksA-regulated genes presumably depends on the promoter strength of the RNAP. Based on the intracellular concentrations of DksA, the importance of the secondary channel of the RNAP has been proposed to provide a new mode for regulating transcription (38). Regulation of the \textit{fimB} promoter (2) and the data presented here lend strong support to this idea. However, further biochemical and in vitro studies would be required to corroborate the interplay among those regulatory factors and its role in the regulation of transcription in bacteria. With respect to competition of the secondary channel for regulatory purposes, Lamour et al. (25) have recently shown that Rnk, which does not affect the RNAP activity per se, has a C-terminal structure similar to that of the Gre family of proteins, and they suggest that it may compete with the Gre factors and DksA for binding to the RNAP secondary channel.

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