

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

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3 Avantika Lal<sup>1</sup>, Sandeep Krishna<sup>2</sup>, and Aswin Sai Narain Seshasayee<sup>1#</sup>

4 <sup>1</sup>National Centre for Biological Sciences, TIFR, Bangalore 560065, Karnataka, India; <sup>2</sup>Simons

5 Centre for the Study of Living Machines, National Centre for Biological Sciences, TIFR,

6 Bangalore 560065, Karnataka, India

7 #aswin@ncbs.res.in

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22 **Abstract**

23

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

35

36 **Importance**

37

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

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

44

## 45 **Introduction**

46

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

51

52 Seven sigma factors –  $\sigma^{70}$  (RpoD),  $\sigma^{38}$  (RpoS),  $\sigma^{32}$ ,  $\sigma^{54}$ ,  $\sigma^{28}$ ,  $\sigma^{24}$  and  $\sigma^{19}$  - have been identified in the  
53 model bacterium *Escherichia coli* (1). *In vivo* studies have shown that when one sigma factor is  
54 overproduced, underproduced, or mutated, not only is there a change in transcription of its target  
55 genes, but increased activity of one sigma factor decreases the activity of others and vice-versa  
56 (2–4). *In vitro* assays have demonstrated competition between sigma factors when E is limiting  
57 (3–6). Though it was previously reported that the cellular concentration of E exceeded that of  
58 sigma factors (6, 7), recent quantitation has shown that sigma factors exceed E under common  
59 culture conditions (8, 9), and therefore compete to bind to limited E.

60

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

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

71

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

77

78 On the other hand, two regulators - the protein Rsd and the non-coding 6S RNA - act on  $\sigma^{70}$ . Rsd  
79 binds to  $\sigma^{70}$ , sequestering it from E, and inhibits  $E\sigma^{70}$ -dependent transcription at several  
80 promoters *in vitro* (25). An Rsd null strain showed increased transcription from a  $\sigma^{70}$  dependent  
81 promoter, and reduced transcription from a  $\sigma^{38}$  dependent promoter, in stationary phase, whereas  
82 Rsd overexpression had the opposite effect (26). It was hypothesized that in stationary phase,  
83 Rsd reduces  $E\sigma^{70}$  formation, and, by freeing E to bind  $\sigma^{38}$ , 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  $\sigma^{38}$ -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  $\sigma^{70}$  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  $E\sigma^{70}$  holoenzyme (28). It has been shown to block  $E\sigma^{70}$  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  $E\sigma^{70}$  promoters containing extended -10  
96 elements, and reduced expression from a few  $E\sigma^{38}$  promoters, in stationary phase (32). It was  
97 suggested that 6S RNA blocks  $E\sigma^{70}$  from binding to certain target promoters, reducing their  
98 transcription, and that sequestration of  $E\sigma^{70}$  by 6S RNA allows  $\sigma^{38}$  to compete more effectively  
99 for E, increasing transcription by  $E\sigma^{38}$ . An alternative hypothesis was that 6S RNA regulates a  
100 trans-acting factor important for  $E\sigma^{38}$  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  $\sigma^{70}$  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  $\sigma^{38}$  target genes, with 6S RNA  
118 also regulating hundreds of  $\sigma^{70}$  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

125 **Results**

126

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

128

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

134

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

141

142 **Rsd increases  $\sigma^{38}$ /RpoS activity throughout growth**

143

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

146 the  $\Delta rsd$  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  $\Delta rsd$  during  
148 stationary phase. The expression of 6S RNA was also altered in  $\Delta rsd$ ; 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  $\Delta rsd$ , 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 ( $\geq 2$ -fold,  $p < 0.05$ ) in  $\Delta rpoS$  to also display slightly reduced expression in  $\Delta rsd$ . This is  
155 illustrated in Figure 1B-C. These boxplots show the distribution of  $\log_2$  fold change in gene  
156 expression in  $\Delta rsd$  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  $1.5 \times$  IQR. For genes whose  
158 expression is significantly reduced in  $\Delta rpoS$ , 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  $\sim 75\%$  of the genes whose expression was significantly reduced in  $\Delta rpoS$  also  
161 showed reduced expression in  $\Delta rsd$ . 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  $\Delta rsd$ . 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

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

176

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

182

### 183 **6S RNA regulates distinct sets of genes in all phases of growth**

184

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

187 very different from the Rsd knockout. To begin with, it showed  $\geq 2$ -fold differential expression  
188 of a total of 447 genes.

189

190 Figure 2A and B show that there was little overlap between genes differentially expressed in  
191  $\Delta ssrS$  in successive growth phases. In particular, 186 of 221 genes regulated by 6S RNA during  
192 late stationary phase were regulated only in that phase. The only genes upregulated  $\geq 2$ -fold in  
193  $\Delta ssrS$  throughout growth were *cusR*, encoding a copper-sensing regulator, and its target genes  
194 (*cusCFB*) encoding a copper/silver efflux system. The only gene downregulated  $\geq 2$ -fold in  
195  $\Delta ssrS$  throughout growth was *fau* (*ygfA*), which is downstream of 6S RNA in the same operon  
196 and encodes a putative 5-formyltetrahydrofolate cyclo-ligase. *fau* expression was increased when  
197 6S RNA was overexpressed from a plasmid in a wild-type background (Figure 2C and D),  
198 indicating that this was at least in part a regulatory effect of 6S RNA and not merely a polar  
199 effect. The mechanism is unclear; however, it could indicate an autoregulatory role of 6S RNA.

200

201 During exponential phase, the expression of several genes encoding amino acid transporters  
202 (*artM*, *artI*, *hisP*, *hisQ*, *hisJ*, *hisM*, *tdcC*) and genes for amino acid biosynthesis (*argH*, *argB*,  
203 *thrA*, *thrB*, *thrC*, *asnB*, *glyA*) was increased in  $\Delta ssrS$ , while expression of genes involved in  
204 stress responses (*rmf*, *appY*, *yadC*, *ybcM*, *yciF*, *gadW*, *ydeI*, *yodD*, *dps*, *hdeA*, *hslV*, *oppA*, *osmE*,  
205 *dosC*) was reduced. These functional changes are consistent with previous reports (24, 33),  
206 though most of the specific genes are different. In LS phase, genes encoding global  
207 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  $\Delta$ *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 $\sigma^{38}$ /RpoS activity throughout growth**

215

216 Like  $\Delta$ *rsd*, the  $\Delta$ *ssrS* strain showed reduced expression of  $\sigma^{38}$  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  $\sigma^{38}$  protein, as western blots in stationary phase showed that the  $\Delta$ *ssrS* strain had  
219 higher  $\sigma^{38}$  protein than the wild-type (Figure S4). On the other hand, expression of genes under  
220 constitutive  $\sigma^{70}$  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

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

234

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

247

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

250 previously (33); here, their expression was increased in  $\Delta ssrS$  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  $\Delta ssrS$  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  $\sigma^{38}$  protein and Crl may be a means to compensate for reduced  $E\sigma^{38}$   
256 activity in  $\Delta ssrS$  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  $\Delta ssrS$ . 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  $\Delta ssrS$  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  $\Delta ssrS$  tended to be highly expressed in the wild-type, and genes upregulated in  $\Delta ssrS$  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  $\sigma^{70}$  in different forms, each regulates the other's expression, and both favor the  
281 activity of  $\sigma^{38}$ . 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  $\Delta rsd\Delta ssrS$  strain showed several of the features described for  $\Delta ssrS$  (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  $\sigma^{70}$ , increase transcription of  $\sigma^{38}$  target genes without as much effect on  $\sigma^{70}$   
301 target genes? How does 6S RNA, which sequesters not only  $\sigma^{70}$ , but also the core RNA  
302 polymerase which is required for transcription by all sigma factors, nevertheless increase  
303 transcription by  $\sigma^{38}$ ?

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  $\sigma^{38}$ , 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

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

318

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

323

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

328

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

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

338

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

344

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

352

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

358

359 What is the effect of 6S RNA? Again, we initially modeled the effect of 6S RNA on sigma factor  
360 competition without DNA. Figure 6C(i) predicts that 6S RNA sequesters  $E\sigma^{70}$ , reducing the  
361 available E for binding to both sigma factors, thus inhibiting formation of both holoenzymes. By  
362 sequestering  $E\sigma^{70}$ , 6S RNA also reduces the ratio of  $\sigma^{70}$  to  $\sigma^{38}$ , and so the decline is sharper for  
363  $E\sigma^{70}$ . Similarly, Figure 6C(ii) shows the predicted effect of 6S RNA on transcription; 6S RNA  
364 decreases the rate of transcription by both holoenzymes, though the decline is greater for  $E\sigma^{70}$ .  
365

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

370

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

374 in the  $\Delta ssrS$  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  $\sigma^{38}$  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  $\mu\text{M}$  to 8  $\mu\text{M}$  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  $\Delta ssrS$ . 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  $\Delta ssrS$  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  $\Delta ssrS$  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

395 instance, increasing Rsd expression in  $\Delta$ ssrS back to the wild-type level should largely mitigate  
396 the reduced expression of  $\sigma^{38}$  target genes.

397

## 398 **Discussion**

399

400 6S RNA and Rsd have long been known to regulate RNA polymerase in *E. coli*. It was originally  
401 hypothesized, based on expression studies using single promoters, that Rsd reduces the  
402 association of E with  $\sigma^{70}$  and correspondingly increases its association with  $\sigma^{38}$  (26). However, a  
403 microarray experiment failed to find any significant effect of deleting Rsd (27). All studies so far  
404 have found effects of Rsd only in stationary phase.

405

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

413

414 6S RNA is more complex, regulating hundreds of genes controlled by multiple sigma factors. We  
415 identified 447 6S RNA regulated genes, of which 203 are upregulated in the 6S RNA knockout,

416 243 are downregulated, and 1 (*rzoD*) is upregulated in EE phase but downregulated in LS phase.  
417 However, our data does not show associations between promoter sequence and 6S RNA  
418 susceptibility as previously reported (30). Our data has somewhat greater similarity with that of  
419 (33), who reported reduced expression of *rpoB* and ribosomal genes in  $\Delta$ *ssrS* in stationary phase,  
420 and observed increased ppGpp without an increase in *relA*; however, there is still relatively low  
421 overlap in the list of 6S RNA regulated genes (46 genes). This is likely because these studies  
422 were carried out in different time points and media from ours. Even within our dataset, there is  
423 little overlap between genes regulated by 6S RNA at different time points. Therefore, it seems  
424 that there is generally a large difference in the 6S RNA regulon under different conditions.  
425 Nevertheless, certain patterns stand out; 6S RNA increases  $\sigma^{38}$  mediated transcription, and  
426 modulates the expression of the transcription and translation machinery. This supports a model in  
427 which 6S RNA acts as a background-level regulator operating on RNA polymerase, with gene-  
428 level outcomes depending strongly upon the cellular environment; this could potentially involve  
429 DNA topology, transcription factors, and other RNA polymerase-binding factors, all of which  
430 vary with growth phase (40).  
431  
432 Previous work using a few promoters (32) had suggested that 6S RNA might increase  
433 transcription by  $E\sigma^{38}$  during stationary phase. It was suggested that this was either a direct effect  
434 of 6S RNA, allowing  $\sigma^{38}$  to compete more effectively for E, or indirect, by means of a trans-  
435 acting factor important for  $\sigma^{38}$  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  $\Delta$ ssrS strain, where the cell reduces RNA polymerase expression to compensate for the loss of 6S  
442 RNA, and increases  $\sigma^{38}$  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  $\sigma^{38}$   
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  $\sigma^{38}$   
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  $\alpha$ - and  $\gamma$ -proteobacterial  
455 genomes as well as some  $\beta$ -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

478 Luria-Bertani broth and agar (20 g/L) were used for routine growth. M9 defined medium (0.6%  
479 Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.05% NaCl, 0.01% NH<sub>4</sub>Cl, 0.1 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 5 x 10<sup>-4</sup>%  
480 Thiamin) supplemented with 0.5% glucose and 0.1% casamino acids was used for RNA-seq and  
481 validation. During strain construction, ampicillin or kanamycin were used at final concentrations  
482 of 100 µg/ml and 50 µg/ml respectively.

483

#### 484 **Strain construction**

485

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

496

#### 497 **RNA extraction and mRNA enrichment**

498

499 Overnight cultures in M9 glucose were inoculated in 100 mL fresh M9 glucose to a final OD<sub>600</sub>  
500 of 0.02 and incubated at 37 °C with shaking. Two biological replicates were performed for each  
501 strain. Cells were collected by centrifugation at the early exponential (OD<sub>600</sub> ~0.3), mid-  
502 exponential (OD<sub>600</sub> ~0.8), transition to stationary (OD<sub>600</sub> ~1.6), stationary (16 hrs, OD<sub>600</sub> ~2), and  
503 late stationary (48 hrs, OD<sub>600</sub> ~1.6) phases of growth. RNA was extracted using TRIzol  
504 (Invitrogen), following the manufacturer's protocol. Total RNA was treated with DNase I  
505 (Invitrogen, 18068-015) according to the manufacturer's protocol. Further precipitation of RNA  
506 and ribosomal RNA cleanup was achieved using the MICROBExpress bacterial mRNA  
507 purification Kit (Ambion, AM1905) according to the manufacturer's protocol. RNA was finally  
508 suspended in 10 µL RNase free water. The concentration was determined using a Nanodrop  
509 2000 (Thermo Scientific) and quality was checked by visualization on agarose gels.

510

#### 511 **RNA-Seq**

512

513 Sequencing libraries were prepared using TruSeq RNA sample preparation kit v2 (Illumina, RS-  
514 122-2001) according to the manufacturer's guidelines, checked for quality on an Agilent 2100  
515 Bioanalyzer, and sequenced for 50 cycles from one end on an Illumina HiSeq1000 platform at  
516 the Centre for Cellular and Molecular Platforms, Bangalore. The RNA-Seq data is summarized  
517 in Table S3.

518

#### 519 **qRT-PCR for RNA-Seq validation**

520

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

525

## 526 **Western Blotting**

527

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

541

## 542 **Cloning**

543

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

550

## 551 **Data sources**

552

553 The *E. coli* K-12 MG1655 genome was downloaded from NCBI (NC\_000913.2). Gene  
554 coordinates were taken from RegulonDB v8.0 (53). Lists of  $\sigma^{38}$  target genes and ppGpp regulated  
555 genes were obtained from (18) and (54) respectively. Previous lists of genes regulated by 6S  
556 RNA were obtained from (30) and (33). Coordinates of RNA polymerase binding regions and  
557 their occupancy were obtained from (36). A list of 501 genes under the control of constitutive  $\sigma^{70}$   
558 promoters was obtained from (34). Of these, we selected 270 genes which were not regulated by  
559  $\sigma^{38}$  or other sigma factors according to RegulonDB (53), (18), or our RNA-Seq data from the  $\sigma^{38}$   
560 knockout.

561

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  $\leq 10$  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.

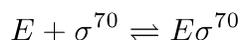
579 Parameters that determine the specific steady-state include the dissociation constants of the  
580 various complexes, the total levels of Rsd, 6S RNA, E, sigma factors, and some others, listed in  
581 Table 2 with their default values. Some values are altered in specific simulations as mentioned in

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

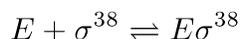
583 reactions:

584

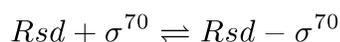
585



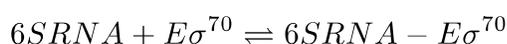
586



587



588



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

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

597 
$$\sigma_{total}^{70} = [\sigma^{70}] + [E\sigma^{70}] + [Rsd - \sigma^{70}] + [6SRNA - E\sigma^{70}] \quad (\text{Equation 6})$$

598 
$$\sigma_{total}^{38} = [\sigma^{38}] + [E\sigma^{38}] \quad (\text{Equation 7})$$

599 
$$Rsd_{total} = [Rsd] + [Rsd - \sigma^{70}] \quad (\text{Equation 8})$$

600 
$$6SRNA_{total} = [6SRNA] + [6SRNA - E\sigma^{70}] \quad (\text{Equation 9})$$

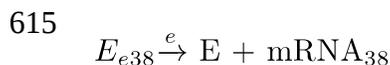
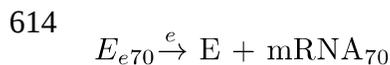
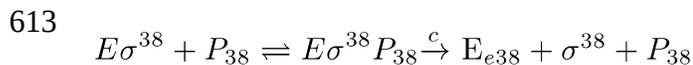
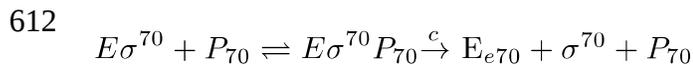
601

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

605

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

608



616

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

621

622

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

623

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

624

$$E_{total} = [E] + [E\sigma^{70}] + [E\sigma^{38}] + [6SRNA - E\sigma^{70}] + [EDNA] + [E\sigma^{70}DNA] \\ + [E\sigma^{38}DNA] + [E\sigma^{70}P_{70}] + [E\sigma^{38}P_{38}] + [E_{e70}] + [E_{e38}]$$

625 (Equation 12)

626

$$\sigma_{total}^{70} = [\sigma^{70}] + [E\sigma^{70}] + [Rsd - \sigma^{70}] + [6SRNA - E\sigma^{70}] + [E\sigma^{70}DNA] + [E\sigma^{70}P_{70}]$$

627 (Equation13)

628

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

629

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

630

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

631

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

632

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

### 640 **Acknowledgments**

641

642 We thank the Centre for Cellular and Molecular Platforms for Illumina sequencing, CGSC for  
643 strains and plasmids, and Prof. Satyajit Mayor's laboratory for the pCR2.1-TOPO vector. We  
644 thank Prof. Dipankar Chatterji, Prof. Akira Ishihama, Prof. Karen Wassarman, Prof. Steve Busby,  
645 Dr. Dasaradhi Palakodeti, and Prof. M.M. Panicker for discussions.

646 A.S.N. is supported by the NCBS Young Investigator and Ramanujan Fellowships. S.K. is  
647 funded by the Simons Foundation. A.L. is supported by the NCBS graduate programme  
648 fellowship.

649

### 650 **Funding Statement**

651 This work was supported by core NCBS funds and by DBT grant BT/PR3695/BRB/10/979/2011  
652 from the Department of Biotechnology, Government of India.

653

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655

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

<b>Molecule</b>	<b>Exponential</b>	<b>Stationary</b>	<b>Reference</b>
Core RNA Polymerase	~4.3 $\mu\text{M}$	~4.3 $\mu\text{M}$	(9)
$\sigma^{70}$	~12.1 $\mu\text{M}$	~12.0 $\mu\text{M}$	
$\sigma^{38}$	Not Detected	~2.7 $\mu\text{M}$	
Rsd	~5.5 $\mu\text{M}$	~10.4 $\mu\text{M}$	
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.

<b>Parameter</b>	<b>Meaning</b>	<b>Value</b>	<b>Reference</b>
Volume	Average cell volume	$10^{-15}$ L	(57)
$E_{total}$	Total cellular concentration of RNA Polymerase	4.3 $\mu$ M	(9)
$\sigma^{70}_{total}$	Total cellular concentration of $\sigma^{70}$	12.0 $\mu$ M	
$\sigma^{38}_{total}$	Total cellular concentration of $\sigma^{38}$	2.7 $\mu$ M	
$Rsd_{total}$	Total cellular concentration of Rsd	10.4 $\mu$ M	
6S RNA <sub>total</sub>	Total cellular concentration of 6S RNA	13 $\mu$ M	(28)
DNA <sub>total</sub>	Nonspecific binding sites per cell	$4.6 \times 10^6$	MG1655 genome size
$P_{70}, P_{38}$	$\sigma$ -cognate promoters per cell	200	(38)
$K_{E\sigma^{70}}$	Dissociation constant for E - $\sigma^{70}$ binding	3.3 nM	(19)
$K_{E\sigma^{38}}$	Dissociation constant for E - $\sigma^{38}$ 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 - $\sigma^{70}$ 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	$0.005 \text{ s}^{-1}$	(38, 63)
$e$	Rate of escape from elongation	$0.021 \text{ s}^{-1}$	(64)

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Figure 1

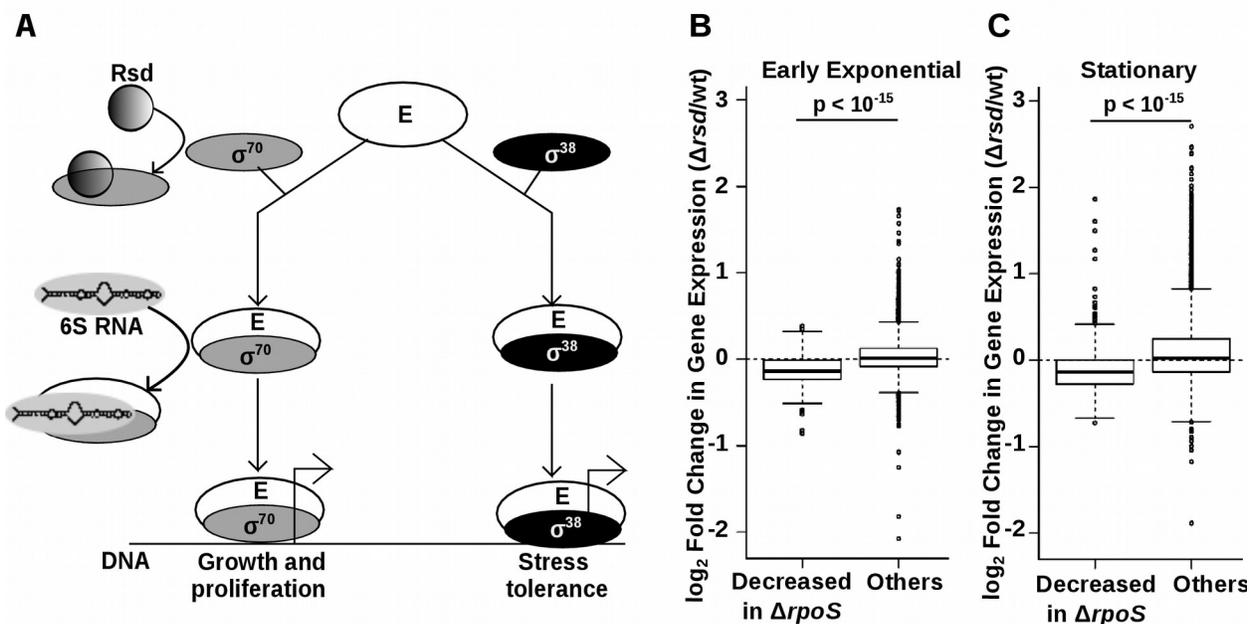


Figure 1: A) Schematic showing the binding activity of Rsd and 6S RNA. B) Boxplot of  $\log_2$  fold change in gene expression ( $\Delta rsd/wild$ -type, EE phase) for 313 genes whose expression is reduced at least twofold in  $\Delta rpoS/wild$ -type in EE phase, compared to all other genes. C) Boxplot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

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Figure 3

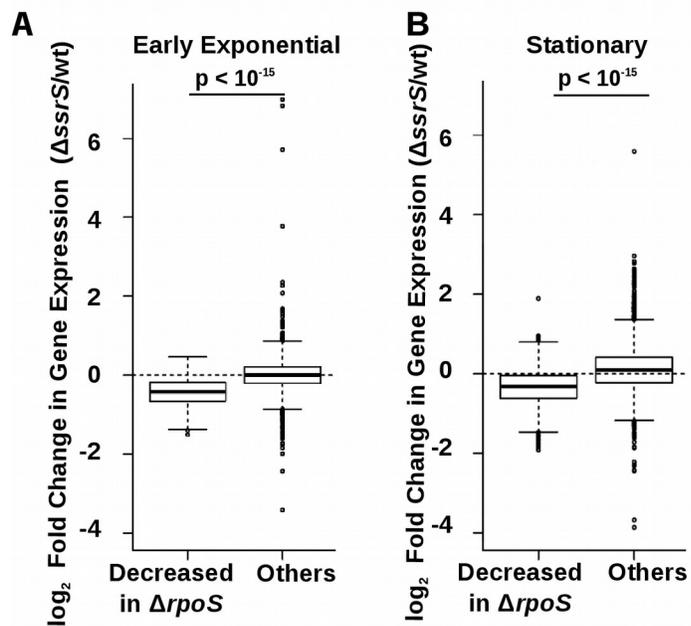


Figure 3: A) Boxplot of log<sub>2</sub> fold change in gene expression (ΔssrS/wild-type, EE phase) for 313 genes whose expression is reduced at least twofold in ΔrpoS/wild-type in EE phase, compared to all other genes. B) Boxplot of log<sub>2</sub> fold change in gene expression (ΔssrS/wild-type, stationary phase) for 634 genes whose expression is reduced at least twofold in ΔrpoS/wild-type in stationary phase, compared to all other genes. p-values are for Wilcoxon Test.

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Figure 4

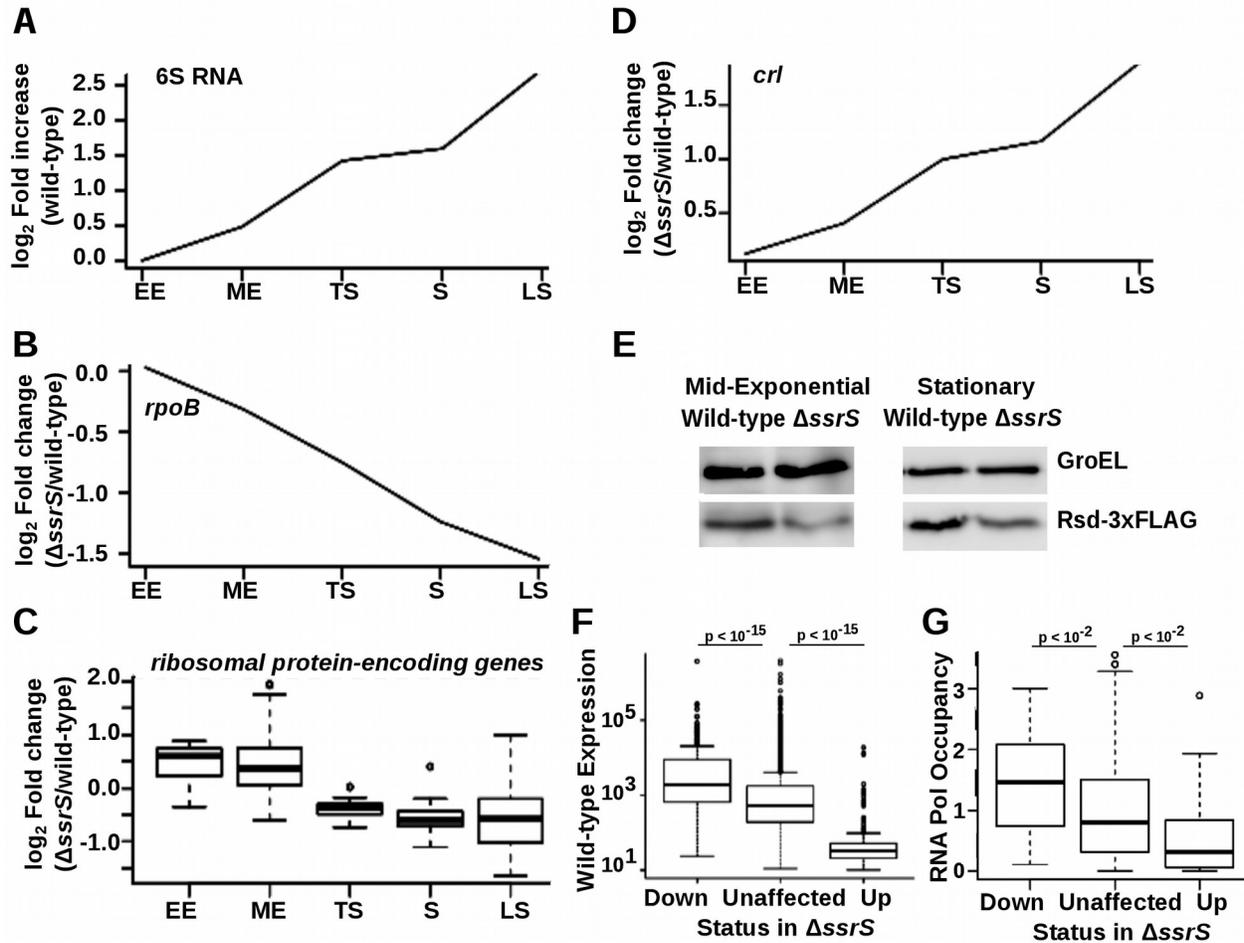


Figure 4: A)  $\log_2$  fold increase in 6S RNA levels in wild-type *E. coli* over successive growth phases (relative to its expression in EE phase), based on RNA-Seq. B)  $\log_2$  fold change in *rpoB* gene expression in  $\Delta ssrS$ /wild-type, in successive growth phases. C)  $\log_2$  fold change in the expression of 46 ribosomal protein genes in  $\Delta ssrS$ /wild-type, in successive growth phases. D)  $\log_2$  fold change in *crl* expression in  $\Delta ssrS$ /wild-type, in successive growth phases. E) Western blot for 3xFLAG-tagged Rsd in wild-type and  $\Delta ssrS$  backgrounds, in mid-exponential and stationary phases. F) Boxplots showing the wild-type expression level of genes that are reduced  $\geq 2$ -fold in  $\Delta ssrS$ /wild-type, genes that are not differentially expressed in  $\Delta ssrS$ , and genes that are increased  $\geq 2$ -fold in  $\Delta ssrS$ /wild-type, during stationary phase. G) Boxplots showing the RNA polymerase occupancy (measured by ChIP-chip) of promoters belonging to genes that are reduced  $\geq 2$ -fold in  $\Delta ssrS$ /wild-type, genes that are not differentially expressed in  $\Delta ssrS$ , and genes that are increased  $\geq 2$ -fold in  $\Delta ssrS$ /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.

Figure 5

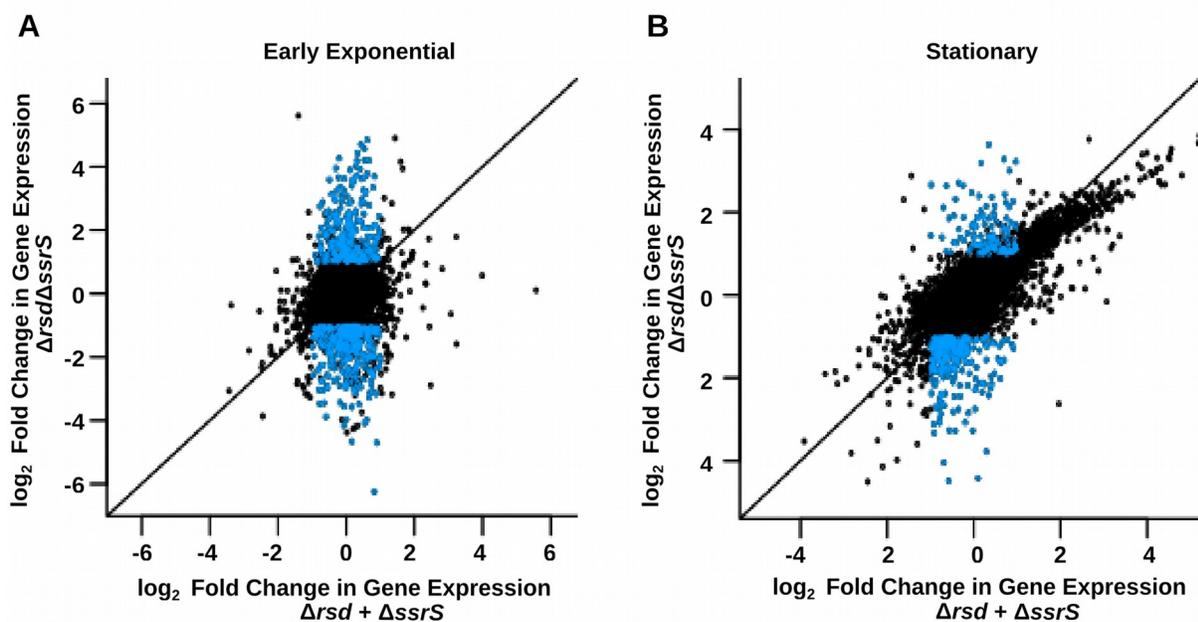


Figure 5: Scatterplots of log<sub>2</sub> fold change in gene expression in the  $\Delta rsd\Delta ssrS$  double knockout versus the sum of log<sub>2</sub> fold changes in the  $\Delta rsd$  and  $\Delta ssrS$  single knockouts, for each gene, in A) Early exponential and B) Stationary phase. Blue points represent genes that show differential expression ( $\geq 2$ -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.

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Figure 6

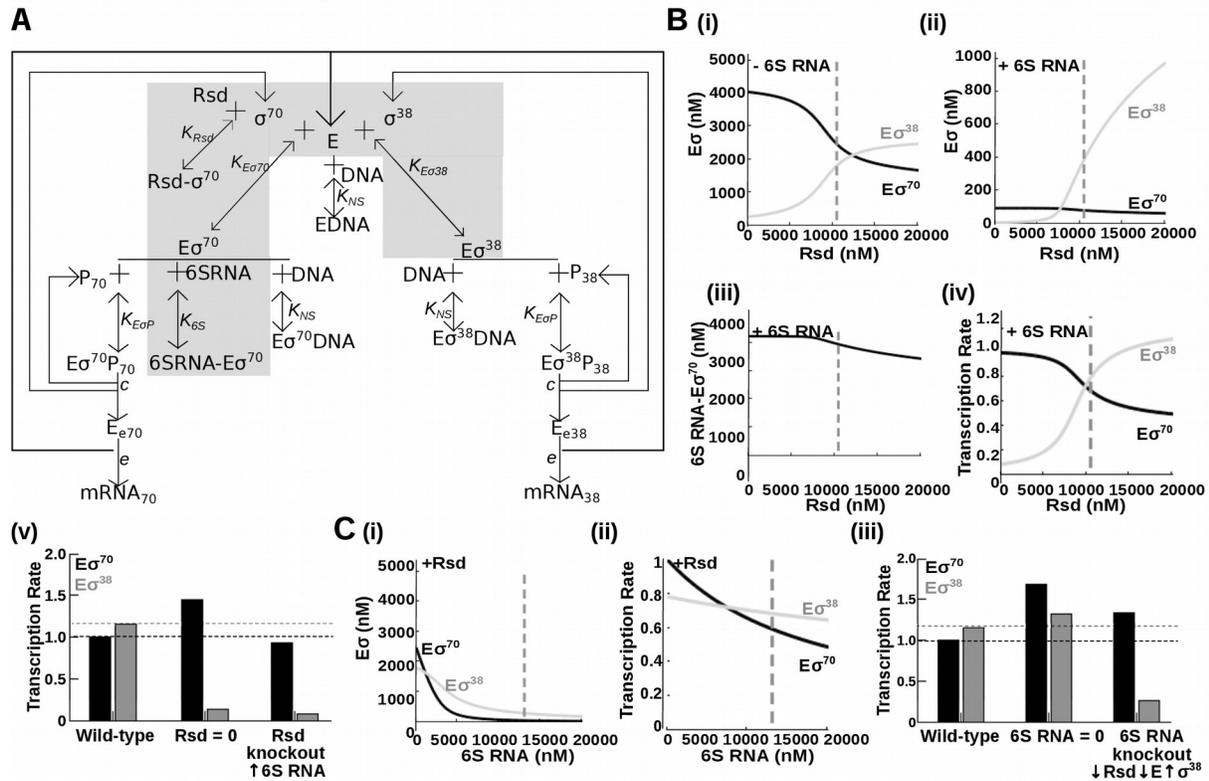


Figure 6: A) Schematic of the model. Shaded area represents reactions involved in holoenzyme formation (without DNA) B) (i) Steady-state levels of  $E\sigma^{70}$  (black) and  $E\sigma^{38}$  (gray), computed from equations (1) - (9), as a function of total Rsd, when 6S RNA = 0. (ii) Same when total 6S RNA = 13  $\mu$ M. (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.

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731 **Supplementary Material Legends**

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

736

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

744

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

748

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

751

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

759

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

763

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

767

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

772

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

775

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

784

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

792

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

801

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

807

808 Figure S14: A schematic showing various pathways by which Rsd and 6S RNA have been  
809 proposed to regulate RNA polymerase. Bold lines represent sequestration into inactive  
810 complexes. Blue lines represent pathways which are affected only in the Rsd-6S RNA double  
811 knockout. The three arrows connecting Rsd and 6S RNA show that Rsd inhibits expression of  
812 6S RNA (in stationary phase) and activates it (in ME phase), while 6S RNA increases expression  
813 of Rsd. The question mark indicates the possibility that 6S RNA is autoregulatory.

- 814 Table S1: Strains and plasmids used in this study
- 815 Table S2: Primers used for knockouts
- 816 Table S3: Primers used for flag-tagging
- 817 Table S4: Primers used for detection of knockouts
- 818 Table S5: Summary of RNA-Seq
- 819 Table S6: Primers used for qRT-PCR validation
- 820 Table S7: Results of qRT-PCR validation
- 821 Table S8: Primers used for cloning
- 822
- 823 Text S1: Selection of model parameters
- 824 Text S2: Differential Equations for Model