Convergence of the Transcriptional Responses to Heat Shock and Singlet Oxygen Stresses

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Abstract

Cells often mount transcriptional responses and activate specific sets of genes in response to stress-inducing signals such as heat or reactive oxygen species. Transcription factors in the RpoH family of bacterial alternative σ factors usually control gene expression during a heat shock response. Interestingly, several α-proteobacteria possess two or more paralogs of RpoH, suggesting some functional distinction. We investigated the target promoters of Rhodobacter sphaeroides RpoH1 and RpoH2 using genome-scale data derived from gene expression profiling and the direct interactions of each protein with DNA in vivo. We found that the RpoH1 and RpoH2 regulons have both distinct and overlapping gene sets. We predicted DNA sequence elements that dictate promoter recognition specificity by each RpoH paralog. We found that several bases in the highly conserved TTG in the −35 element are important for activity with both RpoH homologs; that the T-9 position, which is over-represented in the RpoH1 promoter sequence logo, is critical for RpoH1-dependent transcription; and that several bases in the predicted −10 element were important for activity with either RpoH1 or both RpoH homologs. Genes that are transcribed by both RpoH1 and RpoH2 are predicted to encode for functions involved in general cell maintenance. The functions specific to the RpoH1 regulon are associated with a classic heat shock response, while those specific to RpoH2 are associated with the response to the reactive oxygen species, singlet oxygen. We propose that a gene duplication event followed by changes in promoter recognition by RpoH1 and RpoH2 allowed convergence of the transcriptional responses to heat and singlet oxygen stress in R. sphaeroides and possibly other bacteria.

Introduction

Transcriptional responses to stress are critical to cell growth and survival. In bacteria, stress responses are often controlled by alternative σ factors that direct RNA polymerase to transcribe promoters different from those recognized by the primary σ factor [1,2]. Therefore, identifying the target genes for a particular alternative σ factor can help identify the functions necessary to respond to a given stress. For example, the transcriptional response to heat shock in Escherichia coli uses the alternative σ factor σ32 to increase synthesis of gene products involved in protein homeostasis or membrane integrity [3]. From available genome sequences, proteins related to E. coli σ32 are conserved across virtually all proteobacteria. This so-called RpoH family of alternative σ factors is characterized by a conserved amino acid sequence (the “RpoH box”) that is involved in RNA polymerase interactions [4,5]. RpoH family members also possess conserved amino acid sequences in σ factor regions 2.4 and 4.2 that interact with promoter sequences situated approximately −10 and −35 base pairs upstream of the transcriptional start sites, respectively [6]. However, the definition of functional promoters for this family of alternative σ factor using only the presence or the extent of sequence identity for the predicted −10 and −35 binding regions is not a sufficient predictor of transcription activity [7].

While bacteria often possess many alternative σ factors, they usually possess only one member of the RpoH family. However, several α-proteobacteria, including Brucella melitensis [9], Sinorhizobium meliloti [9,10], Bradyrhizobium japonicum [11,12], Rhizobium etli [13] and Rhodobacter sphaeroides [14], possess two or more RpoH homologs. In some cases, one or more of these RpoH homologs completely or partially complement the phenotypes of E. coli ΔrpoH mutants, suggesting that these proteins can functionally interact with RNA polymerase and recognize similar promoter elements [9–11,14,15]. However, in the nitrogen-fixing plant symbiont Rhizobium etli, the ΔrpoH mutant was sensitive to heat and oxidative stress while the ΔrpoH2 mutant was sensitive to osmotic stress [13]. Therefore, the additional members of the RpoH family in α-proteobacteria may have roles in other stress responses.

Previous work demonstrated that either R. sphaeroides RpoH1 or RpoH2 can complement the temperature sensitive phenotype of
Author Summary

An important property of living systems is their ability to survive under conditions of stress such as increased temperature or the presence of reactive oxygen species. Central to the function of these stress responses are transcription factors that activate specific sets of genes needed for this response. Despite the central role of stress responses across all forms of life, the processes driving their organization and evolution across organisms are poorly understood. This paper uses genomic, computational, and mutational analyses to dissect stress responses controlled by two proteins that are each members of the RpoH family of alternative σ factors. RpoH family members usually control gene expression during a heat shock response. However, the photosynthetic bacterium *Rhodobacter sphaeroides* and several other α-proteobacteria possess two or more paralogs of RpoH, suggesting some functional distinction. Our findings predict that a gene duplication event followed by changes in DNA recognition by RpoH1 and RpoH2 allowed convergence of the transcriptional responses to heat and singlet oxygen stress in *R. sphaeroides* and possibly other bacteria. Our approach and findings should interest those studying the evolution of transcription factors or the signal transduction pathways that control stress responses.

an *E. coli* Δ*σH* mutant; that singly mutant *R. sphaeroides* strains lacking either *rpoH1* or *rpoH2* are able to mount a heat shock response; and that RNA polymerase containing either RpoH1 or RpoH2 can initiate transcription from a common set of promoters in vitro [14–16]. Combined, these observations suggest that RpoH1 and RpoH2 have some overlapping functions in *R. sphaeroides*. On the other hand, in vitro transcription assays identified promoters that were selectively transcribed by either RpoH1 or RpoH2 [14,15]. Moreover, *rpoH1* is under direct transcriptional control of RpoE, a Group IV alternative σ factor that acts as the master regulator of the response of *R. sphaeroides* to singlet oxygen stress [17–19]. These later results and the recent observation that a Δ*σH* mutant is more sensitive to singlet oxygen stress than the wild-type strain [15,17] suggest that RpoH1 and RpoH2 also have distinct functions in *R. sphaeroides*. Finally, global protein profiles of *R. sphaeroides* mutants lacking *rpoH1* or *rpoH2* or both genes, suggested that RpoH1 and RpoH2 have distinct and overlapping regulons [15,17,20]. However, the extent of genes that are direct targets for RpoH1 and RpoH2 is still unknown because past studies have been unable to distinguish direct from indirect effects on gene expression or identify all the direct targets for either of these σ factors.

In this study, we characterized the RpoH1 and RpoH2 regulons using a combination of expression microarrays, chromatin immunoprecipitation and computational methods which have been previously been shown to predict correctly direct targets for other alternative σ factors or DNA binding proteins [19,21]. We found that the genes predicted to be common to the RpoH1 and RpoH2 regulons function in protein repair or turnover, membrane maintenance, and DNA repair. Genes specific to the RpoH1 regulon encode other proteins involved in protein maintenance and DNA repair, whereas genes specific to the RpoH2 regulon include proteins involved in maintaining the oxidation-reduction state of the cytoplasmic thiol pool. We used information on the members of each regulon to generate and test hypotheses about DNA sequences that determine promoter specificity of these two RpoH homologs. The observed properties of these two *R. sphaeroides* RpoH homologs illustrate how duplication of an alternative σ factor and subsequent changes in promoter recognition could have allowed convergence of transcriptional responses to separate signals. In the case of *R. sphaeroides*, we predict that these events allowed convergence of the transcriptional responses to heat shock and singlet oxygen stresses to be under control of these two RpoH paralogs.

Results

Defining the distinct and overlapping regulons of *R. sphaeroides* RpoH1 and RpoH2

To define members of the RpoH1 and RpoH2 regulons, we monitored transcript levels and protein-DNA interactions in *R. sphaeroides* strains ectopically expressing either RpoH1 or RpoH2. To generate these strains, we constructed low copy plasmids carrying *rpoH1* or *rpoH2* under the control of an IPTG-inducible promoter [22] and conjugated them into *R. sphaeroides* mutant strains lacking *rpoH1* [16] or *rpoH2* [15], respectively. To induce target gene expression, we exposed exponentially growing aerobic cultures to IPTG for one generation before cells were either harvested to extract total RNA for analysis of transcript levels or treated with formaldehyde to prepare samples for chromatin immunoprecipitation on a chip (ChIP-chip) assays. The Western blot analysis used to measure levels of these alternative σ factors demonstrates that cells ectopically expressing RpoH1 and RpoH2 contained each protein at levels comparable to those following either heat shock or singlet oxygen stress (Figure 1). Thus, these strains can be used to characterize members of the RpoH1 and RpoH2 regulons.

As controls for this experiment, we measured the abundance of individual RpoH proteins and a control transcription factor (PrrA) [23], which is not known to be dependent on either alternative σ factor for its expression, when wild type cells were exposed to either heat or singlet oxygen stress. This analysis showed that RpoH1 is detectable prior to heat stress, but its levels increase 10 and 20 minutes after the shift to increased temperature (Figure 1A). RpoH1 levels remain elevated after the temperature shift but they decline within 60 minutes after heat shock, suggesting that as in the case of *E. coli* σ32, there is an initial rise in RpoH1 levels immediately on heat shock before they return to a new steady state level at elevated temperature [24]. RpoH2 was also detected prior to exposure to singlet oxygen and within 10 minutes of exposure to this reactive oxygen species, levels of this protein were increased (Figure 1B). Levels of RpoH2 found within 20 minutes after exposure to singlet oxygen remained relatively constant over the time course of this experiment, suggesting a continuous requirement for RpoH2 during this stress response (Figure 1B). The abundance of the control transcription factor PrrA did not follow these same trends, suggesting that the observed increases in individual RpoH proteins was associated with these stress responses. In addition, the abundance of individual RpoH proteins did not increase significantly to both stress responses, as expected if these increases were not due to a general increase in protein levels in response to different signals.

To identify transcripts that were increased in abundance as a result of RpoH1 or RpoH2 activity, we compared mRNA levels of cells expressing RpoH1 or RpoH2 ectopically to those of control cells lacking either *rpoH1* or *rpoH2*. We selected differentially expressed genes with a significance level set for a false discovery rate ≤5% and that displayed at least 1.5-fold higher transcript levels in cells expressing either RpoH family member. This analysis revealed that transcripts from 241 and 186 genes were increased by expression of RpoH1 and RpoH2, respectively (Figure 2). These two sets of differentially expressed genes have 60 genes in common.
RpoHI and RpoHII obtained from ectopic expression vectors used in the singlet oxygen stress). On the same western blots, the levels of FLAG- of the photosensitizer methylene blue in the presence of oxygen detectable cross-reaction between FLAG-RpoHI and the antibody S1). In addition, other control experiments showed there was no version of RpoHI with cells expressing wild-type RpoHI (Figure A) a shift of temperature from 30 °C to 42 °C (heat shock) or (B) addition of the photosensitizer methylene blue in the presence of oxygen (singlet oxygen stress). On the same western blots, the levels of FLAG-RpoHI and RpoHII obtained from ectopic expression vectors used in the expression profiling and ChIP-chip experiments under normal conditions. Note that because of the addition of the FLAG polypeptide, RpoHII-FLAG migrates slower than the wild-type RpoHII. The abundance of RpoHI and RpoHII in wild-type cells in the absence of added stress are shown in the first lane. As a gel loading control, the membranes were also subsequently treated polyclonal antibodies against the response regulator PrrA, a control transcription factor who’s expression is not known to be dependent on either of the RpoHI homologs. The experiment was designed to analyze changes in levels of RpoHI, RpoHII and PrrA before and after a stress, so the differences between panels reflect different exposure times used when developing the Western blots.

Figure 1. RpoHI and RpoHII accumulation following heat and singlet oxygen stresses. Western blots illustrating the levels of RpoHI and RpoHII in wild-type R. sphaeroides (WT) at different times following (A) a shift of temperature from 30 °C to 42 °C (heat shock) or (B) addition of the photosensitizer methylene blue in the presence of oxygen (singlet oxygen stress). On the same western blots, the levels of FLAG-RpoHI and RpoHII obtained from ectopic expression vectors used in the expression profiling and ChIP-chip experiments under normal conditions. Note that because of the addition of the FLAG polypeptide, RpoHII-FLAG migrates slower than the wild-type RpoHII. The abundance of RpoHI and RpoHII in wild-type cells in the absence of added stress are shown in the first lane. As a gel loading control, the membranes were also subsequently treated polyclonal antibodies against the response regulator PrrA, a control transcription factor who’s expression is not known to be dependent on either of the RpoHI homologs. The experiment was designed to analyze changes in levels of RpoHI, RpoHII and PrrA before and after a stress, so the differences between panels reflect different exposure times used when developing the Western blots.

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We recognize that some of these differentially-expressed transcripts might be not be direct targets for RpoHII and RpoHII. Therefore, to determine which of the above genes were directly transcribed by RNA polymerase holoenzyme containing either RpoHII or RpoHII, we performed ChIP-chip assays from comparable cultures to map direct interactions of RpoHII or RpoHII with genomic DNA. We were able to raise specific antibodies against RpoHII, and vice versa (data not shown). From the ChIP-chip analysis we identified 812 and 1353 genomic regions enriched after immunoprecipitation with antibodies against RpoHII and RpoHII, respectively, using a significance level set for a false discovery rate ≤5%. Because the signal from a single factor binding site extends on average over a 1 kb region, some enriched regions may contain multiple binding sites. To increase the resolution of the putative RpoHII and RpoHII binding sites, we identified the modes of the ChIP-chip signal distributions within each enriched region. This adjustment increased the number of putative binding sites for RpoHII and RpoHII to 1085 and 1765, respectively.

We then identified all the annotated genes that contained a ChIP-chip peak within 300 base pairs upstream of their start codons as a way to define candidate genes or operons in the RpoHII or RpoHII regulons. Included in this list of potential regulon members were genes that are predicted to be co-transcribed using a previous computational analysis of R. sphaeroides operon organization (http://www.microbesonline.org/operons/) [26]. Therefore, by these criteria, the upper limits of the total numbers of genes potentially regulated by RpoHII or RpoHII are 1120 and 1616, respectively (Figure 2). We recognized that a significant number of the putative RpoHII or RpoHII promoters may not be assigned from the ChIP-chip dataset alone, especially because promoter orientation needs to be considered and that because factor or RNA polymerase binding events do not always promote transcription. Therefore, we refined the respective RpoHII and RpoHII regulons by intersecting the lists of target genes identified from the ChIP-chip analysis with the lists of candidate genes identified from the expression profiling analysis. After this intersection, we predict that the RpoHII regulon contains 175 genes and the RpoHII regulon contains 144 genes with 45 genes common to both regulons (Figure 2).

Upon examining the annotations of these predicted target genes, the 45 genes that are members of both the RpoHII and RpoHII regulons are predicted to encode mainly for functions related to the electron transport chain, protein homeostasis, and DNA repair (Table 1 and Table S1). The 130 predicted members of the RpoHII regulon also encode functions in these three groups,
Table 1. Compositions of the RpoH\textsubscript{I} and RpoH\textsubscript{II} regulons.

| Mainrole\textsuperscript{1} | Subrole\textsuperscript{2} | Locus\textsuperscript{3} |
|---------------------------|---------------------------|--------------------------|
| **RpoH\textsubscript{II} regulon (45 genes)** | | |
| Energy metabolism | Electron transport | RSP\_0100, RSP\_0101, RSP\_0102, RSP\_0103, RSP\_0104, RSP\_0105, RSP\_0106, RSP\_0107, RSP\_2805 |
| | Other | RSP\_0472 |
| Protein synthesis/fate | Degradation of proteins, peptides, and glycopeptides | RSP\_0665, RSP\_1076, RSP\_1174, RSP\_2710, RSP\_2806 |
| | Other | RSP\_1825 |
| Protein folding and stabilization | RSP\_1207 |
| Ribosomal proteins: synthesis and modification | RSP\_0570 |
| Serine family | RSP\_2481 |
| **RpoH\textsubscript{I} regulon (130 genes)** | | |
| Protein synthesis/fate | Amino acid biosynthesis | RSP\_0244, RSP\_0377, RSP\_1475 |
| | Degradation of proteins, peptides, and glycopeptides | RSP\_0357, RSP\_0554, RSP\_1408, RSP\_1531, RSP\_1742, RSP\_2412, RSP\_2649 |
| | Protein and peptide secretion and trafficking | RSP\_1169, RSP\_1797, RSP\_1798, RSP\_1799, RSP\_1843, RSP\_2540, RSP\_2541 |
| | Protein folding and stabilization | RSP\_1016, RSP\_1173, RSP\_1532, RSP\_1572, RSP\_1805, RSP\_4043 |
| | Protein modification and repair | RSP\_0559, RSP\_0872, RSP\_0873, RSP\_0874, RSP\_0923 |
| | tRNA aminoacylation | RSP\_0875 |
| Energy metabolism | Amino acids and amines | RSP\_3957 |
| | Electron transport | RSP\_0296, RSP\_0610, RSP\_1194, RSP\_1489, RSP\_1529, RSP\_1576, RSP\_2375, RSP\_2685, RSP\_2945 |
| | Glycolysis/gluconeogenesis | RSP\_0361 |
| Fatty acid and cell envelope | Biosynthesis | RSP\_0720, RSP\_0929, RSP\_2776 |
| | Biosynthesis and degradation of murein sacculus and peptidoglycan | RSP\_1240 |
| | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides | RSP\_0125, RSP\_3187 |
| | Degradation | RSP\_0409 |
| | Other | RSP\_1889 |
| Biosynthesis of cofactors, prosthetic groups, and carriers | Folic acid | RSP\_0930 |
| | Lipoate | RSP\_2783 |
| | Molybdopterin | RSP\_0235, RSP\_1071, RSP\_1072 |
| | Other | RSP\_2658 |
| | Pyridoxine | RSP\_1672 |
| Regulatory functions | DNA interactions | RSP\_0014, RSP\_2200 |
| | Other | RSP\_2236 |
| | Protein interactions | RSP\_4193 |
| | Transcription factors | RSP\_2410 |
| Transport and binding proteins | Amino acids, peptides and amines | RSP\_1564 |
| | Cations and iron carrying compounds | RSP\_2542, RSP\_2891 |
### Table 1. Cont.

| Mainrole | Subrole | Locus |
|----------|---------|-------|
| Unknown substrate | RSP_2696, RSP_2897 |
| DNA metabolism | DNA replication, recombination, and repair | RSP_1074, RSP_2815, RSP_4199 |
| Purine ribonucleotide biosynthesis | RSP_2454 |
| Cellular processes | Adaptations to atypical conditions | RSP_4198 |
| Detoxification | RSP_0890, RSP_1058 |
| Central intermediary metabolism | Other | RSP_1196, RSP_1949 |
| Sulfur metabolism | RSP_2738 |
| Signal transduction | Two-component systems | RSP_2130, RSP_3105 |
| Mobile and extra-chromosomal element functions | Transposon functions | RSP_3007 |
| Unknown function | Unknown function | RSP_0126, RSP_0362, RSP_0363, RSP_0408, RSP_0719, RSP_0999, RSP_1104, RSP_1193, RSP_1204, RSP_1238, RSP_1241, RSP_1360, RSP_1406, RSP_1549, RSP_1563, RSP_1573, RSP_1581, RSP_1615, RSP_1671, RSP_1684, RSP_1743, RSP_1852, RSP_2121, RSP_2125, RSP_2214, RSP_2219, RSP_2387, RSP_2638, RSP_2640, RSP_2641, RSP_2739, RSP_2763, RSP_2764, RSP_2816, RSP_2952, RSP_2953, RSP_3067, RSP_3068, RSP_3378, RSP_3426, RSP_3552, RSP_3597, RSP_3598, RSP_3634, RSP_3809, RSP_3810, RSP_4244, RSP_4245, RSP_4248, RSP_4305 |
| RpoHII regulon (99 genes) | |
| Energy metabolism | Biosynthesis and degradation of polysaccharides | RSP_0482 |
| Electron transport | RSP_0108, RSP_0109, RSP_0110, RSP_0112, RSP_0474, RSP_2785, RSP_3212, RSP_3305, RSP_3537 |
| Entner-Doudoroff | RSP_2646 |
| Fermentation | RSP_3164 |
| Glycolysis/gluconeogenesis | RSP_2736, RSP_4045, RSP_4211 |
| Other | RSP_0392, RSP_2294 |
| Pentose phosphate pathway | RSP_2734, RSP_2735 |
| Sugars | RSP_2937, RSP_3138 |
| Biosynthesis of cofactors, prosthetic groups, and carriers | Glutathione and analogs | RSP_3272 |
| Heme, porphyrin, and cobalamin | RSP_1197, RSP_1692, RSP_2831 |
| Menaquinone and ubiquinone | RSP_1175, RSP_1338, RSP_1492, RSP_1869 |
| Other | RSP_0750, RSP_0898, RSP_2314 |
| Transport and binding proteins | Amino acids, peptides and amines | RSP_1542, RSP_3274 |
| Carbohydrates, organic alcohols, and acids | RSP_0149, RSP_0150 |
| Cations and iron carrying compounds | RSP_1546, RSP_2608 |
| Unknown substrate | RSP_1895, RSP_2802, RSP_3160 |
| DNA metabolism | DNA replication, recombination, and repair | RSP_1466, RSP_2083, RSP_2414, RSP_2850, RSP_3077, RSP_3423 |
| Pyrimidine ribonucleotide biosynthesis | RSP_3722 |
| Fatty acid and cell envelope | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides | RSP_1491, RSP_2163, RSP_3721 |
| Degradation | RSP_0119 |
| Other | RSP_0422, RSP_0595, RSP_0855 |
| Regulatory functions | DNA interactions | RSP_1083, RSP_4210 |
| Other | RSP_0148, RSP_2631, RSP_3430, RSP_3431 |
| Transcription factors | RSP_0601 |
| Cellular processes | Detoxification | RSP_1057, RSP_2389, RSP_2693, RSP_3263 |
| Toxic production and resistance | RSP_2803 |
| Central intermediary metabolism | Other | RSP_0897, RSP_1258, RSP_1397, RSP_3072 |
| Phosphorus compounds | RSP_0782 |
| Protein synthesis/fate | Amino acid biosynthesis | RSP_0398 |
Table 1. Cont.

| Mainrole  | Subrole  | Locus  |
|-----------|----------|--------|
| Degradation of proteins, peptides, and glycopeptides | RSP_0686, RSP_1490 |
| Protein folding and stabilization | RSP_1219 |
| tRNA and tRNA base modification | RSP_2971 |
| Unknown function | Unknown function | RSP_0151, RSP_0152, RSP_0269, RSP_0423, RSP_0557, RSP_0799, RSP_0896, RSP_1591, RSP_1956, RSP_1985, RSP_2225, RSP_2268, RSP_3075, RSP_3076, RSP_3089, RSP_3310, RSP_3329, RSP_4144, RSP_4209 |

Summary of the functional annotations of members of the RpoHI and RpoHII regulons in *R. sphaeroides* defined by the intersections of the results from the expression profiling and chromatin immunoprecipitation experiments.

1Classification of the functional main categories according to the JCVI-CRM database (http://cmr.jcvi.org/).

2Classification of the functional sub-categories according to the JCVI-CRM database.

3Unique locus identifiers for *R. sphaeroides* 2.4.1.

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but with a larger representation for functions associated with protein homeostasis. The 99 predicted members of the RpoHII regulon include fewer proteins predicted to play a role in protein homeostasis and a larger number of proteins predicted to help maintain the oxidation-reduction state of the cytoplasmic thiol pool. However, a large number of genes in both the unique and overlapping RpoHII and RpoHII regulons are annotated as having no predicted functions. Overall, this analysis revealed that RpoHII or RpoHII activate a large set of distinct and overlapping sets of target genes.

Predicted differences in promoter sequences recognized by RpoHII or RpoHII

Previous work indicated that RpoHII and RpoHII can recognize and initiate transcription from similar promoter sequences [14,15,20]. The characterization of their respective regulons also suggests that some promoters can be transcribed by both σ factors while others are specific to either RpoHII or RpoHII. Therefore, we hypothesized that while the promoter sequences of the two σ factors may be similar, different sequence-specific interactions of RpoHII or RpoHII with promoter elements are the basis of promoter specificity for transcription initiation by RNA polymerase.

To overcome the limited resolution of the ChIP-chip experiment and predict determinants of promoter specificity for RpoHII or RpoHII, we searched the regions upstream of genes in each regulon for conserved sequence elements (137 sequences for RpoHII and 120 sequences for RpoHII). The conserved sequence elements we identified mapped to putative promoter elements that were within 100 bp of the coordinates of the modes of the distributions of the ChIP-chip signal. Thus, the predictions of these searches identified conserved sequence elements that were in agreement with the experimental data. In addition, even though we analyzed the individual RpoHII and RpoHII regulons independently for these motifs, the sequence alignment algorithm converged to the same sequence elements for promoters that were predicted to be recognized by both RpoHII and RpoHII. This result is not surprising given that both σ factors have similar amino acid sequences in their DNA recognition regions and are thus expected to recognize similar promoter sequences. However, this observation supports the hypothesis that RpoHII and RpoHII recognize common promoter sequences in their respective target genes as opposed to distinct promoters.

To predict specificity sequence determinants for each RpoHII paralog, the putative distinct and overlapping promoter sequences were sorted into three groups according to the expression profiling and ChIP-chip data sets and converted into sequence logos (Figure 3, Table S2). The sequence logos derived from the three groups include: two groups that are preferentially or selectively bound and transcribed by either RpoHII or RpoHII and one group that is bound and transcribed by both σ factors. As noted above, some promoters appear to be bound by RpoHII or RpoHII without inducing detectable changes in transcript levels. We aligned these promoters separately to determine if they possessed unique characteristics, but no significant differences were detected (data not shown).

The conservation of a TTG motif in the −35 region in all three logos is consistent with the importance of this triplet in a previous analysis of at least one promoter known to be recognized by both RpoHII and RpoHII [27]. However, there was also evidence for sequence-specific elements in the logos for each RpoH paralog. In the logo for the RpoHII-dependent promoters, a cytosine is overrepresented at position −37 and a thymine is overrepresented at position −9. In the logo for RpoHII-dependent promoters, cytosine and thymine are overrepresented at positions −14 and −13, respectively.

Overall, the comparison between RpoHII and RpoHII-specific promoter logos allowed us to identify significant differences in the promoter sequences that may be used to adjust promoter selectivity and strength for RpoHII or RpoHII. In addition, the predicted sequence elements for RpoHII or RpoHII promoters are not mutually exclusive. Rather, it appears that promoter specificities for RpoHII or RpoHII are distributed along a gradient using a combination of specific bases at various positions of the −35 or −10 promoter elements.

Degrees of promoter specificity of RpoHII and RpoHII

To test predictions about specificity determinants derived from these logos, we cloned several putative promoters upstream of a lacZ reporter gene and integrated these into the genome of a *R. sphaeroides* ΔrpoHII ΔrpoHII mutant [15] via homologous recombination. The activity of each promoter was measured by assaying β-galactosidase activity in these *R. sphaeroides* reporter strains ectopically expressing either RpoHII or RpoHII (Figure 4) at levels comparable to those found during a stress response (see above and Figure 1). The RSP_1173, RSP_1408, and RSP_1531 promoters (which were either predicted to be members of the RpoHII regulon or, in the case of RSP_1173, known to be heat inducible and transcribed by RpoHII [16], had significant activity in the strain expressing RpoHII, but not when the same strain expressed RpoHII.
Figure 3. Conserved promoter sequences recognized by RpoHI and RpoHII. The logos were constructed from promoter sequences alignments sorted into three categories according to their predicted specificity. The consensus sequence for α32-dependent promoters in E. coli as determined by Nomaka et al. [37], is shown as a reference. The heights of the letters represent the degree of conservation across sequences (information in bits, logos generated using WebLogo: http://weblogo.berkeley.edu/). The coordinates on the x-axes represent the positions relative to the predicted transcription start site. The numbers of promoter sequences used to create the logos are indicated in parentheses on the left of the logos. Below the logos are the sequence of promoter sequences used to create the logos generated using WebLogo. (Figure 3).

(Figure 4). In contrast, the RSP_2314, RSP_2389, and RSP_3274 promoters (which were either predicted to be members of the RpoHII regulon by our analysis or known to be induced by conditions that generate singlet oxygen [17,18,20]) showed activity in the presence of RpoHII but not RpoHI (Figure 4). Finally, the RSP_1207 and RSP_2617 promoters (which were predicted to be transcribed by both RpoH proteins and, in the case of RSP_1207, known to be transcribed by RNA polymerase holoenzyme containing either RpoHI homolog [15] showed activity in cells containing either RpoHI or RpoHII (Figure 4). Overall, these results support predictions about members of the RpoHII or RpoHIII regulons derived from combining the transcription profiling, ChIP-chip and computational analyses.

To test the predictions about the contributions of individual bases to promoter recognition, we measured the activity of R. sphaeroides RpoHII with an existing library of mutant E. coli gndE promoters fused to a lacZ reporter in an E. coli tester strain [7]. The data from this analysis revealed that base substitutions in the TTG motif of the −35 region of this RpoHII-dependent promoter (positions −36, −35, and −34) reduced its activity by at least 80% with RpoHII (Figure 5A), as expected from the predictions of promoter logo. We also found a slight increase in promoter activity when position −32 was changed to a cytosine, even though the C-32 is not conserved in RpoHII promoters. This observation is consistent with the results of a previous mutational analysis showing that E. coli α32 prefers a cytosine at position −32 when the alanine at position 264 of its amino acid sequence is substituted to an arginine (corresponding to R267 of RpoHI) [28], but also suggests that the −32 position is not utilized to distinguish between RpoHI- and RpoHII-specific promoters. In the −10 region of the gndE promoter, substitutions of the cytosine at position −14 for an adenine or guanine, the cytosine at position −13 for an adenine, or substitution of the thymine at position −11 for a cytosine, each reduced RpoHII-dependent promoter activity. In addition, a substitution of the adenine at position −12 for a cytosine or changing the thymine at position −9 for any other base reduced RpoHII-dependent activity by ≥90%. These observations are consistent with the conservation of a thymine at position −9 of the derived RpoHII promoter logo (Figure 3).

To test the predicted requirement of RpoHII for a thymine at position −9, we also analyzed the properties of two R. sphaeroides promoters in this E. coli tester strain. Activity of the RpoHII-dependent RSP_1531 promoter was reduced by 90% when the thymine at position −9 was changed to a cytosine, whereas the RpoHII-dependent RSP_2314 promoter had higher RpoHII-dependent activity when a thymine was placed at position −9 (Figure 5B). Therefore, this analysis confirmed that position −9 plays a critical role in promoter specificity for RpoHII. In conclusion, the measured effects of mutations in the E. coli gndE promoter on RpoHII-dependent transcription confirmed that our
models captured elements that are critical for promoter recognition by RpoHII.

We were unable to test activity of R. sphaeroides RpoHII against this groEL promoter library in the same E. coli tester strain (data not shown). Instead, we generated a small set of point mutations in the P1 promoter of the R. sphaeroides cycA promoter (Figure 3) which was previously shown to be transcribed by both RpoHII and RpoHII [27] and measured activity from single-copy fusions of these mutant promoters to lacZ in cells that either lacked both RpoH homologs or that contained a single rpoH gene under control of an IPTG-inducible promoter (Materials and Methods).

By analyzing this promoter library, we found that a G to T mutation at position -36 of cycA P1 (G-36T) increased its transcription by both RpoHII and RpoHII (Figure 5C). This result is consistent with the high predicted information content for T at this position for both RpoHII and RpoHII, as well as the previous observation that the overall increase in activity of cycA P1 is caused by the G-36T mutation [27]. While our RpoHII and RpoHII promoter models (Figure 3) predict that a C could be allowed at position -36, a G-36C mutation lowered activity with RpoHII and had no positive impact on transcription by RpoHII (Figure 5C). Due to the significantly increased in activity from the G-36T mutation in cycA P1, all of the other promoter mutations we tested were generated in this background. Mutations we tested in the -35 region, T-35C and G-34C, resulted in virtually complete loss of cycA P1 activity with either RpoHII and RpoHII when compared to their G-36T parent promoter (Figure 5C), indicating that these bases are essential for transcription initiation by both RpoH homologs. Based on the relatively low information content predicted by our models for other positions in the -35 element (Figure 3), we did not test the effects of other mutations in this region on promoter selectivity by RpoHII homologs.

In the predicted -10 region, A-12 has very high information content for both RpoHII and RpoHII, but the sequence logo suggests a T at this position might allow selective recognition by RpoHII (Figure 3). Indeed, a promoter containing a T at position -12 is still active only with RpoHII, suggesting that A-12 is essential for RpoHII activity but not RpoHII activity. The T at position -9 of cycA P1 is also predicted to have significantly higher information content for RpoHII than RpoHII, while a C at this position should have more information content for RpoHII than RpoHII (Figure 3). As predicted, we found RpoHII retained significant activity after placing a T-9C mutation in the context of the G-36T cycA P1 promoter. Furthermore, we found that this mutation completely abolished its activity with RpoHII, illustrating the high information content of a T at this position for transcription by this RpoH homolog. The importance for a T at the analogous position was also observed when testing activity of mutant E. coli groE promoters with RpoHII (T-9C mutation Figure 5A) or assaying function of the R. sphaeroides RSP_1531 operon fusions integrated into the genome containing the wild type of indicated mutant R. sphaeroides cycA P1 promoter in a tester strain expressing the indicated RpoH homolog. Most of the promoter mutations were made in the G-36T cycA P1 background, as this promoter had activity with RpoHII or RpoHII than its wild type (WT) counterpart. Base substitutions are indicated on the x-axis. Error bars represents the standard error of the mean from three independent replicates.

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Discussion

When organisms encounter environmental or internal stress they often increase the transcription of genes encoding proteins that help mitigate damage to cellular components. Therefore, identifying functions that are involved in transcriptional stress responses is critical to understand both the nature of the damage caused to cellular components and how organisms respond to these challenges. Singlet oxygen and increased temperature are very different phenomena, but in R. sphaeroides the transcriptional responses to these two stresses involve two alternative σ factors, RpoHII and RpoHII, that each belong to the RpoH family [15,16,18]. Several other σ-proteobacteria contain two or more members of the RpoH family that appear to control different stress responses [13,29,30]. However, as it is the case in R. sphaeroides,
little is known about the target genes for these multiple RpoH homologs. In this work, we characterized genes that are directly transcribed by \textit{R. sphaeroides} RpoH\textsubscript{I} and RpoH\textsubscript{II} to gain a better understanding of the biological response to heat shock and singlet oxygen stresses. We found that each of these \textit{RpoH} paralogs control transcription of over 100 genes, suggesting that each of these phenomena lead to large changes in gene expression. However, we also found that there is significant overlap in the RpoH\textsubscript{I} and RpoH\textsubscript{II} regulons, creating an unexpectedly extensive connection between the transcriptional responses to these two signals. In addition, we investigated the characteristics of RpoH\textsubscript{I}- and RpoH\textsubscript{II}-dependent promoters. This effort allowed us to identify sequence elements that define promoter specificity for each \(\sigma\) factor, thereby allowing cells to selectively partition target genes for each RpoH paralog into different stress responses.

\textit{R. sphaeroides} RpoH\textsubscript{I} and RpoH\textsubscript{II} control the expression of a common set of functions

This work revealed a surprisingly extensive overlap of the RpoH\textsubscript{I} and RpoH\textsubscript{II} regulons even though these two homologs activate transcriptional responses to different signals in \textit{R. sphaeroides}. This suggests that genes activated by these two pathways of the transcriptional regulation network play a role in the physiological response to both these, and even possibly, other stresses. Indeed, the genes regulated by both RpoH\textsubscript{I} and RpoH\textsubscript{II} encode known or annotated functions involved in protein homeostasis, DNA repair, and maintenance of cell membrane integrity (Table 1). These types of functions are central to cell viability and may be relevant for the physiological responses to multiple stresses that can have broad primary and secondary effects on cells. Indeed, the predicted functions of the overlapping members of the RpoH\textsubscript{I} and RpoH\textsubscript{II} regulons encode functions that are also part of the general stress response regulons for \(\sigma^{+}\) in \textit{E. coli} or \(\sigma^{32}\) in \textit{Bacillus subtilis} [31,32]. Interestingly, \(\sigma^{+}\) homologs are mostly present in \(\beta\)- and \(\gamma\)-proteobacteria, but to date absent from sequenced genomes of \(\alpha\)-proteobacteria like \textit{R. sphaeroides} (http://img.jgi.doe.gov/ [33]). Thus, it is possible that the set of genes controlled by both RpoH\textsubscript{I} and RpoH\textsubscript{II} is part of a general stress response that is common to the heat shock, singlet oxygen and possibly other uncharacterized signals in \textit{R. sphaeroides} [14,15,17,19,20]. This hypothesis is supported by the observation that \textit{R. sphaeroides} and \textit{R. elti} strains lacking both RpoH\textsubscript{I} and RpoH\textsubscript{II} are more sensitive to several conditions than strains lacking only one of these proteins [13,15,20].

In considering the scope of functions that are regulated by both RpoH\textsubscript{I} and RpoH\textsubscript{II}, it is also important to note that this set of genes may be larger than the one we characterized because some promoters known to be transcribed by both \(\sigma\) factors were only marginally affected by ectopic expression of either RpoH\textsubscript{I} or RpoH\textsubscript{II}. For example, the RSP 2310 (\textit{groES}) promoter was shown to be transcribed by both RpoH\textsubscript{I} and RpoH\textsubscript{II} in previous in vitro experiments [14] and was detected by our ChiP-chip experiment to be bound by both RpoH\textsubscript{I} and RpoH\textsubscript{II}, but did not meet all the criteria of our analysis. Thus, the \textit{groES} promoter, like other promoters, may be subject to complex regulation in vivo.

RpoH\textsubscript{I} and RpoH\textsubscript{II} each control functions specific to heat shock or singlet oxygen stresses, respectively

Our data also significantly extend the number and types of functions that are specifically controlled by RpoH\textsubscript{I} or RpoH\textsubscript{II} (Table 1). We expected to find specific sets of target genes because strains lacking either RpoH\textsubscript{I} or RpoH\textsubscript{II} displayed different phenotypes [14,15,17,20]. While previous results indicated that accumulation of \(~25\) proteins was dependent on RpoH\textsubscript{I} [17], our data indicate that some \(150\) genes are directly controlled by each \textit{R. sphaeroides} RpoH\textsubscript{II} paralog.

Genes in the direct but RpoH\textsubscript{I}-specific regulon encode functions that are involved in protein homeostasis, maintaining membrane integrity, and DNA repair, as is found for the \textit{E. coli} \(\sigma^{32}\) regulon [3] (Table 1) The RpoH\textsubscript{I} specific regulon is also predicted to encode cation transporters and proteins in the thioredoxin-dependent reduction system (Table 1). Ion transporters can aid the heat shock stress response since exporting cations like iron, which may be released by thermal denaturation of damaged iron-sulfur or other metalloproteins, decreases secondary effects caused by formation of toxic reactive oxygen species [34]. The thioredoxin-dependent reduction system reduces disulfide bonds and peroxydes, which are created by protein oxidation, and thereby helps maintain cytoplasmic proteins in a reduced state [35]. Inclusion of these functions in the RpoH\textsubscript{I} regulon suggests that oxidative damage may be an important secondary effect of heat shock, perhaps caused by protein denaturation or permeabilization of the cell envelope. Overall, these results support the hypothesis that the function of RpoH\textsubscript{I} in \textit{R. sphaeroides} is similar to that of \(\sigma^{32}\) in \textit{E. coli} for the response to heat shock stress. In addition, it is also possible that RpoH\textsubscript{I} plays a role in the \textit{R. sphaeroides} response to other forms of stress. There is precedent for roles of \(\sigma^{32}\) homologs in other stress responses by other bacteria since the activity of RpoH in \textit{Caalobacter crescentus} is increased by heavy metal stress [36].

In contrast, \textit{rpoH}\textsubscript{II} transcription is under direct control of a Group IV alternative \(\sigma\) factor (RpoE) that serves as the master regulator of the singlet oxygen stress response [18]. In addition, an \textit{R. sphaeroides} \textit{ApoHII} mutant is more sensitive to singlet oxygen than a wild-type or \textit{ApoHII} strain [15,17]. Therefore, members of the direct RpoH\textsubscript{II}-specific regulon might be expected to play an important role in the response to singlet oxygen stress. Among the genes in the RpoH\textsubscript{II}-specific regulon are others predicted to function in maintaining membrane integrity and performing DNA repair, both potential targets for damage by singlet oxygen. However, the RpoH\textsubscript{II} specific regulon contains fewer genes encoding functions related to protein homeostasis than found in the RpoH\textsubscript{II} regulon (Table 1). Other functions apparently unique to the RpoH\textsubscript{II} regulon include the glutathione-dependent reduction system, which like the thioredoxin-dependent system repair oxidized protein residues and maintain a reduced cytoplasm (Table 1). Even though the thioredoxin- and glutathione-dependent reduction systems serve similar cellular functions, they are apparently under the control of different RpoH-dependent transcriptional networks in \textit{R. sphaeroides}. Thus, it is possible that the thioredoxin- and glutathione-dependent reduction systems preferentially function on different oxidized substrates. Glutathione-dependent reduction systems are known to function on lipids or other types of protein oxidative damage that might be experienced by the cell following singlet oxygen damage [35]. We also found that the RpoH\textsubscript{II}-specific regulon includes the multi-subunit NADH:quinone oxidoreductase and genes encoding enzymes in heme and quinone biosynthesis (Table 1). Each of these functions are critical for the respiratory and photosynthetic electron transport chains of \textit{R. sphaeroides} and are known or predicted to contain one or more oxidant-sensitive metal centers. Thus, placement of these genes in the RpoH\textsubscript{II}-specific regulon suggests that these membrane or bioenergetic functions are damaged by and need to be replaced in the presence of singlet oxygen. Overall, our data indicates that the RpoH\textsubscript{II}-specific regulon controls expression of functions in the repair of oxidized proteins and replacement or assembly of critical electron transport factors.
RpoH$_I$ and RpoH$_II$ recognize different but compatible promoter sequence elements

Our global gene expression data, results from analysis of gene fusions, as well as previously reported in vitro experiments [14,15] all indicate that RNA polymerase containing either RpoH$_I$ or RpoH$_II$ can recognize some promoters in common. This observation is not surprising considering that RpoH$_I$ and RpoH$_II$ have similar amino acid sequences in their respective promoter recognition regions and are each able to rescue growth of *E. coli* $\sigma^{32}$ mutants [14–16]. Likewise, the sequence logos derived here revealed that the promoter sequences recognized by each of the *R. sphaeroides* RpoH homologs are similar to both each other and to that recognized by *E. coli* $\sigma^{32}$ [37].

Our experiments provide definitive evidence that some promoters are transcribed either exclusively or predominantly by RpoH$_I$ or by RpoH$_II$. We were also able to predict and confirm the importance of bases for activity with individual RpoH homologs (particularly those in the $\sim$9 element). We have computational and experimental observations that can explain some aspects of promoter selectivity by RpoH$_I$ and RpoH$_II$. For example, our experiments identify T-9 and other positions in the $\sim$10 element as potential candidates in this discrimination, as one or more substitutions have larger effects on activity with individual RpoH$_I$ homologs. Mutation of T-9 to any other base reduced or more substitutions have larger effects on activity with individual RpoH$_II$ homologs. Together, these data suggest that T-9 is a determinant of promoter selectivity by RpoH$_I$. These results also predict that other bases, which are overrepresented in the RpoH$_II$ promoters, could be critical for regulation by RpoH$_II$. As a result of these events, the promoters were transcribed either exclusively or predominantly by RpoH$_I$ or by RpoH$_II$. We were also able to predict and confirm functions are also useful for survival in the presence of other forms of stress because these target genes also contain promoters that are recognized by RpoH$_II$. We propose that the duplication of an ancestral RpoH protein to create a second homolog of this alternative $\sigma$ factor provided *R. sphaeroides* the opportunity to connect stress response functions to another stimulus. In this model, $rpoH_I$ was placed under the control of the master regulator of the singlet oxygen stress response and the two RpoH proteins evolved to recognize somewhat different but compatible promoter elements to assure the optimal regulation of distinct but overlapping stress regulons. As a result of these events, the transcriptional responses of *R. sphaeroides* to heat shock and singlet oxygen stress were separable but allowed to converge and contain a common set of functions. It will be interesting to identify and examine other examples of such convergence across bacteria and other organisms that possess multiple homologs of RpoH or other transcription factors.

Materials and Methods

Bacterial strains and growth conditions

*E. coli* strains were grown in Luria-Bertani medium [38] at 30°C or 37°C. *R. sphaeroides* strains were grown at 30°C in Sistrom’s succinate-based medium [39]. *E. coli* DH5x was used as a plasmid host, and *E. coli* S17-1 was used as a donor for plasmid conjugation into *R. sphaeroides*. The media were supplemented with kanamycin (25 µg/ml), ampicillin (100 mg/ml), chloramphenicol (30 mg/ml), spectinomycin (50 mg/ml), tetracycline (10 mg/ml for *E. coli* and 1 mg/ml for *R. sphaeroides*), trimethoprim (30 µg/ml), or 0.1% of L(+)-arabinose when required. Unless noted, all reagents were used according to the manufacturer’s specifications. The list of bacterial strains and plasmids used in this study are summarized in Table S3.

Construction of plasmids for controlled expression of RpoH$_I$ and RpoH$_II$ in *R. sphaeroides*

Plasmids for ectopically expressing RpoH$_I$ or RpoH$_II$ were constructed by separately cloning the $rpoH_I$ or $rpoH_II$ genes downstream of the IPTG-inducible promoter in pLND4 [22]. DNA fragments containing $rpoH_I$ or $rpoH_II$ were amplified from *R. sphaeroides* 2.4.1 genomic DNA using oligonucleotides containing *E. coli* translation-termination signals (Table S3).

Western blot analysis for the expression of RpoH$_I$ and RpoH$_II$

To monitor levels of RpoH$_I$ and RpoH$_II$ after heat shock, exponential phase aerobic cultures (69% nitrogen, 30% oxygen and 1% carbon dioxide) of wild type *R. sphaeroides* strain 2.4.1 grown at 30°C, were transferred to a 42°C warm bath with samples collected before heat treatment and at 10 min time intervals after heat shock, up to 60 min. To assess induction resulting from singlet oxygen stress, similarly grown wild type cells were treated with 1 µM methylene blue and exposed to 10 W/m$^2$ incandescent light with samples collected before treatment and at 10 min time intervals after treatment, up to 60 min. Exponentially growing aerobic cultures of *R. sphaeroides* $\Delta rpoH_I$ and $\Delta rpoH_II$ mutants carrying the pYSD40 or pYSD42 plasmids respectively, were treated with 100 µM IPTG for one generation and harvested. All cell samples were resuspended in 3 M urea containing 1 X protease inhibitor cocktail (Thermo Scientific,
Gene expression microarrays

Triplicate 500 ml cultures were grown aerobically with bubbling (30% O2, 69% N2, 1% CO2) until they reached early exponential phase (OD at 600 nm of 0.15). At this point IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 100 μM to induce gene expression from the pLND4 derivatives. After 3 hours incubation (OD at 600 nm of 0.30), 44 ml of cell culture were collected and 6 ml of 5% v/v phenol in ethanol was immediately added. Cells were collected by centrifugation at 6,000 g and frozen at −80°C until sample preparation. RNA extraction, cDNA synthesis, labeling, and hybridization were performed as previously described on Genechip Custom Express microarrays (Affymetrix, Santa Clara, CA) [40]. Processing, normalization, and statistical analysis of the expression profile data were performed in the R statistical software environment (http://www.r-project.org/) [41]. Data were normalized using the affyPLM package with default settings [42–44]. The expression microarray data have been deposited in the NCBI’s Gene Expression Omnibus [45] and are accessible through GEO Series accession number GSE39806 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39806).

Chromatin immunoprecipitation on a chip

Cells were harvested at mid-exponential growth (OD at 600 nm of 0.30) from the same cell cultures used for the expression microarray experiment to prepare samples for a ChiP-chip assay [19]. RpoHII-FLAG was immunoprecipitated using commercial monoclonal antibodies against the FLAG polypeptide (DYKDDDDK) (Sigma Aldrich, St Louis MO). RpoH2 was immunoprecipitated with anti-R. sphaeroides RpoHII rabbit serum. Labeled DNA was hybridized on a custom-made tiling microarray, synthesized by NimbleGen (Roche Dure, Hercules, CA). An equal amount of total protein for each strain for each promoter fusion containing only the empty pIND4 plasmid (i.e. not expressing either RpoHI or RpoHII) was subtracted from the average of three independent replicates. Background LacZ activity from control strains for each promoter fusion containing only the empty pLND4 plasmid (i.e. not expressing either RpoHII or RpoHII) was subtracted from the measured LacZ activity for each mutant promoter.

The construction of the E. coli CAG57102 mutant strain, the promoter library, and the β-galactosidase assay used to test the activity of R. sphaeroides RpoHII in vivo on mutant promoters were described previously [7]. To express R. sphaeroides RpoHII the E. coli rpoH II gene of pSAKT32 [7] was replaced with the R. sphaeroides rpoHII gene. At least triplicate assays for β-galactosidase activity were performed on all strains.

Supporting Information

Figure S1 Scatter plot of RpoHII versus FLAG-RpoHII dependent change in gene transcription levels.

Table S1 RpoHII and RpoHII target genes.

Table S2 Predicted promoter sequences of RpoHII and RpoHII target genes.

Table S3 Bacterial strains and plasmids.
Author Contributions
Conceived and designed the experiments: YSD B-MK SI HAG TJD. Performed the experiments: YSD B-MK SI HAG. Analyzed the data: YSD

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