ChIP-Seq Analysis of the $\sigma^E$ Regulon of \emph{Salmonella enterica} Serovar Typhimurium Reveals New Genes Implicated in Heat Shock and Oxidative Stress Response

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Abstract

The alternative sigma factor $\sigma^E$ functions to maintain bacterial homeostasis and membrane integrity in response to extracytoplasmic stress by regulating thousands of genes both directly and indirectly. The transcriptional regulatory network governed by $\sigma^E$ in \emph{Salmonella} and \emph{E. coli} has been examined using microarray, however a genome-wide analysis of $\sigma^E$–binding sites in \emph{Salmonella} has not yet been reported. We infected macrophages with \emph{Salmonella} Typhimurium over a select time course. Using chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChIP-seq), 31 $\sigma^E$–binding sites were identified. Seventeen sites were new, which included outer membrane proteins, a quorum-sensing protein, a cell division factor, and a signal transduction modulator. The consensus sequence identified for $\sigma^E$ in vivo binding was similar to the one previously reported, except for a conserved G and A between the -35 and -10 regions. One third of the $\sigma^E$–binding sites did not contain the consensus sequence, suggesting there may be alternative mechanisms by which $\sigma^E$ modulates transcription. By dissecting direct and indirect modes of $\sigma^E$–mediated regulation, we found that $\sigma^E$ activates gene expression through recognition of both canonical and reversed consensus sequence. New $\sigma^E$ regulated genes (\emph{greA}, \emph{luxS}, \emph{ompA} and \emph{ompX}) are shown to be involved in heat shock and oxidative stress responses.

Introduction

Where household sigma factor $\sigma^{70}$ is responsible for promoting transcription of a large number of genes in a bacterial cell, alternative sigma factors are produced or activated when cells undergo particular physiological stresses [1] [2]. The alternative sigma factor E ($\sigma^E$) responds to extracytoplasmic cues generated by temperature, osmotic, or oxidative stress, which releases
σE from sequestration at the inner membrane through a series of protein cleavage events. Free σE in the cytoplasm binds core RNA polymerase and initiates transcription at σE-dependent promoters, resulting in a specific response to promote restoration of homeostasis in the cell [3] [4]. In Salmonella enterica serovar Typhimurium (referred to as STM or Salmonella hereafter), deletion of σE (ΔrpoE) is lethal for intracellular survival in macrophages; the strongest phenotype among regulator deletion strains examined [5].

The transcriptional regulatory network controlled by σE has been investigated in previous studies [6] [7] [8] [9]. Recent findings from our group indicated that approximately 58% of the entire Salmonella genome was regulated by σE, an effect most likely produced by modulation of the expression of multiple general regulators [10]. Aided by sample-matched global proteomic and transcriptomic analyses, we found that σE regulated Salmonella gene expression not only at the transcriptional level, but also by a post-transcriptional mechanism, which was partially dependent on the RNA-binding protein Hfq [11]. The above studies suggested that the majority of transcriptional regulation mediated by σE occurred indirectly, driven by a small number of genes that were directly regulated by σE. Therefore, it is essential to identify σE-binding sites globally to understand the intricacies of σE-mediated gene regulation.

Chromatin-immunoprecipitation followed by high-throughput DNA sequencing (ChIP-seq) has been successfully used in the identification of chromosomal binding sites of various regulators in bacteria [12] [13] [14]. It offers higher resolution, lower noise, and greater coverage than its array-based predecessor ChIP-chip [15]. We selected ChIP-seq as a method for genome-wide profiling of σE-binding sites in STM. Our results indicated some σE-binding sites resided 5’ to divergent flanking genes, and raised the question whether σE regulated these genes equally. Moreover, a previous study from the Gross group mentioned the possibility that a reversed consensus sequence of σE might be involved in repressing gene expression, exemplified by ompX [8]. We elucidated the above questions by constructing consensus sequence substituted strains, and dissecting direct from indirect regulatory effects of σE.

In this study we identified 31 σE-binding sites on the STM genome during in vivo infection of Raw264.7 macrophages with STM and determined a consensus sequence for σE-binding. However, this consensus sequence was not the only mechanism utilized by σE in transcriptional regulation. Moreover, σE did not regulate its flanking genes equally when the σE-binding site resided between bi-directional promoters. σE activated gene expression through binding the consensus or reversed consensus sequence. Finally, we found that new targets directly regulated by σE were involved in response to heat shock and oxidative stress.

Materials and Methods

Bacterial strains and growth conditions

Salmonella enterica Serovar Typhimurium ATCC 14028s was used as the parent strain in this study. The rpoE-deletion strain (ΔrpoE) was constructed using λ red recombination system as described [5]. The consensus sequence substituted mutants were constructed by replacing the σE-binding motif of greA, ompX, ompA, luxS and rpoE with a substitutive consensus sequence consisting of nucleotides with the least prevalence at the corresponding positions in the σE consensus sequence (ATTTGCGGGAACATGCGAAGACTGACTG). The substitutive consensus sequence was synthesized with SacI and AvrII restriction sites at two ends, and ligated into pKD13 modified plasmid [16], resulting in a new plasmid pKD13-RpoE1. λ red recombination was used to construct the consensus sequence substituted mutants with pKD13-RpoE1 plasmid similar to the ΔrpoE strain. All substitutions were validated by DNA sequencing. Primers used for construction of the above mutants are shown in S1 Table.
For *in vitro* study, the bacteria were grown overnight in Luria-Bertani (LB) medium, washed twice in pH 5.8, low phosphate, low magnesium-containing medium (LPM), and resuspended in LPM at 1:10 dilution for an additional 4, 8 or 20 h [17]. All bacterial cultures were grown in triplicate.

**Anti-σ\(^E\) antibody generation**

The anti-σ\(^E\) antibody (Ab) was generated as previously described [10]. Briefly, the *rpoE* gene of STM was cloned into the plasmid pET200/D-TOPO (Invitrogen) and transformed into BL21 (DE3) *E. coli* strain (Invitrogen). Transformed bacteria were induced using Isopropyl β-D-1-thiogalactopyranoside (IPTG), lysed and sonicated for purification of recombinant σ\(^E\) using HisPur\(^\text{TM}\) Cobalt resin (Pierce). The eluted protein was separated on SDS-PAGE, and stained with Coomassie blue. The single gel bands at the size of recombinant RpoE (His tagged) were excised and sent to Pacific Immunology Corp. (Ramona, CA) for polyclonal antisera production. The σ\(^E\) antisera generated in rabbits were purified by affinity chromatography. CH Sepharose 4B (GE Healthcare) was coupled with recombinant σ\(^E\), then σ\(^E\) antisera was loaded in a chromatography column (Bio-Rad). After washes, the column was eluted with 200 mM glycine pH 2.0, then adjusted to pH 7.0 with 5 N NaOH. Fractions containing purified anti-σ\(^E\) Ab as judged by SDS-PAGE were frozen at -20°C. Protein concentration determination was performed according to modified Lowry method using bovine serum albumin (BSA) as reference protein [18].

**Immunoblot analysis**

The WT and Δ*rpoE* strains were cultured in LB for 4 h. Cells were washed and approximately 5 x 10^7 colony-forming units were pelleted and re-suspended in Laemmli sample buffer, boiled for 5 min, and then separated on SDS-PAGE. Proteins on the gel were next transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking in Tris-buffered saline (TBS) plus 5% nonfat dry milk for 1 h, membranes were probed with anti-σ\(^E\) Ab or anti-GFP Ab at 1:1000, 1:3000, and 1:5000 dilutions. The flow-through of rabbit immune sera during affinity chromatography purification of anti-σ\(^E\) Ab was also used to probe the membrane as control. Membranes were washed and probed with secondary antibody (anti-rabbit IgG conjugated with peroxidase) (Cell Signaling Technology). The immune complexes were detected via chemiluminescence using Western Lightning\(^\text{TM}\) (PerkinElmer), and then exposed to XAR Biofilm (Kodak).

**Salmonella infection**

The Raw264.7 macrophage cell line was purchased from ATCC and grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum in 6- well plates until reaching confluency of 80–90%. STM grown overnight in LB was washed and diluted with PBS to an appropriate concentration. Raw264.7 cells were infected with STM at MOI = 100 for 4, 8, or 18 h. Infections were initiated by centrifuging the bacteria onto the cell monolayers at 1,000 × g for 5 min and plates were incubated at 37°C with 5% CO2 for 1 h. To remove extracellular bacteria after internalization, cells were washed with PBS and incubated in DMEM containing 10% fetal calf serum and gentamicin (100 μg/ml) for 1 h. The cells were then washed with PBS and overlaid with DMEM containing 10% fetal calf serum and 20 μg/ml gentamicin for the remainder of the experiment.
Chromatin Immunoprecipitation followed by sequencing (ChIP-seq)

After 4, 8, or 18 h infection with STM, Raw264.7 cells were washed with PBS and scraped off the plates. The cells were collected and cross-linked by 1% formaldehyde at room temperature for 25 min with shaking, then quenched using 125 mM glycine for an additional 5 min of incubation at room temperature. Chromatin Immunoprecipitation assay was performed as described [10]. Cells were lysed and sonicated, and the supernatant was split into two samples. One was mixed with affinity purified rabbit anti-σE Ab to immunoprecipitate σE–DNA complex, and the other sample was mixed with rabbit monoclonal Ab to GFP as control. After overnight incubation at 4°C, 50 μl of the Dynabead M-280 sheep anti-rabbit IgG (Invitrogen) was added into the mixture and further incubated at 4°C for 6 h. Beads were washed and resuspended in elution buffer (50 mM Tris-HCl at pH 8.0, 10 mM EDTA, and 1% SDS) and incubated at 65°C overnight to reverse the cross-linking. Protein and RNA were removed from the samples and DNA was purified with a PCR purification kit (QIAGEN). Gene-specific quantitative PCR was carried out using the DNA samples (primers in S2 Table).

The DNA was then combined with 5x Sequenase buffer and Random 9-Ns primer and cycled to 94°C for 2 min then cooled to 10°C. A mix of 5x Sequenase buffer, dNTP mix, BSA, DTT, and Sequenase was added to the DNA and ramped up from 10°C to 37°C at 0.1°C/s, held at 37°C for 8 min, heated to 94°C for 2 min and then cooled to 10°C. Sequenase dilution buffer and Sequenase enzyme were added and the sample was ramped up from 10°C to 37°C at 0.1°C/s, held at 37°C for 8 min and then cooled to 4°C. Samples were diluted and combined with a mix of pfu buffer, dNTP mix, Rand universal primer, and pfu polymerase. The DNA was cycled to 94°C for 30 s, 40°C for 30 s, 50°C for 30 s, and 72°C for 2 min for 25 cycles. The amplified DNA was purified using the Qiagen PCR purification kit according to protocol. The purified DNA was precipitated using 3M sodium acetate and ethanol overnight. Samples were centrifuged at 4°C for 1 h and subsequently washed with 70% ethanol, dried and resuspended in water.

Libraries for sequence analysis on the Illumina HiSeq 2000 using V3 chemistry were generated per the Illumina TruSeq standard protocol.

Peak calling

HiSeq fastq files were generated and loaded into the CLC Genomics Workbench 7 software (CLCBio, Boston MA) for ChIP peak identification. Imported reads were mapped to the reference genome retrieved from NCBI. Default mismatch rates were applied, with length and similarity fractions both at 90%. All experimental samples were compared to the reference controls to identify binding events of interest. The most statistically significant peak calls at each time point were then visually verified using the GenomeView genome browser [19].

Quantitative RT-PCR analysis

Total RNA was isolated by RNeasy mini kit, combined with RNA-free DNase for on-column DNase digestion (Qiagen) according to manufacturer’s instructions. cDNA was synthesized using the iScript cDNA synthesis kit (Bio Rad). The amount of cDNA corresponding to 10 ng of input RNA was used as template for real-time reaction containing Power SYBR green (Applied Biosystems) and gene-specific primers. The primers were designed with Primer Express 3.0 software and tested for amplification efficiencies (S2 Table). The gyrB gene, encoding for the B subunit of the DNA gyrase, was utilized as endogenous control. The RT-PCR reactions were carried out at 95°C for 10 min, 95°C for 15 s and 60°C for 1 min for 40 cycles (ABI 7700, Applied Biosystems). The expression ratio of each gene was the average from three independent RNA samples and was normalized to the level of gyrB.
Results

Identification of σE-binding sites using ChIP-seq

We recently characterized the set of genes affected by σE in STM 14028s by microarray and identified 2533 genes exhibiting σE-dependent transcription during growth in nutrient-rich and acidic minimal media. However, only 81 genes (3%) were regulated by σE in all of the growth conditions examined, suggesting genes regulated by σE were tuned by growth conditions that activated σE via different environmental cues [10]. In order to study the genomic binding sites of σE under conditions approximating the phagosomal environment, we infected murine macrophage Raw264.7 with STM and performed ChIP-seq on intracellular bacteria to identify genes directly bound by σE on the STM genome. To avoid the possible influence of epitope tagging on protein function, we generated primary anti-σE Ab, purified with affinity chromatography, and assessed its specificity by Western blot before applying it to ChIP. A single band at the correct size of σE (22 kDa) was detected in the cell lysate of the WT strain and absent in the ΔrpoE using anti-σE Ab (S1 Fig). When diluted 1:5000, the anti-σE Ab yielded a single reactive band. This evidence indicated that the generated anti-σE Ab was highly specific and had strong affinity. The monoclonal anti-GFP Ab, which did not show any cross reaction with STM, was used as negative control in the ChIP-seq experiments (S1 Fig).

After peaks were called, the most statistically significant from each time point were visually verified. To pass this step, the read counts composing a peak were required to be substantially higher than background and also needed to be distributed evenly on both sides of the transcript (Fig 1). The peaks were ambiguous for the in vivo 4 h infection. We believe that the signal at this early time was not strong enough to provide robust results, so we excluded it from the analysis. A total of 31 ChIP-enriched peaks were identified for the 8 h and 18 h infections. Fourteen of the peak-associated genes were previously shown to be directly regulated by σE [8,9], supporting the effectiveness of the ChIP-seq procedure (Table 1). The remaining 17 genes represented new targets of varying function; encoding outer membrane proteins (ompA, ompF, and ompN), a quorum sensing protein (luxS) [20], a cell division factor (minD/miniE) [21], a signal transduction modulator (sixA) [22], and hypothetical proteins (stm14_1163, stm14_1514, stm14_1018, stm14_2321, stm14_2952).

Validation of in vivo σE-binding sites

To validate σE-binding, we performed ChIP-qPCR on target genomic loci and determined their fold enrichment in pulldowns using anti-σE Ab versus anti-GFP Ab (control). A known σE-binding site, rpoE promoter 3, was pulled down in our ChIP-seq assay and used as positive control, and a non-target site, the promoter region of hns, was chosen as negative control [10]. For the 8 h infection, except for the binding site upstream of spf, all the ChIP-seq peaks exhibited more than 2-fold enrichment when compared to control samples, and were therefore considered authentic σE-binding sites (Fig 2). For the 18 h infection, all of the ChIP-seq peaks were validated. For both conditions, binding sites upstream of luxS, stm14_1018, ompX, stm14_2321, and yhjJ exhibited enrichment as high as the positive control (rpoE, 64-fold), hence were considered high-affinity binding sites for σE (Fig 2).

Identification of the consensus sequence of σE-binding in vivo

The sequences of the 31 binding regions determined by our ChIP-seq experiments were analyzed with the MEME suite tool to identify a consensus sequence for σE-binding [23]. For 20 of these peak regions, a consensus motif was identified (p-value < 0.0001). This A-T rich σE consensus sequence is largely similar to the one that was identified previously for STM [9], except...
that the nucleotides between the -35 and -10 regions appeared to contain conserved G and A in our in vivo σE-binding consensus sequence. Except for the six genes (rpoE, rpoH, fkpA, htrA, yfiO, and ygiM) that were previously identified to contain σE-dependent promoters [9], the σE consensus sequence was also found upstream of genes encoding outer membrane proteins (ompA, ompF, ompN, and ompX), putative permease (perM), putative cytoplasmic protein (ybhP) [24], transcription elongation factor (greA) [25], and quorum sensing autoinducer 2 synthase (luxS) [20] (Fig 3).

There were 11 σE-binding sites (cspE, galF, minD, rpoD, sixA, spf, stm14_2321, stm14_2952, surA, yggN, yhjI) validated in our study that did not contain any recognizable consensus sequence. However, some of these sites exhibited high σE-binding capacity, such as stm14_2321, ybfJ (> 64-fold enrichment), and stm14_1514, surA, sixA, stm14_2952, galF, minD (> 16-fold enrichment) (Fig 2). This observation suggested that, besides the major regulatory mechanism of using the canonical consensus sequence, σE may utilize additional complementary mechanisms to regulate gene transcription.

Establishing in vitro condition to mimic the in vivo σE-binding

The transcriptional profile of σE for STM intracellular infection is elusive because the rpoE mutation is so deleterious that the mutant can survive for no more than 30 min post-infection [5]. Therefore, we sought an in vitro method that could reproduce in vivo σE-binding conditions. STM grown in LPM was used for mimicking in vivo infection [26], here the bacteria were cultured in LPM for 4, 8, and 18 h and then subjected to ChIP-qPCR as performed on in vivo samples. σE-binding was evaluated on 15 sites selected from the in vivo ChIP-seq results (Fig 4). None of the three in vitro conditions could achieve similar binding capacity of σE on these sites as was seen in the in vivo conditions. However the LPM 4 h condition produced the most favorable result among them, where except for htrA, remaining binding sites were detected at levels higher than the 2-fold threshold. Hence, we used the LPM 4 h condition to study σE-mediated gene regulation in an effort to approximate in vivo binding conditions.
Dissecting direct versus indirect regulation mediated by $\sigma^E$

Although 31 $\sigma^E$-binding sites were recognized as directly regulated by $\sigma^E$ during \textit{in vivo} infection (Table 1, Fig 2), microarray was not sufficient to evaluate the regulatory effects of $\sigma^E$ on these genes through comparing WT and $\Delta rpoE$ because this comparison measured total regulation mediated by $\sigma^E$. Indirect regulation mediated by $\sigma^E$, largely through controlled expression of multiple general regulators, accounted for the majority of the total regulation observed [10].

To dissect direct from indirect $\sigma^E$–mediated regulation for \textit{greA}, \textit{ompX}, \textit{ompA}, \textit{luxS}, and \textit{rpoE}, we replaced the authentic consensus sequence upstream of these genes with a scrambled consensus sequence, which resulted in 5 mutants designated \textit{greA-RpoE1}, \textit{ompX-RpoE1}, \textit{ompA-RpoE1}, \textit{luxS-RpoE1}, and \textit{rpoE-RpoE1}. Mutant strains were cultured in LPM for 4 h and the $\sigma^E$ binding capacity on 5 designated binding sites (\textit{greA}, \textit{ompX}, \textit{ompA}, \textit{luxS}, and \textit{rpoE} promoter region) was examined using ChIP-qPCR (Fig 5). As expected, mutation of the $\sigma^E$–binding motif upstream of each gene only eliminated (\textit{greA}, \textit{ompA}, \textit{luxS}) or decreased (\textit{ompX}) $\sigma^E$ binding of each mutant to its corresponding sites, while leaving the other sites intact for $\sigma^E$-
These results further confirmed the direct occupation of $\sigma^E$ on these selected $\sigma^E$-binding motifs.

Of the 31 $\sigma^E$-binding sites identified, 16 of them were located between bi-directional promoter elements (Table 1). Since the $\sigma^E$-binding site was $5'$ to genes in both directions, we further investigated if $\sigma^E$ regulated these genes equally. Two $\sigma^E$-binding sites were chosen, one located between $\text{dacB}$ and $\text{greA}$, with a second between $\text{ompX}$ and $\text{ybiF}$ (Fig 6A). WT, $\Delta rpoE$, and consensus sequence substituted mutants ($\text{greA}-\text{RpoE1}$ or $\text{ompX}-\text{RpoE1}$) were cultivated in LPM for 4 h, and expression of the genes in question was measured using qRT-PCR with $\text{gyrB}$ serving as the internal control. The total regulatory effects of $\sigma^E$ on a particular gene was calculated by comparing its expression ratio in WT versus $\Delta rpoE$. The direct contribution of $\sigma^E$ was measured by the expression ratio in WT versus the consensus sequence substituted mutant, and the indirect contribution of $\sigma^E$ was measured by the expression ratio in $\Delta rpoE$ versus the consensus sequence substituted mutant. We found that based on total regulation, it was apparent that $\sigma^E$ regulated $\text{dacB}$ instead of $\text{greA}$. However the indirect regulation of $\sigma^E$ on $\text{greA}$ was much higher than that on $\text{dacB}$, leading to the net regulation directly mediated by $\sigma^E$ significant on $\text{greA}$, but not $\text{dacB}$ (Fig 6B). Similarly, dissecting $\sigma^E$ regulation indicated that $\sigma^E$ activated $\text{ompX}$ instead of $\text{ybiF}$ (Fig 6C). Three more $\sigma^E$-binding sites ($5'$ to $\text{ompA}$, $\text{luxS}$, and $\text{rpoE}$) were examined. $\sigma^E$ activated each locus through direct occupation of their $\sigma^E$ consensus sequence (Fig 6D). Moreover, the direct effect of $\sigma^E$ in regulating its own expression ($\text{rpoE}$) was much lower than the total and the indirect contributions of $\sigma^E$. The dissection of $\sigma^E$-mediated regulation clarified that $\sigma^E$ activated gene expression by binding to the consensus sequence (such as $\text{greA}$) or reverse consensus sequence (such as $\text{ompX}$). The previous findings of $\sigma^E$ repressing gene expression by comparing the level of expression in WT and $\Delta rpoE$ was therefore misleading, in that indirect regulation of $\sigma^E$ expression itself played a large role in defining activation versus repression. When the indirect regulation of $\sigma^E$ is subtracted from the total regulation mediated by $\sigma^E$, the resulting direct regulation of $\sigma^E$ is activation of gene expression. Together,
our results clearly elucidated σE-mediated regulation by dissecting direct versus indirect regulation.

Genes regulated by σE are involved in heat shock and oxidative stress response

The alternative sigma factor σE is activated under extracytoplasmic stress such as heat shock, ethanol, osmotic stress, immune response etc. [4]. By regulating gene expression at both the transcriptional and post-transcriptional levels [11], σE functions to maintain the integrity and homeostasis of the cell. We further investigated if newly identified targets of σE-binding served to fulfill this purpose. The WT and consensus sequence substituted mutants (greA-RpoE1, ompX-RpoE1, ompA-RpoE1, luxS-RpoE1, and rpoE-RpoE1) were challenged by heat shock and oxidative stress (Fig 7). Compared to WT, the susceptibility of all mutants to heat shock was significantly altered at 90 min post-challenge. Except for greA-RpoE1, all other mutants exhibited lower resistance to heat shock. At the 3 h time point, the greA-RpoE1 strain recovered to WT levels, whereas the remaining mutants were significantly hyper-sensitive to heat shock when compared to WT (Fig 7A). Under oxidative stress, the mutant strains exhibited
higher (greA-RpoE1) or lower (rpoE-RpoE1 and ompX-RpoE1) susceptibility than WT at 60 min post-challenge. At the 2 h time point, all mutant strains showed significantly altered sensitivity to oxidative stress compared to WT (Fig 7B). These results suggested the genes regulated by σE (greA, luxS, ompA, and ompX) were involved in heat shock and oxidative stress response.

**Fig 4.** Comparison of σE-binding sites between *in vitro* cultured conditions and *in vivo* infection. WT bacteria were cultured in acidic minimal medium (LPM) for 4 h, 8 h, or 20 h. ChIP and qPCR followed the same procedures as described in Fig 2. The fold enrichment of σE-binding sites was determined in pulldowns with anti-σE Ab (sample) versus anti-GFP Ab (control). The *in vivo* infection results were imported from Fig 2.

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**Fig 5.** Scrambled σE consensus sequence decreases/eliminates σE binding. The substitutive consensus sequence consisted of nucleotides with the least prevalence at the corresponding positions in the consensus sequence. The σE consensus sequences upstream greA, ompX, ompA, luxS, and rpoE genes were replaced by the substitutive sequence (RpoE1), and the resulting mutants were named greA-RpoE1, ompX-RpoE1, ompA-RpoE1, luxS-RpoE1, and rpoE-RpoE1 respectively. These mutants and WT bacteria were grown in LPM for 4 h, crosslinked, sonicated, and immunoprecipitated with anti-σE Ab (sample) or anti-GFP Ab (control). qPCR was used to determine the binding of σE at each original site. The mean and S.D. values were obtained from independent biological triplicates.

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Discussion

The transcriptional profile of σE has been studied using microarray by comparing WT to ΔrpoE strain or by comparing WT to rpoE-overexpressed strain [10] [8]. Since microarray cannot distinguish direct from indirect regulation, a large amount of genes were found to be regulated by σE, and the number of genes activated by σE was similar to those repressed [10]. Although the Salmonella σE-regulon associated with direct binding has been studied using an E. coli two-plasmid screening system [9], the genome-wide identification of σE-binding sites during Salmonella in vivo infection has not been investigated. The in vivo σE-binding sites are likely different from in vitro because the signals perceived by σE from these two environments are different. Thirty-one σE-binding sites were identified during Salmonella in vivo infection in this study, and when compared with in vitro culture on fifteen of these sites, the binding capacity of σE in LPM 4, 8, and 20 h culture was lower on the majority of the sites measured (Fig 4). How σE selectively binds to some sites but not other potential sites under certain conditions is not known. However, other general regulators such as H-NS, IHF, and Fis might play a role in
affecting the direct binding of $\sigma^E$ to these sites [14]. Out of the thirty-one $\sigma^E$-binding sites, sixteen were located upstream of bi-directional promoter elements (Table 1). We found that those flanking genes were not equally regulated by $\sigma^E$ under the conditions examined (Fig 6).

The $\sigma^E$ consensus sequence predicted here during Salmonella infection was similar to the consensus sequence identified by the E. coli two-plasmid system [9], and also comparable to the consensus sequence identified in E. coli [8], suggesting conservation of sequence features related to $\sigma^E$-binding. Both this and previous studies found that the $\sigma^E$ consensus sequence located upstream of $ompX$ was reversed, and the microarray data indicated that $\sigma^E$ represses $ompX$ expression [8] [10]. Since $ompX$ was found to be directly regulated by $\sigma^E$, the repression effect of $\sigma^E$ on outer membrane proteins was hypothesized as a conserved feature [8]. We constructed a consensus sequence substituted strain and by comparing with WT and $\Delta rpoE$ strains, successfully dissected the direct versus indirect contribution of $\sigma^E$. The results clearly demonstrated that $\sigma^E$ directly activated $ompX$ expression through the reversed consensus sequence. The perception of $\sigma^E$-mediated repression was due to the high-level of indirect effects of $\sigma^E$. Except for $ompX$, other genes encoding outer membrane proteins ($ompA$, $ompF$, and $ompN$) were additionally directly regulated by $\sigma^E$. Similar to $ompX$, the total effect of $\sigma^E$ on the expression of these genes is not activation [10], likely mediated by $\sigma^E$-dependent small RNAs that accelerate global $omp$ mRNA decay upon membrane stress [27].

We found that a considerable amount of $\sigma^E$-binding sites were associated with genes involved in transcriptional circuity ($rpoE$, $rpoH$, $rpoD$, $rseA$, $greA$, and $sixA$) [25] [22], protein folding ($fkpA$, $surA$, and $htrA$) [28] [29] [30], protein biosynthesis ($pth$) [31] and assembly ($yfO$) [32], stress adaptation ($cspE$, $galF$, and $luxS$) [33] [34,35], cell division ($\text{minD/minE}$) [21], and lipid A biosynthesis ($ddg$) [36]. Upon activation of $\sigma^E$, a positive feedback on itself ensures sufficient $\sigma^E$ is produced to accelerate the protein synthesis required for regaining homeostasis. As a strategy to avoid imbalances, $\sigma^E$ also regulates the anti-sigma factor $rseA$, which dampens the $\sigma^E$ response as a negative feedback loop [8]. Other alternative factors are also activated by $\sigma^E$, not only because some of the stresses they respond to are overlapping, but

![Fig 7. Heat shock and oxidative stress susceptibility.](http://plosone journa l.pone.0138466.g007)
also to expand the protection to other stresses the bacteria may encounter in later stages [37]. A previous study identified a cell division protein FtsZ is regulated by $\sigma^E$ in eight organisms closely related to E. coli [8]. Here, we found $\sigma^E$ regulates other cell division factors (minD/minE), further suggesting that $\sigma^E$ may orchestrate factors associated with cell division. Both outer membrane proteins (OmpA, OmpF, OmpN, and OmpX) and proteins promoting OMP assembly (FkpA, HtrA, and YfiO) are regulated by $\sigma^E$, hence facilitating the proper assembly and insertion of the newly synthesized OMPs into the outer membrane of Salmonella.

Activation of $\sigma^E$ is thought to maintain bacterial homeostasis under extracytoplasmic stresses. Five $\sigma^E$-binding sites (greA, ompX, ompA, luxS, and rpoE) were tested for the fulfillment of this purpose using heat shock and oxidative stress challenges (Fig 7). All of them exhibited significantly altered susceptibility to these stresses, consistent with the traditional role of $\sigma^E$. Moreover, this finding supported the observation that the transcription elongation factor GreA has functional chaperone activity [38], which is likely regulated by $\sigma^E$. Why the quorum sensing protein LuxS is involved in extracytoplasmic stress response needs to be further investigated. The $\sigma^E$ regulatory system is flexible and efficient, hence playing a significant role for Salmonella to survive in various environments.

### Supporting Information

**S1 Fig. The quality of antibodies for ChIP experiments.** The rabbit Ab to $\sigma^E$ and monoclonal Ab to GFP were tested for specificity by western blot. Cell lysates from WT or $\Delta rpoE$ strains were loaded on the gel, and different Abs were used to develop the membrane. Lane 1, the flow through of rabbit immune sera during affinity chromatography purification of anti-$\sigma^E$ Ab. Lane 2–4, Ab to $\sigma^E$ diluted at 1:1000, 1:3000, and 1:5000 respectively. Lane 5–7, monoclonal Ab to GFP diluted at 1:1000, 1:3000, and 1:5000 respectively. (TIF)

**S1 Table. List of primers used for mutant construction.** (XLSX)

**S2 Table. List of quantitative PCR primers used in this study.** (XLSX)

### Author Contributions

Conceived and designed the experiments: FH JNA JL. Performed the experiments: JL RCJ MBJ. Analyzed the data: CCO JL. Contributed reagents/materials/analysis tools: JEM EDC. Wrote the paper: JL EDC.

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