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Transcription Profiling of the Stringent Response in *Escherichia coli*▽†

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The bacterial stringent response serves as a paradigm for understanding global regulatory processes. It can be triggered by nutrient downshifts or starvation and is characterized by a rapid RelA-dependent increase in the alarmone (p)ppGpp. One hallmark of the response is the switch from maximum-growth-promoting to biosynthesis-related gene expression. However, the global transcription patterns accompanying the stringent response in *Escherichia coli* have not been analyzed comprehensively. Here, we present a time series of gene expression profiles for two serine hydroxymate-treated cultures: (i) MG1655, a wild-type *E. coli* K-12 strain, and (ii) an isogenic *relAΔ251* derivative defective in the stringent response. The stringent response in MG1655 develops in a hierarchical manner, ultimately involving almost 500 differentially expressed genes, while the *relAΔ251* mutant response is both delayed and limited in scope. We show that in addition to the down-regulation of stable RNA-encoding genes, flagellar and chemotaxis gene expression is also under stringent control. Reduced transcription of these systems, as well as metabolic and transporter-encoding genes, constitutes much of the down-regulated expression pattern. Conversely, a significantly larger number of genes are up-regulated. Under the conditions used, induction of amino acid biosynthetic genes is limited to the leader sequences of attenuator-regulated operons. Instead, up-regulated genes with known functions, including both regulators (e.g., *rpoE*, *rpoH*, and *rpoS*) and effectors, are largely involved in stress responses. However, one-half of the up-regulated genes have unknown functions. How these results are correlated with the various effects of (p)ppGpp (in particular, RNA polymerase redistribution) is discussed.

In rapidly dividing *Escherichia coli* cells grown in rich media, most RNA polymerase (RNAP) molecules are engaged in transcribing a small set of genes whose products are primarily involved in translation, most notably rRNAs and tRNAs (stable RNAs) (9, 27, 69). However, when cells are subjected to abrupt amino acid starvation, a coordinated program known as the stringent response is induced (14). This response is characterized by a rapid transcriptional switch whereby highly expressed genes required for rapid growth are down-regulated and amino acid biosynthetic operons are induced. How the transcriptional switch is regulated is now understood in some detail and defines a fundamental strategy for coping with rapidly changing nutrient environments.

Amino acid starvation results in an increased uncharged tRNA/charged tRNA ratio for the depleted amino acid, leading to ribosome stalling, which in turn activates the ribosome-associated enzyme RelA (34, 64, 76, 99). RelA is the major synthetase of the small-molecule effectors guanosine tetra- and pentaphosphates [(p)ppGpp] (13, 14). (p)ppGpp levels are also controlled by the *spoT* gene product, a bifunctional enzyme having both a regulated hydrolase activity for degrading (p)ppGpp and a weak synthetase activity (28). *relA spoT* double null mutants are unable to produce (p)ppGpp and have multiple defects, including auxotrophies for several amino acids (100). Although the ability of *relA* mutants to initiate a stringent response is severely compromised and hence these mutants are referred to as "relaxed" mutants (86), they are amino acid prototrophs due to (p)ppGpp produced by SpoT.

Although (p)ppGpp has been reported to affect transcription elongation (52, 54, 97), the mode of action of these small effectors is best understood in transcriptional initiation. (p)ppGpp binds directly to RNAP (2, 16, 91) and, together with the DksA coregulator, both reduces open complex half-lives at all promoters (5, 48, 71) and increases forward isomerization rates for at least some promoters (71, 72). (p)ppGpp/DksA effects on stable RNA synthesis are especially crucial in setting the bounds of global transcription patterns. Estimates have indicated that a cell contains approximately 2,000 RNAP molecules and more than 3,000 promoters per genome equivalent (7, 40, 79). Although stable RNA operons represent only ~1% of the genome, they can engage >60% of the RNAP pool, severely constraining RNAP availability for the remainder of the genome (9). During rapid growth, the RNAP concentration at stable RNA operons is sufficient to be visualized as transcription foci in the nucleoid (12). However, stable RNA promoters are regulated by open complex stability, making them especially sensitive to the inhibitory effects of (p)ppGpp/DksA (5, 71).

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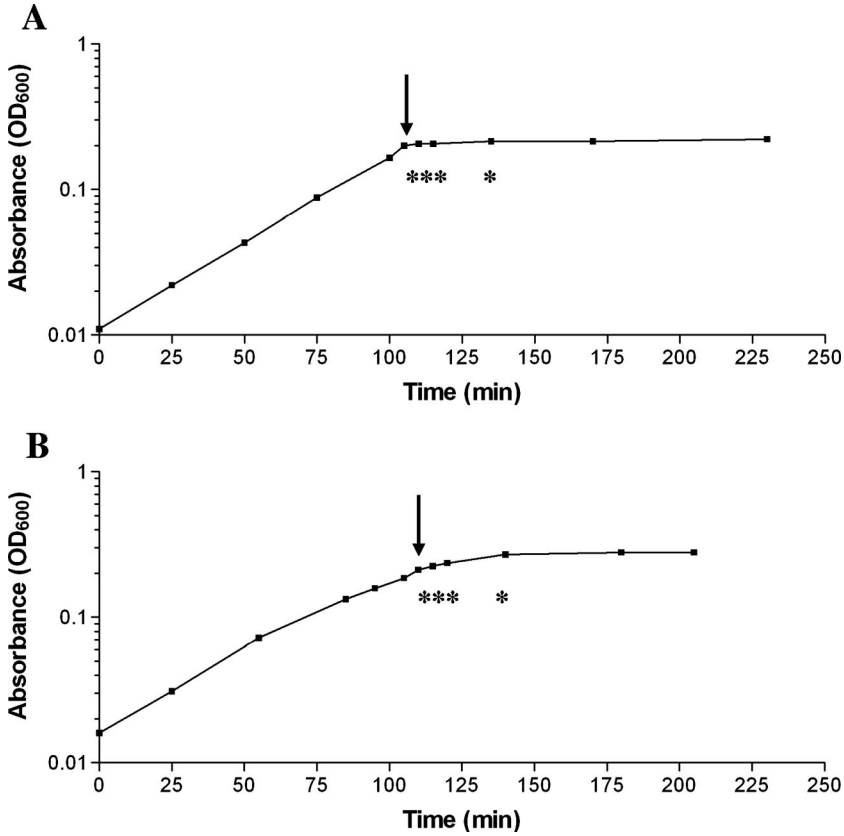

FIG. 1. Growth curves for MG1655 (A) and the *relA*Δ*251* derivative (B) during an SHX-mediated stringent response. The arrow indicates the time when SHX was added. Asterisks indicate times when expression profiles of each strain were determined. OD_{600}, optical density at 600 nm.

RNAP mutants that are capable of restoring amino acid prototrophy to *relA spoT* double null cells and have a constitutive stringent phenotype have been isolated; they have reduced stable RNA expression and increased amino acid biosynthetic gene expression in the absence of (p)ppGpp or nutrient starvation (104, 105). From in vivo and in vitro studies of these "stringent" RNAP mutants, Zhou and Jin formulated a model in which reduced stable RNA synthesis upon starvation increases a pool of "free" RNAP for transcription of appropriate response genes, such as the genes for amino acid biosynthesis (105). Critical support for this model was subsequently obtained by Gourse and coworkers using wild-type polymerase (4). New gene expression was originally envisioned to result mainly from the "passive" activation of promoters sensitive to the RNAP concentration, but data have demonstrated that at least some promoters can be directly activated by (p)ppGpp/DksA (72, 75). This intimate coupling of inhibition of stable RNA transcription to RNAP (re)distribution across the genome can be visualized microscopically as the loss of transcription foci and the appearance of a diffuse RNAP signal throughout the nucleoid (12, 44).

Genome-wide transcriptional profiles during amino acid starvation for two gram-positive bacteria, *Bacillus subtilis* and *Corynebacterium glutamicum*, and one gram-negative bacterium, *Vibrio cholerae*, have been reported (10, 25, 77). In *E. coli*, gene expression following treatment with acivicin, 4-azaleucine, or mupirocin has been shown to partially mimic the stringent response (82, 85). In each of these studies, the profiles for a single time point from 10 to 30 min after treatment were determined. Time course profiles for diauxic shift and carbon source depletion studies of *E. coli*, both of which involved increased (p)ppGpp levels, have also been described (15, 50, 95). One key missing data set, however, is a time course describing the classic *E. coli* stringent response to amino acid starvation. In this study, we determined the gene expression profiles for both wild-type and *relA* mutant cells at three different time points after induction of the stringent response by serine hydroxymate (SHX) treatment. The results allowed the hierarchical nature of the response over time to be followed and should aid further studies to distinguish primary effects of (p)ppGpp/DksA from secondary effects. Moreover, the large number of differentially expressed genes provides a transcription level rationale for the pleiotropic nature of the response. The profiles for the *relA*Δ*251* mutant showed a far more limited and delayed transcriptional response consistent with the "relaxed" phenotype of the mutant.

MATERIALS AND METHODS

Bacterial growth conditions and RNA isolation. Cultures of MG1655 (7) and an isogenic derivative of this strain containing the *relA*Δ*251* mutation (65) were grown aerobically at 37°C in morpholineethanesulfonic acid (MOPS)-based medium supplemented with all 20 amino acids (50 μg/ml each) and 0.1% glucose (66) in shake flasks. The stringent response was induced by addition of 100 μg/ml SHX (Sigma) when cultures reached an optical density at 600 nm of 0.2. At four

time points during growth (Fig. 1), samples were processed for transcriptional profiling. Detailed protocols for sample preparation are available on our website (www.genome.wisc.edu). Briefly, 15-ml aliquots were harvested, mixed with 30 ml RNAprotect bacterial reagent (Qiagen), and pelleted. Total nucleic acid was isolated using MasterPure kits according to the manufacturer's specifications (Epicenter Technologies). Nucleic acid pellets were treated with 0.05 U/μl DNase I for 45 min at 37°C and then repurified with MasterPure.

Probe preparation and hybridization. Conversion of RNA to cDNA, labeling, and hybridization were conducted essentially as described previously (www.affymetrix.com). Briefly, 10 μg of purified total RNA was reverse transcribed using Superscript II (Invitrogen), and this was followed by RNase digestion and cDNA purification using Qiaquick PCR purification columns (Qiagen). Note that these columns did not retain DNA smaller than 40 bp and had a lower affinity for molecules smaller than 100 bp, a property which affected quantitative recovery of small RNAs, including tRNAs. Isolated cDNA was fragmented by partial DNase I digestion to obtain an average length of 50 to 100 bp. Fragmented cDNA was 3′ end labeled with biotin-N_6-ddATP (Enzo Biochem), mixed with hybridization solution, and loaded onto Affymetrix GeneChip *E. coli* antisense genome arrays. Following 16 h of hybridization at 45°C, each array was washed and stained with streptavidin-phycoerythrin (Molecular Probes) using an antibody intermediate to enhance the signal. Arrays were scanned at 570 nm with 3-μm resolution using a confocal laser scanner (Hewlett-Packard). Three replicates from independent experiments were analyzed for MG1655, and two replicates were analyzed for the *relAΔ251* strain.

Primer extension analyses. MG1655 and the *relAΔ251* mutant strain were cultured in the same medium, and RNAs were isolated as described above. The method used for primer extension analysis to detect the transcripts from P1 promoters of four *E. coli* rRNA operons and from an *ompA* control promoter and the associated primers have been described previously (103).

Data analysis. Raw data were imported into a relational database and first normalized by scaling the average of the fluorescence intensities for each gene to a constant correcting for variations in sample loading, hybridization, and staining. The average of the normalized intensity values across replicates corresponded to the abundance of each gene. Genes showing a $\log_2$ ratio ≥ 1.5 with an average $\log_2$ signal intensity greater than 9 were considered induced. Conversely, genes with a $\log_2$ ratio ≤ -1.5 and had average $\log_2$ signal intensity greater than 9 in the control were considered repressed. The signal intensity cutoff was used to restrict the analysis to the genes that were unambiguously expressed. The apparent fold changes for some genes should be interpreted with caution when the signal intensity for a gene in either of the profiles being compared is low ($\log_2$ value, <7). Complete data sets are available at our website (http://www.genome.wisc.edu/tools/asap.htm) and have been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/).

RESULTS

Growth profiles of *E. coli* K-12 wild-type and *relAΔ251* cells upon induction of the stringent response. To characterize the expression profile changes during a stringent response, MG1655 (*relA*$^+$) and an isogenic derivative containing the *relAΔ251* allele were grown aerobically in a MOPS-based defined medium containing all 20 amino acids (50 μg/ml each) and 0.1% glucose at 37°C. Growth curves were determined for both strains, and they showed that the two strains grew with almost identical doubling times (~26 min) in this medium (Fig. 1). At an optical density at 600 nm of 0.2, cultures were treated with 100 μg/ml SHX, a serine analogue that inhibits serine tRNA synthetase and thus mimics serine starvation (14, 90). The absorbance of the MG1655 culture stopped increasing almost immediately following SHX addition, consistent with induction of the stringent response, and remained nearly constant over the next 2 h. In contrast, the absorbance of the *relAΔ251* culture continued to increase for 30 to 45 min, albeit at a greatly reduced rate, before reaching a plateau.

For expression profiling, samples were taken just prior to SHX addition (time zero), as well as 5, 10, and 30 min after treatment. We expected that the earlier time points might identify direct targets of (p)ppGpp/DksA, while the 30-min profile would reflect more complex regulation. As SHX blocks translation almost completely (90), transcriptional changes are expected to be dependent mainly on RNAP and preexisting transcription factors, some of which may be regulated post-translationally. Total RNA was converted to cDNA, end labeled, and hybridized to Affymetrix GeneChip *E. coli* antisense arrays. Normalized data for three MG1655 replicates and two *relAΔ251* replicates were averaged, and differentially expressed genes were identified by comparing each profile after SHX addition to the pretreatment control (see Materials and Methods).

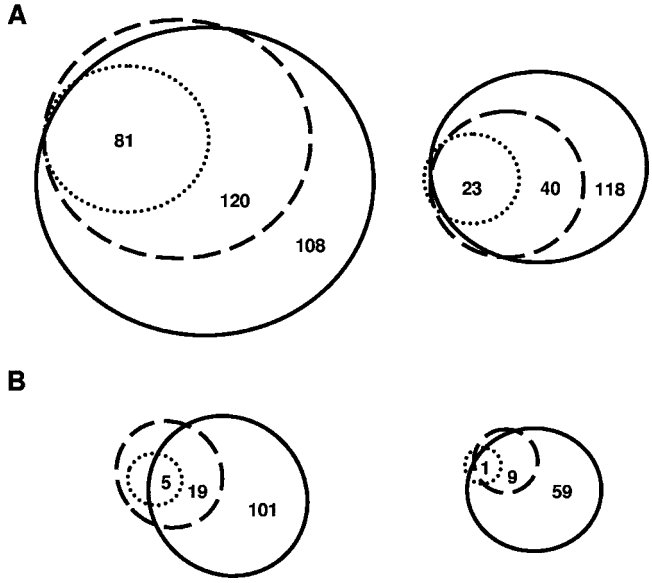

FIG. 2. Differentially expressed gene sets expand hierarchically in MG1655 but are aberrant both in scope and timing in *relAΔ251*: Venn diagrams of overlapping up- and down-regulated gene sets (left and right diagrams, respectively) in MG1655 (A) and the *relAΔ251* strain (B). Time points are indicated as follows: dotted circles, 5 min; dashed circles, 10 min; and solid circles, 30 min. The numbers indicate the numbers of core genes affected at each time point (i.e., not affected at previous time points).

Expression profile overview. Initial comparisons indicated that SHX treatment resulted in rapid and extensive reprogramming of transcription in MG1655 (Fig. 2A and Table 1). A total of 110 genes were differentially regulated within 5 min after treatment, and there were approximately three times more up-regulated genes than down-regulated genes. By 30 min, the number of differentially expressed genes was 490, although the up-regulated/down-regulated ratio was less than 2:1. Comparison of the up- and down-regulated gene sets across the time series demonstrated that the response occurred in a hierarchical manner (Fig. 2A). In contrast, the *relAΔ251* response was substantially reduced both temporally and in scope and did not show as much consistency across time as the wild-type response (Fig. 2B and Table 1).

Differentially expressed genes were grouped into the functional categories of Serres et al. (84) and then further merged into nine related groups (Table 1). Genes were generally spread across functional categories throughout the experiment, with one exception: genes with unknown functions were dramatically overrepresented among up-regulated genes (~50%

TABLE 1. Functional grouping of differentially expressed genes

Functional group[a]	No. of genes											
	5 min				10 min				30 min			
	MG1655		*relA*Δ*251* mutant		MG1655		*relA*Δ*251* mutant		MG1655		*relA*Δ*251* mutant	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Metabolism and cofactor biosynthesis	4	1	1	0	19	4	2	2	29	41	8	20
Membrane	3	6	0	1	8	14	2	1	11	20	8	8
Transport	3	1	0	0	7	9	5	1	15	23	6	13
Translation	6	1	0	1	12	3	1	0	15	17	7	2
DNA and RNA metabolism	4	7	1	0	13	7	2	1	14	15	3	3
Regulation	7	0	0	0	14	1	0	3	22	0	11	3
Other	7	6	0	0	30	14	4	4	42	27	4	13
Unknown	49	2	6	0	107	5	23	1	161	14	78	7
tRNAs	0	3	0	0	0	11	0	0	0	24	0	0
Total	83	27	8	2	210	68	39	13	309	181	125	69

[a] Genes with putative functions are classified as unknown.

at each time point) and underrepresented among down-regulated genes (~7% at each time point).

Inhibition of stable RNA synthesis. A central feature of the stringent response is the rapid inhibition of stable RNA synthesis. To confirm that the genes responded to SHX treatment as expected, we first monitored the *rrnB* P1 leader sequence abundance at each time point in both wild-type and *relA*Δ*251* cultures using primer extension (Fig. 3). In contrast to the stability of ribosome-bound rRNAs, rRNA leader sequences are rapidly degraded (30, 83) and therefore provide an accurate reflection of rRNA promoter activity (103). The two strains showed similar levels of *rrnB* P1 activity prior to SHX treatment, as expected from their identical growth rates (Fig. 3, lanes 1 and 5). However, while *rrnB* P1 activity declined dramatically (10-fold) following SHX addition in MG1655 cultures (Fig. 3, lanes 2 to 4), synthesis in the *relA*Δ*251* mutant cultures was only modestly reduced (20%) (Fig. 3, lanes 6 to 8). A similar trend was obtained using the microarray data sets when probe features corresponding to the leaders of all seven *rrn* operons were analyzed (data not shown).

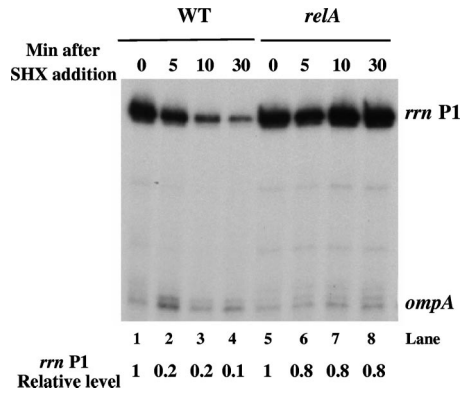

FIG. 3. P1 transcripts of rRNA operons in MG1655, but not in the *relA*Δ*251* strain, rapidly decline during the stringent response: autoradiograph of *rrn* P1 primer extension products from the strains determined at the time points used for expression profiling. The *ompA* products, which were maintained at constant levels (as shown by array data), were used as an internal control to normalize rRNA levels. The relative amounts of rRNA synthesized from the P1 promoters were determined by comparing the normalized values at each time to the time zero control. The experiments were repeated at least twice, and similar results were obtained. WT, wild type.

Transcription of tRNA-encoding genes is also under stringent control. Indeed, significant declines in transcript abundance for 37 tRNA-encoding genes, including the 11 genes embedded in rRNA operons, were observed during the first 30 min following SHX treatment. Further, 49 tRNA-encoding genes showed at least twofold down-regulation after 30 min, and most of the remaining genes displayed some degree of repression. In contrast, no tRNAs were significantly affected in *relA*Δ*251* cells. These results are in agreement with previous work (39), although they must be interpreted qualitatively for technical reasons related to sample purification (see Materials and Methods).

Induction of attenuator-regulated amino acid biosynthetic operons. The stringent response also leads to induction of multiple amino acid biosynthetic operons (14). Under the conditions used, eight operons were activated at different times following SHX treatment, and seven of them are also regulated by attenuation. Activation of the attenuator-regulated operons was restricted to the leader sequence (Fig. 4A), while the structural genes were modestly down-regulated. This implies that attenuation was enhanced under the conditions used, which was expected due to the presence of all 20 amino acids in the medium and the lack of serine codons at crucial sites in the affected leader elements. One exception was the *ilvLG_1G_2MEDA* operon, where accumulation of the *ilvG_1* and *ilvM* genes was also observed. The only attenuator-regulated operon not up-regulated was *trpLEDCBA*, whose transcription was blocked under the conditions used by TrpR. In the *relA*Δ*251* mutant, only *ilvL* and *leuL* showed significant increases after 30 min (Fig. 4B).

MG1655 response to SHX treatment at 5 min. Within 5 min after SHX treatment, 27 down-regulated and 83 up-regulated genes were detected in MG1655 (Table 1; see Table S1 in the

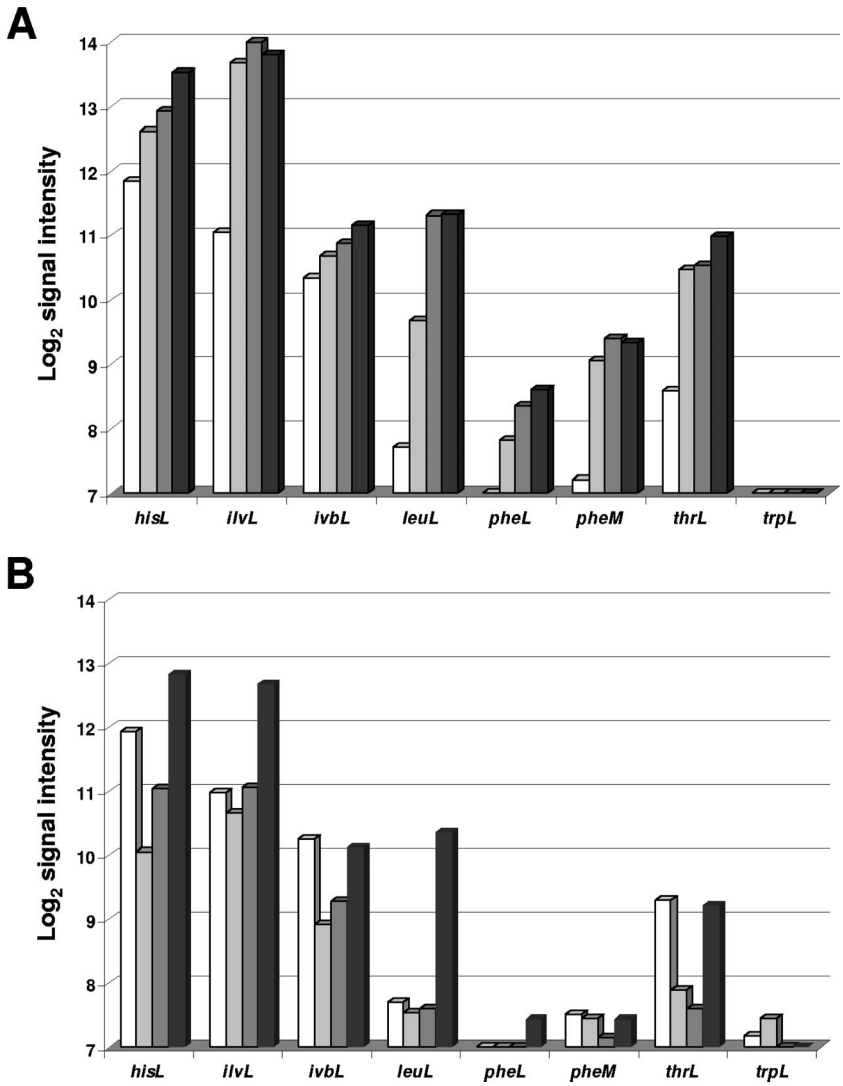

FIG. 4. Expression of attenuator-regulated amino acid biosynthetic operons is steadily up-regulated in MG1655 but shows aberrant activation in the *relAΔ251* strain: histograms of signal intensities ($\log_2$ scale) for each of the eight attenuator-regulated amino acid biosynthetic leader elements in MG1655 (A) and *relAΔ251* (B). Time points are indicated as follows: open bars, zero time; light gray bars, 5 min; dark gray bars, 10 min; and black bars, 30 min.

supplemental material). Among the down-regulated genes was the *flhDC* operon encoding the master transcriptional regulator for flagellum-related gene expression, as well as three downstream operons in the FlhDC regulon (Fig. 5). Together, these genes accounted for one-third of the down-regulated genes at 5 min.

The other genes that were immediately repressed included two genes encoding nucleoid-associated factors: *fis*, a known stringent gene (68), and *stpA* (102). Fis positively regulates rRNA and certain tRNA-encoding operons (67, 80), and its down-regulation is coordinated with an effect on stable RNA synthesis (62). Expression of *rnpA*, encoding a subunit of RNase P which is required for processing of both 4.5S RNA and tRNAs, was also down-regulated, although the *rnpB* gene, which was previously shown to be stringently regulated (49), was not significantly affected in these experiments. Additionally, three genes involved in nucleotide biosynthesis (*pyrF*, *purU*, and *apt*) were repressed, as was the gene encoding the delta subunit of DNA polymerase III, *holA*.

In addition to four up-regulated amino acid biosynthetic operons, genes involved in transcriptional regulation were induced. Two sigma factors (*rpoE* and *rpoH*) and one anti-sigma factor (*rsd*) were among this group. The known σ^E regulon consists of 55 members (1, 23, 78), 4 of which (*rpoE*, *rpoH*, *ecfI*, and *smpA*) were immediately activated. *ecfI* and *smpA* two encode a conserved outer membrane protein and a lipoprotein, respectively. Nine additional predicted membrane proteins encoded by the large set of genes with unknown functions were also induced. No effect on the σ^H regulon was observed at this time point. The anti-σ^{70}-encoding gene, *rsd*, like several other early induced genes (e.g., *bolA* and *rmf*), is also up-regulated upon entry into stationary phase when (p)ppGpp levels rise dramatically (45).

Eleven genes encoding nonsigma transcription factors were

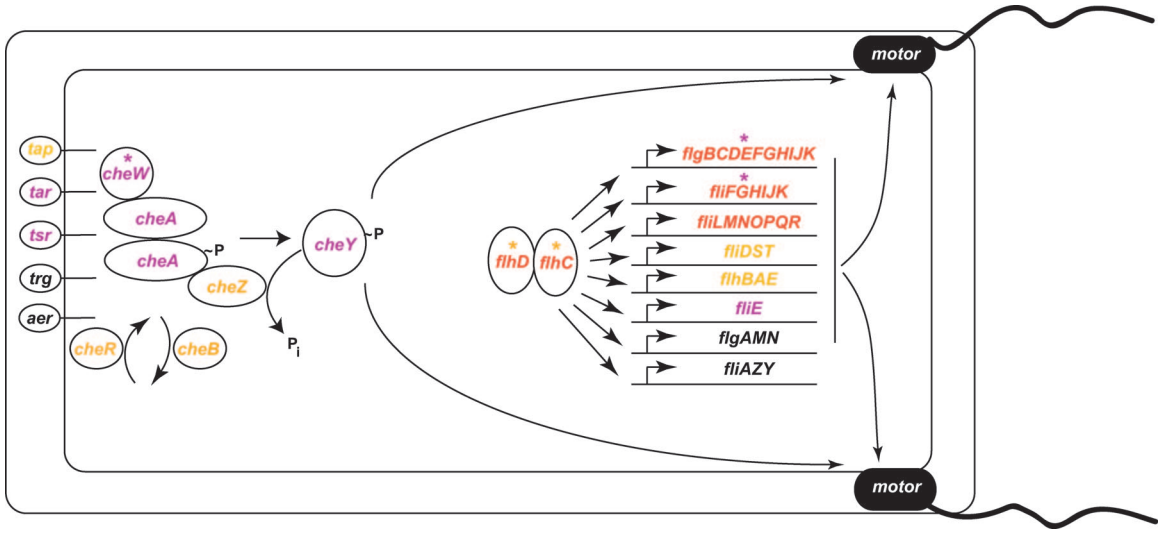

FIG. 5. Schematic diagram of down-regulated genes involved in flagellum synthesis and chemotaxis. For flagellum-related expression, the key master regulator, encoded by *flhDC*, activates expression of eight operons (indicated on the right). Chemotaxic signal transduction involves sensing by one of five chemoreceptors (shown across the inner membrane) which in complex with CheW and the CheA kinase activate the CheY response regulator via phosphorylation. CheY then influences flagellum activity through interaction with the motor. Dephosphorylation of CheY by CheZ resets the system. CheB and CheR control the methylation state of the chemoreceptors. Colored letters indicate when the gene is first down-regulated in MG1655, as follows: red, 5 min; gold, 10 min; purple, 30 min; and black, not affected. Colored asterisks indicate when the gene is first down-regulated in *relAΔ251*.

also up-regulated; seven of these genes (*bolA*, *crp*, *lexA*, *lrp*, *phoB*, *pspB*, and *zntR*) have known functions, and four (*yddM*, *yfeD*, *yjeB* and *ygjM*) have predicted regulatory functions (in this study, putative genes were categorized as unknown [Table 1]). *yddM* was the most highly induced gene (18.8-fold) at this time point.

Response of *relAΔ251* cells at 5 min. In contrast to the significant changes observed in MG1655, very few genes were differentially expressed in *relAΔ251* cells immediately following SHX treatment (Table 1; see Table S2 in the supplemental material). Only two genes showed significant down-regulation; one of these genes was *hisL*, which was up-regulated in MG1655 (Fig. 4). Five of the six up-regulated genes, including *yddM*, were are induced in MG1655, although the level of induction was substantially reduced (3-fold compared to 18.8-fold in the wild type). These results suggest that a very rudimentary response is initiated in the absence of RelA, possibly by SpoT-mediated (p)ppGpp production and/or by (p)ppGpp-independent mechanisms.

MG1655 response to SHX treatment at 10 min. The 10-min profile is characterized by down-regulation of flagellum/chemotaxis and metabolic gene expression and by up-regulation of alternative sigma factor regulon genes and other stress-related genes (see Table S3 in the supplemental material).

Two additional FlhDC-regulated flagellar operons (*fliDST* and *flhBAE*) (Fig. 5) were down-regulated together with four genes in the chemotaxis pathway (Fig. 5). The affected chemotaxis-related genes included the genes for the Tap chemoreceptor, the CheR and CheB methylation/demethylation enzymes for the chemoreceptors, and the CheZ phosphatase, which dephosphorylates and inactivates the CheY response regulator (3). These chemotaxic genes are not directly regulated by FlhDC but are controlled by the "flagellum sigma factor," σ^F, encoded by *fliA*. The *fliAZY* operon is regulated by FlhDC, although its transcript level did not change significantly across the time series (Fig. 5). However, a second FlhDC operon not affected by SHX treatment, *flgAMN*, encodes the FlgM anti-σ^F factor. How the balance between these factors and the stringent response controls flagellum and chemotaxis gene expression clearly warrants further investigation.

Some effect of the stringent response on metabolism-related gene expression was also seen as transcription of ATP synthase-encoding genes, as well as five transporter-encoding operons, decreased (Fig. 6). The transcript levels of transporters for alternative carbon compounds, as well as arginine and oligopeptides, were also reduced. The *lac* operon has been shown to be under direct positive regulation by (p)ppGpp (75), although the promoter was repressed in our experiments because of LacI activity.

Alternative sigma factor-dependent expression increased, with additional members of the σ^E, σ^H, and σ^S regulons showing significant increases in transcript abundance. The transcript levels for *rpoS*, encoding σ^S, also increased significantly. These findings are consistent with a dependence on increased (p)ppGpp levels for production of both σ^S and its targets (29, 57, 58). The number of additional up-regulated transcription factors increased as well. Among this group were 14 known factors (including all 7 factors induced at 5 min) and six factors with putative functions (including all four factors induced at 5 min). Again, *yddM* was the most highly up-regulated gene at 10 min (33-fold). The multitude of induced transcriptional regulator-encoding genes implies that these factors play an important role in directing the overall stringent response.

Consistent with the up-regulation of genes encoding stress response regulators, expression of other stress response subsystems was induced. First, the following four members of the

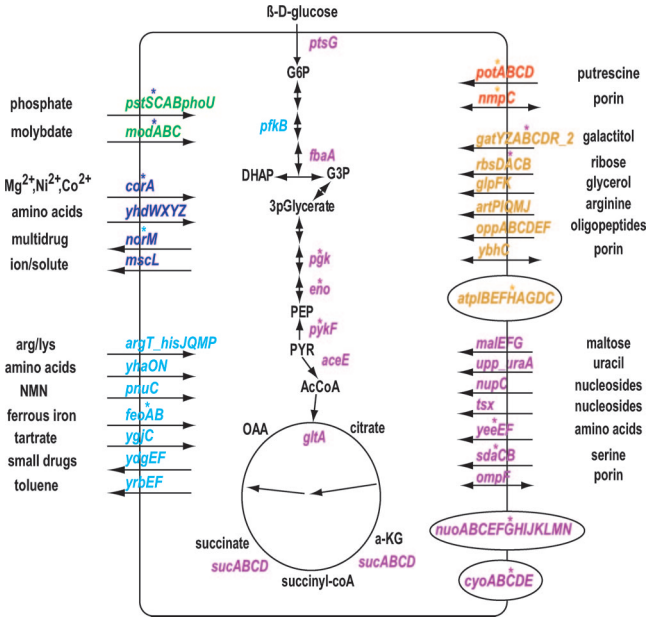

FIG. 6. Schematic diagram of up- and down-regulated genes involved in central metabolism, respiration, and transport. For clarity in central metabolism, only glycolysis/gluconeogenesis and the tricarboxylic acid cycle are shown. For up- and down-regulated transporters (shown on the left and right, respectively) the arrows indicate the direction of transport. Colored letters indicate when the gene is first up- or down-regulated in MG1655, as follows: green, up-regulated at 5 min; dark blue, up-regulated at 10 min; light blue, up-regulated at 30 min; red, down-regulated at 5 min; gold, down-regulated at 10 min; purple, down-regulated at 30 min. Colored asterisks indicate when the gene is first down-regulated in *relA*Δ*251*. Abbreviations: G6P, glucose-6-phosphate; DHAP, dihydroxyacetone-phosphate; G3P, D-glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl coenzyme A; α-KG, α-ketoglutarate; OAA, oxaloacetate; NMN, nicotinamide mononucleotide.

universal stress protein (Usp) family were induced: *uspA* (induced by 5 min), *uspB*, *uspD*, and *uspG*. UspA, UspD, and UspG are part of a structurally related group and appear to have nonredundant functions involved in the response to DNA damage (33). The corresponding genes are not σ^S dependent but instead require (p)ppGpp (33). In contrast, *uspB* is σ^S regulated and encodes a structurally unrelated protein involved in ethanol resistance (26).

Second, additional genes involved in DNA damage repair were up-regulated; these genes included six genes involved in the SOS response (*recA*, *ruvA*, *sbmC*, *sulA*, *umuD*, and *yebG*), as well as *lexA*. These genes are all negatively regulated by LexA, arguing that the repressor is inactivated during the stringent response. Further, three genes with functions related to repair of mismatched or inappropriately modified bases (*mrr*, *ung*, and *ygjF*) were up-regulated.

Third, several cold response genes were up-regulated. In particular, three members of the CspA family (*cspA*, *cspB*, and *cspG*), encoding RNA chaperones/transcription antiterminators, were induced. These genes are among the most highly induced genes during severe temperature downshifts, although their induction was relatively modest in our experiments (e.g., *cspB* was induced 12-fold, compared to >300-fold during a temperature downshift). The cold shock and stationary-phase-induced *yfiA* gene, encoding a ribosome-associated factor, was also induced. The up-regulation of these genes is intriguing given that decreased (p)ppGpp levels are necessary for the cold shock response (47). Finally, the *cspD* gene, which encodes a protein structurally related to CspA but which functions as an inhibitor of DNA replication (101), was also up-regulated. *cspD* is induced upon entry into stationary phase, but it is down-regulated during cold shock.

Response of *relA*Δ*251* cells at 10 min. While stable RNA expression was not dramatically altered in the *relA*Δ*251* strain, *flhDC* expression was down-regulated and led to a reduction in flagellar operon expression similar to that in MG1655 (see Table S4 in the supplemental material). Note that most of the genes were not included in the overall count of differentially expressed genes in *relA*Δ*251* because of signal levels below the threshold used. Of the 39 up-regulated genes, 26 were also induced in MG1655, including multiple genes having unknown or putative functions.

MG1655 response to SHX treatment at 30 min. The 30-min profile (see Table S5 in the supplemental material) revealed a continued reduction in the expression of flagellum/chemotaxis and metabolic pathways, while stress response systems showed continued induction and increased toxin-antitoxin (TA) gene expression was also observed. Both translation and phospholipid-related pathways showed mixed up- and down-regulated expression patterns suggestive of modifications in tRNAs and ribosomes and in membrane composition, respectively.

Expression of flagellum- and chemotaxis-related gene expression was further reduced (Fig. 5). A fifth FlhDC-regulated flagellar operon, *fliE*, was repressed, while both the FliA- and FlgM-encoding operons remained unaffected. The additional reduction of chemotaxis gene expression included genes for two chemoreceptors, Tar, which senses aspartate and maltose, and Tsr, which senses serine. Also repressed were two genes, *cheA* and *cheW*, encoding the histidine kinase and its associated factor. CheW and CheA form a chemoreceptor complex that transmits information to the response regulator CheY. *cheY* expression was also repressed. Each of the affected operons has a σ^F-regulated promoter, arguing that the activity of the alternative sigma factor may have been inhibited by FlgM. Some operons also have σ^{70}-regulated promoters which could be repressed by (p)ppGpp/DksA activity.

The down-regulation of metabolic gene expression (Fig. 6) included five steps in the glycolytic/gluconeogenic pathway, as well as two operons controlling conversions in the tricarboxylic acid cycle. Respiration-related gene expression was affected, and the NADH dehydrogenase I-encoding operon, *nuoABCEFGHIJKLMN*, was repressed as much as eightfold. The NADH dehydrogenase II-encoding gene, *ndh*, was expressed at much lower levels in the untreated wild-type control and was not affected by SHX treatment. The cytochrome *bo* oxidase-encoding operon, *cyoABCDE*, was also significantly repressed, as were the cytochrome *bd* oxidase genes, although they were expressed at lower levels. How these changes are coordinated with the down-regulation of two prime proton motive force consumers, flagella and ATP synthase, warrants further study. The transcript levels of seven more transporters, including the serine transporter encoded by *sdaCB* and the glucose-specific phosphotransferase system enzymes IIB and IIC encoded by *ptsG*, were also reduced (Fig. 6).

Expression of the σ^S regulon expanded with activation of 10 more members, including five genes that were also induced upon osmotic shock (*osmB*, *osmC*, *osmE*, *osmY*, and *mscL*). In contrast, multiple genes in both the σ^E and σ^H regulons were down-regulated. In the case of σ^H, this included two key chaperone-encoding operons, *dnaKJ* and *groSL*, whose products are also present in anti-σ^H factor complexes 32, 89). Finally, *rpoD*, encoding σ^{70}, was down-regulated at this time point. Together with continued expression of *rsd*, these findings support the switching from predominantly σ^{70}-dependent gene expression to a pattern that reflects the contribution of stress-induced sigma factors, in particular, σ^S.

The number of induced genes encoding known and putative regulators increased to 26, including 17 of the 18 genes induced at 10 min. In this set are response regulators from five two-component systems (TCS) (*evgA*, *narP*, *uvrY*, *yedW*, and *ypdB*). Expression of the cognate TCS sensor gene increased modestly when it was cotranscribed with the regulator. No change in expression was seen when the sensor was in a separate operon.

Stress response gene expression also increases, including induction of the final member of the UspA family, *uspE*, and two more members of the LexA regulon (*ssb* and *uvrY*). Continued up-regulation of the LexA regulon suggests that blockage of DNA replication and/or DNA damage persists, leading to SOS signaling.

Genes for four TA pairs, including *relBE* and *chpBI*/*chpBK*, were up-regulated. RelE and ChpBK both function by cleaving mRNA in stalled ribosome complexes, allowing tmRNA-dependent ribosome recycling (21, 74). The related *chpA* (also known as *mazEF*) locus was also modestly up-regulated. Antitoxin-encoding genes for two other TA loci, *dinJ-yafQ* and *yefM-yoeB*, were induced, although the *yafQ* toxin gene was not induced (*yoeB* was not represented on the array). Similar to RelB and the Chp toxins, YoeB has been shown to cleave translated mRNAs and to be activated by Lon (19). Finally, the newly identified *yncN-ydcQ* TA pair (61) was not differentially expressed. Together, the activation of these various TA loci is consistent with their proposed roles in rescuing stalled ribosome complexes and replenishing amino acid pools by degrading the truncated polypeptides (18).

For translation-related functions there were both up- and down-regulated genes. Five ribosomal protein-encoding operons, mostly single-gene operons, and elongation factor Ts (encoded by *tsf*) were repressed, although there were relatively modest declines in the transcript levels other ribosomal protein-encoding operons. Genes for three tRNA synthetases (*glnS*, *valS*, and *aspS*), as well as two tRNA-modifying enzymes (*dusB* and *queA*), were also down-regulated. In contrast, 15 genes related to translation and posttranslational modification were up-regulated. This included continuing synthesis of amino acid biosynthetic operon leaders, as well as the *pheM* tRNA synthetase leader peptide and the *miaA* gene which encodes a δ(2)-isopentenylpyrophosphate tRNA-adenosine transferase that modifies several tRNAs and enhances reading frame maintenance (96). In addition, ribosome-modifying genes were induced. The S22 ribosomal subunit-encoding gene, *sra* (*s*tationary-phase-induced *r*ibosome *a*ssociated), and *rmf* have been shown to be regulated by (p)ppGpp during stationary phase (41, 42).

For the phospholipid synthesis and associated pathways there was also a mixture of up- and down-regulated genes (Fig. 7). First, key genes in the fatty acid biosynthetic pathway responsible for the conversion of acetyl coenzyme A to acetoacetyl-acyl carrier protein were down-regulated. Acetoacetyl-acyl carrier protein and glycerol-3-phosphate are condensed to 1-acyl-*sn*-glycerol-3-phosphate in the first step of phospholipid synthesis by the *plsB*-encoded glycerol-3-phosphate acyltransferase. *plsB* was also down-regulated, and previous studies have suggested that this is dependent on (p)ppGpp (37). Further downstream, the pathway branches, with one path leading to L-1-phosphatidylethanolamine and the other path leading to L-1-phosphatidylglycerol (PG) and cardiolipin (CL). Gene expression in the L-1-phosphatidylethanolamine branch of the pathway was down-regulated, while gene expression in the PG/CL branch was up-regulated. This should favor the accumulation of CL, in agreement with results demonstrating that there was increased CL content upon entry into stationary phase when (p)ppGpp levels also rise (38). This was further reinforced at the transcript level by down-regulation of the membrane-derived oligosaccharide (MDO) pathway which normally uses PG to modify MDO using the phosphoglycerol transferase I enzyme encoded by *mdoB*. Additionally, the up-regulation of the *dgkA* gene, encoding diacylglycerol kinase, suggests that diacylglycerol generated as a by-product of the MdoB-mediated reaction would be recycled into the phospholipid pathway.

Response of *relAΔ251* cells at 30 min. Although the transcriptional response of *relAΔ251* cultures remained limited relative to that of MG1655 cultures, significant increases in differentially expressed genes were observed by 30 min (Table 1 and Fig. 2; see Table S6 in the supplemental material). Secondary effects of reduced *flhDC* expression were observed with down-regulation of two flagellum-related operons (Fig. 5). There was also overlap between the mutant and the wild type in reduced expression of metabolic, respiratory, and transport genes (Fig. 6), as well as membrane-associated gene expression, although the latter was more limited (Fig. 7). Four σ^H-regulated genes were also down-regulated (*dnaK*, *groL*, *groS*, and *hslV*), as they were in the wild type.

Similarly, 107 of the 125 up-regulated genes in the mutant were shared with MG1655. These genes included both *rpoS* and *rpoH* (*rpoE* was only modestly induced), as well as numerous other transcription factors, including *arcA*, *bolA*, *crp*, *lexA*, and *lrp*. Accumulation of transcripts of these factors did not generally translate into activation of regulon members, although, for example, *ruvA* (in addition to *lexA* itself) of the LexA regulon was up-regulated, suggesting that the SOS response was initiated. Also, *relBE* was activated in the mutant, as were the *dinJ* and *yefM* antitoxin-encoding genes. Finally, approximately one-half of the up-regulated genes had unknown or putative functions, similar to the proportion observed in MG1655.

Together, the *relAΔ251* profiles show significant overlap with the MG1655 profiles, but they are limited in scope and timing. Similar overlaps in transcription profiles between wild-type and *relA* mutant strains 30 min after other stresses have been reported (82, 95). The finding that the mutant response increased substantially by 30 min is consistent with the small rise in (p)ppGpp levels seen at that time in *relAΔ251* cells (65);

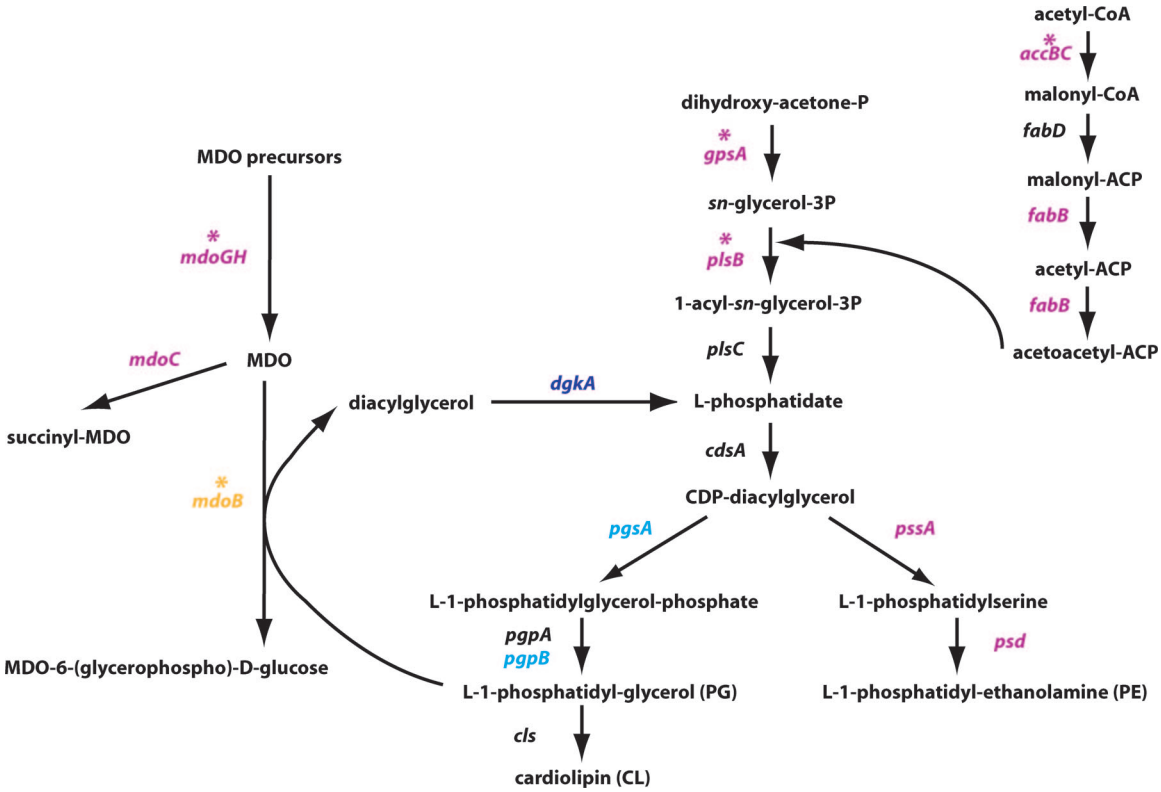

FIG. 7. Affected pathways involved in fatty acid, phospholipid, and MDO synthesis. Colored letters indicate when the gene is first up- or down-regulated in MG1655, as follows: dark blue, up-regulated at 10 min; light blue, up-regulated at 30 min; gold, down-regulated at 10 min; purple, down-regulated at 30 min; black letters, not affected. Colored asterisks indicate when the gene is first affected in the *relA*Δ*251* mutant. Alternative paths not affected at the transcript level are omitted for clarity. Abbreviations: CoA, coenzyme A; ACP, acyl carrier protein.

however, a (p)ppGpp-independent mechanism(s) may also be involved.

DISCUSSION

The stringent response is a complex global gene regulation system that has been intensively studied for more than four decades. The expression profiles presented here reflect the pleiotropic nature of the stringent response and provide a temporal framework for understanding how the response develops. Several different models have been proposed to account for the global changes in gene expression during the stringent response (17, 24, 43, 73, 81, 105). Although some of the expression changes observed in this study could be explained by other models, overall, our results appear to be most consistent with the RNAP redistribution model. This model was initially proposed in 1998 based on studies of several constitutive stringent RNAP mutants (104, 105) and has been supported by subsequent reports on different aspects of the response (4, 5). Recently, a very similar model was proposed to explain the (p)ppGpp effect on gene expression changes occurring during a diauxic shift (95).

The RNAP redistribution model is based not only on the premise that RNAP is limiting in the cell (see Introduction) (11) but also on the changing RNAP-promoter kinetics in response to nutrient starvation. As such, it has ramifications for how the genome, and promoter sequences in particular, coevolved with the (p)ppGpp/DksA regulator. The model implicitly assumes that the *E. coli* genome is composed of at least four different classes of promoters: (i) promoters inhibited by (p)ppGpp/DksA ("stringent promoters"; e.g., stable RNA promoters), (ii) promoters insensitive to both RNAP concentration and (p)ppGpp/DksA ("unaffected promoters"), (iii) promoters sensitive to RNAP concentration but not to (p)ppGpp/DksA ("passively induced promoters"), and (iv) promoters sensitive to RNAP concentration and directly induced by (p)ppGpp/DksA ("directly induced promoters"). The sequence features that define each class are largely unknown, with the exception of the stringent promoters, which contain a GC-rich region between the −10 hexamer and the start of transcription known as the discriminator (93). However, dissection of the *rrnB* P1 promoter and other stringent promoters has demonstrated that the set of sequence features required for stringent regulation is significantly more complex than the mere discriminator G+C content (35, 70).

The discovery that *flhDC* is rapidly down-regulated by the stringent response and the existence of a GC-rich discriminator in its promoter suggest that the operon is under direct negative control by (p)ppGpp/DksA. Indeed, the Gourse lab has shown that ppGpp/DksA directly inhibits the *flhDC* promoter in vivo and in vitro (J. Lemke, T. Durfee, and R. Gourse, unpublished results). This finding makes physiological sense in that flagellum production and motility impose a high energy burden on the cell (60) and limiting their expression in star-

vation conditions would be advantageous for cell survival. The promoters of the other down-regulated genes identified in this study represent candidates for direct stringent control and should aid in dissecting the subtleties that make these sequences especially sensitive to (p)ppGpp/DksA regulation.

Limited expression from these strong stringent promoters during nutrient downshifts or starvation is accompanied by an increase in the total number of genes being expressed compared to the number in nutrient-rich environments, as shown in this study and elsewhere (15, 59, 95, 98). Up-regulated gene expression can result from several mechanisms that act either alone or in combination. The most straightforward of these mechanisms are exemplified by promoters that depend only on RNAP-promoter interactions. Several promoters have been shown be sensitive to RNAP concentrations in vitro and can be envisioned to represent "passively" activated promoters (36). Alternatively, (p)ppGpp/DksA can directly stimulate initiation from some promoters in vitro, including two amino acid biosynthetic operons up-regulated in these experiments, *hisLGDCBHAFI* and *thrLABC* (72, 87).

Gene expression patterns are also influenced by (p)ppGpp-dependent effects on sigma factor availability and activity. Active σ^{70} levels are limited by first rapidly up-regulating *rsd*, encoding the anti-σ^{70} factor, and later down-regulating the stringently controlled promoters for *rpoD*, which encodes σ^{70} (31). Conversely, genes for three key alternative sigma factors, σ^{E}, σ^{H}, and σ^{S}, are activated. σ^{E} activation by (p)ppGpp has recently been demonstrated independent of cell envelope stress (22), although the extensive changes in membrane modification and composition that occur during the stringent response suggest that envelope stress may also occur. Activation of σ^{S} and its regulon has been shown to require (p)ppGpp (29, 57). Recently, (p)ppGpp has been shown to regulate σ^{S} degradation (8). At present, it is not known how increased (p)ppGpp levels lead to increased alternative sigma factor association with RNAP core complexes (46). One possibility is that simply increasing "free" RNAP availability by reducing stringent gene expression together with an increased level of alternative sigma factor is sufficient. A similar model was proposed in several recent reports (6, 88). Despite the apparent activation of the alternative sigma factors, only a small subset of each regulon is up-regulated, consistent with other studies in which additional stresses were not imposed (15, 59, 95).

Many promoters are also regulated by transcriptional repressors and activators, a large number of which were induced at the transcription level in our experiments. Activators and repressors commonly respond to specific intracellular cues to precisely coordinate transcription changes with the current physiological state of the cell. The factors induced by the stringent response provide additional direction for RNAP redistribution other than that solely determined by RNAP-promoter (or RNAP-DNA) interactions. Transcriptional changes during the stringent response represent a composite of each of these inputs (RNAP-promoter, sigma factor-core associations, and activators/repressors) and the effects that (p)ppGpp/DksA have on each of them.

Moreover, the structure of the nucleoid is affected during the stringent response (12), indicating that the distribution of RNAP, global gene expression, and the dynamic structure of the nucleoid are coupled (44). An altered nucleoid structure may also have secondarily contributed to the profiles described here because transcription and nucleoid structure are likely to be interrelated (94).

In addition to controlling promoter selection and initiation, (p)ppGpp can increase the pause time of transcription elongation complexes at some positions (52, 54). This not only slows transcription of targeted operons but could also provide the rationale for induction of the SOS response during the stringent response. Stalled transcription complexes have been shown to block proper fork migration during DNA replication, resulting in increased regions of single-stranded DNA (63, 92). SOS induction occurs when single-stranded DNA is recognized by RecA, resulting in cleavage of the LexA transcriptional repressor and activation of the LexA regulon, which consists largely of DNA repair enzymes (51). It is conceivable that (p)ppGpp-induced stalling of RNAP either at pause sites or at promoters could lead to the same regulatory cascade. The finding that induction of LexA regulon members is severely delayed in *relA*Δ*251* cells supports such a hypothesis.

(p)ppGpp has also been shown to increase polyphosphate levels through inhibition of the exopolyphosphatase, Ppx (55). Polyphosphate, in turn, activates the Lon protease, which degrades both antitoxin proteins, such as RelB (20), and free ribosomal proteins (56). This leads to rescue of stalled ribosomes by the RelBE system, both recycling the ribosomes and replenishing amino acid pools (56). Activation of other TA pairs in addition to *relBE* further indicates the importance of this strategy in overcoming amino acid starvation during the stringent response.

Ideally, direct comparison of the profiles described here and those reported previously (10, 25, 77, 82, 85) would provide a comprehensive view of the stringent response across organisms and nutritional challenges. However, this is not possible because of the many differences in experimental setup (microorganisms, media, sampling time points, etc.). Moreover, there is evidence that the mechanism of (p)ppGpp action may be different in some organisms (53). Nevertheless, some common themes have emerged. In each case, a large effect on global gene expression was observed, with the negatively controlled genes typically involved in cell growth (stable RNAs and translational machinery) and DNA replication and the positively controlled genes consistently including amino acid biosynthetic operon, transcriptional factor, and/or alternative sigma factor genes. Finally, a large number of unknown genes are induced, whose expression is generally negligible during rapid growth in rich media.

The (p)ppGpp-dependent regulatory system is used to solve a range of nutrient and environmental challenges commonly faced by bacteria such as *E. coli* (14). Targeting the sole RNAP, which is required for transcription at all promoters, provides a facile means for rapidly adapting global transcription to a sudden stress such as amino acid starvation. While other transcription factors clearly have a role in the overall response by providing direction and amplification, the majority of the response may be based simply on differential interactions between RNAP and promoters across the genome.

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