Regulation of global transcription in *E. coli* by Rsd and 6S RNA

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Abstract

In *Escherichia coli*, the housekeeping sigma factor σ^{70} directs RNA polymerase to transcribe growth-related genes, whereas the alternative sigma factor σ^{38} directs it to transcribe stress response genes during stationary phase. Two molecules hypothesized to regulate RNA polymerase activity are Rsd, which sequesters free σ^{70}, and 6S RNA, which sequesters the RNA polymerase-σ^{70} holoenzyme. Despite multiple studies, their function remains controversial. Here we use genome-wide expression studies in five phases of growth, along with theoretical modeling, to investigate the functions of Rsd and 6S RNA. We show that 6S RNA and Rsd act as global regulators of gene expression throughout bacterial growth, and that both increase transcription of σ^{38} dependent genes. We also find several instances of crosstalk between 6S RNA and Rsd, and propose a model in which this crosstalk is important in regulating sigma factor competition.

Importance

Bacteria possess a single RNA polymerase which transcribes all genes. This RNA polymerase is a crucial point for regulation, as changes in its level, distribution or activity could have rapid and widespread effects on gene expression. In the model bacterium *E. coli*, Rsd and 6S RNA are two abundant molecules that regulate the activity of RNA polymerase. Here we identify genes

regulated by Rsd and 6S RNA in five phases of bacterial growth and suggest a model to explain their regulatory activity.

Introduction

In bacteria, all transcription is dependent on a single core RNA polymerase. This multisubunit enzyme ($\alpha_2\beta\beta'\omega$, referred to as E) cannot bind specifically to promoters. This ability is conferred by a sigma (σ) factor, which binds to E forming an Eσ holoenzyme, and directs it to transcribe RNA from specific promoters.

Seven sigma factors – σ^{70} (RpoD), σ^{38} (RpoS), σ^{32}, σ^{54}, σ^{28}, σ^{24} and σ^{19} - have been identified in the model bacterium *Escherichia coli* (1). *In vivo* studies have shown that when one sigma factor is overproduced, underproduced, or mutated, not only is there a change in transcription of its target genes, but increased activity of one sigma factor decreases the activity of others and vice-versa (2–4). *In vitro* assays have demonstrated competition between sigma factors when E is limiting (3–6). Though it was previously reported that the cellular concentration of E exceeded that of sigma factors (6, 7), recent quantitation has shown that sigma factors exceed E under common culture conditions (8, 9), and therefore compete to bind to limited E.

σ^{70} (RpoD) is the housekeeping sigma factor, directing transcription of genes essential for growth and proliferation. The alternative sigma factors are produced under specific conditions, and

direct transcription of genes required under those conditions. The major alternative sigma factor is σ^{38} (RpoS). This is present at low concentration during exponential growth, but nevertheless regulates several hundred genes (10, 11). Upon entry into stationary phase, while the concentrations of E and σ^{70} show little change, σ^{38} accumulates (9, 12, 13; Table 1) and directs transcription of genes responsible for multiple stress tolerance (14–18). However, σ^{70} remains the most abundant sigma factor even in stationary phase, and has higher affinity for E than any other sigma factor (6, 9, 19, 20), implying that additional regulators are needed for σ^{38} and other sigma factors to compete effectively with σ^{70}.

Such regulators are known. The Crl protein binds to σ^{38} and increases its affinity for E, and promotes transcription by the $E\sigma^{38}$ holoenzyme at some promoters (21–23). The nucleotide ppGpp increases the ability of alternative sigma factors to compete with σ^{70} (3). However, ppGpp is produced transiently on entry into stationary phase (24), and Crl has also been shown to decrease during extended stationary phase (21).

On the other hand, two regulators - the protein Rsd and the non-coding 6S RNA - act on σ^{70}. Rsd binds to σ^{70}, sequestering it from E, and inhibits $E\sigma^{70}$-dependent transcription at several promoters *in vitro* (25). An Rsd null strain showed increased transcription from a σ^{70} dependent promoter, and reduced transcription from a σ^{38} dependent promoter, in stationary phase, whereas Rsd overexpression had the opposite effect (26). It was hypothesized that in stationary phase, Rsd reduces $E\sigma^{70}$ formation, and, by freeing E to bind σ^{38}, increases $E\sigma^{38}$ formation. However, a

84 microarray experiment found no significant difference in gene expression between an Rsd
85 knockout and wild-type *E. coli*, and even comparison of the knockout with an Rsd
86 overexpressing strain found changed expression of only a few σ^{38}-dependent genes (27). All
87 effects of Rsd *in vivo* were observed only during stationary phase. This was initially attributed to
88 low Rsd levels during exponential phase. However, recent quantitation has shown that Rsd is
89 present at ~50% of the level of σ^{70} in exponential phase and ~90% in stationary phase (9) –
90 raising the question of why no change in expression is seen in its knockout.
91
92 6S RNA is a 184-nucleotide non-coding RNA expressed from the *ssrS* gene. 6S RNA binds to
93 the Eσ^{70} holoenzyme (28). It has been shown to block Eσ^{70} binding to a target promoter (29), and
94 inhibit transcription from several promoters *in vitro* and *in vivo* (24, 28, 30–32). A 6S RNA
95 knockout showed increased expression from some Eσ^{70} promoters containing extended -10
96 elements, and reduced expression from a few Eσ^{38} promoters, in stationary phase (32). It was
97 suggested that 6S RNA blocks Eσ^{70} from binding to certain target promoters, reducing their
98 transcription, and that sequestration of Eσ^{70} by 6S RNA allows σ^{38} to compete more effectively
99 for E, increasing transcription by Eσ^{38}. An alternative hypothesis was that 6S RNA regulates a
100 trans-acting factor important for Eσ^{38} activity (32). A later study showed that 6S RNA regulates
101 hundreds of genes in stationary phase, and, by mutating promoter sequences, showed that an
102 extended −10 element and a weak -35 element could make a promoter sensitive to 6S RNA (30).
103 But contrary to these, another expression study found no correlation of 6S RNA sensitivity with

104 promoter sequence or sigma factor preference (33), and there was little overlap between the
105 regulated genes found in the two experiments.
106
107 Rsd and 6S RNA are present at high concentrations during growth, increase in stationary phase,
108 and are thereafter at high levels (9, 25, 28). Yet their effects on gene expression, especially
109 during growth, remain controversial. Both have been hypothesized to reduce $E\sigma^{70}$ and increase
110 formation of $E\sigma^{38}$; however, they act at different levels, one sequestering free σ^{70} and the other
111 sequestering the $E\sigma^{70}$ holoenzyme. We must therefore ask what impact this difference has on their
112 regulatory effects. Do Rsd and 6S RNA possess similar regulatory functions?
113
114 Here, we present a genome-wide investigation of the functions of Rsd and 6S RNA in *E. coli*. We
115 used RNA-seq to identify genes regulated by Rsd and 6S RNA in five phases of growth, and
116 demonstrated that both function as global regulators of transcription during exponential as well
117 as stationary phase. We showed that both increase transcription of σ^{38} target genes, with 6S RNA
118 also regulating hundreds of σ^{70} targets, including genes encoding subunits of RNA polymerase,
119 ribosomes and other global regulators, and substantiated these findings by qRT-PCR and western
120 blotting. We found evidence of substantial crosstalk between Rsd and 6S RNA, with each
121 regulating the other's expression and non-additive effects on over a thousand genes. Finally, we
122 developed a mathematical model of sigma factor competition in *E. coli*, which suggested a
123 theoretical basis for our experimental results.
124

Results

RNA-seq to identify genes regulated by Rsd and 6S RNA

Figure 1A shows a schematic of the binding activity of 6S RNA and Rsd. Rsd sequesters σ^{70} and prevents it from binding to core RNA polymerase (E), while 6S RNA binds to the Eσ^{70} holoenzyme and prevents it from binding to promoters. To find their effects on gene expression, we carried out RNA-seq to identify genes regulated by Rsd and / or 6S RNA in five growth phases.

Table S1 lists strains and plasmids used in this study. Five strains: *E. coli* K-12 MG1655 (Wild-type), Rsd knockout (Δ*rsd*), 6S RNA knockout (Δ*ssrS*), 6S RNA-Rsd double knockout (Δ*rsd*Δ*ssrS*), and σ^{38}/RpoS knockout (Δ*rpoS*) were used for RNA-seq. These strains had similar growth rates in M9 glucose (Figure S1). RNA-seq was performed at five growth phases: early exponential (EE), mid-exponential (ME), transition to stationary (TS), stationary (S), and late stationary (LS; time points in Methods).

Rsd increases σ^{38}/RpoS activity throughout growth

We defined differentially expressed genes as genes whose expression changed >=2-fold in a mutant strain relative to the wild-type, with an FDR-adjusted p-value < 0.05. Using these criteria,

146 the Δ*rsd* strain showed only 16 differentially expressed genes. These included several non-
147 coding RNAs (*ryfD*, *sokA*, *oxyS*, *sroH*, *sibD*) which were increased 2-6 fold in Δ*rsd* during
148 stationary phase. The expression of 6S RNA was also altered in Δ*rsd*; 6S RNA was increased to
149 more than twice the wild-type level in stationary phase, but in mid-exponential phase was
150 reduced to about half its wild-type level (Table S2).
151
152 As very few genes were differentially expressed in Δ*rsd*, we looked for smaller changes. We
153 found that in all growth phases, there was a trend for genes whose expression was significantly
154 reduced (>=2-fold, $p < 0.05$) in Δ*rpoS* to also display slightly reduced expression in Δ*rsd*. This is
155 illustrated in Figure 1B-C. These boxplots show the distribution of $\log_2$ fold change in gene
156 expression in Δ*rsd* relative to wild-type, for all genes. The central line represents the median, box
157 edges represent the inter-quartile range, and the whiskers represent 1.5 x IQR. For genes whose
158 expression is significantly reduced in Δ*rpoS*, the distribution is shifted downward, indicating a
159 tendency toward reduced expression ($\log_2$ fold change < 0). The upper edge of the IQR is below
160 0, indicating that ~75% of the genes whose expression was significantly reduced in Δ*rpoS* also
161 showed reduced expression in Δ*rsd*. Plots for other growth phases are in Figure S2A.
162
163 This reduced expression was less than twofold in magnitude and so was not seen when searching
164 for differentially expressed genes directly in Δ*rsd*. Though these fold changes are small, we
165 consider them important for several reasons. They represent a consistent and highly significant
166 (Wilcoxon test $p < 10^{-15}$ in all growth phases except LS) decrease in expression across hundreds

of *rpoS* affected genes, in five growth phases. The average overlap between the set of *rpoS*-regulated genes in successive growth phases is only 54%; so it is not a single set of genes whose expression was reduced in Δ*rsd*, but a substantially different set in each growth phase. The Δ*rsd* samples also showed high inter-replicate correlation in all growth phases (Table S3). This trend of decreased expression held true when only previously reported σ^{38} targets (18) were considered (Figure S2B). Conversely, genes whose expression was increased >=2-fold in Δ*rpoS* showed increased expression in Δ*rsd*, during the early exponential, mid-exponential and stationary phases (Figure S2C). However, expression of genes under the control of constitutive σ^{70} target promoters (34) was not substantially altered in any phase (Figure S3).

Thus the Rsd knockout behaved like a σ^{38} knockout, only with smaller changes in gene expression. As western blots in stationary phase did not show changed σ^{38} protein level in Δ*rsd* (Figure S4), this is likely due to reduced binding of σ^{38} to E. Therefore, although Rsd binds to σ^{70}, we hypothesize that its function is to increase σ^{38} binding to E, and therefore transcription of σ^{38} targets, in exponential as well as stationary phase.

6S RNA regulates distinct sets of genes in all phases of growth

What role does the $E\sigma^{70}$ - sequestering 6S RNA play in gene regulation? Is its function similar to that of the σ^{70}-sequestering Rsd? Our RNA-seq showed that the 6S RNA knockout (Δ*ssrS*) was

very different from the Rsd knockout. To begin with, it showed >=2-fold differential expression of a total of 447 genes.

Figure 2A and B show that there was little overlap between genes differentially expressed in Δ*ssrS* in successive growth phases. In particular, 186 of 221 genes regulated by 6S RNA during late stationary phase were regulated only in that phase. The only genes upregulated >=2-fold in Δ*ssrS* throughout growth were *cusR*, encoding a copper-sensing regulator, and its target genes (*cusCFB)* encoding a copper/silver efflux system. The only gene downregulated >=2-fold in Δ*ssrS* throughout growth was *fau* (*ygfA*), which is downstream of 6S RNA in the same operon and encodes a putative 5-formyltetrahydrofolate cyclo-ligase. *fau* expression was increased when 6S RNA was overexpressed from a plasmid in a wild-type background (Figure 2C and D), indicating that this was at least in part a regulatory effect of 6S RNA and not merely a polar effect. The mechanism is unclear; however, it could indicate an autoregulatory role of 6S RNA.

During exponential phase, the expression of several genes encoding amino acid transporters (*artM*, *artI*, *hisP*, *hisQ*, *hisJ*, *hisM*, *tdcC*) and genes for amino acid biosynthesis (*argH*, *argB*, *thrA*, *thrB*, *thrC*, *asnB*, *glyA*) was increased in Δ*ssrS*, while expression of genes involved in stress responses (*rmf*, *appY*, *yadC*, *ybcM*, *yciF*, *gadW*, *ydeI*, *yodD*, *dps*, *hdeA*, *hslV*, *oppA*, *osmE*, *dosC*) was reduced. These functional changes are consistent with previous reports (24, 33), though most of the specific genes are different. In LS phase, genes encoding global transcriptional regulators including *crp*, *crl* and *hha* and genes linked to the TCA cycle (*sdhD*,

208 *sdhC*, *gltA*, *aceB*, *ppc*, *sucA*) were upregulated, while downregulated genes included *iraP*, *csrB*,
209 and 9 subunits of NADH dehydrogenase.
210
211 While the effects of the Δ*ssrS* deletion appeared largely growth phase-dependent, we observed
212 certain patterns throughout growth. These are discussed in the following sections.
213
214 **6S RNA increases σ^{38}/RpoS activity throughout growth**
215
216 Like *Δrsd*, the *ΔssrS* strain showed reduced expression of σ^{38} target genes (Figure 3A and B,
217 Figure S5, Wilcoxon test $p < 10^{-15}$ in all growth phases except late stationary). This was not due
218 to reduced σ^{38} protein, as western blots in stationary phase showed that the *ΔssrS* strain had
219 higher σ^{38} protein than the wild-type (Figure S4). On the other hand, expression of genes under
220 constitutive σ^{70} promoters was slightly increased in the mid-exponential, transition to stationary
221 and late stationary phases (Figure S6).
222
223 **6S RNA regulates the expression of RNA polymerase, ribosomal genes, and Rsd**
224
225 Figure 4A shows that the wild-type expression of 6S RNA increased in successive growth
226 phases, as reported (28). Therefore, the effect of 6S RNA on its target genes should be greater in
227 each successive growth phase. Indeed, with the exception of the transition to stationary phase,
228 the number of 6S RNA regulated genes increased with growth phase (Figure 2A and B). A

previous study (24) showed increased ppGpp in a 6S RNA knockout during the transition to stationary phase; consistent with this, we observed slightly increased expression of ppGpp-activated genes and reduced expression of ppGpp-repressed genes in the *ΔssrS* strain during this phase (Figure S7). Since ppGpp also favors the competition of alternative sigma factors with σ^{70} (3), increased ppGpp may reduce the effects of the *ΔssrS* deletion during this phase.

Are there genes where 6S RNA has a dose-dependent effect throughout growth, increasing with its expression level? Of 447 6S RNA regulated genes, 36 show such an effect, with the magnitude of their repression or activation by 6S RNA increasing in each successive growth phase (Figure S8). For example, Figure 4B shows the $\log_2$ fold change in expression of *rpoB* (encoding the β subunit of core RNA polymerase) in Δ*ssrS*. *rpoB* expression was slightly reduced in mid-exponential phase, and the magnitude of this reduction increased with time, as the level of 6S RNA in the wild-type increased. Reduced RpoB expression was validated in stationary phase by qRT-PCR (Table S7) and western blotting (Figure S9). Since excessive $E\sigma^{70}$ inhibits *rpoB* transcription (35), we suggest that deleting 6S RNA leads to higher free $E\sigma^{70}$, which proportionally represses *rpoB* transcription. Since RpoB is the limiting subunit for the formation of core RNA polymerase (9), this implies that the cell compensates for the loss of 6S RNA by reducing RNA polymerase synthesis.

Similarly, we observed changed expression of genes encoding ribosomal proteins (Figure 4C). Decreased expression of these genes in a 6S RNA knockout during stationary phase was reported

250 previously (33); here, their expression was increased in Δ*ssrS* during early and mid-exponential
251 phases, then decreased steadily. 6S RNA also represses some genes similarly. For example,
252 Figure 4D shows that *crl* expression is slightly increased in Δ*ssrS* during early exponential
253 phase, and the magnitude of this increase keeps growing, paralleling the increase in 6S RNA in
254 the wild-type. As with the effect of reducing RNA polymerase synthesis to compensate for the
255 loss of 6S RNA, increasing σ^{38} protein and Crl may be a means to compensate for reduced $E\sigma^{38}$
256 activity in Δ*ssrS* bacteria.
257
258 Since we observed that Rsd regulated 6S RNA expression, we checked whether 6S RNA in turn
259 regulated Rsd expression. Our RNA-Seq showed that *rsd* was not differentially expressed in
260 Δ*ssrS*. To check if 6S RNA regulated Rsd post-transcriptionally, we added a 3xFLAG tag to the
261 C-terminal of the Rsd protein. Indeed, western blots showed that the expression of 3xFLAG-
262 tagged Rsd was reduced in the Δ*ssrS* background relative to wild-type, in both mid-exponential
263 and stationary phases (Figure 4E).
264
265 Finally, though 6S RNA binds to $E\sigma^{70}$, its effects are highly promoter-specific (24, 30, 32, 33).
266 Therefore we asked what features of a gene might be responsible for its response to 6S RNA.
267 However, we did not observe any link between 6S RNA sensitivity and an extended -10 or weak
268 -35 promoter sequence as reported in (30) (Figure S10). Instead, during stationary phase, the 6S
269 RNA sensitivity of a gene was correlated with its expression level. Genes downregulated in
270 Δ*ssrS* tended to be highly expressed in the wild-type, and genes upregulated in Δ*ssrS* had low

271 expression in the wild-type (Figure 4F). It was also correlated with the occupancy of the gene
272 promoter by RNA polymerase, measured by ChIP-chip in (36) (Figure 4G). Thus, our data
273 support a model in which sequestration of RNA polymerase by 6S RNA primarily represses
274 promoters that are weak in binding to RNA polymerase.
275
276 **The Rsd/6S RNA double knockout shows differential expression of a distinct set of**
277 **genes**
278
279 We have discovered several instances of crosstalk between 6S RNA and Rsd; apart from the fact
280 that both sequester σ^{70} in different forms, each regulates the other's expression, and both favor the
281 activity of σ^{38}. We therefore asked whether the double knockout of Rsd and 6S RNA showed
282 effects on gene expression distinct from the single knockouts.
283
284 The Δ*rsd*Δ*ssrS* strain showed several of the features described for Δ*ssrS* (Figure S11 and S12).
285 In addition, in each growth phase, hundreds of genes were differentially expressed in the double
286 knockout relative to the wild-type. This far exceeded the number of differentially expressed
287 genes in both single knockouts, suggesting significant crosstalk between the two regulators.
288
289 Figure 5A and B show genes that showed differential expression in the double knockout, but less
290 than twofold change in both single knockouts added together; there were 1780 such genes in
291 total. These included genes encoding DNA Gyrase and Topoisomerase I, which maintain DNA

292 supercoiling and regulate expression of hundreds of genes (37), the nucleoid-associated proteins
293 HU, H-NS and StpA, the global transcriptional regulators ArcA, LRP and IHF, the small RNA
294 chaperone Hfq, and the F_0-F_1 ATP synthase.
295
296 **A theoretical model suggests possible explanations for the behavior of Rsd and 6S RNA**
297
298 Our RNA-Seq demonstrated that Rsd and 6S RNA are regulators of sigma factor competition and
299 gene expression at a global scale; however, several results appear counter-intuitive. How does
300 Rsd, which sequesters σ^{70}, increase transcription of σ^{38} target genes without as much effect on σ^{70}
301 target genes? How does 6S RNA, which sequesters not only σ^{70}, but also the core RNA
302 polymerase which is required for transcription by all sigma factors, nevertheless increase
303 transcription by σ^{38}?
304
305 To suggest possible answers, we constructed a mathematical model of transcription during
306 stationary phase, using parameters from literature (Table 2.2). We focused on stationary phase as
307 that is when σ^{38}, 6S RNA and Rsd are at high concentrations. Our model is similar in structure to
308 previous studies (8, 38), that have attempted to model sigma factor competition. However, this is
309 the first model of stationary phase conditions and the first to include both 6S RNA and Rsd.
310 Consequently, our results differ from previous models.
311

A schematic of reactions in the model is given in Figure 6A. Core RNA polymerase (E) binds to sigma factors (σ^{70} and σ^{38}) forming holoenzymes ($E\sigma^{70}$ and $E\sigma^{38}$). Holoenzymes recognize target promoters (P_{70} and P_{38} respectively) and initiate transcription, releasing the sigma factor. The elongating RNA polymerase (E_{e70} and E_{e38}) transcribes until released. Holoenzymes and E can also bind to DNA non-specifically. We focus on the steady state of this model, determined byequations (1) - (4) and (8) – (17) in Methods.

To understand how Rsd and 6S RNA regulate competition between sigma factors for RNA polymerase, we initially modeled the formation of $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes in the absence of DNA. This is represented by the shaded area in Figure 6A. The corresponding steady-state equations are (1) - (9) in Methods.

Figure 6B(i) depicts what happens when Rsd is added to a system containing only E, σ^{70} and σ^{38}. We emphasize that this represents the steady-state behavior of the model. That is, each value of Rsd on the x-axis corresponds to a separate 'run' of the model where we compute the steady-state for those fixed parameter values, before moving on to the next Rsd value.

Initially, our model predicts that ~94% of total E would be bound to σ^{70}. As Rsd is increased, it sequesters σ^{70}, reducing $E\sigma^{70}$ formation. This allows more E to bind to σ^{38} and thus increases the formation of $E\sigma^{38}$ by an equal amount, consistent with previous predictions of the function of Rsd (26). However, Figure 6B(ii) predicts that when 6S RNA is present in the system, Rsd

increases $E\sigma^{38}$ with relatively little effect on $E\sigma^{70}$. How? This paradoxical result can be understood with Figure 6B(iii), which shows that the concentration of the 6S RNA–$E\sigma^{70}$ complex decreases as Rsd is increased. When 6S RNA is present, increasing Rsd still reduces E-σ^{70} association; however, the reduction in $E\sigma^{70}$ is partially compensated for by the release of $E\sigma^{70}$ from its complex with 6S RNA, and so there is little change in the overall $E\sigma^{70}$ level.

Next, we included DNA in the model (represented by the complete schematic in Figure 6A) and observed how Rsd's effects on holoenzyme formation lead to changes in transcription. Here, we modeled 200 promoters specific to each sigma factor. As expected from Figure 6B(ii), the model predicts that Rsd increases the rate of transcription by $E\sigma^{38}$ with less effect on $E\sigma^{70}$ transcription (Figure 6B(iv)).

Apart from losing Rsd, our Δ*rsd* strain also displayed a ~2.3-fold increase in 6S RNA during stationary phase. Figure 6B(v) shows the predicted rate of transcription from $E\sigma^{70}$ and $E\sigma^{38}$ target promoters in the wild-type and when Rsd = 0. The third pair of bars is an approximation of conditions in the Δ*rsd* strain, where 6S RNA is increased 2.3-fold. We see that increased expression of 6S RNA could reduce $E\sigma^{70}$ dependent transcription in the Δ*rsd* strain to almost the wild-type level, such that the main observable effect of knocking out Rsd would be reduced transcription by $E\sigma^{38}$.

We therefore hypothesize that in the presence of 6S RNA, Rsd increases the formation of $E\sigma^{38}$ and therefore transcription of $E\sigma^{38}$ target genes, with less effect on $E\sigma^{70}$, consistent with both our RNA-seq and a previous study (27). We note that increasing Rsd is predicted to be a considerably more effective way to increase $E\sigma^{38}$ transcription than increasing σ^{38} itself (Figure S13).

What is the effect of 6S RNA? Again, we initially modeled the effect of 6S RNA on sigma factor competition without DNA. Figure 6C(i) predicts that 6S RNA sequesters $E\sigma^{70}$, reducing the available E for binding to both sigma factors, thus inhibiting formation of both holoenzymes. By sequestering $E\sigma^{70}$, 6S RNA also reduces the ratio of σ^{70} to σ^{38}, and so the decline is sharper for $E\sigma^{70}$. Similarly, Figure 6C(ii) shows the predicted effect of 6S RNA on transcription; 6S RNA decreases the rate of transcription by both holoenzymes, though the decline is greater for $E\sigma^{70}$.

However, our RNA-seq showed that deleting 6S RNA actually results in reduced transcription of $E\sigma^{38}$ target genes, i.e. 6S RNA causes increased transcription by $E\sigma^{38}$. How is this possible? A 10-fold increase or decrease in any of the parameters was not sufficient to reproduce this observation.

Apart from losing 6S RNA, our Δ*ssrS* strain also displayed changed levels of Rsd, RpoB, and σ^{38}. Figure 6C(iii) shows the predicted rate of transcription from $E\sigma^{70}$ and $E\sigma^{38}$ target promoters in the wild-type and when 6S RNA = 0. The third pair of bars is an approximation of conditions

374 in the Δ*ssrS* strain (based on western blots in Figure 4, Figure S4 and Figure S9). Here, Rsd and
375 E are reduced to 50% of their wild-type levels and σ^{38} is increased by 50%. We see that $E\sigma^{38}$
376 transcription is now reduced to less than the wild-type level. In fact, within the default
377 parameters of our model, reducing Rsd alone from 10.4 μM to 8 μM is sufficient to lower $E\sigma^{38}$
378 transcription in the 6S RNA knockout below its wild-type level.
379
380 We also observed increased *crl* mRNA in Δ*ssrS*. We have not modeled this due to lack of
381 quantitative data on Crl. However, if the change in mRNA corresponds to increased Crl protein,
382 it could partially mitigate the effect of reduced Rsd in the Δ*ssrS* strain.
383
384 Thus, from our base model we can make the strong claim that the binding reactions of Rsd and
385 6S RNA shown in Figure 6A cannot explain the reduced transcription of $E\sigma^{38}$ target genes in the
386 Δ*ssrS* strain. However, adding reduced Rsd levels to the model is sufficient. We therefore
387 hypothesize that the reduced $E\sigma^{38}$ transcription in the 6S RNA knockout could be due to indirect
388 effects, primarily via Rsd.
389
390 The predictions of this model would ideally be validated by *in vitro* transcription experiments
391 with Rsd and 6S RNA in varying concentrations; to our knowledge, such studies have never been
392 carried out. However, such experiments are necessarily limited to a few promoters, and previous
393 studies have not succeeded in distinguishing specific transcription inhibition by 6S RNA *in vitro*.
394 Some predictions may be testable by looking at distributions of gene expression *in vivo*; for

instance, increasing Rsd expression in Δ*ssrS* back to the wild-type level should largely mitigate the reduced expression of σ^{38} target genes.

Discussion

6S RNA and Rsd have long been known to regulate RNA polymerase in *E. coli*. It was originally hypothesized, based on expression studies using single promoters, that Rsd reduces the association of E with σ^{70} and correspondingly increases its association with σ^{38} (26). However, a microarray experiment failed to find any significant effect of deleting Rsd (27). All studies so far have found effects of Rsd only in stationary phase.

Here we report for the first time that Rsd regulates gene expression from early exponential to stationary phase. Though it sequesters σ^{70}, our data leads us to hypothesize that its primary function is to increase the association of the alternative sigma factor σ^{38} with RNA polymerase. Based on theoretical modeling, we suggest that this is due to the presence of 6S RNA, which minimizes the effect of Rsd on $E\sigma^{70}$ levels. Since Rsd overexpression has been seen to increase transcription directed by σ^{24} and σ^{54} in a $ppGpp^0$ background (4, 39), Rsd may generally assist alternative sigma factors in associating with RNA polymerase, under suitable conditions.

6S RNA is more complex, regulating hundreds of genes controlled by multiple sigma factors. We identified 447 6S RNA regulated genes, of which 203 are upregulated in the 6S RNA knockout,

243 are downregulated, and 1 (*rzoD)* is upregulated in EE phase but downregulated in LS phase. However, our data does not show associations between promoter sequence and 6S RNA susceptibility as previously reported (30). Our data has somewhat greater similarity with that of (33), who reported reduced expression of *rpoB* and ribosomal genes in Δ*ssrS* in stationary phase, and observed increased ppGpp without an increase in *relA;* however, there is still relatively low overlap in the list of 6S RNA regulated genes (46 genes). This is likely because these studies were carried out in different time points and media from ours. Even within our dataset, there is little overlap between genes regulated by 6S RNA at different time points. Therefore, it seems that there is generally a large difference in the 6S RNA regulon under different conditions. Nevertheless, certain patterns stand out; 6S RNA increases σ^{38} mediated transcription, and modulates the expression of the transcription and translation machinery. This supports a model in which 6S RNA acts as a background-level regulator operating on RNA polymerase, with gene-level outcomes depending strongly upon the cellular environment; this could potentially involve DNA topology, transcription factors, and other RNA polymerase-binding factors, all of which vary with growth phase (40).

Previous work using a few promoters (32) had suggested that 6S RNA might increase transcription by $E\sigma^{38}$ during stationary phase. It was suggested that this was either a direct effect of 6S RNA, allowing σ^{38} to compete more effectively for E, or indirect, by means of a trans-acting factor important for σ^{38} activity. However, another study (33) found no evidence of this

436 link. We show that 6S RNA increases transcription by $E\sigma^{38}$ globally, from early exponential to
437 stationary phase, and suggest that it does so through indirect effects, such as increasing Rsd.
438
439 It has been asked, given 6S RNA's function as a global regulator, why its deletion does not cause
440 a growth defect. Our data, along with others (24, 33), shows multiple feedback effects in the
441 Δ*ssrS* strain, where the cell reduces RNA polymerase expression to compensate for the loss of 6S
442 RNA, and increases σ^{38} and possibly Crl to compensate for reduced $E\sigma^{38}$ activity; increased
443 ppGpp may play a part during the TS phase. Our data also shows that in LS phase, 6S RNA
444 represses *ppk*, responsible for the synthesis of inorganic polyphosphate - which increases σ^{38}
445 expression and inhibits transcription by $E\sigma^{70}$ (41). In stationary phase, the Rsd/6S RNA double
446 knockout shows reduced expression of DNA supercoiling enzymes; supercoiling regulates
447 promoter binding by $E\sigma^{70}$ and $E\sigma^{38}$ (42, 43); in ME phase, it shows reduced expression of the
448 gene encoding the small RNA chaperone Hfq, which, among many functions, increases σ^{38}
449 expression (44). These examples, illustrated in Figure S14, demonstrate that RNA polymerase
450 activity is very carefully controlled, and 6S RNA is connected to multiple pathways involved in
451 this process.
452
453 We also report for the first time that 6S RNA positively regulates the expression of its
454 downstream gene *fau*. The *ssrS-fau* operon arrangement is conserved in α- and γ-proteobacterial
455 genomes as well as some β-proteobacteria (45), suggesting that it is functionally relevant,
456 perhaps for linking *fau* expression to 6S RNA, and thus to the nutritional state of the cell. As the

457 *fau* gene product has been linked to folate metabolism (46), biofilm formation (47) and persister
458 cell formation (48), further investigation of the link between 6S RNA and *fau* is warranted.
459
460 Lastly, we report that 6S RNA and Rsd regulate each other. Rsd activates 6S RNA expression in
461 mid-exponential phase and inhibits it in stationary phase, and 6S RNA activates Rsd protein
462 expression in both mid-exponential and stationary phase. In addition, 1780 genes across 5
463 growth phases – almost 40% of the genes in the cell - are differentially expressed in the 6S RNA-
464 Rsd double knockout but not in the single knockouts added together, indicating some degree to
465 which 6S RNA and Rsd can compensate for each other's absence.
466
467 Given that 6S RNA homologs are widespread in bacteria, co-occurring with Rsd and with other
468 RNA polymerase regulators such as the actinobacterial RbpA, and many bacterial species have
469 two or three 6S RNA homologs with different expression patterns and potentially different
470 binding partners (45, 49), we suggest that studying the relationships between these regulators of
471 RNA polymerase will give greater insights into transcriptional control across the bacterial
472 kingdom.
473
474 **Materials and Methods**
475
476 **Growth conditions**
477

Luria-Bertani broth and agar (20 g/L) were used for routine growth. M9 defined medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.01% NH_4Cl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 x 10^{-4}% Thiamin) supplemented with 0.5% glucose and 0.1% casamino acids was used for RNA-seq and validation. During strain construction, ampicillin or kanamycin were used at final concentrations of 100 µg/ml and 50 µg/ml respectively.

Strain construction

Single gene deletions were achieved by the λ Red recombination system (50), using plasmids pKD46 and pKD13 and specific primers (Table S4). This method introduced a kanamycin resistance cassette into the chromosome. Knockout strains were selected on LB Kanamycin plates. In the *rsd* knockout, the resistance cassette was removed by FLP-mediated site-specific recombination using plasmid pCP20. The Δ*rsd*Δ*ssrS* double knockout was generated by P1 transduction from single knockouts (51). The 3x-FLAG epitope was added to the C-terminus of Rsd by a PCR-based method using plasmid pSUB11 as template (52), and introduced onto the MG1655 chromosome by λ Red recombination using specific primers (Table S5). The *ssrS* knockout was moved into this strain using P1 transduction. Strain constructions were verified by PCR using specific primers (Table S6) and Sanger sequencing.

RNA extraction and mRNA enrichment

Overnight cultures in M9 glucose were inoculated in 100 mL fresh M9 glucose to a final OD_{600} of 0.02 and incubated at 37 °C with shaking. Two biological replicates were performed for each strain. Cells were collected by centrifugation at the early exponential (OD_{600} ~0.3), mid-exponential (OD_{600} ~0.8), transition to stationary (OD_{600} ~1.6), stationary (16 hrs, OD_{600} ~2), and late stationary (48 hrs, OD_{600} ~1.6) phases of growth. RNA was extracted using TRIzol (Invitrogen), following the manufacturer's protocol. Total RNA was treated with DNase I (Invitrogen, 18068-015) according to the manufacturer's protocol. Further precipitation of RNA and ribosomal RNA cleanup was achieved using the MICROBExpress bacterial mRNA purification Kit (Ambion, AM1905) according to the manufacturer's protocol. RNA was finally suspended in 10 µL RNAse free water. The concentration was determined using a Nanodrop 2000 (Thermo Scientific) and quality was checked by visualization on agarose gels.

RNA-Seq

Sequencing libraries were prepared using TruSeq RNA sample preparation kit v2 (Illumina, RS-122-2001) according to the manufacturer's guidelines, checked for quality on an Agilent 2100 Bioanalyzer, and sequenced for 50 cycles from one end on an Illumina HiSeq1000 platform at the Centre for Cellular and Molecular Platforms, Bangalore. The RNA-Seq data is summarized in Table S3.

qRT-PCR for RNA-Seq validation

qRT–PCR was carried out using specific primers to selected mRNA targets (Table S2 and S7). 5 ng of RNA was used for each RT-PCR reaction. TAKARA One-step SYBR PrimeScript RT-PCR kit II (RR086A) was used according to the manufacturer's protocol, on an Applied Biosystems ViiA 7 Real-Time PCR system.

Western Blotting

Cells were grown as for RNA-seq. For stationary phase samples, 5 ml of culture was harvested by centrifugation. For mid-exponential phase, 10 ml was harvested. Lysates were prepared and protein concentration was estimated using BCA assay (Thermo Fisher Scientific, 23227). Lysates containing equal amounts of protein were loaded onto an SDS-PAGE gel. Proteins were elecroblotted onto a nitrocellulose membrane and probed with mouse primary antibody against the protein of interest followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. The primary antibodies used were: Mouse monoclonal antibody to RpoB (Neoclone, WP023), Mouse monoclonal antibody to σ^{70} (Neoclone, WP004), Mouse monoclonal antibody to σ^{38} (Neoclone, WP009), Mouse monoclonal anti-FLAG antibody (Sigma-Aldrich, F3165), Mouse monoclonal antibody to GroEL (Abcam, ab82592). Bands were visualized using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, 34076) and imaged using an ImageQuant LAS 4000 system (GE Healthcare Life Sciences). Band intensities were quantified using ImageJ (http://imagej.nih.gov/ij).

Cloning

A segment of DNA constaining the *ssrS* gene, both its upstream promoters and its downstream terminator was amplified from MG1655 genomic DNA by PCR using specific primers (Table S8), and cloned into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen, K4500), to produce the circular plasmid pTOPO-s*srS*. pTOPO-s*srS* was digested with EcoRI to remove the insert, and the digested vector was eluted and self-ligated to produce the circular empty vector pTOPO-EV.

Data sources

The *E. coli* K-12 MG1655 genome was downloaded from NCBI (NC_000913.2). Gene coordinates were taken from RegulonDB v8.0 (53). Lists of σ^{38} target genes and ppGpp regulated genes were obtained from (18) and (54) respectively. Previous lists of genes regulated by 6S RNA were obtained from (30) and (33). Coordinates of RNA polymerase binding regions and their occupancy were obtained from (36). A list of 501 genes under the control of constitutive σ^{70} promoters was obtained from (34). Of these, we selected 270 genes which were not regulated by σ^{38} or other sigma factors according to RegulonDB (53), (18), or our RNA-Seq data from the σ^{38} knockout.

562 **Data Analysis**

563

564 RNA-Seq reads were mapped to the *E. coli* K-12 MG1655 genome using BWA (55), and reads
565 mapping uniquely were used for further analysis. The number of reads mapping to each gene was
566 calculated and a matrix of read counts was generated with 50 columns, one per sample, and one
567 row per gene. This matrix was fed into the Bioconductor package DESeq (56) for differential
568 expression analysis. Genes with <= 10 reads mapping to them under all conditions were
569 excluded from all analyses and plots. All statistical analyses were performed in R version 3.0.1.

570

571 **Mathematical model**

572

573 The reactions shown in Figure 6A can be represented by a set of differential equations that
574 determine how the dynamical variables (levels of sigma factors, holoenzymes, etc.) change with
575 time. These equations are given in Text S2. We are interested in steady-state conditions as the
576 timescales on which these reactions occur is much faster than typical timescales of cell division,
577 or processes such as stress responses in stationary phase. Therefore, it is reasonable to assume
578 that the levels of sigma factors, holoenzymes, etc., are in quasi steady-state in the cell.
579 Parameters that determine the specific steady-state include the dissociation constants of the
580 various complexes, the total levels of Rsd, 6S RNA, E, sigma factors, and some others, listed in
581 Table 2 with their default values. Some values are altered in specific simulations as mentioned in

582 the results. The model of holoenzyme formation (shaded in Figure 6A) describes the following

583 reactions:

584

585 $$E + \sigma^{70} \rightleftharpoons E\sigma^{70}$$

586 $$E + \sigma^{38} \rightleftharpoons E\sigma^{38}$$

587 $$Rsd + \sigma^{70} \rightleftharpoons Rsd - \sigma^{70}$$

588 $$6SRNA + E\sigma^{70} \rightleftharpoons 6SRNA - E\sigma^{70}$$

589

590 In steady state, the following equations must be fulfilled:

591

592 $$\frac{[E][\sigma^{70}]}{[E\sigma^{70}]} = K_{E\sigma^{70}} \quad \text{(Equation 1)}$$

593 $$\frac{[E][\sigma^{38}]}{[E\sigma^{38}]} = K_{E\sigma^{38}} \quad \text{(Equation 2)}$$

594 $$\frac{[Rsd][\sigma^{70}]}{[Rsd - \sigma^{70}]} = K_{Rsd} \quad \text{(Equation 3)}$$

595 $$\frac{[6SRNA][E\sigma^{70}]}{[6SRNA - E\sigma^{70}]} = K_{6S} \quad \text{(Equation 4)}$$

596 $$E_{total} = [E] + [E\sigma^{70}] + [E\sigma^{38}] + [6SRNA - E\sigma^{70}] \quad \text{(Equation 5)}$$

$$\sigma^{70}_{total} = [\sigma^{70}] + [E\sigma^{70}] + [Rsd - \sigma^{70}] + [6SRNA - E\sigma^{70}] \quad \text{(Equation 6)}$$

$$\sigma^{38}_{total} = [\sigma^{38}] + [E\sigma^{38}] \quad \text{(Equation 7)}$$

$$Rsd_{total} = [Rsd] + [Rsd - \sigma^{70}] \quad \text{(Equation 8)}$$

$$6SRNA_{total} = [6SRNA] + [6SRNA - E\sigma^{70}] \quad \text{(Equation 9)}$$

Steady-state levels of the dynamical variables were obtained from the above equations both by solving them numerically and by integrating the corresponding differential equations until they reached steady-state.

The model with DNA (represented by the full schematic in Figure 6A) includes, in addition, the following reactions:

$$E + DNA \rightleftharpoons EDNA$$

$$E\sigma^{70} + DNA \rightleftharpoons E\sigma^{70}DNA$$

$$E\sigma^{38} + DNA \rightleftharpoons E\sigma^{38}DNA$$

$$E\sigma^{70} + P_{70} \rightleftharpoons E\sigma^{70}P_{70} \xrightarrow{c} \mathrm{E}_{e70} + \sigma^{70} + P_{70}$$

$$E\sigma^{38} + P_{38} \rightleftharpoons E\sigma^{38}P_{38} \xrightarrow{c} \mathrm{E}_{e38} + \sigma^{38} + P_{38}$$

$$E_{e70} \xrightarrow{e} \mathrm{E} + \mathrm{mRNA}_{70}$$

$$E_{e38} \xrightarrow{e} \mathrm{E} + \mathrm{mRNA}_{38}$$

Here, *c* represents the rate of promoter clearance and *e* represents the rate of transcript elongation. At steady state these fulfil the following equations, in addition to equations (1) - (4) and (8) – (9). K_{NS} represents the dissociation constant for non-specific binding of RNA polymerase to DNA, which we assume is equal for E, $E\sigma^{70}$ and $E\sigma^{38}$.

$$\frac{[E][DNA]}{[EDNA]} = \frac{[E\sigma^{70}][DNA]}{[E\sigma^{70}DNA]} = \frac{[E\sigma^{38}][DNA]}{[E\sigma^{38}DNA]} = K_{NS} \quad \text{(Equation 10)}$$

$$\frac{[E\sigma^{70}][P_{70}]}{[E\sigma^{70}P_{70}]} = \frac{[E\sigma^{38}][P_{38}]}{[E\sigma^{38}P_{38}]} = K_{E\sigma P} \quad \text{(Equation 11)}$$

$$E_{total} = [E] + [E\sigma^{70}] + [E\sigma^{38}] + [6SRNA - E\sigma^{70}] + [EDNA] + [E\sigma^{70}DNA] + [E\sigma^{38}DNA] + [E\sigma^{70}P_{70}] + [E\sigma^{38}P_{38}] + [E_{e70}] + [E_{e38}] \quad \text{(Equation 12)}$$

$$\sigma^{70}_{total} = [\sigma^{70}] + [E\sigma^{70}] + [Rsd - \sigma^{70}] + [6SRNA - E\sigma^{70}] + [E\sigma^{70}DNA] + [E\sigma^{70}P_{70}] \quad \text{(Equation13)}$$

$$\sigma^{38}_{total} = [\sigma^{38}] + [E\sigma^{38}] + [E\sigma^{38}DNA] + [E\sigma^{38}P_{38}] \quad \text{(Equation 14)}$$

$$DNA_{total} = [DNA] + [EDNA] + [E\sigma^{70}DNA] + [E\sigma^{38}DNA] \quad \text{(Equation 15)}$$

$$P_{70total} = [P_{70}] + [E\sigma^{70}P_{70}] \quad \text{(Equation 16)}$$

$$P_{38total} = [P_{38}] + [E\sigma^{38}P_{38}] \quad \text{(Equation 17)}$$

633 Here, steady-state levels were obtained by integrating the corresponding differential equations
634 until they reached steady-state.
635
636 **Accession Numbers**
637
638 RNA-Seq data have been deposited with NCBI GEO under the accession number GSE74809.
639
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641
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653

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655

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667 Table 1: Cellular concentrations of RNA polymerase, sigma factors, Rsd and 6S RNA.

Molecule	**Exponential**	**Stationary**	**Reference**
Core RNA Polymerase	~4.3 μM	~4.3 μM	(9)
σ^{70}	~12.1 μM	~12.0 μM	
σ^{38}	Not Detected	~2.7 μM	
Rsd	~5.5 μM	~10.4 μM	
6S RNA	~1000 molecules/cell	~10,000 molecules/cell	(28)

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682 Table 2: Default values of parameters used for simulations. Wherever possible, values used are

683 specific to stationary phase. These values are discussed in Text S1.

Parameter	Meaning	Value	Reference
Volume	Average cell volume	10^{-15} L	(57)
E_{total}	Total cellular concentration of RNA Polymerase	4.3 μM	(9)
σ^{70}_{total}	Total cellular concentration of σ^{70}	12.0 μM	
σ^{38}_{total}	Total cellular concentration of σ^{38}	2.7 μM	
Rsd_{total}	Total cellular concentration of Rsd	10.4 μM	
6S RNA_{total}	Total cellular concentration of 6S RNA	13 μM	(28)
DNA_{total}	Nonspecific binding sites per cell	4.6 x 10^6	MG1655 genome size
P_{70}, P_{38}	σ-cognate promoters per cell	200	(38)
$K_{E\sigma 70}$	Dissociation constant for E - σ^{70} binding	3.3 nM	(19)
$K_{E\sigma 38}$	Dissociation constant for E - σ^{38} binding	15.2 nM	
K_{NS}	Dissociation constant for nonspecific binding of RNA Polymerase and DNA	10^{-4} M	(58)
K_{Rsd}	Dissociation constant for Rsd - σ^{70} binding	32 nM	(59)
K_{6S}	Dissociation constant for 6S RNA - $E\sigma^{70}$ binding	131 nM	(60)
$K_{E\sigma P}$	Dissociation constant for holoenzyme - promoter binding	10^{-7} M	(61)
Operon length	Average operon length	1000 nt	(62)
c	Rate of promoter clearance	0.005 s^{-1}	(38, 63)
e	Rate of escape from elongation	0.021 s^{-1}	(64)

Figure 1

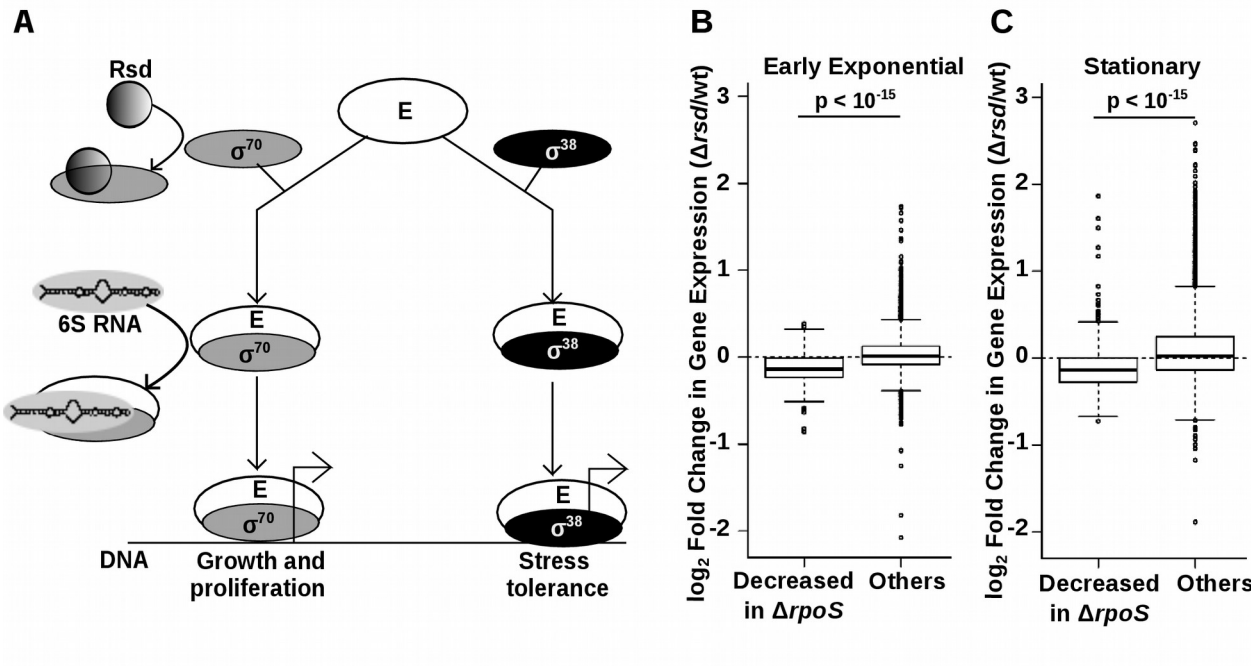

Figure 1: A) Schematic showing the binding activity of Rsd and 6S RNA. B) Boxplot of $\log_2$ fold change in gene expression (Δ*rsd*/wild-type, EE phase) for 313 genes whose expression is reduced at least twofold in Δ*rpoS*/wild-type in EE phase, compared to all other genes. C) Boxplot of $\log_2$ fold change in gene expression (Δ*rsd*/wild-type, stationary phase) for 634 genes whose expression is reduced at least twofold in Δ*rpoS*/wild-type in stationary phase, compared to all other genes. p-values are for Wilcoxon Test.

Figure 2

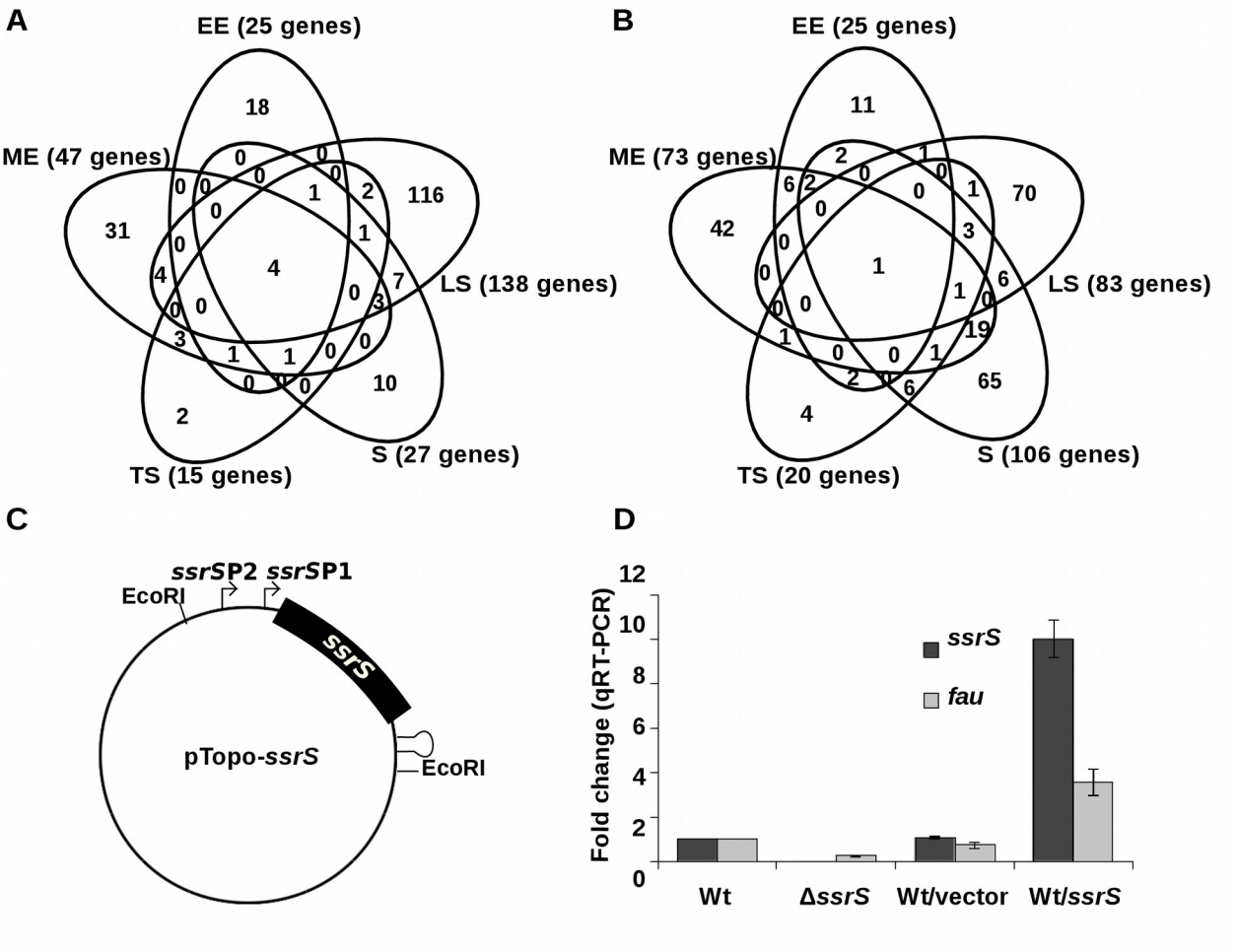

Figure 2: A) Venn diagram showing genes whose expression is increased at least twofold in Δ*ssrS* relative to wild-type, in different phases of growth. B) Venn diagram showing genes whose expression is reduced at least twofold in Δ*ssrS* relative to wild-type, in different phases of growth. C) Schematic of plasmid pTopo-*ssrS*, used for overexpressing 6S RNA. D) Fold change in expression (qRT-PCR) of *ssrS* (dark gray) and *fau* (light gray) RNA in wild-type (both set to 1), Δ*ssrS*, wild-type transformed with empty vector, and wild-type transformed with pTopo-*ssrS*, during stationary phase. Data represent mean +/- SEM for 3 biological replicates.

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Figure 3

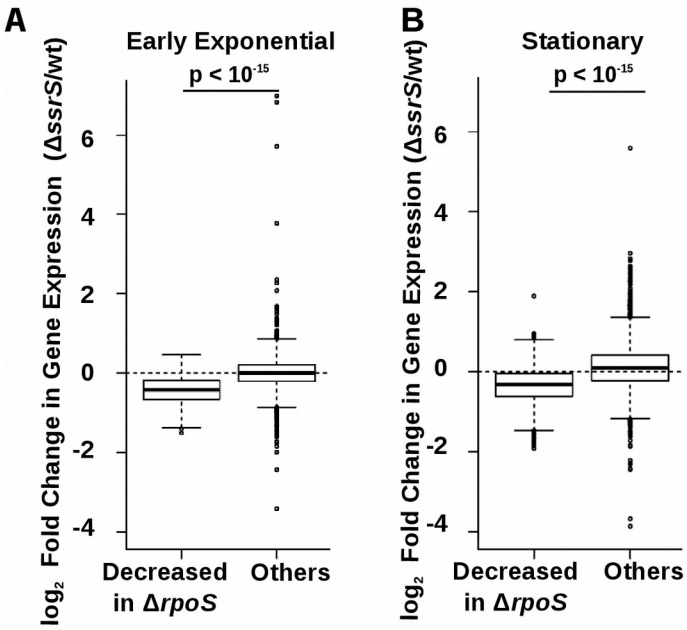

Figure 3: A) Boxplot of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type, EE phase) for 313 genes whose expression is reduced at least twofold in Δ*rpoS*/wild-type in EE phase, compared to all other genes. B) Boxplot of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type, stationary phase) for 634 genes whose expression is reduced at least twofold in Δ*rpoS*/wild-type in stationary phase, compared to all other genes. p-values are for Wilcoxon Test.

Figure 4

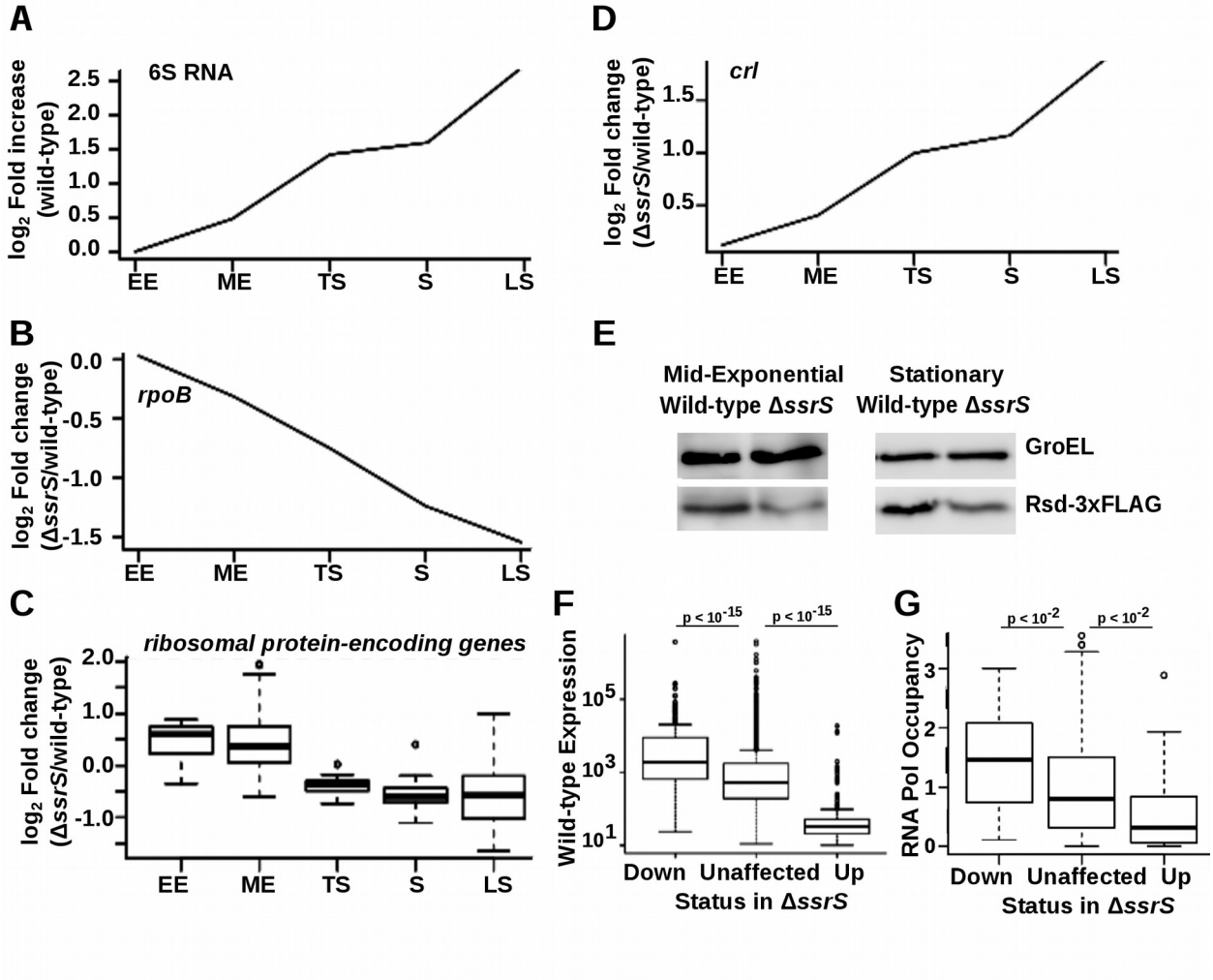

Figure 4: A) $\log_2$ fold increase in 6S RNA levels in wild-type *E. coli* over successive growth phases (relative to its expression in EE phase), based on RNA-Seq. B) log2 fold change in *rpoB* gene expression in Δ*ssrS*/wild-type, in successive growth phases. C) $\log_2$ fold change in the expression of 46 ribosomal protein genes in Δ*ssrS*/wild-type, in successive growth phases. D) $\log_2$ fold change in *crl* expression in Δ*ssrS*/wild-type, in successive growth phases. E) Western blot for 3xFLAG-tagged Rsd in wild-type and Δ*ssrS* backgrounds, in mid-exponential and stationary phases. F) Boxplots showing the wild-type expression level of genes that are reduced >=2-fold in Δ*ssrS*/wild-type, genes that are not differentially expressed in Δ*ssrS*, and genes that are increased >=2-fold in Δ*ssrS*/wild-type, during stationary phase. G) Boxplots showing the RNA polymerase occupancy (measured by ChIP-chip) of promoters beloning to genes that are reduced >=2-fold in Δ*ssrS*/wild-type, genes that are not differentially expressed in Δ*ssrS*, and genes that are increased >=2-fold in Δ*ssrS*/wild-type, during stationary phase. Only genes that were the first genes in their transcription unit, and were associated with a single RNA polymerase binding site, were included in (G). All p-values are for Wilcoxon Test.

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Figure 5

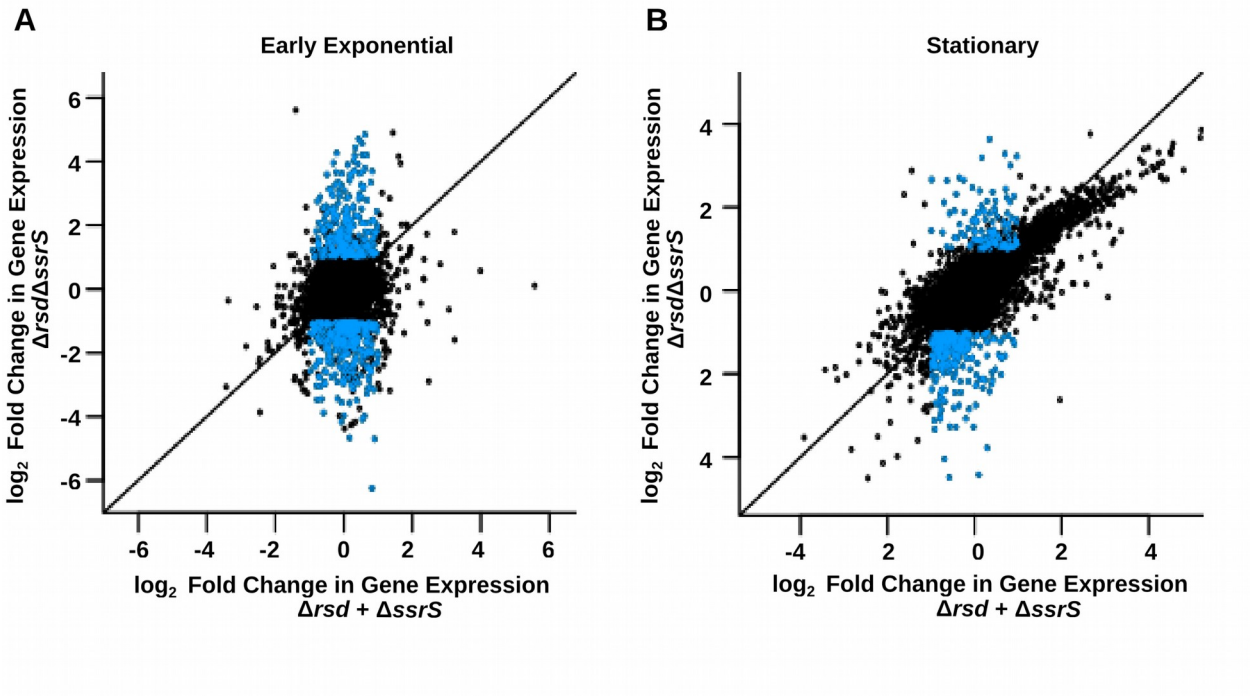

Figure 5: Scatterplots of $\log_2$ fold change in gene expression in the Δ*rsd*Δ*ssrS* double knockout versus the sum of $\log_2$ fold changes in the Δ*rsd* and Δ*ssrS* single knockouts, for each gene, in A) Early exponential and B) Stationary phase. Blue points represent genes that show differential expression (>=2-fold increase or decrease, FDR-adjusted p-value<0.05) in the double knockout, but less than twofold change in expression in both single knockouts added together.

Figure 6

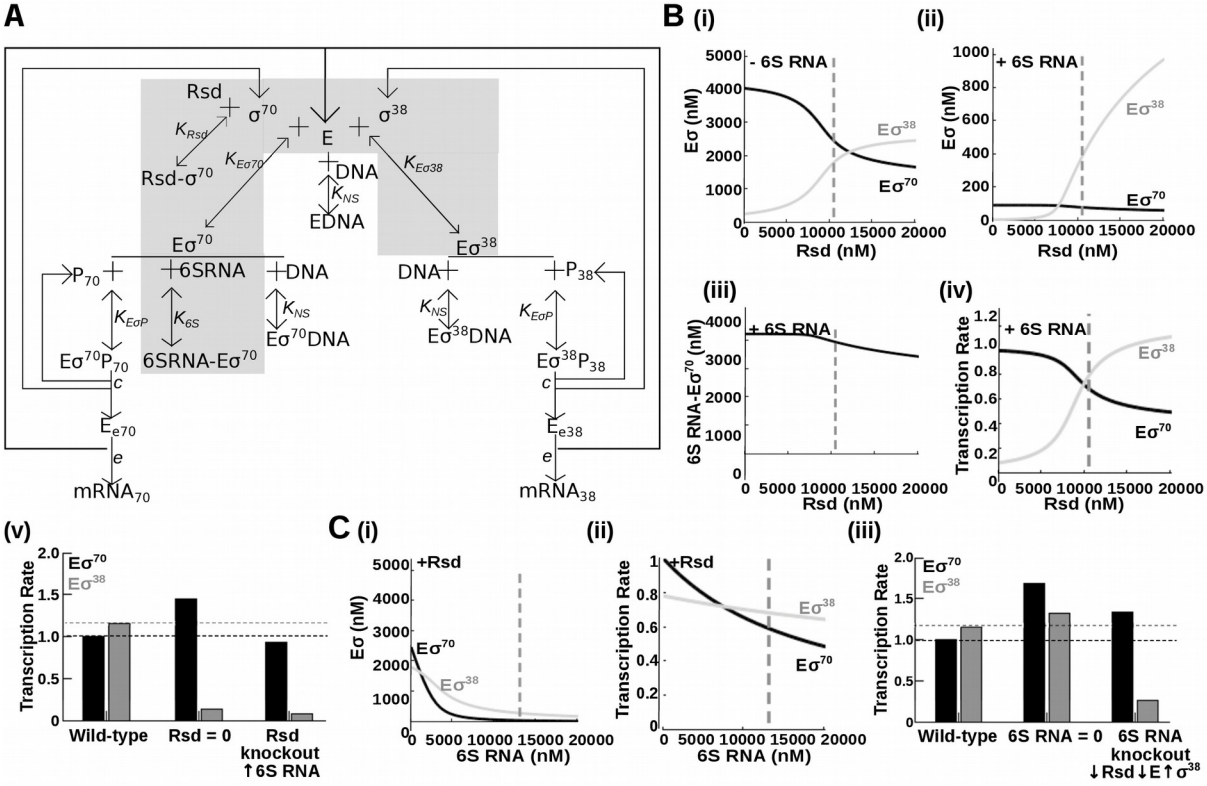

Figure 6: A) Schematic of the model. Shaded area represents reactions involved in holoenzyme formation (without DNA) B) (i) Steady-state levels of $E\sigma^{70}$ (black) and $E\sigma^{38}$ (gray), computed from equations (1) - (9), as a function of total Rsd, when 6S RNA = 0. (ii) Same when total 6S RNA = 13 μM. (iii) Steady-state levels of the 6S RNA-$E\sigma^{70}$ complex, as a function of total Rsd. (iv) Steady-state rate of transcription from $E\sigma^{70}$ dependent promoters (black) and $E\sigma^{38}$ dependent promoters (gray), as a function of total Rsd. Vertical dashed lines represent wild-type cellular concentrations in stationary phase. (v) Steady-state rate of transcription from $E\sigma^{70}$ dependent promoters (black bars) and $E\sigma^{38}$ dependent promoters (gray bars) in the wild-type, absence of Rsd, and simulated Rsd knockout. C) (i) Steady-state levels of $E\sigma^{70}$ (black) and $E\sigma^{38}$ (gray), computed from equations (1) – (9), as a function of total 6S RNA. (ii) Steady-state rate of transcription from $E\sigma^{70}$ dependent promoters (black) and $E\sigma^{38}$ dependent promoters (gray), as a function of total 6S RNA. Vertical dashed lines represent wild-type cellular concentrations in stationary phase. (iii) Steady-state rate of transcription from $E\sigma^{70}$ dependent promoters (black bars) and $E\sigma^{38}$ dependent promoters (gray bars) in the wild-type, absence of 6S RNA, and simulated 6S RNA knockout.

Supplementary Material Legends

Figure S1: Sample growth curves of wild-type *E. coli* and the mutant strains used for RNA-Seq. Overnight cultures were diluted 1:100 in fresh M9 Glucose medium and growth was estimated by measuring optical density at 600 nm.

Figure S2: (A) Boxplots of $\log_2$ fold change in gene expression (Δ*rsd*/wild-type) for genes whose expression is significantly reduced in Δ*rpoS* in the indicated growth phase, compared to all other genes. (B) Boxplots of $\log_2$ fold change in gene expression (Δ*rsd*/wild-type) for reported σ^{38} targets (18) in the indicated growth phase, compared to all other genes. (C) Boxplots of $\log_2$ fold change in gene expression (Δ*rsd*/wild-type) for genes whose expression is significantly increased in Δ*rpoS* in the indicated growth phase, compared to all other genes. p-values are for Wilcoxon Test.

Figure S3: Boxplots of $\log_2$ fold change in gene expression (Δ*rsd*/wild-type) for 270 genes whose expression is controlled by constitutive σ^{70}-dependent promoters, compared to all other genes, in the indicated growth phase. p-values are for Wilcoxon Test.

Figure S4: Western blot showing expression of σ^{38} (RpoS) during stationary phase, in the five strains used for RNA-Seq. GroEL was used as a loading control.

Figure S5: (A) Boxplots of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) for genes whose expression is significantly reduced in Δ*rpoS* in the indicated growth phase, compared to all other genes. (B) Boxplots of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) for reported σ^{38} targets (18) in the indicated growth phase, compared to all other genes. (C) Boxplots of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) for genes whose expression is significantly increased in Δ*rpoS* in the indicated growth phase, compared to all other genes. p-values are for Wilcoxon Test.

Figure S6: Boxplots of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) for 270 genes whose expression is controlled by constitutive σ^{70}-dependent promoters, compared to all other genes, in the indicated growth phase. p-values are for Wilcoxon Test.

Figure S7: Boxplots of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) during the transition to stationary phase, for 710 genes repressed by ppGpp, 3159 genes unaffected by ppGpp, and 704 genes activated by ppGpp (54). p-values are for Wilcoxon Test.

Figure S8: Heatmaps showing the $\log_2$ fold change in expression, in successive growth phases, of (A) 16 genes whose expression was increased in Δ*ssrS*/wild-type, with the magnitude of this increase increasing in successive growth phases, and (B) 20 genes whose expression was reduced in Δ*ssrS*/wild-type, with the magnitude of this reduction increasing in successive growth phases.

Figure S9: Western blot showing expression of RNA polymerase β subunit (RpoB) during stationary phase, in the five strains used for RNA-Seq. GroEL was used as a loading control.

Figure S10: (A) Boxplots of $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) for genes as a function of the number of nucleotides in their promoter -35 sequence that match to consensus, for a set of 312 mapped σ^{70} promoters, based on the data of (30) (B) Boxplots showing $\log_2$ fold change in expression (Δ*ssrS*/wild-type) of the same genes in our dataset. (C) Boxplots showing $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) for 77 genes whose promoters have a weak -35 element and extended -10 element, compared to 72 genes with a weak -35 element only, based on the data of (30) (D) Boxplots showing $\log_2$ fold change in gene expression (Δ*ssrS*/wild-type) for the same genes in our dataset. p-values are for Wilcoxon Test.

Figure S11: (A) Boxplots of $\log_2$ fold change in gene expression (Δ*rsd*Δ*ssrS*/wild-type) for genes whose expression is significantly reduced in Δ*rpoS* in the indicated growth phase, compared to all other genes. (B) Boxplots of $\log_2$ fold change in gene expression (Δ*rsd*Δ*ssrS*/wild-type) for reported σ^{38} targets (18) in the indicated growth phase, compared to all other genes. (C) Boxplots of $\log_2$ fold change in gene expression (Δ*rsd*Δ*ssrS*/wild-type) for genes whose expression is significantly increased in Δ*rpoS* in the indicated growth phase, compared to all other genes. p-values are for Wilcoxon Test.

Figure S12: (A) $\log_2$ fold change in gene expression of *rpoB* in successive growth phases, in Δ*ssrS*/wild-type (black) and Δ*rsd*Δ*ssrS*/wild-type (blue) (B) Boxplots showing $\log_2$ fold change in gene expression of 46 ribosomal protein-encoding genes in successive growth phases, in Δ*ssrS*/wild-type (black) and Δ*rsd*Δ*ssrS*/wild-type (C) $\log_2$ fold change in gene expression of *crl* in successive growth phases, in Δ*ssrS*/wild-type (black) and Δ*rsd*Δ*ssrS*/wild-type (blue). (D) Boxplots showing the wild-type expression level of genes that are downregulated by twofold or more, genes that are not differentially expressed, and genes that are upregulated by twofold or more, in Δ*ssrS*/wild-type (black) and Δ*rsd*Δ*ssrS*/wild-type (blue) during stationary phase.

Figure S13: (A) Steady-state rate of transcription from $E\sigma^{70}$ dependent promoters (black) and $E\sigma^{38}$ dependent promoters (gray), as a function of total σ^{38}, in the absence of Rsd. (B) Steady-state rate of transcription from $E\sigma^{70}$ dependent promoters (black) and $E\sigma^{38}$ dependent promoters (gray), as a function of total Rsd. Vertical dashed lines represent wild-type cellular concentrations in stationary phase.

Figure S14: A schematic showing various pathways by which Rsd and 6S RNA have been proposed to regulate RNA polymerase. Bold lines represent sequestration into inactive complexes. Blue lines represent pathways which are affected only in the Rsd-6S RNA double knockout. The three arrows connecting Rsd and 6S RNA show that Rsd inhibits expression of 6S RNA (in stationary phase) and activates it (in ME phase), while 6S RNA increases expression of Rsd. The question mark indicates the possibility that 6S RNA is autoregulatory.