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Expanding the RpoS/σ^S-Network by RNA Sequencing and Identification of σ^S-Controlled Small RNAs in Salmonella

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Abstract

The RpoS/σ^S sigma subunit of RNA polymerase (RNAP) controls a global adaptive response that allows many Gram-negative bacteria to survive starvation and various stresses. σ^S also contributes to biofilm formation and virulence of the food-borne pathogen Salmonella enterica serovar Typhimurium (S. Typhimurium). In this study, we used directional RNA-sequencing and complementary assays to explore the σ^S-dependent transcriptome of S. Typhimurium during late stationary phase in rich medium. This study confirms the large regulatory scope of σ^S and provides insights into the physiological functions of σ^S in Salmonella. Extensive regulation by σ^S of genes involved in metabolism and membrane composition, and down-regulation of the respiratory chain functions, were important features of the σ^S effects on gene transcription that might confer fitness advantages to bacterial cells and/or populations under starving conditions. As an example, we show that arginine catabolism confers a competitive fitness advantage in stationary phase. This study also provides a firm basis for future studies to address molecular mechanisms of indirect regulation of gene expression by σ^S. Importantly, the σ^S-controlled downstream network includes small RNAs that might endow resistance of σ^S in Salmonella. Extensive regulation by σ^S of genes involved in metabolism and membrane composition, and down-regulation of the respiratory chain functions, were important features of the σ^S effects on gene transcription that might confer fitness advantages to bacterial cells and/or populations under starving conditions. As an example, we show that arginine catabolism confers a competitive fitness advantage in stationary phase. This study also provides a firm basis for future studies to address molecular mechanisms of indirect regulation of gene expression by σ^S.

Introduction

In eubacteria, a single multi-subunit RNA polymerase (RNAP) is responsible for transcription. Although the core RNAP (E, α2ββ’ω) is capable of transcription elongation and termination, it cannot specifically initiate transcription from a promoter site. Promoter recognition relies on an additional subunit, σ, which associates with E to form the holoenzyme Eσ [1]. σ directs RNAP to specific promoters, is involved in promoter melting, and dissociates stochastically once sequence-specific promoter DNA contacts are no longer required. All bacteria have a primary house-keeping sigma factor, known as σ^70 (RpoD) in Escherichia coli (E. coli) and Salmonella, which promotes the transcription of genes required for the essential functions in the cell. Most bacteria also have one or more alternative σ factors that direct transcription of specific subsets of genes [1]. The alternative sigma factor σ^S (RpoS) controls a global adaptive response allowing many Gram-negative bacteria to survive nutrient deprivation and environmental stresses [1–3]. σ^S also contributes to virulence and biofilm formation of Salmonella enterica serovar Typhimurium (S. Typhimurium) [3–5], a wide host-range pathogen and a major cause of human gastroenteritis and foodborne disease.

Previous works have focused on the complex regulation of rpoS in E. coli K-12 and on σ^70 promoter specificity [2,3]. In contrast to σ^70, σ^S is almost undetectable in early exponential phase and is induced in stationary phase or in response to various stresses by a fine-tuned combination of transcriptional, translational and post-translational controls [2,3]. σ^S and σ^70 bind to almost identical – 35 and –10 promoter elements, a finding consistent with the high degree of sequence similarity between these two sigma in their DNA binding regions [3,6]. The activity of Eσ^S and Eσ^70 holoenzymes can be modulated by additional regulatory proteins that bind to the promoter region and can also contribute to σ factor selectivity at a given promoter [2,3].

σ^S regulons have been characterized using microarrays in E. coli and occasionally in other bacterial species [3,7,8,9], but not in Salmonella. Indeed, previous transcriptional profiling using a S. Typhimurium rpsS mutant only focussed on σ^S-activated genes requiring σ^S for maximal expression [10]. More than 10% of the E. coli genes were found to be under positive control by σ^S [3]. In addition, negative effects of σ^S on gene expression is an important but poorly understood aspect of σ^S-dependent control in E. coli [2,3,7]. Elimination of these negative effects in rpsS mutants likely contributes to the growth advantage of these mutants in some
environments in the absence of stress [11,12]. Our previous studies suggest that σs exerts negative effects on gene expression and growth capabilities in Salmonella as well [13–15], although it is not known to which extent.

In this study, we used directional RNA-sequencing and complementary assays to explore the σs-dependent transcriptome of S. Typhimurium. Our data confirm the large impact of σs on gene transcription in stationary phase bacteria, including gene repression by σs, and provide insights into the main physiological functions of σs in S. Typhimurium. Further, we show that the σs-controlled downstream network includes small RNAs that might mediate σs activity by post-transcriptional regulatory functions and might be intermediate regulators in the down-regulation of gene expression by σs. This study provides a firm basis for future studies to address molecular mechanisms used by σs to control gene expression indirectly and to assess the physiological impact of negative regulation by σs.

Materials and Methods

Bacterial Strains, Bacteriophage and Growth Conditions

Strains are listed in Table S1. Bacteriophage P22HT105/1int was used to transfer mutations and lacZ fusions between Salmonella strains by transduction [16]. Green plates, for screening for P22-infected cells or lysogens, were prepared as described previously [17]. Bacteria were routinely grown in Luria-Bertani medium (LB) at 37°C under aeration. Antibiotics were used at the following concentrations (μg ml⁻¹): carbenicillin (Cb), 100; kanamycin (Km), 50; and tetracycline (Tet), 20.

DNA Manipulations and Inactivation of Chromosomal Genes

Standard molecular biology techniques were used [4,18]. Oligonucleotides were obtained from Sigma-Aldrich and are listed in Table S2. DNA sequencing was performed by Beckman Coulter Genomics. Chromosomal deletions in the σsR, σsL and csrC loci of Salmonella ATCC14028 were generated using tetAR PCR-generated linear DNA fragments (Table S2) and the λ-Red recombinase-based redcombinase linking procedure [19,20]. The scarless in frame deletion of rpoS in strain VFC331 was achieved with a two-step Red-recombinase-based recombineering procedure [20]. The procedure involves 1) replacement of the rpoS coding sequence by a tetAR module (produced by PCR, Table S2) and 2) replacement of the tetR module by a DNA fragment obtained by PCR (Table S2) and carrying the desired deletion through positive selection of tetracycline-sensitive recombinants [21]. All strains were confirmed to contain the expected mutation by DNA sequencing. Transcriptional lacZ fusions in the astA, katE and katN genes were previously described [14,22].

Isolation of Total RNA from S. Typhimurium

Total RNA was isolated from cells grown aerobically until late stationary phase (18 h growth) in LB at 37°C, using TRizol. Pellets of cells were resuspended in 12.5 mM Tris-HCl (pH 7.6), 10 mM EDTA, 10% glucose. After addition of 1/5 volume of 0.5 M EDTA, disruption of cells was performed by vigorous shaking using glass beads (G1277, Sigma-Aldrich) in acid phenol (10 mM EDTA, 10% glucose). After centrifugation, the aqueous phase was carefully mixed with 2 volumes of TRizol (Invitrogen), and five minutes later with a chloroform:isoamyl alcohol mixture (24:1). After centrifugation, chloroform:isoamyl alcohol was added to the aqueous phase and the mixture was allowed to stand for five minutes before centrifugation. Total RNA present in the aqueous phase was precipitated with isopropanol. After centrifugation, the pellet was washed in 70% Ethanol, air-dried and resuspended in RNase-free water. The RNA was subsequently treated with DNaseI (Ambion) and its quality was analyzed using an Agilent BioAnalyzer.

cDNA Library Preparation, Sequencing and Analysis of Sequences

Total RNA from three biological replicates of each strain was isolated from late stationary phase cultures as described above and its quality checked with an Agilent BioAnalyzer. Starting from 10 μg of total RNA, rRNA content was depleted using MicrobExpress kit (Ambion). The rRNA depleted fraction was used for construction of strand specific single end cDNA libraries according to manufacturer’s instructions (using Truseq Small RNA sample prep kit, Illumina). Libraries were sequenced using an Illumina Hiseq2000 sequencer (multiplexing 3 samples per lane) according to manufacturer’s instructions (Illumina). Sequences were demultiplexed using the Illumina pipeline (Gerald, included in CASAVA version 1.7) giving FASTQ formatted reads. Those reads were cleaned from adapter sequences and sequences of low quality using an in-house program. Only sequences with a minimum length of 30 nucleotides were considered for further analysis. Bowtie [23] (version 0.12.7, –chunkmbs 200, –m 50, –e 50, –a –best, –solexa1.3-quals) was used to align to the reference genome (CP0013631.1 and CP0013621.1). HTSeq-count (Simon Anders, www-huber.embl.de/users/anders/HTSeq/doc/count.html, parameters: -m intersection-nonempty, -s yes, -t gene) was used for counting genes. Statistical analyses were performed using R version 2.15.1 [24] and Bioconductor packages (http://www.bioconductor.org/). Genes with null raw counts in all samples were excluded from the data table. Normalization and differential analysis were performed using DESeq version 1.8.3 [25]. The whole dataset was first normalized using the normalization function of DESeq and dispersion was estimated with default parameters. The statistical test was then applied on pairs of strains. Resulting p-values were adjusted for multiple comparisons according to the BH method [26]. Two significance thresholds (0.05 and 0.001) were applied on adjusted p-values in order to declare genes as differentially expressed. The mapped reads were formatted into graph files for visualization using COV2HTML [27] (https://monomut.euc/COV2HTML) and GBrowse (http://genopole.pasteur.fr/GBrowse/).

RNA-seq Transcriptome Accession Number

The RNA-seq data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE46380 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46380). The RpoS/σs-Netword in Salmonella

Analysis of sRNAs Expression from RNAseq Data

Normalization and differential analysis of expression of sRNAs previously annotated in the genome of S. Typhimurium SL1344 [28] were performed using DESeq2 version 1.2.5 [25] then integrated in COV2HTML [27] for data analysis with a cut off ratio of 2.

Northern Analysis

Total RNA from Salmonella strains grown for 18 H in LB at 37°C was fractionated on an 8% polyacrylamide–7 M urea gel and transferred to Hybond-N+membranes (RPNI520B GE Healthcare). Blots were hybridized to DNA oligonucleotides (Table S2) labeled at the 5’ ends with T4 polynucleotide kinase using the UltraHyb-OLIGO buffer (AM8663, Ambion).
Quantitative Real-time PCR

Quantitative real-time PCR was performed to verify the transcriptomic data using Applied Biosystems 7300 Real-Time PCR system. Total RNA was extracted from cells grown to stationary phase in LB as described above. The RNA (1 μg) was reverse-transcribed 2 hours at 37°C in 50 μl of reverse transcriptase buffer in the presence of 2 mM dNTPs, 1 μl of random hexamers (1 μg/ml p(dN)₆, Roche) and 10 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega) and 40 U of recombinant ribonuclease inhibitor (Rnasin, Promega). The

Figure 1. Directional RNAseq data analyses. (A) Relative expression level and σ^{S}-dependency of σ^{S}-dependent genes (p<0.001). The x axis shows reads counts in the wild-type strain VF7969 normalized to the length of the gene. The y axis shows the fold change in the expression levels of the gene in the ΔrpoS strain VF9356 compared to the wild-type strain (as reported in Dataset S2). Red and green dots represent genes negatively and positively controlled by σ^{S} respectively. (B) Functional categories of σ^{S}-controlled genes (p<0.001). Genes controlled by σ^{S} are grouped according to their functional categories in the COG database (detailed COG assignments are given in Dataset S2). The relative occurrence of genes belonging to each category in the set of genes positively controlled by σ^{S} (left pie chart) and negatively controlled by σ^{S} (right pie chart) is shown. Some of the genes do not currently have a COG functional category assignment (here represented as not in COGs). Note that some genes have multiple COG category assignments (Dataset S2).

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Figure 2. Role of σ^{S} in the transport and catabolism of L-arginine. Schematic representation of σ^{S}-dependent pathways involved in metabolism of L-Arginine, putrescine and 4-aminobutyrate. To assess the contribution of σ^{S} in the expression of the metabolic pathways indicated, genes differentially expressed with a p value of less than 0.05 in the wild-type and ΔrpoS strains of Salmonella were considered (Dataset S2). Genes showing differential expression with p<0.001 are indicated in bold face. Genes in red and green were negatively and positively controlled by σ^{S} respectively. Genes in black did not show differential expression in the wild-type and ΔrpoS strains.

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Figure 3. Motility of Salmonella ΔrpoS mutant. Motility on 0.3% agar LB plates of the wild-type strain ATCC14028 (WT) and its ΔrpoS mutant VFC331 after 5 h at 37°C.

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relative amounts of target mRNA were determined by real-time PCR using the Fast Start Universal SYBR Green Master following the manufacturer’s instructions (Roche). A final dissociation curve analysis step from 60°C to 95°C was performed to confirm the amplification specificity. To check whether contaminating chromosomal DNA was present, each sample was tested in control reactions that did not contain reverse transcriptase. The real-time PCR was performed using 200 nM gene-specific primer pairs (Table S2) designed in silico using Primer3 software (http://primer3.ut.ee) to generate amplicons in the 100–150 bp range. A relative standard curve experiment using a ten-fold dilution series of genomic DNA was performed for each primer pair to determine the amplification efficiency. The efficiency of the amplification for all the genes tested was higher than 1.8. Three biological replicates were analysed in duplicate each. \( \Delta \Delta CT \) was used as reference gene as it displays little variation in the transcriptional studies performed in our lab using wild-type and \( \Delta rpoS \) strains. Gene expression levels were calculated using the comparative Ct method \( (2^{-\Delta \Delta CT}) \) as previously described [29]. P values were calculated using a two-tailed t test.

**Enzymatic Assays**

\( \beta \)-galactosidase activity was measured as described by Miller [30] and is expressed in Miller units.

**Sequence Analyses**

DNA and amino acid sequence analyses were conducted using the BLAST programs at the NCBI (National Center for Biotechnology Information). Functional annotations were obtained from the MicroScope Microbial Genome Annotation & Analysis Platform (www.genoscope.cns.fr/agc/microscope/home/index.php) [31] and the KEGG server (www.genome.jp/kegg/kegg2.html). Functional analysis of genes with significant changes in expression was done using clusters of orthologous groups (COG) functional categories described for *Salmonella enterica* serovar Typhimurium ATCC14028 genes (www.genoscope.cns.fr/agc/microscope/genomic/classifCOG.php).

**Motility Assay**

Three independent stationary phase cultures of strains grown in LB (18 h, 37°C, 200 rpm) were used. 1 μl of culture was inoculated into 0.3% agar LB plates that were incubated at 37°C for 5 h.

**Competition Assays**

Overnight LB cultures were washed and resuspended in phosphate-buffered saline (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1.76 mM) to an OD600 of 1.0. Equal numbers of cells of the wild-type strain ATCC14028 and the mutant strain were then mixed in fresh LB medium to give a total of about 3000 cells ml⁻¹ and the mixture was incubated at 37°C with shaking. Aliquots of bacteria were removed at timed intervals and numbers of viable cells of each strain were determined on LB plates containing the appropriate antibiotics. P values were calculated using a two-tailed t test.

**Results and Discussion**

**Global Gene Expression in Wild-type and \( \Delta rpoS \) Salmonella Strains**

To assess the relative impact of \( \sigma^5 \) at a global level, transcript levels of wild-type and \( \Delta rpoS \) strains of *Salmonella* were measured by directional RNA-sequencing using three biological replicates of strains grown to stationary phase in LB (GEO GSE46380). \( \sigma^5 \) is known to accumulate during entry to stationary phase in rich medium and to reach its maximum level of production in late stationary phase [14]. Consistently, in a previous study using RNA sequencing and chromatin immunoprecipitation methods to evaluate transcription in *S. Typhimurium* in rich medium, \( \sigma^{70} \) was the main \( \sigma \) factor at early stationary phase [28], suggesting that most \( \sigma^5 \)-regulated genes are expressed in late stationary phase. We thus isolated total RNA from cells in late stationary phase. We identified a total of 1071 genes differentially expressed in the wild-type strain and the \( \Delta rpoS \) mutant (\( p<0.05 \)), of which 607 were highly significant (\( p<0.001 \)) (Dataset S1 and Figure 1). In general, genes up-regulated in the \( \Delta rpoS \) mutant (145 genes, \( p<0.001 \)) exhibited lower expression levels and fold-change values than down-regulated genes (462 genes, \( p<0.001 \)) (Figure 1A). Some \( \sigma^5 \)-dependent genes are likely directly regulated through binding of \( \sigma^5 \) to promoters while others are likely regulated indirectly by \( \sigma^5 \).

**Physiological Functions of the \( \sigma^5 \) Network in S. Typhimurium**

Among ATCC14028 genes that have been assigned to a category of orthologous genes (COG), the most prominent categories associated with \( \sigma^5 \) regulation were metabolism, transcription, signal transduction mechanisms and membrane biogenesis and unknown functions (see detailed COG assignments in Dataset S2 and an overview in Figure 1B).

\( \sigma^5 \) had a substantial effect on expression of metabolic genes, primarily for energy production/conversion and transport/metabolism of carbohydrates, amino acids and inorganic ions (Dataset S2). In some cases, most genes in a given pathway are controlled by \( \sigma^5 \), suggesting a role for this pathway in stationary phase physiology (see for instance pathways shown schematically in Figure 2 and Figures S1–S2, with genes activated and down-regulated by \( \sigma^5 \) in green and red, respectively). A number of genes involved in central energy metabolism exhibited positive \( \sigma^5 \) control (phosphotransferase systems, glycolysis, the pentose phosphate pathway, mixed acid fermentation, and acetate metabolism) whereas genes encoding enzymes in the tricarboxylic acid (TCA) cycle and the operons encoding NADH dehydrogenase-1 (nuo) and ATP synthase (atp) were down-regulated by \( \sigma^5 \) (Dataset S2 and Figure S1). \( \sigma^5 \) might thus play a role in transition from aerobic respiration towards more fermentative and/or
an aerobic respiratory energy metabolism in stationary phase *Salmonella*.

Since $\sigma^5$ controls, either positively or negatively, a large number of genes, and it is required for complex phenotypes such as multiple stress resistance and biofilm formation, it is difficult to pinpoint specific genes directly involved in a particular physiological function of $\sigma^5$. However, it is likely that several $\sigma^5$-controlled genes contribute to prevent or repair oxidative damage. First, $\sigma^5$ controls antioxidant pathways involving catalases, superoxide dismutase, glutaredoxins and glutathione-S-transferases (Dataset S2) [3]. Given the complexity of regulatory controls, either positively or negatively, a large number of genes can modulate expression of isoenzymes more likely as a result of increased endogenous oxidative stress [38]. In addition, whereas the $\sigma^5$-mediated induction of genes encoding proteases (including *btrA*, *cpxA*, *psbB*, *yggB*, *tdiD*, *hsIV*) may favor the recycling in stationary phase of mis-folded proteins as nutrients, a low level of expression of these genes in the *ΔrpoS* mutant may contribute to accumulation of damaged proteins.

Along with these, the expression of other genes that are regulated by *ΔrpoS* strain are probably induced in response to cellular damages derived by the lack of a functional $\sigma^5$ protein. For instance, the heat shock protein *groEL/groES* is induced in response to cellular damages derived by the lack of *ΔrpoS* strain possibly because the level of damaged proteins is increased in the absence of $\sigma^5$. Indeed, the levels of carbonylated proteins increased in stationary phase *E. coli* strains lacking $\sigma^5$ likely as a result of increased endogenous oxidative stress [38]. In addition, whereas the $\sigma^5$-mediated induction of genes encoding proteases may modulate expression of isoenzymes more likely as a result of increased endogenous oxidative stress [38]. In addition, whereas the $\sigma^5$-mediated induction of genes encoding proteases may modulate expression of isoenzymes more likely as a result of increased endogenous oxidative stress [38].

### Negative Regulation by $\sigma^5$ and Bacterial Fitness

Whereas $\sigma^5$ has a positive effect on a large number of genes that likely contribute to stress resistance, it has also a negative effect on the expression of several genes under the control of other sigma.

#### Figure 5. $\sigma^5$-dependent expression of small RNAs in *Salmonella*

(A) The indicated sRNAs were detected in Northern experiments in the wild-type strain ATCC14028 (+) and its ΔrpoS derivative VFC331 (−). The positions of bands were in agreement with the expected transcript lengths [28,64,65] (Table S3) except for STnc1110 (195 nt, detected with positions of bands were in agreement with the expected transcript lengths). Blots were stripped and re-probed with 5S RNA probe to confirm loading of each quantities of wild type and ΔrpoS RNA. (B) Mapped reads, in the wild-type and ΔrpoS strains, VF7969 and VF9356 respectively, of the $\sigma^5$-dependent RNAs assessed in Northern experiments. The mapped reads were formatted into graph files for visualization at a strand-specific manner using COV2HTML. The annotated sRNA genes are indicated as grey arrows and open-reading frames annotated in ATCC14028 are shown as blue arrows. Open-reading frames overlapping the sRNAs are small putative CDS of unknown function annotated in ATCC14028. STnc2080 is located upstream of STM14_3200, 1–250 for SVaR and S60R and up-regulated in response to cellular damages derived by the lack of a functional $\sigma^5$ protein. For instance, the heat shock protein genes encoding *groEL/groES* are up-regulated in the *ΔrpoS* strain possibly because the level of damaged proteins is increased in the absence of $\sigma^5$. Indeed, the levels of carbonylated proteins increased in stationary phase *E. coli* strains lacking $\sigma^5$ likely as a result of increased endogenous oxidative stress [38]. In addition, whereas the $\sigma^5$-mediated induction of genes encoding proteases (including *btrA*, *cpxA*, *psbB*, *yggB*, *tdiD*, *hsIV*) may favor the recycling in stationary phase of mis-folded proteins as nutrients, a low level of expression of these genes in the *ΔrpoS* mutant may contribute to accumulation of damaged proteins. (Dataset S2) [3] and seems to play an important role in the transport and utilization of amino acids such as L-arginine (Dataset S2, Figure 3). Given the complexity of regulatory controls, either positively or negatively, a large number of genes may positively or negatively, a large number of genes contribute to prevent or repair oxidative damage. First, $\sigma^5$ controls gene expression of small RNAs in *Salmonella*. (A) The indicated sRNAs were detected in Northern experiments in the wild-type strain ATCC14028 (+) and its ΔrpoS derivative VFC331 (−). The positions of bands were in agreement with the expected transcript lengths [28,64,65] (Table S3) except for STnc1110 (195 nt, detected with positions of bands were in agreement with the expected transcript lengths). Blots were stripped and re-probed with 5S RNA probe to confirm loading of each quantities of wild type and ΔrpoS RNA. 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factors (Dataset S2) [3,7,12,40]. These negative effects of σ5 on gene expression likely drive the selection of non-functional rpoS alleles in environments with no stress, where reduced σ5 activity confers a growth advantage [3,11–15,41]. These observations have led to the proposal that the acquisition of stress resistance mediated by σ5 comes at the expense of growth capabilities as a consequence of a regulatory antagonism between σ5 and other σi, mainly σ70 [3,41,42]. The implications of negative regulation by σ5 in the stationary phase physiology have not been studied in detail.

Table 1. sRNAs differentially expressed in wild-type and ΔrpoS strains.

| sRNA      | Mean rpoS | Mean WT | Fold Change* | Start    | End      | Strand |
|-----------|------------|---------|--------------|----------|----------|--------|
| CsrC      | 4333       | 19747   | 0.26         | 4223741  | 4223984  | +      |
| CyaR      | 345        | 56      | 4.99         | 2282684  | 2282769  | +      |
| DsrA      | 694        | 217     | 2.83         | 2080053  | 2080139  | –      |
| GcvB      | 2          | 17      | 0.24         | 3155126  | 3155326  | +      |
| GlmY      | 7111       | 17770   | 0.43         | 2759217  | 2759400  | –      |
| IsrI      | 7          | 248     | 0.05         | 2812865  | 2813112  | –      |
| IsrR-1,2  | 67         | 283     | 0.29         | 4011708  | 4011839  | –      |
| MicA      | 99         | 494     | 0.24         | 2987088  | 2987161  | +      |
| Omra      | 28         | 621     | 0.07         | 3189331  | 3190017  | –      |
| OxyS      | 9          | 41      | 0.29         | 4356452  | 4356750  | –      |
| RybA      | 104        | 334     | 0.36         | 903092   | 903188   | –      |
| RybB      | 59         | 229     | 0.3          | 943606   | 943684   | –      |
| RybD      | 90         | 10      | 5.26         | 808431   | 808515   | +      |
| RyDC      | 2789       | 402     | 5.33         | 1739650  | 1739715  | +      |
| RyeF      | 72         | 419     | 0.21         | 2012350  | 2012656  | –      |
| RygC      | 466        | 2565    | 0.22         | 3242088  | 3242323  | +      |
| RygD      | 913        | 3695    | 0.28         | 3380548  | 3380692  | –      |
| RybH-1    | 124        | 1238    | 0.14         | 3729100  | 3729194  | –      |
| RybH-2    | 165        | 4480    | 0.06         | 1362850  | 1362950  | –      |
| SdrR      | 3          | 4823    | <0.001       | 1979458  | 1979560  | –      |
| SraC      | 122        | 17      | 5.37         | 1979380  | 1979690  | +      |
| Srl       | 38         | 23180   | <0.001       | 4518412  | 4518552  | –      |
| sRNA10    | 6          | 117     | 0.09         | 680323   | 680422   | –      |
| SroC      | 13056      | 49251   | 0.32         | 729258   | 729410   | –      |
| STnc1060  | 32         | 109     | 0.35         | 467925   | 467990   | –      |
| STnc1080  | 133        | 42      | 2.76         | 1064537  | 1064598  | –      |
| STnc1110  | 13         | 204     | 0.1          | 1696589  | 1696782  | +      |
| STnc1200  | 12         | 2       | 3.79         | 926560   | 926629   | –      |
| STnc1220  | 1          | 9       | 0.26         | 1501842  | 1501914  | –      |
| STnc1280  | 763        | 290     | 2.41         | 2093821  | 2093893  | +      |
| STnc1300  | 373        | 129     | 2.57         | 2125107  | 2125245  | +      |
| STnc1330  | 6          | 1246    | 0.01         | 2322111  | 2322325  | +      |
| STnc1380  | 84         | 14      | 4.47         | 2783476  | 2783543  | –      |
| STnc1390  | 41         | 137     | 0.35         | 1294132  | 1294195  | –      |
| STnc150   | 4          | 30      | 0.2          | 1335643  | 1335799  | –      |
| STnc1560  | 97         | 312     | 0.36         | 2502911  | 2503019  | +      |
| STnc2080  | 45         | 3081    | 0.03         | 2813257  | 2813365  | –      |
| STnc290   | 144        | 26      | 3.81         | 3214058  | 3214136  | –      |
| STnc540   | 46         | 181     | 0.3          | 1429394  | 1429487  | +      |
| STnc570   | 461        | 2600    | 0.22         | 1603700  | 1604389  | –      |
| STnc580   | 13         | 1       | 4.66         | 1759908  | 1760029  | –      |
| STnc750   | 36         | 1258    | 0.05         | 3259604  | 3259692  | –      |

*Fold change estimated by DESeq2 using normalized means. (p<0.001).

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We believe that an important issue to be addressed in future experiments is whether and how the negative effects of $\sigma^S$ on gene expression confer any fitness advantage to the bacteria. Genes down-regulated by $\sigma^S$ may show antagonistic phenotypic pleiotropy (i.e. their expression is advantageous in some environmental conditions and detrimental in others). Optimizing bacterial fitness in a defined constant environment would require selection for and against these genes, and this evolutionary force would drive gene loss or inactivation, in line with the selection of $rpoS$ mutants in some environmental conditions [11,12]. However, in fluctuating environments, fine-tuning regulatory processes by $\sigma^S$ might be used to adapt bacterial fitness to a variety of natural habitats, including host niches.

Negative effects of $\sigma^S$ on the respiratory chain might contribute to the antioxidant defenses by reducing the production of ROS as toxic by-products of aerobic metabolism [3,32] and might redirect NADH usage to fuel the activity of antioxidant enzymes. Indeed, accumulation of NADH following the inhibition of $Salmonella$’s electron transport chain by nitric oxide has been identified as an antioxidant strategy [43]. Interestingly also, inhibition of ATP synthase-promoted proton translocation and ATP synthesis is a strategy utilized by $Salmonella$ during infection to control ATP levels and maintain physiological cytoplasmic pH, and membrane potential [44]. Therefore, $\sigma^S$-mediated reduction in the synthesis of the ATP synthase might enable $Salmonella$ to maintain a physiological cytosolic pH and modulate its membrane potential for optimal survival under starvation conditions. Furthermore, down-regulation by $\sigma^S$ of the respiratory complexes I (NADH dehydrogenase Nuo) and II (succinate dehydrogenase Sdh) and the $\sigma^S$-dependent switch from the Isc to the Suf Fe-S cluster biosynthesis machinery might reduce the uptake of antibiotics. Indeed, it has been recently shown that, during iron limitation, $E. coli$ cells become intrinsically resistant to aminoglycosides by switching the Fe-S cluster biosynthesis machinery from Isc to Suf and down-regulating respiratory complexes I and II [45]. The Suf system cannot efficiently mature these respiratory complexes, resulting in impairment of the proton motive force, which is required for bactericidal aminoglycoside uptake [45].

Besides effects of $\sigma^S$ in metabolism and the respiratory chain functions, additional negative effects of $\sigma^S$ on gene expression
might contribute to bacterial fitness, σ^8 controls mutagenesis induced by subinhibitory concentrations of antibiotics via the down-regulation of mutS, a gene involved in mismatch-repair [46]. It is tempting to speculate that negative regulation of mutS by σ^8 (Dataset S2) might contribute to the appearance of antibiotic resistant mutants, and consequently the survival of bacterial populations in environments containing antibiotics. Down-regulation of porins (for example encoded by ompC, ompF, ampD, ampG, and ompW) (Dataset S2) might also confer resistance to antibiotics and other toxic compounds and bacteriophages [47]. More generally, σ^8 controls genes encoding membrane proteins and transporters, especially those belonging to the ATP-Binding Cassette transporter family, suggesting altered membrane composition and traffic in stationary phase (Dataset S2). This membrane remodeling may be directed towards nutrients scavenging and increased resistance against toxic compounds and physical assaults, an hypothesis consistent with the observed positive effect of σ^8 in cell envelope resilience in E. coli [48]. Negative control by σ^8 of surface determinants that are targets for a protective antibody response, such as OmpD [49], may also contribute in the escape of immune response during host infection.

Hierarchical Regulation and Regulatory Loops in the σ^70 Network and Interplay with other Global Regulators

Because the number of sigma factors exceeds that of the core RNAP, sigma factors compete for binding to the core RNAP available in the cell [2,3]. Many genes down-regulated by σ^8 (Dataset S2) are transcribed in Salmonella from promoters showing characteristics of σ^70-dependent promoters [28,50]. Negative control by σ^8 is likely in part an indirect effect. According to the current model of negative regulation by σ^8 invoking σ competition [3,40,42], σ^70-dependent genes are up-regulated in the absence of σ^8 because they are expressed from promoters that are sensitive to the increase in the cellular concentration of Eσ^70 that might result from a lack of competition between σ^8 and σ^70 for E binding. However, global regulation by σ^8 might also involve intermediate regulators in the σ^70 network, including repressor molecules.

As a first step to explore indirect regulation by σ^8, the possible regulatory functions of the σ^8-controlled downstream network were examined. σ^8 affected the transcript levels of numerous genes encoding known or putative signal transducing and/or DNA-binding proteins (Figure 1B and COGs T and K, Dataset S2), suggesting that σ^8 controls the transcription of many secondary transcription factors. Genes for global regulators (csrA, ssaS, arcA and to a lesser extent ampR), abundant nucleoid-associated proteins (hofAB, clpX, hupAB and to a lesser extent stpA), and modulators of RNAP activity (dksA and to a lesser extent ybaA, yaaG) have similarly been reported in E. coli K-12 [51–54] and arcA appeared down-regulated in a E. coli ΔpoS mutant [8]. Consistent with the RNA-seq data, quantitative reverse transcriptase-polymerase chain reaction showed that transcription of the regulatory genes csaA, dksA, clfX, hupA and ybaB is positively controlled by σ^8 whereas the hupA and hupB genes are down-regulated by σ^8 (Figure 4, Dataset S2). Also, the clfX gene, encoding a subunit of the ATP-dependent complex ClpXP protease, involved in proteolysis of many proteins including σ^8 [2,3] is activated by σ^8 (Figure 4, Dataset S2). Although these data showed that transcripts levels for csaA, dksA, clfX, hupA, hupB and clfX are modulated by σ^8 in late stationary phase in Salmonella, additional experiments are required to determine whether wild-type and ΔpoS cells differ in the global activity of the corresponding gene products. Indeed, other factors capable of differentially influencing protein levels and activity of these regulators might compensate for the observed variations in their transcript levels in the absence of σ^8.

As an example, interesting regulatory antagonisms were observed. csrA, encoding a post-transcriptional global regulator, and the small RNA CsrC are both positively controlled by σ^8 (Dataset S2, Figures 4–5 and see paragraph below). CsrA acts mostly negatively by binding and destabilizing mRNAs [53–56]. CsrC binds and sequesters CsrA, thereby inhibiting its activity [53,57]. The other components of the Csr system include the sRNA CsrB, that also binds and sequesters CsrA, and CsrD, a protein that participates in degradation of CsrC and CsrB [53,56]. In the conditions used, csrB and csrD were detected to low and similar levels in wild-type and ΔpoS strains (data not shown). The regulation of expression of the Csr system is complex [53,54]. CsrA indirectly activates its own transcription while repressing its own translation and also controls production of CsrC/CsrB/CsrD. Transcription of csrA in E. coli is controlled by several promoters, two of which are σ^8-dependent [54]. It is conceivable that σ^8 modulates the fine-tuned balance of the Csr system and uses this system to indirectly regulate target genes at the post-transcriptional level. Also, transcript levels for σ^E and for its antisigma factor RseA and its coantisigma factor RseB, encoded by the same operon, are all reduced in the ΔpoS strain, compared to the wild-type (3.5 fold p<0.001 for rseA and about 2 fold p<0.05 for rpoE and rseB, Dataset S2). Since σ^8 has a positive effect on σ^E expression in stationary phase [10], a possible control of σ^E expression and/or activity by σ^8 would not be unexpected. σ^8 may have several self-regulatory circuits by controlling the expression of numerous genes that modulate its expression [2,3], for instance, clpX, rssB/hnr, arcA, hupAB, dksA, and dksC (p<0.001, Dataset S2). Since these regulators may work either cooperatively or independently, or even have opposing effects on σ^8 expression [2,3], σ^8 self regulatory control may be important for maintaining a proper level of σ^8 and for integration of signal inputs. Future experiments will assess whether these changes in expression of global regulators at the transcriptome level are transferred at the functional level and whether some of the σ^8-controlled secondary regulators are intermediate regulators in regulatory cascades and/or contribute to Eσ^8-mediated regulation in feedforward regulatory loops.

Besides the control of regulatory proteins, control of metabolic/signalizing enzymes by σ^8 might lead to variations in levels of signalling molecules and affect protein modifications and/or gene expression at different levels. For instance, genes involved in the metabolism of the second messenger C-di-GMP [3,55,58] showed differential transcript levels in the wild-type and ΔpoS strains (ydiC, ydiF, yegE, STIM14_2408, STIM14_2209, STIM14_5555, STIM14_4086, STIM14_2047, Dataset S2); Also, putrescine affects global gene expression [37] and the control of its intracellular levels (Figure 2) might be a mechanism of indirect gene regulation by σ^8. Since σ^8 plays a central role in metabolism, it may affect the levels of intermediate metabolites with signalling functions such as CoA derivatives, NADH/NADPH, glutamate, acetate and acetyl-phosphate.

σ^8-dependent sRNAs

sRNAs are important pleiotropic regulatory elements [59–61]. Some sRNAs are positive regulators but the majority of sRNAs negatively regulate their targets by translational repression and/or destabilization of the mRNA [59–61]. Thus, sRNAs might be important contributors to negative regulation of gene expression by σ^8, as recently shown for σ^8 [62]. More than one hundred sRNAs have been recently annotated in S. Typhimurium SL1344
conditions and it has been proposed that this regulation enables iron sparing for essential pathways [60]. Experiments are underway to assess to which extent RydB sRNAs might play a role in σ5-dependent modulation of iron use in late stationary phase. During the preparation of this manuscript, SraL was shown to be controlled by σ5 and to down-regulate the expression of a chaperone encoded by the tig gene and involved in protein folding [63]. Under the conditions used in our study, the σ5-dependent control of SraL (Figure 5) did not significantly affect tig transcripts or this effect was masked by compensatory regulations in the network. A few sRNAs appeared down-regulated by σ5 (Table 1). Among those displaying the highest fold change in their expression levels between wild-type and ΔpoS strains, RydC activates translation of the cfa mRNA produced from a σ5-dependent promoter [69]. cfa encodes a cyclopropane fatty acid synthase which modifies phospholipids and contributes to the stability of the bacterial membrane and acid resistance [3,69]. In stationary phase, σ5 activates expression of the cfa gene [Dataset S2] [3] and the σ5-dependent promoter yields a shorter isoform of the cfa mRNA, insensitive to RydC regulation [69]. Thus, in stationary phase, when cfa transcription relies on σ5, RydC might be dispensable and possibly detrimental for expression of other stationary phase genes and its expression might be downregulated accordingly by σ5. Alternatively, RydC might be up-regulated in the ΔpoS strain to activate synthesis of cyclopropane fatty acid synthase and compensate for the absence of σ5-induction of cfa transcription (i.e. RydC-mediated activation of cfa might be a backup mechanism).

σ5-dependent sRNAs might also target proteins instead of mRNAs [59]. As mentioned above the finding that csrC and csrA were both positively controlled by σ5 (Figures 4–5, Dataset S2) reveals an interesting regulatory antagonism and suggests that σ5 modulates the fine-tuned balance of the Csr system in late stationary phase. Whereas expression of the CsrC and CsrB sRNAs is coordinated by positive transcriptional control mediated by the two-component regulatory system BarA/SirA in Salmonella [57], csrC but not csrB was found to be controlled by σ5, suggesting that these two sRNAs are differentially regulated in the stationary phase of growth.

The physiological impact of the σ5-dependent regulatory RNAs network and its possible connections with the hierarchical σ5-dependent transcriptional network will be an exciting issue for future studies.

Inactivation of the astA and sdsR Loci has a Fitness Cost in Stationary Phase

Many σ5-dependent genes are of unknown functions (Figure 1B) and physiological roles in starved populations of most of the σ5-dependent genes are unexplored. Understanding what genes regulated by σ5 may do for the cell is an important issue for future studies. Even for genes with known functions, understanding whether and how they help bacteria deal with survival in stationary phase and stress conditions is far from being complete. Our RNAseq data pinpoint to metabolic functions as key characteristics of σ5 activity in Salmonella, as its was previously suggested in E. coli K-12 [3]. However, the contribution of σ5-regulated metabolic functions in the physiology of non/slow growing bacteria needs to be further evaluated through construction of mutations in relevant pathways. For instance, σ5 might activate the transport and utilization of L-arginine (Figure 2). The astCADDBE operon required for the degradation of arginine is transcribed from two promoters, one is dependent on σ5, the other on σ70 [70,71]. We previously isolated a mutant of Salmonella carrying a Tn5B21 transposon insertion in the astA gene, creating
a astA-lacZ gene fusion [22] (Table S1). Consistent with the RNAseq data, expression of the astA-lacZ fusion in stationary phase was dependent on σ8 (Figure 6A).

We previously showed that the wild-type strain of Salmonella has a competitive advantage over the ΔrpoS mutant in stationary phase [14] (Figure 6B). To assess the impact of L-arginine degradation in maintenance metabolism, we performed similar competition experiments in which the wild-type strain and astA mutant were in equal cell numbers in LB liquid medium and the numbers of each were followed for several days (Figure 6C). The wild-type strain ATCC14028 showed a competitive advantage during stationary phase over the astA mutant (Figure 6C). Three days after inoculation of the medium, more than 98% of the cells population was wild-type. In similar control experiments, the wild-type strain ATCC14028 showed similar fitness as the wild-type strain 2922 K (Figure 6D) [14]. The fitness disadvantage of the astA mutant was not due to the Tn5R21 insertion since strains carrying Tn5R21 insertions in the katE and katN genes showed similar fitness as the wild-type strain (Figure 6EF). The σ5-dependent katE and katN genes encode catalases [72] involved in the destruction of hydrogen peroxide (H2O2). Cellular respiration using oxygen may result in the accumulation of ROS [32]. The inactivation of catalases did not affect Salmonella fitness under the conditions used, possibly due to redundant functions in Salmonella [73]. Alternatively, as discussed above, σ5 may set up conditions minimizing endogenous oxidative stress and, under these conditions, catalase production might be a preventive σ5 response. These data showed that inactivation of the ast pathway has a fitness cost and thus arginine degradation might have a protective role in oxidative stress. The data presented here will inspire future studies to address these questions.

Supporting Information

Figure S1 Central metabolic pathways controlled by σ8 in LB stationary phase cultures of Salmonella. Central metabolic pathways, including glycolysis and gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, acetate and pyruvate metabolism are shown schematically. To assess the contribution of σ8 in the expression of the metabolic pathways indicated, genes differentially expressed with a p value of less than 0.05 in the wild-type and ΔrpoS strains of Salmonella were considered (Dataset S2). Genes showing differential expression with p<0.001 are indicated in bold face. Genes in red and green were negatively and positively controlled by σ8 respectively. Genes in black did not show differential expression in the wild-type and ΔrpoS strains. (TIF)

Figure S2 Central metabolic pathways controlled by σ8 in LB stationary phase cultures of Salmonella. Schematic representation of pathways controlled by σ8. (A) degradation of N-acetylneuraminic acid, N-acetyl-β-D-mannosamine and N-acetyl-D-glucosamine, (B) 4-hydroxyphenylacetate catabolism, (C) L-arabinose degradation, (D) propionate degradation, (E) Glycogen biosynthesis and degradation, (F) galactose degradation, (G) Ethanolamine utilization, (H) trehalose biosynthesis and degradation, (I) Glycine metabolism, (J) Glutamine transport and metabolism, (K) Glutathione metabolism, (L) Aspartate degradation, (M) L-serine degradation, (N) L-cysteine degradation and hydrogen sulfite biosynthesis. See also legend of Figure S1. (TIF)

Table S1 Bacterial strains used in this study. (DOC)

Table S2 Oligonucleotides used in this study. (DOC)

Table S3 Coordinates in the ATCC14028 genome of the sRNAs annotated in SL1344. (XLS)

Dataset S1 Differential gene expression in wild-type and ΔrpoS strains. (XLS)

Dataset S2 Annotation of σ8-controlled genes (from Dataset S1, p<0.05). Genes differentially expressed in the wild-type strain and the ΔrpoS mutant with p<0.001 are indicated in bold face. (XLS)
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Author Contributions

Conceived and designed the experiments: JYC FN. Performed the experiments: CLM VM OS FN. Analyzed the data: CLM VM MAD BJ MM FN. Contributed reagents/materials/analysis tools: MB BD. Wrote the paper: CLM VM FN.

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