The genome-wide transcriptional response to varying RpoS levels in *Escherichia coli* K-12.

Garrett T. Wong[1,2], Richard P. Bonocora[3], Alicia N. Schep[1,4], Suzannah M. Beeler[1,5], Anna Lee[1,6], Lauren Shull[1,7], Lakshmi Batachari[1], Moira Dillon[1], Ciaran Evans[8,9], Johanna Hardin[8], Joseph T. Wade[3,10,#], and Daniel M. Stoebel[1,#]

1 Department of Biology, Harvey Mudd College, Claremont CA

2 Current address: Departments of Neuroscience, Genetics and Genome Sciences, Icahn School of Medicine at Mount Sinai.

3 Wadsworth Center, New York State Department of Health, Albany NY

4 Current address: Department of Genetics, Stanford University

5 Current address: Division of Biology and Biological Engineering, California Institute of Technology

6 Current address: a-tek, Washington DC

7 Current address: Sackler School of Graduate Biomedical Sciences, Tufts University

8 Department of Mathematics, Pomona College, Claremont CA

9 Current address: Department of Statistics, Carnegie Mellon University

10 Department of Biomedical Sciences, School of Public Health, University at Albany, SUNY, Albany NY

22 # Address correspondence to Joseph T. Wade, joseph.wade@health.ny.gov, or Daniel

23 M. Stoebel, stoebel@g.hmc.edu

24

25 Running title: Effect of RpoS concentration on *E. coli* transcriptome

26

Abstract

The alternative sigma factor RpoS is a central regulator of a many stress responses in *Escherichia coli*. The level of functional RpoS differs depending on the stress. The effect of these differing concentrations of RpoS on global transcriptional responses remains unclear. We investigated the effect of RpoS concentration on the transcriptome during stationary phase in rich media. We show that 23% of genes in the *E. coli* genome are regulated by RpoS level, and we identify many RpoS-transcribed genes and promoters. We observe three distinct classes of response to RpoS by genes in the regulon: genes whose expression changes linearly with increasing RpoS level, genes whose expression changes dramatically with the production of only a little RpoS ("sensitive" genes), and genes whose expression changes very little with the production of a little RpoS ("insensitive"). We show that sequences outside the core promoter region determine whether a RpoS-regulated gene in sensitive or insensitive. Moreover, we show that sensitive and insensitive genes are enriched for specific functional classes, and that the (in)sensitivity of a gene to RpoS corresponds to the timing of induction as cells enter stationary phase. Thus, promoter sensitivity to RpoS is a mechanism to coordinate specific cellular processes with growth phase, and may also contribute to the diversity of stress responses directed by RpoS.

Importance

The sigma factor RpoS is a global regulator that controls the response to many stresses in *Escherichia coli*. Different stresses result in different levels of RpoS

production, but the consequences of this variation are unknown. We describe how changing the level of RpoS does not influence all genes equally. The cause of this variation is likely the action of transcription factors that bind the promoters of the genes. We show that the sensitivity of a gene to RpoS levels explains the timing of expression as cells enter stationary phase, and that genes with different RpoS sensitivities are enriched for specific functional groups. Thus, promoter sensitivity to RpoS is a mechanism to coordinate specific cellular processes in response to stresses.

Introduction

Genome-wide measurements of RNA levels have revolutionized our understanding of how cells organize their patterns of transcription. These studies have given us snapshots of how patterns of gene expression change in response to changes in the external environment. They have also allowed us to define the regulons controlled by specific transcription factors. A major weakness of the vast majority of these studies is that they explore the function of a regulatory protein only by comparing expression of target genes in a wild-type strain to a gene knock-out, or to a mutant with a single diminished or increased level of activity. While some genetic regulatory networks are certainly switch-like (1, 2) and can be fully characterized by only two levels of activity, many other regulatory proteins vary continuously in their abundance and/or activity. How regulons respond to a range of regulator levels is a largely unstudied question (3). Should we expect all genes in a regulon to increase or decrease expression by the same relative amount following a change in abundance of a regulatory protein? Or

72 should we expect genes to respond in different ways? These questions motivate this
73 study.

74 A paradigmatic example of a bacterial regulatory protein whose abundance and
75 activity vary continuously in response to different conditions is the alternative sigma
76 factor RpoS in *Escherichia coli*. Transcription by RNA polymerase containing RpoS is
77 responsible for the general stress response (4–6). Under conditions of optimal growth in
78 the laboratory (such as exponential phase growth in rich media at 37°C), RpoS levels
79 are nearly undetectable in the model *E. coli* strain K-12. As conditions become poorer
80 for growth, either because cells begin to starve for various nutrients, or because they
81 face physical challenges such as low temperature or elevated osmolarity, RpoS levels
82 rise (4–6). RpoS coordinates the transcription of genes that are critical for the response
83 to these stresses.

84 RpoS expression is not an all-or-none phenomenon. For example, RpoS levels rise
85 continuously as cells transition from exponential growth to stationary phase (7).
86 Moreover, starvation for different nutrients upregulates RpoS to differing levels (8, 9).
87 This level of control over RpoS levels is accomplished by regulating transcription,
88 translation, and degradation (4, 7, 10), allowing for careful control over protein levels. In
89 addition to regulation of protein abundance, RpoS activity can also be directly
90 modulated by a number of factors, such as Crl (11).

91 Not only do RpoS levels vary across conditions for a single strain, but different
92 strains of *E. coli* also differ in their patterns of expression of RpoS. For example,
93 naturally occurring strains can differ in the amount of RpoS they produce during

94 exponential phase (12) or stationary phase (13). All studies that have measured RpoS
95 levels in naturally occurring strains of *E. coli* have detected variation (13–17), though the
96 extent and cause of this variation in RpoS between strains is still a matter of some
97 controversy (16, 17).

98 Microarray studies (18–20) have shown that RpoS controls the expression of at least
99 500 genes (over 10% of the genome) either directly or indirectly, but the set of RpoS-
100 regulated genes differs across environmental conditions. For most RpoS-controlled
101 genes, it is not known whether the gene is directly transcribed by RpoS or regulated
102 indirectly, as a consequence of RpoS transcribing other genes. Previous studies have
103 not investigated the impact of changes in RpoS levels on the RpoS regulon, or whether
104 quantitative differences in RpoS levels between environmental conditions influence the
105 observed differences in what genes are RpoS-regulated. It is clear that *E. coli* has a
106 complicated regulatory network to fine-tune RpoS levels to different conditions, but we
107 do not yet know the consequences of this regulation.

108 In this study, we tested the hypothesis that members of the RpoS regulon vary in
109 their response to RpoS levels. Using a combination of ChIP-seq and RNA-seq, we
110 identified RpoS-regulated genes, and we showed that genes vary in their sensitivity to
111 RpoS levels in a manner dependent on sequences outside of the core promoter region.
112 Sensitivity of genes to RpoS levels corresponds to the order in which genes are induced
113 during the transition into stationary phase, and genes with different levels of sensitivity
114 are enriched for specific functional groups. Thus, the level of sensitivity of genes to
115 RpoS controls the physiological response to different stress conditions.

116 **Materials and Methods**
117

118 *Culture conditions*

119 Cells were grown in 5mL of LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl) in
120 150 x 18 mm tubes shaking at 225 rpm at 37°C in a water bath unless otherwise
121 specified in the text. When required, antibiotics were used at the following final
122 concentrations: ampicillin at 100 *μ*g/ml (for plasmids) or 25 *μ*g/ml (for chromosomal
123 integration); chloramphenicol at 20 *μ*g/ml; kanamycin at 50 *μ*g/ml.

124

125 *Strains and genetic manipulations*

126 Strains and plasmids used in this study are listed in Table S1. The wild-type genetic
127 background for this all experiments except for ChIP-seq is BW27786, a strain designed
128 to give a graded transcriptional response to increasing arabinose concentration (21). To
129 create a strain of this background lacking *rpoS*, the *ΔrpoS746::kan* allele of JW5437
130 (22) was moved by P1 transduction into BW27786, creating strain DMS2545.

131 The arabinose-inducible RpoS strain was created by PCR amplifying the *kan* gene
132 and the P_{araB} promoter of plasmid pAH150 (23) using primers ParaBRpoSRecomb-F
133 and ParaBRpoSRecomb-R (Table S2). This PCR product was then integrated into the
134 *nlpD* gene (i.e. 5' of *rpoS*) in a MG1655 background using plasmid pKD46 (24), and P1
135 transduced into BW27786, creating strain DMS2564. This strain thus lacks both the
136 native transcriptional and translational control of RpoS.

137 For flow cytometry experiments, plasmid pDMS123 (25), which contains a *otsB-gfp*
138 transcriptional fusion, was transformed into DMS2564 by the method of Groth et al. (26).

139 ChIP-seq experiments used strain RPB104, an unmarked derivative of MG1655 that
140 expresses a C-terminally SPA-tagged derivative of RpoS from its native locus. This
141 strain was constructed by P1 transduction of kan^R-linked *rpoS*-SPA from a previously
142 described strain (27). The kan^R cassette was removed using the pCP20 plasmid that
143 encodes Flp recombinase (24).
144
145 *Construction and chromosomal integration of lacZ fusions*
146 The *gadB* and *astC* plasmids were built by standard cloning methods. The *gadC*
147 and *astA* promoter regions with transcription factor binding sites were PCR-amplified
148 with primers gadCpromoter +/- and astApromoter +/- (Table S2), which included *Kpn*I
149 and *Eco*RI restriction sites for cloning. Cloning of core promoter regions was performed
150 by annealing oligonucleotides designed to contain the whole RpoS binding region, as
151 predicted by Fraley *et al.* (28) and Castanie-Cornet and Foster (29). Oligonucleotides
152 were annealed by heating 1 μM of forward and reverse primer for one minute at 100°C
153 with 5 mM $MgCl_2$ and 7 mM Tris-HCl and then cooling slowly to room temperature.
154 Inserts and plasmid (pLFX) were digested with *Eco*RI-HF and *Kpn*I-HF (NEB), ligated
155 with T7 ligase (NEB) and cloned into strain BW23473. Transformants were miniprepped
156 and inserts were verified by Sanger sequencing.
157 The *gadA* and *hdeA* plasmids were built using Gibson assembly (30) with the
158 NEBuilder HiFi Assembly kit (New England Biolabs). The *gadA* and *hdeA* promoter
159 regions were PCR amplified with primers hdeAHiFi+/- and gadAHiFi+/-. The core
160 promoter was cloned with the single long oligo hdeAcoreHiFi or gadAcoreHiFi, as

161 predicted by Arnqvist et al (31) and De Biase et al (32). PCR products or
162 oligonucleotides were mixed with pLFX digested with *Kpn*I-HF and *Eco*RI-HF, and
163 assembled according to the manufacturers' instructions. Mixtures were cloned into
164 strain BW23473, transformants were miniprepped, and inserts were verified by Sanger
165 sequencing.
166 *lacZ* fusion plasmids were integrated into strain DMS2564 with helper plasmid
167 pPFINT (33). Single-copy integrants were confirmed using the PCR assay of Haldimann
168 and Wanner (23).
169
170 *Quantitative Western Blotting*
171 Quantitative western blotting was used to measure RpoS levels. Cells were
172 inoculated from frozen cultures into 5 mL of LB, and grown overnight at 37 °C, shaken
173 at 225 rpm. 5 μL of this overnight culture was diluted into 5 mL of LB with the
174 appropriate concentration of arabinose, and grown for 20 h. 100 μL of overnight culture
175 to be assayed was centrifuged and resuspended in 1X Laemmli sample buffer (Sigma-
176 Aldrich) and boiled for 5 minutes. Samples were diluted 1:10 in 1X Laemlli, and 10 μL
177 was electrophoresed on a 10% polyacrylamide gel (Bio-Rad) in tris-glycine running
178 buffer (25 mM Tris base, 250 mM glycine, 0.5% SDS) at 100 V for 90 min at room
179 temperature. Proteins were transferred to an Immobilon-FL PVDF membrane by
180 electrophoresis at 100 V for 45 min at 4 °C in transfer buffer (48 mM Tris base, 39 mM
181 glycine, 20% methanol, and 0.0375% SDS). Membranes were blocked by overnight
182 incubation in Odyssey blocking buffer (Li-Cor) at 4 °C.

183 The blocked membrane was probed with affinity-purified monoclonal antibodies
184 to RpoS (clone 1RS1) and RpoD (clone 2G10) (NeoClone) at a final concentration of 0.4
185 μg / mL in Odyssey blocking buffer plus 0.2% Tween 20 for 1 hour at room temperature.
186 The membrane was washed four times for five minutes each with 15 mL of 1X TBST. A
187 fluorescent secondary antibody (IRDye 800CW goat anti-mouse, Li-Cor) was diluted
188 1:10,000 in a solution of Odyssey Blocking Buffer plus 0.2% Tween 20 and 0.01% SDS
189 and incubated in the dark for 1 hour at room temperature. The membrane was washed
190 as before, dried for 2 hours between sheets of Whatmann 3MM blotting paper, and
191 imaged on a LiCor Clx fluorescent imager.
192 Band intensity was estimated using Image Studio 2.1 (LiCor). RpoS levels were
193 divided by RpoD levels to normalize for differences in total protein levels. The ratio of
194 RpoS to RpoD is biologically meaningful because this ratio, rather than RpoS level
195 alone, dictates levels of transcription from RpoS-dependent promoters due to sigma
196 factor competition (34).
197
198 *RNA-seq experiments and analysis*
199 Cells were inoculated from frozen cultures into 5 mL of LB, and grown overnight. 5
200 μL of this overnight culture was diluted into 5 mL of LB and grown for 20 hours. (For the
201 26% RpoS condition, a final concentration of 10^{-4}% arabinose was also added.) RNA
202 was purified from 200 μL of overnight culture by pelleting and resuspending in 500 μL of
203 Trizol at 65 °C, followed by purification on a column (Direct-Zol, Zymo Research).
204 Samples received two 30-minute DNase treatments using TURBO DNA-free (Ambion)

205 following the manufacturer's instructions. RNA samples were then purified on a column
206 (RNA Clean & Concentrator, Zymo Research). Samples were stored at -80 °C until
207 used. Three samples were prepped from each culture and pooled to generate sufficient
208 RNA. Two biological replicates were prepared for each strain or condition of interest.
209 rRNA depletion, cDNA synthesis, library preparation, and sequencing were performed
210 by a commercial provider (Otogenetics, Norcross, GA). Paired-end, 100 bp sequences
211 were generated for 7-15 million reads per sample.

212 Before reads were mapped, the first ten base pairs of each read were trimmed using
213 FASTX-Toolkit 0.0.13. Reads were mapped to the NCBI K12 reference genome
214 (NC_000913.2 Escherichia coli str. K-12 substr. MG1655) using BWA v0.7.5a (35).The
215 number of read pairs mapped to each gene was counted with HTSeq 0.6.1 (36).
216 Differential expression analysis was performed with DESeq v2.13 (37). All p-values
217 were first FDR adjusted using the procedure of Benjamini and Hochberg (38). p-values
218 were then further Bonferroni-adjusted for the three comparisons between pairs of RpoS
219 levels. All differential expression p-values reported in this paper reflect both the FDR
220 and Bonferroni-adjustments.

221 To determine if a gene differed significantly from the null expectation of linearity, we
222 calculated the probability of the observed read count value at 26% RpoS if the true
223 expression level was given by the linear prediction between the endpoints. Note that to
224 calculate both the expected read count and the probability of the observed value under
225 the null (i.e. the p-value) our model required estimating both the DEseq size factor (for
226 scaling) and the dispersion (a variance factor) for each of the samples. The negative

227 binomial probability model (routinely used to measure count data, (e.g. (37)) was used,
228 with the size factors and dispersion estimated from DESeq (37) to calculate the
229 probability of the observed read count at 26% RpoS.
230 GO term analysis was performed using the topGO package (39) together with the
231 org.EcK12.eg.db annotation package (40) in R 3.1.0. Enrichment was assessed using
232 the weight01 algorithm (39) together with the Fisher's-exact test. The GO hierarchy was
233 pruned to include only nodes with at least five associated genes, as significance tests
234 can be unstable for GO terms with fewer genes (39).
235 A Venn diagram of the number of significant genes in each condition was prepared
236 with EulerAPE 3.0.0 (41).
237
238 *ChIP-seq analysis*
239 *E. coli* strain RPB104 (MG1655 with C-terminally SPA-tagged *rpoS*) was grown
240 overnight in M9 minimal medium with 0.4% glycerol at 30 °C and then subcultured 1:100
241 in the same medium, and grown for 60 hours to saturation (OD_{600} of ~3). ChIP-seq
242 using the M2 monoclonal anti-FLAG antibody was performed as described previously
243 (42). Regions of enrichment ("peaks") were identified as described previously (43).
244 Relative enrichment was reported as a "Fold Above Threshold" (FAT) score.
245 We used MEME-ChIP (Version 4.11.2; default parameters) to analyze enriched
246 regions identified by ChIP-seq (44) Regions within 100 bp were merged. The reported
247 sequence motif was identified by MEME (Version 4.11.2) (45), run within the MEME-
248 ChIP environment.

249 We used MEME (45) within the MEME-ChIP environment (Version 4.11.2; default
250 parameters except that only the given strand was analyzed) to identify enriched
251 sequence motifs in regions surrounding TSSs associated with RpoS ChIP-seq peaks.
252 We analyzed sequences from -45 to +5 relative to each TSS.
253 We identified directly RpoS-transcribed genes by requiring that (i) the gene start is
254 within 300 bp of a ChIP-seq peak, (ii) the gene is positively regulated by RpoS, as
255 determined by RNA-seq, (iii) no other positively regulated gene starts within 300 bp of
256 the ChIP-seq peak, (iv) there is no associated sequence motif or TSS (identified in (46))
257 that would be consistent with transcription in the opposite orientation. For regulated
258 genes, we determined whether other genes in the same operon were also regulated,
259 using a published operon list (47) for *E. coli*. Peaks were associated with transcription
260 start sites from a previous study (46) by identifying transcription start sites within 20 bp
261 of a peak.
262
263 *QPCR*
264 RNA was isolated as for RNA-seq, except that samples underwent three 30-
265 minute DNase treatments using TURBO DNA-free (Ambion). cDNA was made from the
266 RNA samples using SuperScript VILO master mix (Invitrogen) and stored at -20 °C for
267 use in quantitative real-type PCR (qPCR) reactions.
268 qPCR was performed using Power SYBR Green master mix (Invitrogen), 2 μL of
269 cDNA, and primers at 300 nM. Cycling was performed at 95 °C for 10 min; and 40
270 cycles of 95 °C for 15 sec, 58 °C for 15 sec, and 68 °C for 30 sec. Three control genes

271 (*ftsZ, pgm,* and *hemL*) were used in addition genes of interest. These genes were
272 selected using the approach of Vandesompele et al. (48). Details of this selection
273 process, including the other seven genes tested, are available in Supplementary Text 1,
274 Table S3, and Figure S1.
275 For each gene, a standard curve was made using the following amounts of
276 genomic DNA: 200 ng, 20 ng, 2 ng, 200 pg, 20 pg, and 2 pg. Genomic DNA was
277 extracted from overnight cultures using a Puregene Kit (Qiagen), following the
278 manufacturer's instructions. Expression levels for each gene were interpolated from the
279 standard curve. Expression levels of experimental genes were divided by the geometric
280 mean of the three control genes.
281 Statistical assessment of sensitivity was performed by a bootstrapping approach.
282 Bootstrapping, rather than a parametric approach, was appropriate because the data
283 did not conform to parametric assumptions. In this approach, the unit of resampling was
284 the RNA isolated from the 0%, 26% and 100% RpoS conditions on an individual day.
285 For each resampled data set, the median expression at 0% and 100% RpoS was
286 calculated. The linear fit from the 0% RpoS median and the 100% RpoS median was
287 then used to predict the level of expression at the intermediate level of RpoS. Repeating
288 this re-sampling of the data 10,000 times yielded a 95% confidence interval for 26%
289 RpoS. The observed median for 26% was then compared to the confidence interval to
290 test for significance.
291
292 *Beta-galactosidase activity*

293 Beta-galactosidase activity was measured using the method of Miller (49). With
294 lacZ fusion strains, the level of sensitivity was quantified, rather than only categorized as
295 sensitive, linear, or insensitive. To quantify the level of sensitivity, for each replicate we
296 calculated the distance between the observed expression at the intermediate RpoS
297 concentration and the expected level based on a linear pattern, standardized by the
298 difference in expression between high and low RpoS conditions. Testing if sensitivities
299 were different from zero used a one-sample t-test, with p-values adjusted for multiple
300 comparisons using Holm's sequential adjustment method (50). Testing if two
301 sensitivities were different used a two-sample t-test with the same method of adjustment
302 for multiple comparisons.
303
304 *Analysis of published RNA-seq data*
305 We analyzed published RNA-seq data for wild-type and Δ*rpoS E. coli* over a
306 time-course of growth into stationary phase (47). Using normalized genome coverage
307 information extracted from wiggle files, we calculated relative abundance for all genes at
308 each of the four stationary phase time-points in the growth curves (the last four time-
309 points for each strain). We arbitrarily selected a threshold coverage value of 500 and
310 excluded any genes scoring below this threshold at all four time-points in wild-type cells.
311 This reduced variability associated with low expression levels. We excluded any genes
312 for which coverage at the final time-point was 0 since this would have prevented
313 normalization. We also excluded any gene for which the first stationary phase time-point
314 had the highest expression value of the four time-points for wild-type cells, since RpoS-

315 dependent expression of these genes is likely to be masked by other factors. We then
316 selected genes whose expression we had found to be induced by RpoS, and separated
317 these genes into insensitive, linear, and sensitive classes. We calculated expression
318 levels for each of these genes relative to expression at the final time-point.
319
320
321 **Results**
322
323 *The RpoS regulon in late stationary phase.*
324 To understand the role of the RpoS protein in late stationary phase, we used RNA-
325 seq to compare the transcriptome of wild-type and Δ*rpoS* cells. We observed differential
326 expression of 1044 genes (23% of genes) between these two conditions ($p < 0.05$). Of
327 the 1044 genes whose expression is influenced by RpoS, 605 are upregulated, and 439
328 are downregulated (Table S4).
329 Influencing transcription of 23% of the genome could have many potential
330 phenotypic effects. To better understand the function of these genes, we examined
331 which kinds of gene functions, as described by the gene ontology (GO), are more
332 abundant in the regulon than expected by chance. GO enrichment analysis indicates
333 that the RpoS regulon includes many genes involved in metabolic processes (Table 1);
334 17 of 18 significantly enriched GO terms are metabolic terms. This metabolic
335 reorganization includes the upregulation of genes encoding glycolytic enzymes and
336 pathways for metabolism of L-arginine to glutamine, or from L-arginine to putrescine and
337 then into succinate. RpoS also drives the downregulation of genes involved in the TCA

338 cycle. These patterns of metabolic regulation are very similar to those identified in late
339 stationary phase in *Salmonella enterica* (51). The only significant GO term not explicitly
340 linked to metabolism (GO:0006970, “response to osmotic stress”) also includes
341 metabolic genes, such as *otsA* and *otsB,* that are involved in trehalose biosynthesis.
342 Central metabolism is not the only phenotype similarly regulated by RpoS in both *S.*
343 *enterica* and *E. coli*. Other similarities include transcription of genes involved in
344 antioxidant activities, iron regulation, and Fe-S cluster assembly, the upregulation of
345 proteases, and down regulation of porins. As in *S. enterica,* RpoS in *E. coli* influences
346 the expression of many genes encoding other regulatory proteins, including *csrA*, *arcA,*
347 *cra, fur, ihfA, hupA* and *hupB.* Not all of the regulation is identical between *E. coli* and *S.*
348 *enterica,* however. For example, Lévi-Meyrueis *et al.* (51) noted that RpoS appears to
349 direct switching between many pairs of isozymes. While some pairs show a similar
350 pattern in *E. coli* (such as *tktA/B* and *acnA/B*), others (such as *fumA/fumC*) do not show
351 this pattern.
352
353 *Genome-wide binding profile of RpoS*
354 While RNA-seq identifies which genes are regulated by RpoS, it cannot distinguish
355 between direct and indirect effects. To determine sites where RpoS binds (and hence
356 likely plays a direct role in transcription), we used ChIP-seq to map the association of
357 RpoS across the *E. coli* chromosome during stationary phase growth in minimal medium.
358 To facilitate ChIP, RpoS was C-terminally SPA-tagged at its native locus. We reasoned
359 that RpoS would only be identified with promoter regions since it is likely released from

360 elongating RNAP complexes. We identified 284 peaks of RpoS ChIP-seq signal
361 covering 260 genomic regions (peaks within 100 bp of each other were merged). 217 of
362 the RpoS-bound regions are intergenic, and 67 are located within genes. We reasoned
363 that annotated genes that are transcribed by RpoS would be positioned close to an
364 RpoS-bound region. Consistent with this, 213 RpoS-bound regions are ≤300 bp
365 upstream of an annotated gene start. These 213 regions include 27 that are intragenic.
366 In 79 cases, we observed an RpoS-bound region ≤300 bp from the starts of two
367 divergently transcribed genes.
368 We used MEME to search for enriched sequence motifs within the RpoS-bound
369 regions. We detected a highly enriched motif in 107 regions; this motif closely
370 resembles the known -10 hexamer recognized by RpoS (Figure 1A). Moreover,
371 occurrences of the motif are positionally enriched with respect to the ChIP-seq peak
372 center (Figure 1B), indicating that the data have high spatial resolution. Note that the
373 motifs tend to be located just upstream of the peak centers (Figure 1B), as we have
374 observed previously for the *E. coli* flagellar Sigma factor, FliA (43). This presumably
375 reflects the fact that the footprint of initiating RNA polymerase associated with RpoS is
376 not centered on the -10 hexamer.
377 The identification of RpoS binding sites allowed us to better understand the role of
378 RpoS in both positive and negative regulation. While similar proportions of the RpoS
379 regulon are positively and negatively regulated by RpoS, it is not clear if this is true at
380 the level of direct regulation. Only 19 genes within 300 bp of a binding site are
381 negatively regulated by RpoS. 19 of the 286 such genes is *fewer* than we would expect

382 by chance if binding sites were randomly distributed around the genome, given than 439
383 of 4513 genes in the genome are negatively regulated. On the other hand, 111 of 286
384 genes within 300 bp are positively regulated, a highly significant effect (Fisher's exact
385 test, $p < 10^{-16}$). Thus, the binding profile of RpoS is consistent with direct positive
386 regulation of many genes, but provides no evidence of direct negative regulation.
387
388 *Identification of RpoS-transcribed genes and promoters*
389 We combined the ChIP-seq and RNA-seq data to identify genes that are directly
390 transcribed by RNA polymerase containing RpoS. Thus, we identified 123 RpoS-
391 transcribed genes in 99 transcripts (Table S5), and compared them to other published
392 analyses (18–20, 46, 52–55). In some cases, we identified RpoS-bound regions
393 upstream of genes that were not detected as being RpoS-regulated by RNA-seq. These
394 genes may have promoters that bind transcriptionally inactive RNA polymerase (56, 57).
395 Alternatively, the disparity between RpoS binding and regulation could be explained by
396 differences in growth conditions between the ChIP-seq and RNA-seq experiments, or by
397 the possibility that the C-terminal SPA tag affects the function of the C-terminus of the
398 protein in the response to transcription activators (56, 58).
399 The high spatial resolution of sigma factor ChIP-seq can facilitate the
400 identification of specific promoters (43, 59) when combined with nucleotide resolution
401 transcription start site (TSS) maps. Using published TSS data from *E. coli* under
402 stationary phase conditions similar to those used for the ChIP-seq experiment (46), we
403 determined all pairwise distances between RpoS ChIP-seq peaks and stationary phase

404 TSSs. We observed a strong enrichment for peak-TSS distances ≤20 bp (Figure S2).
405 We infer that these 112 TSSs are RpoS-transcribed. Consistent with this, the putative
406 RpoS-transcribed TSSs are associated with -10 hexamers that have features expected
407 of RpoS promoters (Figure 1C). In some cases, RpoS promoters were identified ≤300
408 bp upstream of genes that were not RpoS-regulated in the RNA-seq data set. We
409 presume that these are RpoS-transcribed genes that otherwise escaped detection
410 because of differences in the growth conditions used for ChIP-seq and RNA-seq.
411
412 *The transcriptome at three different RpoS levels*
413 This first view of the RpoS regulon considers RpoS as either present or absent.
414 RpoS levels vary continuously across environmental stresses (7), so we sought to better
415 understand how the level of RpoS in the cell influences transcription of target genes. To
416 do this, we placed the *rpoS* gene under the control of the arabinose-inducible promoter
417 P_{araB}. This promoter was integrated just upstream of the native *rpoS* gene, placing
418 transcription under the control of arabinose concentration and removing the 5' region
419 that regulates translation of the native mRNA (4).
420 To measure the resulting arabinose-induced expression of RpoS, we employed
421 quantitative western blotting. RpoS levels increased with increasing arabinose
422 concentration, from undetectable RpoS levels, to levels similar to those in wild-type cells
423 (Fig 2a). To confirm that expression was graded and not all-or-none in this system (21),
424 we used flow cytometry to measure expression in individual cells. We transformed the
425 arabinose-inducible RpoS strain with the plasmid pDMS123 (25), which contains the

426 RpoS-dependent *otsBA* promoter fused to *gfp*. As expected, *gfp* expression increased
427 with increasing arabinose concentrations, and at each expression level the population
428 was unimodal (Fig 2b).
429 We measured the transcriptome in cells with 26% of wild-type levels of RpoS,
430 achieved with the addition of 10^{-4} % arabinose to cells with our arabinose-inducible *rpoS*
431 strain. Of the genes that are differentially expressed between 26% RpoS and either 0%
432 (ΔrpoS) or 100% (wild-type), 95% are also differentially expressed between 0% and
433 100% (Fig 3) ($p < 0.05$).
434 Nearly all genes that are significantly differentially expressed have monotonically
435 increasing or decreasing patterns of expression between three levels of RpoS. Only
436 two genes (*ytfR* and *ytfT*) have an expression level at 26% rpoS that is significantly
437 higher than the expression at both 100% and 0% RpoS. The only two genes with
438 expression lower in the 26% RpoS condition than either 100% or 0% are *nlpD* and *pcm*.
439 These genes lie immediately upstream of *rpoS. nlpD* was removed from the genome
440 during the construction the arabinose inducible RpoS strain. The *pcm* gene is still
441 present, but the level of transcription was lowered by the genetic modification.
442
443 *Classifying quantitative responses to RpoS levels*
444 To explore how genes respond to changing levels of RpoS, we developed a new
445 metric, *sensitivity.* Our null expectation was that gene expression would increase
446 linearly with increasing RpoS concentration. We observed many genes in our RNA-seq
447 data set (such as *osmY*) whose expression at intermediate RpoS levels falls on, or

448 close, to a line drawn between the 0% and 100% RpoS conditions (Fig 4a). We refer to
449 these genes as *linear* in their response to increasing RpoS levels. Other genes (such as
450 *astA*) are transcribed more at 26% than would be expected based on their expression
451 levels at 0% and 100% (Fig 4b). We refer to these genes as *sensitive*, because only a
452 small amount of RpoS results in relatively high levels of transcription. In contrast, some
453 genes (like *gadC*) are expressed at intermediate RpoS levels less than expected based
454 on expression at 0% and 100% RpoS; such genes are referred to as *insensitive* (Fig 4c).

455 We identified 910 linear, 102 sensitive, and 32 insensitive genes. 96% of sensitive
456 genes and 88% of insensitive genes are positively regulated by RpoS. In contrast, only
457 53% of linear genes are positively regulated by RpoS, a significant difference (chi-
458 square test, $p < 0.001$).

459 To determine whether sensitive or insensitive (hereafter *(in)sensitive*) genes are
460 associated with specific physiological responses to increasing RpoS levels, we again
461 used GO enrichment. We tested the null hypothesis that the functions of these genes
462 are a random sample from the entire RpoS regulon (not the whole genome). The GO
463 terms significantly enriched in the sensitive class are response to osmotic stress,
464 cellular amino acid catabolic process, and fatty acid oxidation (Table 2). Several genes
465 encoding regulators are among the sensitive genes, including *arcA*, which encodes a
466 global regulator of respiratory metabolism, and *rssB,* which encodes the adaptor protein
467 required for degradation of RpoS by ClpXP.

468 GO enrichment is less useful for understanding the possible function of the
469 insensitive gene set. Three GO terms are enriched (Table 3), but only a few genes with

470 these annotations are present in the insensitive gene set, and their enrichment probably
471 reflects the relatively small number of insensitive genes. More strikingly, the insensitive
472 genes include nearly all of the genes required for acid resistance system 2: the
473 structural genes *gadA, gadB,* and, *gadC*, and the regulator of this system *gadE* (60, 61).
474 In addition, the genes *yhiM, yhiD*, *hdeA, hdeB , hdeD, mdtE, mdtF,* all of which have
475 been described as having roles in acid resistance (60, 61), are insensitive.
476 We used reverse transcription coupled to qPCR to confirm the expression
477 patterns of two insensitive genes (*gadC* and *gadE*), and three sensitive genes (*prpR,*
478 *prpD,* and *astA*). All genes were positively regulated by RpoS (Fig 5), and the median
479 expression at 26% RpoS is consistent with RNA-seq expression patterns for all genes.
480 We used a bootstrapping approach to assess if expression at 26% was significantly
481 above or below the linear expectation at 26% RpoS. The expression of *gadC* was
482 significantly insensitive ($p < 10^{-4}$), and *prpR* expression was significantly sensitive ($p <$
483 10^{-4}). *astA* expression was marginally significantly sensitive ($p = 0.06$ for sensitivity),
484 while *gadE* and *prpD* were not significantly different from the linear expectation ($p =$
485 0.10 and $p = 0.56$ respectively).
486
487 *Control of (in)sensitivity of expression*
488 What makes one promoter sensitive to RpoS levels and another promoter insensitive
489 to RpoS levels? We hypothesized three possible mechanisms. First, chromosomal
490 location could determine the response to RpoS levels, as is known to occur in the
491 context of total transcription levels (62). Second, it is possible that the DNA sequence of

492 the core promoter drives the response. Finally, it is possible that the binding of
493 transcription factors upstream of the core promoter influences the response to RpoS
494 levels. To test these hypotheses, we cloned the promoters (including all upstream
495 transcription factor binding sites annotated in EcoCyc (63)) of four operons into the *lacZ*
496 fusion plasmid pLFX (33). The four promoters were the sensitive *astCADBE*, and the
497 insensitive *gadA, gadBC,* and *hdeAB-yhiD.* Plasmid pLFX recombines into the lambda-
498 attachment site, placing the fusion in a novel genomic context. While we did not detect
499 binding of RpoS upstream of *astC, gadA, gadB,* or *hdeA* by ChIP-seq, this is likely due
500 to the difference in growth conditions, since these genes have been previously shown to
501 be directly transcribed by RpoS (31, 64, 65).
502 The pattern of transcription of all four fusions was the same as observed for the
503 respective genes in the RNA-seq data (Fig 6 a-d). *astC* transcription was sensitive to
504 RpoS levels (one sample t-test, $p = 0.04$), while *gadA, gadB*, and *hdeA* transcription
505 were all insensitive (one sample t-test, $p = 2 \times 10^{-6}$, $p = 10^{-5}$, $p = 0.04$ respectively).
506 Since all reporters were placed at the same genomic locus, this result suggests that
507 genomic location is not the determinant of response to RpoS levels.
508 A second potential mechanism to explain the difference between sensitive and
509 insensitive genes is interactions between RpoS and the core promoter sequence. For
510 example, specific nucleotides (or combinations of nucleotides) might tend to confer
511 (in)sensitive patterns of transcription. The majority of both sensitive and insensitive
512 genes were not associated with RpoS-bound regions in the ChIP-seq experiment,
513 suggesting that they are indirectly regulated by RpoS (Table 4). The fact that most

514 sensitive and insensitive genes are not bound by RpoS argues against the hypothesis of
515 direct RpoS-DNA interactions driving (in)sensitivity. To see if specific sequence motifs
516 were consistently associated with (in)sensitivity, we used the discriminative motif search
517 feature of DREME (66) to search for motifs that differed between sensitive and linear, or
518 insensitive and linear regulatory sequences. There are 28 ChIP-seq peaks associated
519 with an operon with at least one sensitive gene, and 4 ChIP-seq peaks associated with
520 an operon with at least one insensitive gene. We found no motifs that distinguished
521 these sets of sequences, although the small number of sequences would have
522 restricted the power of such a test.

523 To directly test the hypothesis that the core promoter region of RpoS-transcribed
524 genes is responsible for determining the sensitivity to RpoS levels, we cloned this short
525 region from the *astC, gadA, gadB, hdeA* promoters into pLFX and recombined the
526 plasmids into the chromosome. These core promoters had absolute levels of
527 transcription much lower than the entire promoter that included upstream transcription
528 factor binding sites (Fig 6e - h). The core promoter sequence for *astC* alone was
529 somewhat less sensitive to RpoS levels than the full-length construct (two sample t-test,
530 $p = 0.08$), although not significantly so, probably due to the variability of expression from
531 the full-length *astC* reporter. The *gadA* and *gadB* core promoters differed significantly
532 from their full-length promoters (two sample t-test, $p= 0.01$ and $p < 10^{-6}$, respectively).
533 The *hdeA* core promoter (Fig 6h) is not RpoS-dependent, showing a decline in
534 expression of approximately 10% in the presence of RpoS. This is consistent with the
535 previous finding that the ability of RpoD (but not RpoS) to transcribe *hdeA* is repressed

536 by H-NS protein bound upstream of the promoter (67). The core promoter construct
537 lacks the native H-NS binding sites upstream, and so the selectivity is apparently lost.
538 Thus, the core promoters do not replicate the RpoS sensitivity of their whole promoter
539 sequences. In addition, the three RpoS-dependent core promoters (Fig 6 e - g) do not
540 differ from each other in sensitivity (ANOVA, $p = 0.32$). We conclude that the core
541 promoter is not responsible for RpoS sensitivity.
542
543 *Sensitivity to RpoS determines the timing of induction during entry into stationary*
544 *phase*
545 We hypothesized that the degree of sensitivity to RpoS could impact the timing of
546 expression under conditions when levels of active RpoS increase, such as during entry
547 into stationary phase. A previous study used RNA-seq to monitor the transcriptome over
548 a time-course of growth, including four time-points in stationary phase (47). We
549 analyzed these data to determine whether insensitive, linear, and sensitive genes show
550 differences in the timing of induction. We selected only those RpoS-induced genes
551 whose transcription increased upon entry into stationary phase. (It total, there were 250
552 such linear genes, 90 sensitive, and 19 insensitive.) We then determined the pattern of
553 expression for each such gene over four time-points beginning at the onset of stationary
554 phase. Although there is considerable variability in the expression patterns of genes, as
555 a group the three classes show clear differences in the timing of induction (Figure 7a).
556 Specifically, sensitive genes are induced most rapidly, followed closely by linear genes.
557 In both cases, expression peaked early in stationary phase and fell between 30 and 180

558 minutes after entering stationary phase. In contrast, insensitive genes showed relatively
559 little change in expression until the final time-point, 180 minutes into stationary phase.
560 To determine the importance of RpoS on the patterns of gene expression, we repeated
561 the above analysis using data generated from a Δ*rpoS* strain. As expected, the large
562 difference in timing between the groups of genes was greatly diminished (Figure 7b).
563 We conclude that the (in)sensitivity of a gene is associated with the timing of expression
564 during stationary phase in an RpoS-dependent manner.
565

566 **Discussion**

567

568 *Expanding the known set of RpoS-transcribed genes and promoters*

569 The *E. coli* RpoS regulon has been widely investigated using targeted and genome-
570 scale approaches. Most genome-scale studies have focused on genes whose
571 expression is altered in the absence of RpoS (18–20, 53, 54). Hence, these studies
572 cannot distinguish between genes that are transcribed by RpoS, and those that are
573 indirectly regulated. ChIP-seq affords a high resolution view of RpoS binding. By
574 combining ChIP-seq with RNA-seq, we have identified 123 RpoS-transcribed genes with
575 high confidence, considerably expanding the known RpoS regulon. Previous studies
576 have suggested a role for RpoS in direct repression of some target genes (46, 56, 68).
577 While we observed negative regulation of 439 genes by RpoS, there were fewer of
578 these repressed genes associated with ChIP-seq peaks than expected by chance,
579 suggesting that direct negative regulation by RpoS is rare.

580 Only two previous studies have used ChIP methods to map RpoS binding genome-
581 wide in *E. coli*. The first used ChIP-chip to identify 868 RpoS-bound regions (46), many
582 more identified in our study but with considerably lower resolution (median peak length
583 of 324 bp for RpoS ChIP-chip). The second used ChIP-seq, but identified relatively few
584 RpoS-bound regions (52). Of the 63 RpoS-bound regions identified in that study, 41 are
585 shared with those from our study.
586 The high resolution of ChIP-seq allowed us to identify specific promoter sequences
587 recognized by RpoS. By combining ChIP-seq data with a TSS map, we identified many
588 high-confidence RpoS promoters. These promoters are strongly enriched for the
589 presence of a -10 hexamer, with sequence preferences consistent with several of the
590 previously described features of RpoS promoters (69). Specifically, we observed a
591 preference for a C at position -8 (within the -10 hexamer), a C at position -13
592 (immediately upstream of the -10 hexamer), and a TAA a positions -6 to -4 (immediately
593 downstream of the -10 hexamer). Previous studies have suggested that RpoS
594 promoters often contain a -35 hexamer (70), although the spacing relative to the -10
595 hexamer is considerably more variable than for σ^{70} promoters. However, we did not
596 detect enrichment of a -35 hexamer-like sequence among RpoS promoters, suggesting
597 that the requirement for this element is weak.
598
599 *Sensitivity to RpoS affects timing of expression, and groups functionally related*
600 *genes*

601 As is true for many transcription factors, RpoS levels vary continuously across a
602 wide range of conditions. Our data show that genes differ in the sensitivity of their
603 response to RpoS levels. Moreover, whether a gene is (in)sensitive to RpoS levels is
604 associated with its function, suggesting a physiological rationale for sensitivity. For
605 example, insensitive genes include many of those involved in the glutamate-dependent
606 acid resistance 2 system (AR2). These genes are of particular interest because AR2
607 allows *E. coli* to survive a pH of 2, an important trait for its ability to pass through the
608 stomach and colonize the gastrointestinal tract (71, 72). To some extent, the shared
609 sensitivity of functionally related genes can be explained by operon structure, i.e. co-
610 transcription of multiple functionally related genes from a single promoter. However, the
611 phenomenon of shared sensitivity for functionally related genes extends beyond
612 operons. For example, the insensitive genes involved in AR2 are transcribed from at
613 least five different promoters (63).
614 The fact that functionally related genes often have similar patterns of sensitivity to
615 RpoS suggests that sensitivity can serve as a mechanism to control the timing of gene
616 expression, and hence to coordinate specific cellular processes as part of a response to
617 environmental stresses. Consistent with this idea, we have shown that the sensitivity of
618 genes to RpoS levels correlates with the timing of their expression. RpoS sensitivity
619 may drive similar patterns of expression in response to other stresses. Different
620 environmental stresses are known to upregulate RpoS to varying levels (8), suggesting
621 that some insensitive genes may only be expressed under certain stresses. In addition
622 to the effects on gene expression, sensitivity to RpoS may also impact the effects of

623 mutations in *rpoS*, which have been seen to evolve in the lab (73, 74). We expect
624 mutations attenuating RpoS to have the strongest effect on insensitive genes, and the
625 weakest effect on sensitive genes.
626
627 *Possible mechanisms of RpoS sensitivity*
628 While the connection between RpoS sensitivity and the timing of gene expression is
629 clear, the molecular basis of sensitivity is less so. Our data indicate that the genomic
630 location of these operons does not determine the expression pattern. Moreover, several
631 lines of evidence suggest that direct interactions between RpoS and the core promoter
632 are also not responsible for determining sensitivity. First, analysis of the ChIP-seq data
633 for the sensitive and insensitive genes finds no motif that distinguishes between them.
634 Second, core promoters from both sensitive and insensitive genes do not replicate the
635 pattern of expression of the full-length promoters. Third, the core promoters of a
636 sensitive operon (*astC*) and two insensitive operons (*gadA* and *gadB*) have
637 indistinguishable patterns of sensitivity, suggesting that what was excluded from those
638 constructs (i.e., binding sites of regulatory proteins) determines the shape of the
639 relationship.
640 Given our finding that core promoter sequences cannot explain the difference in
641 (in)sensitivity between promoters, we suggest that sensitivity is largely due to the action
642 of specific regulatory proteins bound upstream. If this hypothesis is correct, it could also
643 explain the physiological coherence of these groups. For example, many insensitive
644 genes are involved in the AR2 phenotype and are also regulated by GadX, GadW, and

645 GadE (75–77). If one or more of these three regulators was directly responsible for the
646 insensitive pattern of expression, then this could help to explain the physiological
647 coherence of the insensitive group. The sensitive genes, being a larger group, have no
648 obvious single regulator, although relatively little is known about regulators that function
649 in stationary phase.

650 RpoS responds to a wide variety of environmental cues, and regulates genes
651 responsible for many different kinds of responses. This work has demonstrated that one
652 facet of that response, the level of RpoS produced, has varying effects across the
653 entirety of the regulon. The level of RpoS produced in a stress response, together with
654 the action of other transcription factors, may help to tune the RpoS-dependent stress
655 response in ways appropriate for individual stresses.

656

657 **Acknowledgements**

658 We thank Rachael Kretsch for experimental help, and Robert Drewell, Xuelin Wu,
659 Jae Hur, Keith Derbyshire and Todd Gray for helpful discussions. This work was
660 supported by HHMI Undergraduate Science Education award #52007544 to Harvey
661 Mudd College and by the NIH Director's New Innovator Award Program,
662 1DP2OD007188 (JTW).

663

664 **Literature Cited**

665 1. **Novick A**, **Weiner M**. 1957. ENZYME INDUCTION AS AN ALL-OR-NONE
666 PHENOMENON. Proc Natl Acad Sci U S A **43**:553–66.

667 2. **Ptashne M**. 2004. A Genetic Switch, Third Edition: Phage Lambda Revisited3rd
668 edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

669 3. **Brinsmade SR**, **Alexander EL**, **Livny J**, **Stettner AI**, **Segrè D**, **Rhee KY**,
670 **Sonenshein AL**. 2014. Hierarchical expression of genes controlled by the Bacillus
671 subtilis global regulatory protein CodY. Proc Natl Acad Sci **111**:8227–8232.

672 4. **Battesti A**, **Majdalani N**, **Gottesman S**. 2011. The RpoS-Mediated General Stress
673 Response in Escherichia coli. Annu Rev Microbiol 189–213.

674 5. **Hengge R**. 2011. Stationary-Phase Gene Regulation in Escherichia coli, p. . *In* A.
675 Böck, R. Curtiss III, J. B. Kaper, P. D. Karp, F. C. Neidhardt, T. Nyström, J. M.
676 Slauch, C. L. Squires, D. Ussery (eds.), EcoSal - Escherichia coli and Salmonella:
677 Cellular and Molecular Biology. Washington, DC, ASM Press.

678 6. **Schellhorn HE**. 2014. Elucidating the function of the RpoS regulon. Future
679 Microbiol **9**:497–507.

680 7. **Lange R**, **Hengge-Aronis R**. 1994. The cellular concentration of the sigma S
681 subunit of RNA polymerase in Escherichia coli is controlled at the levels of
682 transcription, translation, and protein stability. Genes Dev **8**:1600–1612.

683 8. **Mandel MJ**, **Silhavy TJ**. 2005. Starvation for Different Nutrients in Escherichia Coli
684 Results in Differential Modulation of RpoS Levels and Stability. J Bacteriol
685 **187**:434–442.

686 9. **Zafar MA**, **Carabetta VJ**, **Mandel MJ**, **Silhavy TJ**. 2014. Transcriptional occlusion
687 caused by overlapping promoters. Proc Natl Acad Sci **111**:1557–1561.

688 10. **Hengge R**. 2009. Proteolysis of sigmaS (RpoS) and the general stress response in
689 Escherichia coli. Res Microbiol **160**:667–76.

690 11. **Pratt LA**, **Silhavy TJ**. 1998. Crl stimulates RpoS activity during stationary phase.
691 Mol Microbiol **29**:1225–1236.

692 12. **Hryckowian AJ**, **Battesti A**, **Lemke JJ**, **Meyer ZC**, **Welch RA**. 2014. IraL Is an
693 RssB Anti-adaptor That Stabilizes RpoS during Logarithmic Phase Growth in
694 Escherichia coli and Shigella. mBio **5**:e01043–14.

695 13. **Chiang SM**, **Dong T**, **Edge TA**, **Schellhorn HE**. 2011. Phenotypic diversity
696 caused by differential RpoS activity among environmental Escherichia coli isolates.
697 Appl Environ Microbiol **77**:7915–23.

698 14. **Bhagwat AA**, **Tan J**, **Sharma M**, **Kothary M**, **Low S**, **Tall BD**, **Bhagwat M**. 2006.
699 Functional heterogeneity of RpoS in stress tolerance of enterohemorrhagic
700 Escherichia coli strains. Appl Environ Microbiol **72**:4978–86.

701 15. **Ferenci T**, **Galbiati HF**, **Betteridge T**, **Phan K**, **Spira B**. 2011. The constancy of
702 global regulation across a species: the concentrations of ppGpp and RpoS are
703 strain-specific in Escherichia coli. BMC Microbiol **11**:62.

704 16. **Snyder E**, **Gordon DM**, **Stoebel DM**. 2012. Escherichia coli Lacking RpoS Are
705 Rare in Natural Populations of Non-Pathogens. G3 GenesGenomesGenetics
706 **2**:1341–1344.

707 17. **Bleibtreu A**, **Clermont O**, **Darlu P**, **Glodt J**, **Branger C**, **Picard B**, **Denamur E**.
708 2014. The rpoS Gene Is Predominantly Inactivated during Laboratory Storage and
709 Undergoes Source-Sink Evolution in Escherichia coli Species. J Bacteriol
710 **196**:4276–4284.

711 18. **Patten CL**, **Kirchhof MG**, **Schertzberg MR**, **Morton R a**, **Schellhorn HE**. 2004.
712 Microarray analysis of RpoS-mediated gene expression in Escherichia coli K-12.
713 Mol Genet Genomics MGG **272**:580–91.

714 19. **Weber H**, **Polen T**, **Heuveling J**, **Wendisch VF**, **Hengge R**. 2005. Genome-wide
715 analysis of the general stress response network in Escherichia coli: sigmaS-
716 dependent genes, promoters, and sigma factor selectivity. J Bacteriol **187**:1591–
717 603.

718 20. **Dong T**, **Schellhorn HE**. 2009. Control of RpoS in global gene expression of
719 Escherichia coli in minimal media. Mol Genet Genomics **281**:19–33.

720 21. **Khlebnikov A**, **Datsenko KA**, **Skaug T**, **Wanner BL**, **Keasling JD**. 2001.
721 Homogeneous expression of the PBAD promoter in Escherichia coli by constitutive
722 expression of the low-affinity high-capacity AraE transporter. Microbiology
723 **147**:3241–3247.

724 22. **Baba T**, **Ara T**, **Hasegawa M**, **Takai Y**, **Okumura Y**, **Baba M**, **Datsenko KA**,
725 **Tomita M**, **Wanner BL**, **Mori H**. 2006. Construction of Escherichia coli K-12 in-
726 frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol
727 **2**:2006.0008.

728 23. **Haldimann A**, **Wanner BL**. 2001. Conditional-replication, integration, excision, and
729 retrieval plasmid-host systems for gene structure-function studies of bacteria. J
730 Bacteriol **183**:6384–93.

731 24. **Datsenko KA**, **Wanner BL**. 2000. One-step inactivation of chromosomal genes in
732 Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97**:6640–
733 6645.

734 25. **Stoebel DM**, **Hokamp K**, **Last MS**, **Dorman CJ**. 2009. Compensatory evolution of
735 gene regulation in response to stress by Escherichia coli lacking RpoS. PLoS
736 Genet **5**:e1000671.

737 26. **Groth D**, **Reszka R**, **Schenk JA**. 1996. Polyethylene glycol-mediated
738 transformation of Escherichia coli is increased by room temperature incubation.
739 Anal Biochem **240**:302–304.

740 27. **Butland G**, **Peregrín-Alvarez JM**, **Li J**, **Yang W**, **Yang X**, **Canadien V**,
741 **Starostine A**, **Richards D**, **Beattie B**, **Krogan N**, **Davey M**, **Parkinson J**,
742 **Greenblatt J**, **Emili A**. 2005. Interaction network containing conserved and
743 essential protein complexes in Escherichia coli. Nature **433**:531–537.

744 28. **Fraley CD**, **Kim JH**, **McCann MP**, **Matin A**. 1998. The Escherichia coli Starvation
745 Gene cstC Is Involved in Amino Acid Catabolism. J Bacteriol **180**:4287–4290.

746 29. **Castanie-Cornet M-P**, **Foster JW**. 2001. Escherichia coli acid resistance: cAMP
747 receptor protein and a 20 bp cis-acting sequence control pH and stationary phase
748 expression of the gadA and gadBC glutamate decarboxylase genes. Microbiology
749 **147**:709–715.

750 30. **Gibson DG**, **Young L**, **Chuang R-Y**, **Venter JC**, **Hutchison CA**, **Smith HO**. 2009.
751 Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat
752 Methods **6**:343–345.

753 31. **Arnqvist A**, **Olsén A**, **Normark S**. 1994. Sigma S-dependent growth-phase
754 induction of the csgBA promoter in Escherichia coli can be achieved in vivo by
755 sigma 70 in the absence of the nucleoid-associated protein H-NS. Mol Microbiol
756 **13**:1021–1032.

757 32. **De Biase D**, **Tramonti a**, **Bossa F**, **Visca P**. 1999. The response to stationary-
758 phase stress conditions in Escherichia coli: role and regulation of the glutamic acid
759 decarboxylase system. Mol Microbiol **32**:1198–211.

760 33. **Edwards AN**, **Patterson-Fortin LM**, **Vakulskas CA**, **Mercante JW**, **Potrykus K**,
761 **Vinella D**, **Camacho MI**, **Fields JA**, **Thompson SA**, **Georgellis D**, **Cashel M**,
762 **Babitzke P**, **Romeo T**. 2011. Circuitry Linking the Csr and Stringent Response
763 Global Regulatory Systems. Mol Microbiol **80**:1561–1580.

764 34. **Farewell A**, **Kvint K**, **Nyström T**. 1998. Negative regulation by RpoS: a case of
765 sigma factor competition. Mol Microbiol **29**:1039–51.

766 35. **Li H**, **Durbin R**. 2009. Fast and accurate short read alignment with Burrows–
767 Wheeler transform. Bioinformatics **25**:1754–1760.

768 36. **Anders S**, **Pyl PT**, **Huber W**. 2014. HTSeq - A Python framework to work with
769 high-throughput sequencing data. bioRxiv.

770 37. **Anders S**, **Huber W**. 2010. Differential expression analysis for sequence count
771 data. Genome Biol **11**:R106.

772 38. **Benjamini Y**, **Hochberg Y**. 1995. Controlling the False Discovery Rate: A Practical
773 and Powerful Approach to Multiple Testing. J R Stat Soc Ser B Methodol **57**:289–
774 300.

775 39. **Alexa A**, **Rahnenfuhrer J**. 2010. topGO: Enrichment analysis for Gene Ontology.
776 R package version 2.22.0.

777 40. **Carlson M**. org.EcK12.eg.db: Genome wide annotation for E coli strain K12. R
778 package version 3.0.0.

779 41. **Micallef L**, **Rodgers P**. 2014. eulerAPE: Drawing Area-Proportional 3-Venn
780 Diagrams Using Ellipses. PLoS ONE **9**:e101717.

781 42. **Singh SS**, **Singh N**, **Bonocora RP**, **Fitzgerald DM**, **Wade JT**, **Grainger DC**. 2014.
782 Widespread suppression of intragenic transcription initiation by H-NS. Genes Dev
783 **28**:214–219.

784 43. **Fitzgerald DM**, **Bonocora RP**, **Wade JT**. 2014. Comprehensive mapping of the
785 Escherichia coli flagellar regulatory network. PLoS Genet **10**:e1004649.

786 44. **Machanick P**, **Bailey TL**. 2011. MEME-ChIP: motif analysis of large DNA datasets.
787 Bioinformatics **27**:1696–1697.

788 45. **Bailey TL**, **Elkan C**. 1994. Fitting a mixture model by expectation maximization to
789 discover motifs in biopolymers. Proc Int Conf Intell Syst Mol Biol ISMB Int Conf
790 Intell Syst Mol Biol **2**:28–36.

791 46. **Cho B-K**, **Kim D**, **Knight EM**, **Zengler K**, **Palsson BO**. 2014. Genome-scale
792 reconstruction of the sigma factor network in Escherichia coli: topology and
793 functional states. BMC Biol **12**:4.

794 47. **Conway T**, **Creecy JP**, **Maddox SM**, **Grissom JE**, **Conkle TL**, **Shadid TM**,
795 **Teramoto J**, **San Miguel P**, **Shimada T**, **Ishihama A**, **Mori H**, **Wanner BL**. 2014.
796 Unprecedented high-resolution view of bacterial operon architecture revealed by
797 RNA sequencing. mBio **5**:e01442–01414.

798 48. **Vandesompele J**, **Preter KD**, **Pattyn F**, **Poppe B**, **Roy NV**, **Paepe AD**,
799 **Speleman F**. 2002. Accurate normalization of real-time quantitative RT-PCR data

800 by geometric averaging of multiple internal control genes. Genome Biol
801 **3**:research0034.

802 49. **Miller JH**. 1992. A short course in bacterial genetics. Cold Spring Harbor
803 Laboratory Press.

804 50. **Holm S**. 1979. A simple sequentially rejective multiple test procedure. Scand J Stat
805 **6**:65–70.

806 51. **Lévi-Meyrueis C**, **Monteil V**, **Sismeiro O**, **Dillies M-A**, **Monot M**, **Jagla B**,
807 **Coppée J-Y**, **Dupuy B**, **Norel F**. 2014. Expanding the RpoS/σS-Network by RNA
808 Sequencing and Identification of σS-Controlled Small RNAs in Salmonella. PloS
809 One **9**:e96918.

810 52. **Peano C**, **Wolf J**, **Demol J**, **Rossi E**, **Petiti L**, **De Bellis G**, **Geiselmann J**, **Egli T**,
811 **Lacour S**, **Landini P**. 2015. Characterization of the Escherichia coli σS core
812 regulon by Chromatin Immunoprecipitation-sequencing (ChIP-seq) analysis. Sci
813 Rep **5**.

814 53. **Lacour S**, **Landini P**. 2004. SigmaS-dependent gene expression at the onset of
815 stationary phase in Escherichia coli: function of sigmaS-dependent genes and
816 identification of their promoter sequences. J Bacteriol **186**:7186–7195.

817 54. **Dong T**, **Kirchhof MG**, **Schellhorn HE**. 2008. RpoS regulation of gene expression
818 during exponential growth of Escherichia coli K12. Mol Genet Genomics MGG
819 **279**:267–277.

820 55. **Salgado H**, **Peralta-Gil M**, **Gama-Castro S**, **Santos-Zavaleta A**, **Muñiz-Rascado**
821 **L**, **García-Sotelo JS**, **Weiss V**, **Solano-Lira H**, **Martínez-Flores I**, **Medina-Rivera**
822 **A**, **Salgado-Osorio G**, **Alquicira-Hernández S**, **Alquicira-Hernández K**, **López-**
823 **Fuentes A**, **Porrón-Sotelo L**, **Huerta AM**, **Bonavides-Martínez C**, **Balderas-**
824 **Martínez YI**, **Pannier L**, **Olvera M**, **Labastida A**, **Jiménez-Jacinto V**, **Vega-**
825 **Alvarado L**, **Del Moral-Chávez V**, **Hernández-Alvarez A**, **Morett E**, **Collado-**
826 **Vides J**. 2013. RegulonDB v8.0: omics data sets, evolutionary conservation,
827 regulatory phrases, cross-validated gold standards and more. Nucleic Acids Res
828 **41**:D203–213.

829 56. **Rosenthal AZ**, **Kim Y**, **Gralla JD**. 2008. Poising of Escherichia coli RNA
830 Polymerase and Its Release from the [sigma] 38 C-Terminal Tail for osmY
831 Transcription. J Mol Biol **376**:938–949.

832 57. **Reppas NB**, **Wade JT**, **Church GM**, **Struhl K**. 2006. The Transition between
833 Transcriptional Initiation and Elongation in E. coli Is Highly Variable and Often Rate
834 Limiting. Mol Cell **24**:747–757.

835 58. **Huo Y-X**, **Rosenthal AZ**, **Gralla JD**. 2008. General stress response signalling:
836 unwrapping transcription complexes by DNA relaxation via the sigma38 C-terminal
837 domain. Mol Microbiol **70**:369–378.

838 59. **Bonocora RP**, **Smith C**, **Lapierre P**, **Wade JT**. 2015. Genome-Scale Mapping of
839 Escherichia coli σ 54 Reveals Widespread, Conserved Intragenic Binding. PLOS
840 Genet **11**:e1005552.

841 60. **Mates AK**, **Sayed AK**, **Foster JW**. 2007. Products of the Escherichia coli acid
842 fitness island attenuate metabolite stress at extremely low pH and mediate a cell
843 density-dependent acid resistance. J Bacteriol **189**:2759–2768.

844 61. **Kanjee U**, **Houry WA**. 2013. Mechanisms of Acid Resistance in Escherichia coli.
845 Annu Rev Microbiol **67**:65–81.

846 62. **Bryant JA**, **Sellars LE**, **Busby SJW**, **Lee DJ**. 2014. Chromosome position effects
847 on gene expression in Escherichia coli K-12. Nucleic Acids Res **42**:11383–11392.

848 63. **Keseler IM**, **Mackie A**, **Peralta-Gil M**, **Santos-Zavaleta A**, **Gama-Castro S**,
849 **Bonavides-Martínez C**, **Fulcher C**, **Huerta AM**, **Kothari A**, **Krummenacker M**,
850 **Latendresse M**, **Muñiz-Rascado L**, **Ong Q**, **Paley S**, **Schröder I**, **Shearer AG**,
851 **Subhraveti P**, **Travers M**, **Weerasinghe D**, **Weiss V**, **Collado-Vides J**, **Gunsalus**
852 **RP**, **Paulsen I**, **Karp PD**. 2013. EcoCyc: fusing model organism databases with
853 systems biology. Nucleic Acids Res **41**:D605–D612.

854 64. **Kiupakis AK**, **Reitzer L**. 2002. ArgR-Independent Induction and ArgR-Dependent
855 Superinduction of the astCADBE Operon in Escherichia coli. J Bacteriol **184**:2940–
856 2950.

857 65. **Waterman SR**, **Small PLC**. 2003. Transcriptional expression of Escherichia coli
858 glutamate-dependent acid resistance genes gadA and gadBC in an hns rpoS
859 mutant. J Bacteriol **185**:4644–4647.

860 66. **Bailey TL**. 2011. DREME: motif discovery in transcription factor ChIP-seq data.
861 Bioinformatics **27**:1653–1659.

862 67. **Shin M**, **Song M**, **Rhee JH**, **Hong Y**, **Kim Y-J**, **Seok Y-J**, **Ha K-S**, **Jung S-H**,
863 **Choy HE**. 2005. DNA looping-mediated repression by histone-like protein H-NS:
864 specific requirement of Eσ70 as a cofactor for looping. Genes Dev **19**:2388–2398.

865 68. **Levi-Meyrueis C**, **Monteil V**, **Sismeiro O**, **Dillies M-A**, **Kolb A**, **Monot M**, **Dupuy**
866 **B**, **Duarte SS**, **Jagla B**, **Coppee J-Y**, **Beraud M**, **Norel F**. 2015. Repressor activity
867 of the RpoS/ S-dependent RNA polymerase requires DNA binding. Nucleic Acids
868 Res **43**:1456–1468.

869 69. **Typas A**, **Becker G**, **Hengge R**. 2007. The molecular basis of selective promoter
870 activation by the sigmaS subunit of RNA polymerase. Mol Microbiol **63**:1296–306.

871 70. **Typas A**, **Hengge R**. 2006. Role of the spacer between the –35 and –10 regions in
872 σs promoter selectivity in Escherichia coli. Mol Microbiol **59**:1037–1051.

873 71. **Richard HT**, **Foster JW**. 2003. Acid resistance in Escherichia coli. Adv Appl
874 Microbiol **52**:167–186.

875 72. **Lund P**, **Tramonti A**, **Biase DD**. 2014. Coping with low pH: molecular strategies in
876 neutralophilic bacteria. FEMS Microbiol Rev **38**:1091–1125.

877 73. **Zambrano MM**, **Siegele DA**, **Almirón M**, **Tormo A**, **Kolter R**. 1993. Microbial
878 competition: Escherichia coli mutants that take over stationary phase cultures.
879 Science **259**:1757–60.

880 74. **Spira B**, **de Almeida Toledo R**, **Maharjan RP**, **Ferenci T**. 2011. The uncertain
881 consequences of transferring bacterial strains between laboratories - rpoS
882 instability as an example. BMC Microbiol **11**:248.

883 75. **Hommais F**, **Krin E**, **Coppée J-Y**, **Lacroix C**, **Yeramian E**, **Danchin A**, **Bertin P**.
884 2004. GadE (YhiE): a novel activator involved in the response to acid environment
885 in Escherichia coli. Microbiol Read Engl **150**:61–72.

886 76. **Tramonti A**, **De Canio M**, **Delany I**, **Scarlato V**, **De Biase D**. 2006. Mechanisms
887 of transcription activation exerted by GadX and GadW at the gadA and gadBC
888 gene promoters of the glutamate-based acid resistance system in Escherichia coli.
889 J Bacteriol **188**:8118–27.

890 77. **Seo SW**, **Kim D**, **O'Brien EJ**, **Szubin R**, **Palsson BO**. 2015. Decoding genome-
891 wide GadEWX-transcriptional regulatory networks reveals multifaceted cellular
892 responses to acid stress in Escherichia coli. Nat Commun **6**:7970.

893 78. **Schneider TD**, **Stephens RM**. 1990. Sequence logos: a new way to display
894 consensus sequences. Nucleic Acids Res **18**:6097–6100.

895 79. **Crooks GE**, **Hon G**, **Chandonia J-M**, **Brenner SE**. 2004. WebLogo: A Sequence
896 Logo Generator. Genome Res **14**:1188–1190.

897

898
899

900 **Table 1** Biological processes enriched in the RpoS regulon.
901
902

GO ID	GO Term	Genes in genome	Observed in regulon	Expected in regulon	p-value
GO:0009063	cellular amino acid catabolic process	69	38	18.5	3.5e-07
GO:0006099	tricarboxylic acid cycle	20	15	5.35	8.3e-06
GO:0006096	glycolytic process	18	14	4.81	8.3e-06
GO:0006094	gluconeogenesis	13	9	3.48	0.0016
GO:0009441	glycolate metabolic process	6	5	1.6	0.0063
GO:0006090	pyruvate metabolic process	21	17	5.61	0.0180
GO:0006970	response to osmotic stress	28	13	7.49	0.0190
GO:0006542	glutamine biosynthetic process	10	6	2.67	0.0270
GO:0009060	aerobic respiration	65	33	17.38	0.0285
GO:0006006	glucose metabolic process	36	20	9.62	0.0317
GO:0016052	carbohydrate catabolic process	276	94	73.79	0.0326
GO:0009101	glycoprotein biosynthetic process	13	7	3.48	0.0342
GO:0019395	fatty acid oxidation	30	13	8.02	0.0354
GO:0000162	tryptophan biosynthetic process	11	6	2.94	0.0463
GO:0009246	enterobacterial common antigen biosynthesis	11	6	2.94	0.0463
GO:0042398	cellular modified amino acid biosynthesis	17	7	4.55	0.0472
GO:0009239	enterobactin biosynthetic process	6	4	1.6	0.0472
GO:0000270	peptidoglycan metabolic process	51	14	13.64	0.0475

903 **Table 2** Biological processes enriched in the sensitive genes.

904

GO ID	GO Term	Genes in genome	Observed in sensitive set	Expected in sensitive set	p-value
GO:0006970	response to osmotic stress	13	4	0.79	0.0055
GO:0009063	cellular amino acid catabolic process	38	6	2.31	0.0210
GO:0019395	fatty acid oxidation	13	3	0.79	0.0385

905
906
907
908

Table 3 Biological processes enriched in the insensitive genes.

GO ID	GO Term	Genes in genome	Observed in insensitive set	Expected in insensitive set	p-value[1]
GO:0006412	translation	21	3	0.6	0.018
GO:0006164	purine nucleotide biosynthetic process	10	2	0.28	0.030
GO:0006805	xenobiotic metabolic process	13	2	0.37	0.049

920
921
922
923 **Table 4** Contingency table of the (in)sensitivity of a gene and if is promoter is or is
924 not bound by RpoS.
925

	Sensitive	Linear	Insensitive
Bound by RpoS	31	87	5
Not bound by RpoS	71	823	27

926
927

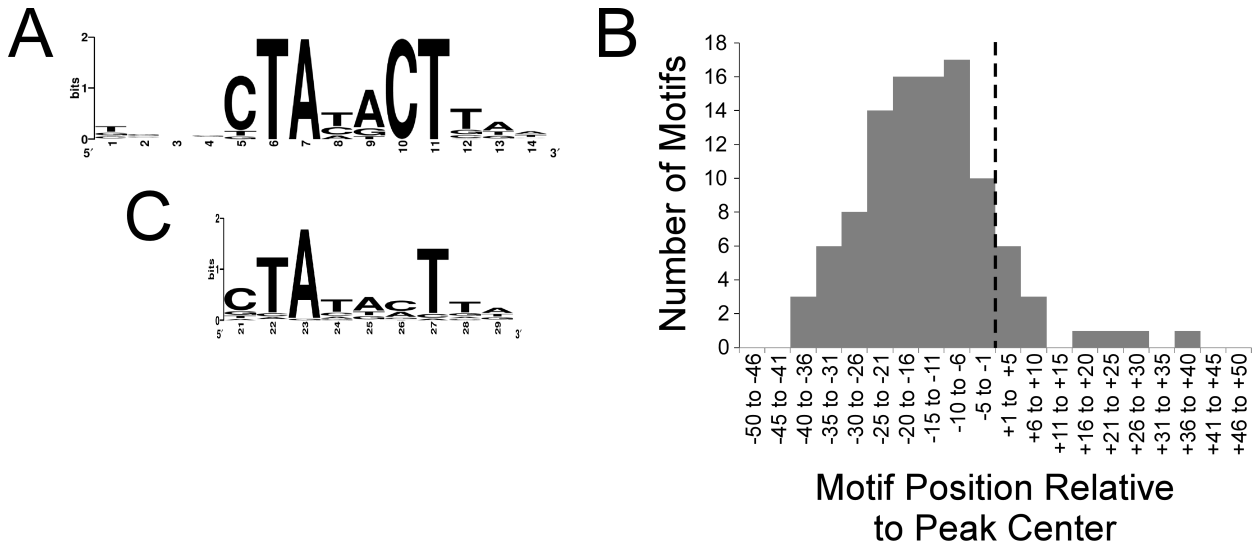


Figure 1. Analysis of RpoS ChIP-seq data. (A) Sequence logo (78) of the enriched sequence motif associated with RpoS-bound regions (MEME E-value 2.0e-40). The sequence logo was generated using WebLogo (79) (B) Histogram showing the frequency distribution of distances between identified motifs and ChIP-seq peak centers. The black, vertical, dashed line indicates the peak center position. (C) Sequence logo (78) of the enriched sequence motif associated with TSSs located within 20 bp of RpoS ChIP-seq peak centers (MEME E-value 2.6e-56). Note that only positions 21-29 of the motif are shown. The sequence logo was generated using WebLogo (79).

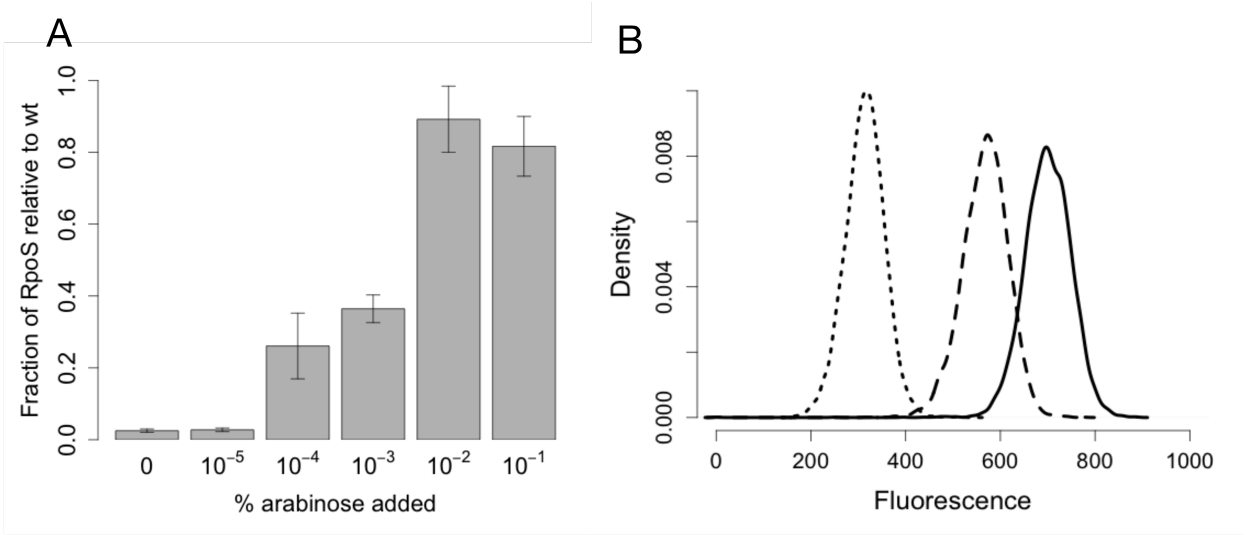


Figure 2. Arabinose control of RpoS protein levels in strain DMS2564. (a) Increasing arabinose results in more RpoS expression in strain DMS2564, as measured by western blotting. n = 4 to 5; error bars represent SEM. (b) Flow cytometric measurement of an *otsB-gfp* fusion under 0%, 10^{-4}%, and 10^{-1}% arabinose. (Dotted line, dashed line, and solid line, respectively.)

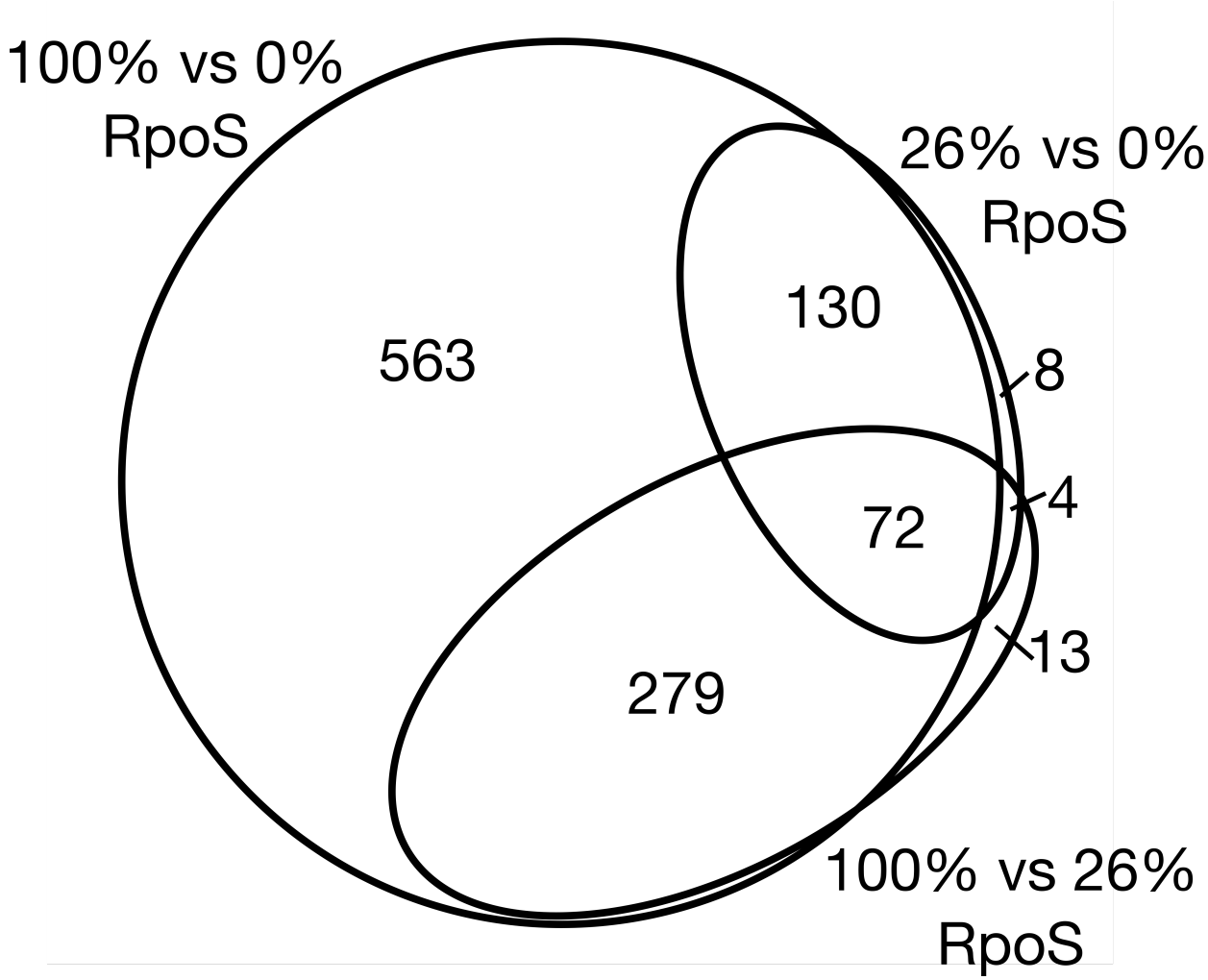


Figure 3: Area proportional Venn diagram showing the number of genes differentially expressed between each of the three conditions.

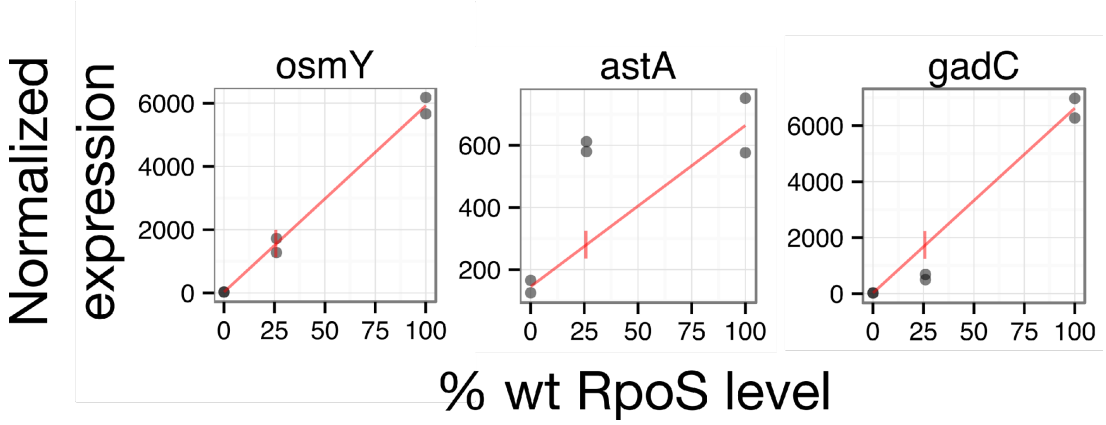


Figure 4: Examples of three classes of patterns of RpoS response. *osmY* expression is linear with RpoS level, *astA* is sensitive to RpoS level, and *gadC* is insensitive to RpoS level. Dots represent normalized expression levels from individual RNA-seq samples. The line is drawn between the mean at 0% and the mean at 100%.

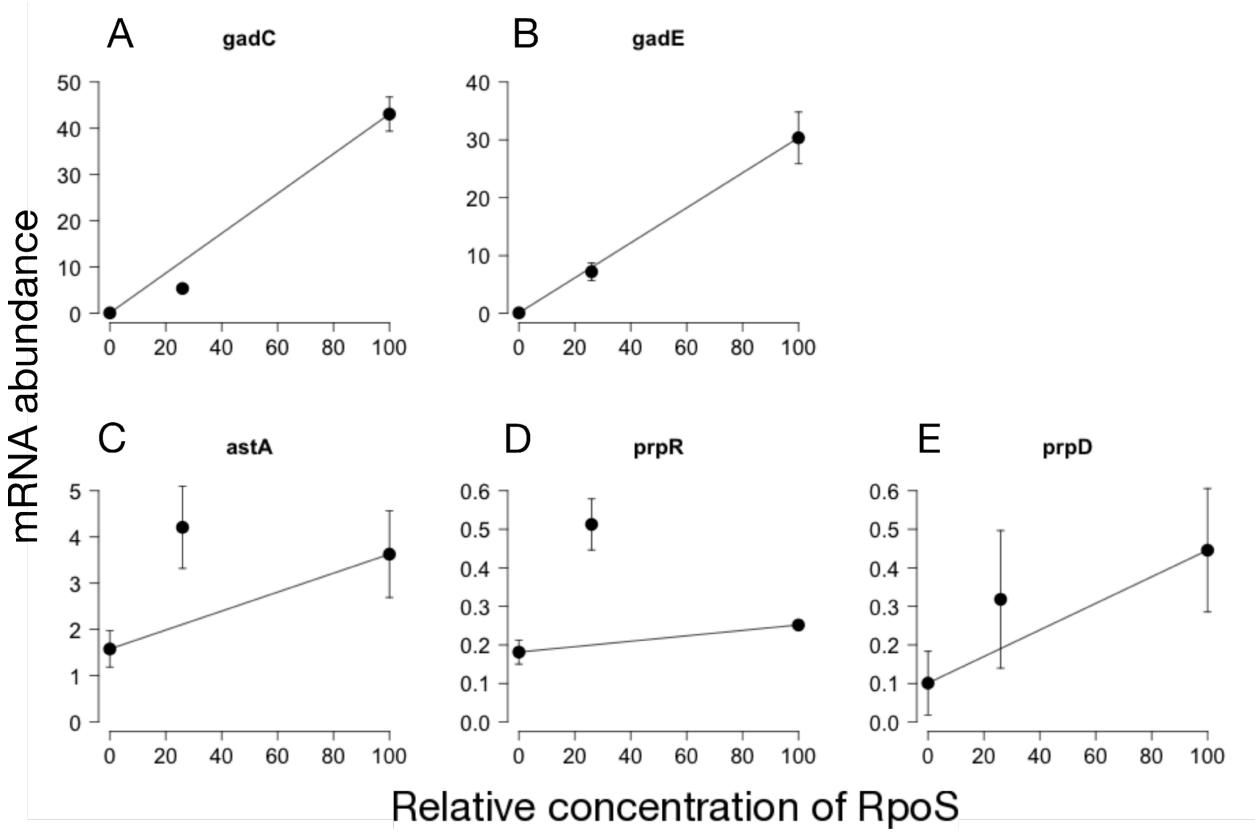


Figure 5. Testing of RNA-seq expression patterns using qPCR. Expression patterns were measured for (a,b) *gadC* and *gadE*, which were insensitive in the RNA-seq data, and (c-e) *astA*, *prpR,* and *prpD*, which were sensitive in the RNA-seq data. In all cases, the level of transcription at 26% RpoS was on the side of the line predicted by the RNA-seq data, though not always significantly so. *gadC* was significantly insensitive ($p < 10^{-4}$), *gadE* was not significantly different from linear ($p = 0.10$), *astA* was (barely) not significantly sensitive ($p = 0.06$), *prpR* was significant sensitive ($p < 10^{-4}$), and *prpD* was not significant ($p = 0.56$). n = 6, error bars = SEM.

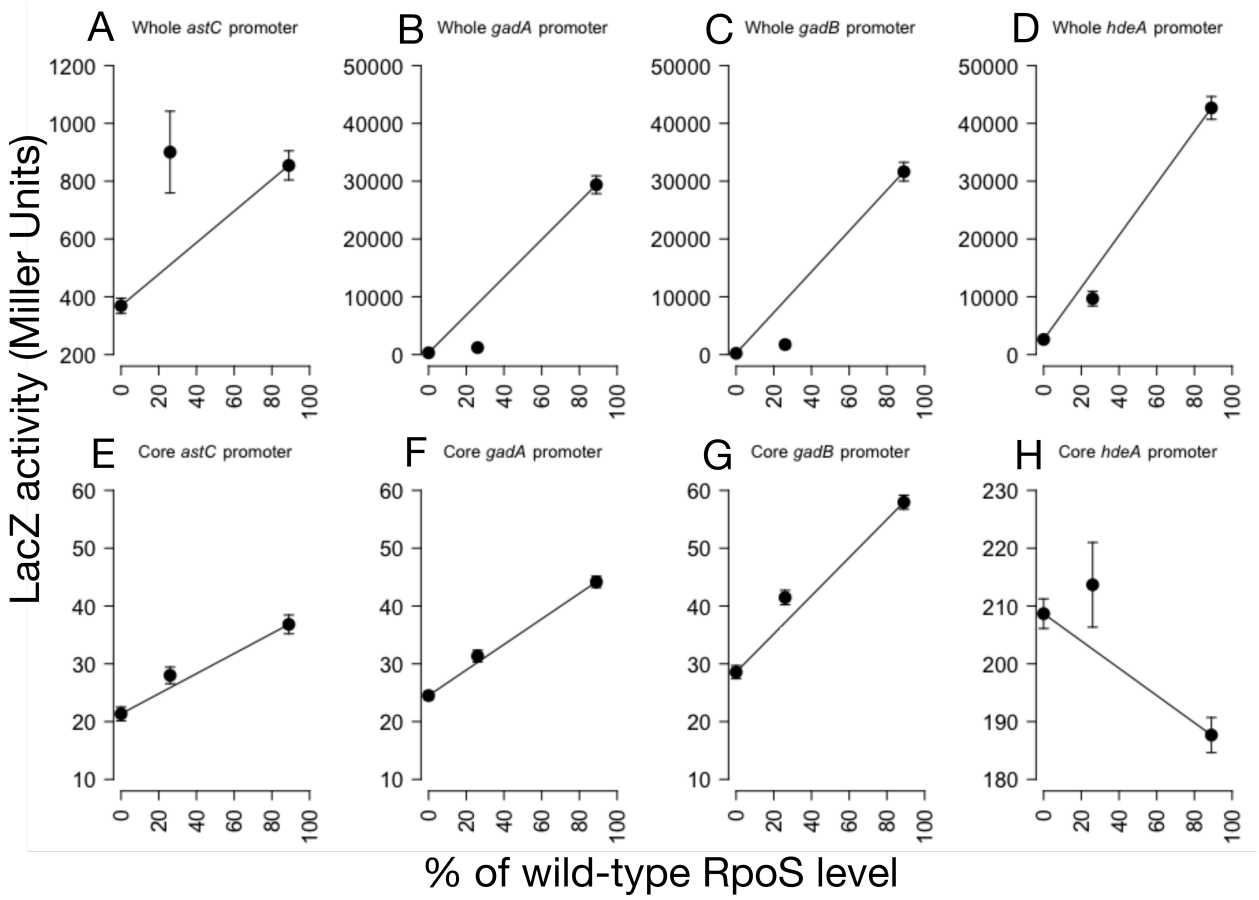
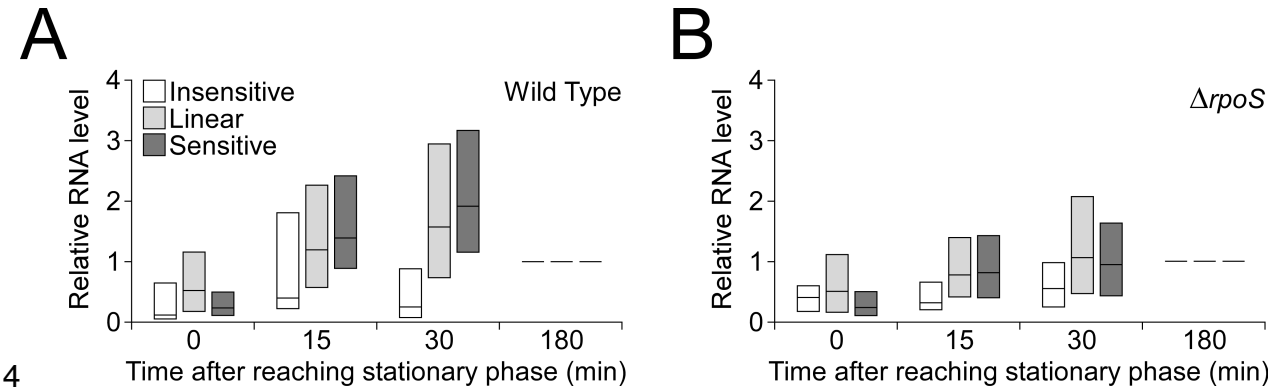


Figure 6. **Expression patterns of whole promoters and core promoters only.** Expression patterns were measured for the (a - d) whole upstream regulatory region and the (e - h) core promoter only of *astC, gadA, gadB,* and *hdeA.* As expected by RNA-seq data, the whole *astC* fusion was sensitive ($p = 0.04$, t-test), and the *gadA*, *gadB,* and *hdeA* fusions were all insensitive ($p = 2 \times 10^{-6}$, $p = 10^{-5}$, $p = 0.04$, all by one sample t-test). The p-values are adjusted for multiple comparisons using Holm's sequential adjustment method (50). The core promoters (e - h) have much lower levels of maximal transcription than the full length promoters. All four have much altered patterns of sensitivity, although only *gadA* and *gadB* was significantly different from the whole promoter ($p = 0.08$ (*astC*), $p = 0.01$ (*gadA*), $p = 10^{-6}$ (*gadB*), $p = 0.21$ (*hdeA*), all by two-sample t-test with Holm's correction.) n = 6 to 8, error bars = SEM.

1012
1013



1014
1015
1016

1017**Figure 7. Expression profiles of genes when cells enter stationary phase. (A)**
1018Relative expression levels of RpoS-regulated genes were taken from a published
1019study (47) for four time-points following entry into stationary phase. All expression
1020values were normalized to those from the final time-point for each gene. The graph
1021shows the range of relative expression values for insensitive (white boxes), linear
1022(light gray boxes), and sensitive (dark gray boxes) genes for each time-point. Boxes
1023represent the 25th-75th percentile range, and horizontal lines indicate the median
1024value. **(B)** As above, but for a Δ*rpoS* strain of *E. coli*.