Characterization of the *Escherichia coli* $\sigma^S$ core regulon by Chromatin Immunoprecipitation-sequencing (ChIP-seq) analysis

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In bacteria, selective promoter recognition by RNA polymerase is achieved by its association with $\sigma$ factors, accessory subunits able to direct RNA polymerase "core enzyme" (E) to different promoter sequences. Using Chromatin Immunoprecipitation-sequencing (ChIP-seq), we searched for promoters bound by the $\sigma^S$-associated RNA polymerase form (E$\sigma^S$) during transition from exponential to stationary phase. We identified 63 binding sites for E$\sigma^S$ overlapping known or putative promoters, often located upstream of genes (encoding either ORFs or non-coding RNAs) showing at least some degree of dependence on the $\sigma^S$-encoding *rpoS* gene. E$\sigma^S$ binding did not always correlate with an increase in transcription level, suggesting that, at some $\sigma^S$-dependent promoters, E$\sigma^S$ might remain poised in a pre-initiation state upon binding. A large fraction of E$\sigma^S$-binding sites corresponded to promoters recognized by RNA polymerase associated with $\sigma^70$ or other $\sigma$ factors, suggesting a considerable overlap in promoter recognition between different forms of RNA polymerase. In particular, E$\sigma^S$ appears to contribute significantly to transcription of genes encoding proteins involved in LPS biosynthesis and in cell surface composition. Finally, our results highlight a direct role of E$\sigma^S$ in the regulation of non-coding RNAs, such as OmrA/B, RyeA/B and SibC.

Bacteria are constantly exposed to changes and fluctuations in their environment, to which they can adapt by reprogramming their gene expression through various mechanisms, including use of alternative $\sigma$ factors. $\sigma$ factors are accessory subunits of bacterial RNA polymerase that associate, in a 1:1 stoichiometric ratio, to the core enzyme (E), *i.e.*, the multi-subunit complex responsible for RNA polymerase catalytic activity. Binding to any of the different alternative $\sigma$ factors creates different RNA polymerase holoenzymes (E$\sigma$), proficient in specific promoter recognition and transcription initiation. After the process of transcription initiation has taken place, the $\sigma$ factor dissociates from the holoenzyme, and the core enzyme carries out transcription elongation$^1$. The number of $\sigma$ factors varies considerably among bacteria: seven $\sigma$ factors are known to be present in *Escherichia coli*, including $\sigma^70$ (or $\sigma^{17}$), the "housekeeping" $\sigma$ factor devoted to transcription of a large part of the genome and of most essential genes. In contrast, alternative $\sigma$ factors are responsible for the transcription of smaller subsets of genes, fulfilling specific roles or belonging to defined functional groups$^2$. One alternative $\sigma$ factor, $\sigma^S$, strongly affects cell survival during stress conditions, such as starvation, oxidative stress, and exposure to either low or high pH, and controls expression of virulence factors in several pathogens$^3$. For its important role in response

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to cellular stresses, σ^5 is considered the master regulator of the so-called “general stress response” and, consistently, it is induced in response to any stressful event leading to reduction in specific growth rate\(^6\).

Interestingly, σ^6 and σ^70 appear to recognize very similar promoter sequences\(^7\). Consequently, several promoters are recognized with similar efficiency by both Eσ^6 and Eσ^70 in vitro, and their preferential recognition by either form of RNA polymerase in vivo is mediated by accessory regulatory proteins\(^8\). Selective promoter recognition by either σ^70 or σ^5 can be achieved by deviations from a common consensus sequence\(^9\) which confer specificity for either σ factor: for instance the presence of a C nucleotide (−13C) immediately upstream of the −10 promoter element is a known determinant for σ^5 binding and it is a common feature in σ^5-dependent promoters\(^9\). In a previous work, we set out to determine which promoters are preferentially bound in vitro by either Eσ^70 or Eσ^5 by run-off transcription microarray (ROMA); we confirmed the importance of sequence elements important for promoter recognition by σ^6, such as the presence of C residues at positions −13 and −12 C element, and suggested that an A/T-rich discriminator region would favour transcription initiation by Eσ^5 in vitro\(^10\).

In this work, we used Chromatin-Immunoprecipitation-sequencing (ChIP-seq) to identify promoters bound by Eσ^5 at early stationary phase, i.e., at a moment in which σ^5 accumulates inside the bacterial cell. Our results led to identification of novel σ^5-dependent genes, and provided insight on regulation of non-coding RNAs by σ^5. We could also show that a significant subset of Eσ^5-bound promoters controls genes whose expression is σ^5-independent, suggesting considerable overlap in promoter recognition by different σ factors.

Results

MG1655-rpoS\(^{His6}\) construction and σ^5-His\(^6\) immunoprecipitation. Since no anti-σ^5 antibodies suitable for immunoprecipitation were available at the time of this study, we decided to utilize anti-6xHis-tag antibodies targeting a histidine-tagged σ^5 protein (σ^5-His\(^6\)). In order to study promoter binding by σ^5-His\(^6\) without perturbing σ^6 physiological levels or rpoS gene expression, we constructed a strain carrying a chromosomal rpoS\(^{His6}\) allele, i.e., an otherwise wild type rpoS allele with 6 codons for histidine at its 3’ end, as described in Materials and Methods. We verified the effects of the rpoS allele replacement on specific growth rate (Fig. 1A) and checked the relative amounts of both the wild type and the σ^5-His\(^6\) proteins at the onset of stationary phase by Western blot, using an anti-σ^5 antibody (Fig. 1A, inset). A Western blot with the anti-6xHis antibody confirmed that the MG1655-rpoS\(^{His6}\) strain did indeed produce a 6xHis-tagged σ^5 protein (data not shown). No differences were detected in either specific growth rate or intracellular σ^5 amounts in the two strains (Fig. 1A). Western blot analysis clearly showed that, as expected, the amount of σ^5 (or σ^5-His\(^6\)) increased significantly at the end of the exponential phase, (compare points 1 and 2): at this point, bacterial cells were growing at a specific growth rate of 0.32 (±0.02) h\(^−1\). Cells were collected at the growth stage corresponding to point 2 in Fig. 1A in all subsequent experiments.

To verify whether the C-terminal histidine tag might affect σ^5 activity in vivo, we tested the activity of HPII catalase, encoded by the rpoS-dependent katE gene and a marker for rpoS functionality\(^11\). No statistically significant difference in HPII specific activity was detected between MG1655 and MG1655-rpoS\(^{His6}\) while, in contrast, HPII catalase specific activity was almost totally abolished in an rpoS null mutant strain, as expected (Fig. 1B). These results indicate that introduction of the 6xHis-tag in the σ^5 protein does not affect its abundance, physiological regulation and activity. Thus, we performed protein-DNA co-immunoprecipitation experiments in the MG1655-rpoS\(^{His6}\) strain, using anti-6xHis antibodies. As a quality control of the co-immunoprecipitation experiment, we verified the enrichment of a known binding site for Eσ^5 in the immunoprecipitated samples compared to sonicated DNA (Input sample). To this purpose, we performed qRT-PCR experiments comparing the relative abundance of the promoter region of the σ^5-dependent dps gene (Pdps) to coding sequences within the rpoB and the yeeJ genes. Both the Pdps/rpoB and Pdps/yeeJ ratios approached 1 in the Input sample, while being 10-fold higher in the σ^5-His\(^6\) immunoprecipitation sample (σ^5-IP; Fig. 1C), thus suggesting strong enrichment in Eσ^5 binding sites by the immunoprecipitation procedure.

Chromatin immunoprecipitation-sequencing (ChIP-seq). Two replicates of the Input sample (MG1655-rpoS\(^{His6}\) chromosomal DNA) and of the σ^5-IP sample (σ^5-His\(^6\) immunoprecipitated DNA) were used to prepare sequencing libraries. The libraries were sequenced into 4 separate lanes of the same GAIIx run. We obtained more than 50 million mapping reads for both the input samples (corresponding to a sequencing depth of 543-fold the E. coli genome); for the first and the second IP samples, more than 26 and 32 million mapping reads were obtained, respectively. Identification of the DNA regions more represented in the σ^5-IP sample, corresponding to potential binding sites for Eσ^5, was carried out using the CisGenome software\(^12\), which yielded 78 “peaks”, i.e., regions of the genome significantly enriched (pval ≤ 0.01) in the σ^5-IP sample as compared to the Input sample. Almost all peaks detected (72/78) corresponded to DNA regions ≤400 bp-long or slightly larger, consistent with the DNA fragment sizes obtained after DNA sonication (see Materials and Methods, “σ^5-His\(^6\) immunoprecipitation”).

Three enriched regions were slightly larger in size (500-700 bp), while only three regions had sizes larger than 1kbp (1049, 1199 and 3149 bp, respectively). The last one encompassed a DNA region including five different ORFs and several non-coding and regulatory elements, making it impossible to identify a
putative binding site for $\sigma^S$; thus, this DNA fragment was excluded from further analysis and is listed, together with intragenic peaks, in Supplementary Table S2 (see below). On the contrary, the two peaks just over 1 kbp overlapped a single known promoter region, and were thus included in the $\sigma^S$ binding site analysis shown in Table 1. The visualization through Integrative Genome Viewer (IGV) of representative $\sigma^S$ binding peaks obtained from the CisGenome analysis is shown in Fig. 2: significantly enriched genomic regions (i.e., peaks) are reported for the known $rpoS$-dependent genes $osmB$, $dps$, $osmE$ and $csrA$ (Fig. 2A) and for loci associated to the small RNAs $sibC/ibsC$, $ryeA/ryeB$, and $omrA/omrB$ (Fig. 2B; see also section “Regulation of non-coding RNA by $\sigma^S$”).

The large majority (63 out of 78) of the $\sigma^S$-IP peaks was located immediately upstream of coding sequences or known regulatory RNAs, consistent with $\sigma^S$ binding to promoter regions. Out of these 63 peaks, 61 were located in intergenic peaks, in Supplementary Table S2 (see below). On the contrary, the two peaks just over 1 kbp overlapped a single known promoter region, and were thus included in the $\sigma^S$ binding site analysis shown in Table 1. The visualization through Integrative Genome Viewer (IGV) of representative $\sigma^S$ binding peaks obtained from the CisGenome analysis is shown in Fig. 2: significantly enriched genomic regions (i.e., peaks) are reported for the known $rpoS$-dependent genes $osmB$, $dps$, $osmE$ and $csrA$ (Fig. 2A) and for loci associated to the small RNAs $sibC/ibsC$, $ryeA/ryeB$, and $omrA/omrB$ (Fig. 2B; see also section “Regulation of non-coding RNA by $\sigma^S$”).

The large majority (63 out of 78) of the $\sigma^S$-IP peaks was located immediately upstream of coding sequences or known regulatory RNAs, consistent with $\sigma^S$ binding to promoter regions. Out of these 63 peaks, 61 were located in intergenic regions, while two peaks lie within the $stfR$ and $wbbH$ ORFs, but upstream, respectively, of the $tfsS$ and $wbbI$ genes, suggesting that they might define internal promoters within operons. The remaining peaks fell into intergenic regions at considerable distance from other ORFs (listed in Supplementary Table S2). Although it is possible that some of these peaks might define $bona fide$ $Es^S$ binding sites (e.g., promoters for yet unknown antisense RNAs), they were not considered for further characterization within this study. However, even assuming that all the intergenic peaks are artefacts of ChIP-seq, the resulting percentage of false positives (19%) would still be lower than what reported for similar studies.

50 out of the 63 peaks corresponding to known or putative promoter regions could unequivocally be attributed to one specific gene, based on the DNA sequence covered by the peak, the direction of transcription of the neighbouring genes, the distance to the nearest ORFs and, when available, the presence of an experimentally determined transcription start site within the boundaries of the peak. Of the 50
genes unequivocally identified, 27 had been shown to be at least partially rpoS-dependent in previous reports, as listed in Table 1. In contrast, 13 peaks, listed in Table 2, lie in intergenic regions between divergently transcribed genes or operons and could not be assigned to a specific gene. However, we often found that one of the two divergent genes (or even both, as for the dsrB-yodD intergenic region, Table 2) had previously been described as rpoS-dependent, thus suggesting that EσS binding was due the presence of an rpoS-dependent promoter within the intergenic region. As an example, we assigned the putative EσS binding site in the osmE-nadE intergenic region to osmE, since its promoter is σS-dependent14–16 (Fig. 2 and Table 2).

Figure 2. Visualization through IGV of the binding peaks obtained from CisGenome analysis. The blue profiles show the IP and Input tag density profiles for the known rpoS-dependent genes osmB, dps, osmE and csrA (A) and for the loci associated to the non-coding RNAs sibC/ibsC, ryeA/ryeB, and omrA/omrB (B). The red profiles show the log2 signal to control enrichment estimates values obtained using spp (peaks) for the same genes and non-coding RNAs. Values on X axis are the genomic coordinates of the peaks; a representation of the corresponding gene/intergenic regions taken from Ecocyc (ecocyc.org) is shown.
| peak start | peak end | downstream gene** | chromosome strand | experimentally validated TSS located inside peak | Gene function | References showing gene regulation by σ\(^{32}\) or by other alternative σ factors |
|------------|----------|-------------------|-------------------|---------------------------------|--------------|----------------------------------------------------------|
| 63400      | 63538    | hepA              | -                 | 106530                          | RNA-polymerase associated ATPase 11 (σ\(^{32}\))      |
| 106436     | 106616   | lpxC              | +                 | 106530                          | UDP-3-O-acetyl-N-acetylglucosamine deacetylase (lipid A biosynthesis) 18 |
| 262040     | 262202   | thrW              | +                 | IS-3 transposase 17              |
| 392250     | 392349   | insEF-2           | -                 | IS-3 transposase 17              |
| 406100     | 406199   | yaiA              | +                 | unknown, oxidative stress 56    |
| 437329     | 437469   | yaoO              | -                 | putative NAD(P)H-dependent xylose reductase 56 |
| 479920     | 480115   | tomb              | -                 | antitoxin in tomb/hha T/A system |
| 574850     | 575099   | insH-2            | -                 | IS-5 transposase 15             |
| 837550     | 837849   | ybl                | -                 | unknown                         |
| 848050     | 848349   | dsz                | -                 | 848173 stationary phase nucleoid component/ferritin 14–16 |
| 1215900    | 1216399  | ymgC              | +                 | involved in biofilm formation 15 |
| 1219400    | 1219949  | ycgH              | pseudogene- autotransporter 15,16,56 |
| 1341304    | 1341480  | yodC              | -                 | unknown 15,16 | osmotically inducible lipoprotein 15,53,54 |
| 1430250    | 1430549  | tfaR              | +                 | Rac prophage tail fiber assembly protein, induced in biofilms |
| 1509526    | 1509687  | ydcS              | +                 | polyanime transporter 15,16,55 |
| 1524000    | 1524199  | amaP              | -                 | arginine transporter 14,18 |
| 1608700    | 1608949  | uxaB              | -                 | galacturonate degradation 16,18 |
| 168744     | 1687907  | ydgA              | +                 | unknown, involved in swarming motility 16,18 |
| 1753530    | 1755499  | lpp                | +                 | Braun lipoprotein 14,18 |
| 1756820    | 1756885  | ynbG              | -                 | transpeptidase, associated to Lpp 15,16 |
| 1894663    | 1894896  | sdaA              | +                 | serine deaminase 15 (σ\(^{32}\)) |
| 1905547    | 1905784  | yobF              | -                 | stress response protein 16,18 |
| 1920033    | 1920203  | yebW              | unknown            |
| 1921150    | 1921299  | ryeB              | -                 | small RNA, antisense of small RNA ryeA 16,20 |
| 2026384    | 2026505  | yodC              | unknown            |
| 2061261    | 2061484  | erfK               | -                 | transpeptidase, associated to Lpp 16 |
| 2103850    | 2104199  | wbbI              | -                 | (β-1,6-galactofuranosyl-transferase, LPS O-antigen 14,15,56 |
| 2104550    | 2105599  | wbbH              | -                 | LPS O-antigen polymerase 15,16,55 |
| 2190800    | 2190949  | yehE              | -                 | unknown 15,16 |
| 2225279    | 2225390  | yohF              | predicted acetoin dehydrogenase 16 |
| 2468677    | 2468882  | tfaS              | +                 | CPS-53 prophage tail protein 16,18 |
| 2663364    | 2663501  | csiE              | +                 | stationary phase inducible gene 15,16,56 |
| 2734910    | 2735081  | raiA              | +                 | ribosome inhibitor, stationary phase-dependent 13 (σ\(^{32}\)), 19 |
| 2735302    | 2735707  | ssaA              | +                 | tmRNA 18,27 |
| 2758300    | 2758999  | yflJ              | +                 | CP4-57 prophage protein 17 |
| 2797100    | 2797249  | alaE              | +                 | alanine exporter 18,27 |
| 2817227    | 2817395  | csrA              | -                 | RNA-binding protein, translational regulator 18,27 |
| 2924252    | 2924570  | ygdH              | +                 | unknown 19 |
| 2974153    | 2974278  | omrA              | -                 | small regulatory RNA 30 |
| 2991100    | 2992299  | ygeI              | +                 | unknown 18,27 |
| 3054792    | 3054952  | sibC              | +                 | small regulatory RNA 30 |
| 3058600    | 3058749  | scpA              | +                 | methyl-malonyl-CoA mutase 18,27 |
| 3066050    | 3066149  | yggE              | -                 | unknown, oxidative stress 14,18 |
| 3235233    | 3235381  | ygiR              | +                 | predicted dehydrogenase 18,27 |
| 3598950    | 3599099  | rpoH              | -                 | alternative sigma factor (sigma32) 35 |

Continued
### Table 1. Location of putative En^5 binding sites attributable to a specific promoter region. *Genes for which regulation by En^5 has already been shown (see last column) are indicated in boldface type; genes with promoter DNA regions that were studied in vitro are underlined.

| peak start | peak end | nearest gene* (= strand) | Gene function | experimentally validated TSS located inside peak | nearest gene* (= strand) | Gene function | References showing gene regulation by σ^S or by other alternative σ factors |
|------------|----------|--------------------------|---------------|-----------------------------------------------|--------------------------|---------------|--------------------------------------------------|
| 3637750    | 3637949  | uspB                     | -             | 3637871 universal stress protein B             | 3637750                  | -             | 36,56,58                                         |
| 3706750    | 3706999  | proK                     | -             | 3707368 prolyl-tRNA                           | 3706750                  | -             |                                                  |
| 4361287    | 4361432  | yycG                     | -             | 4361353 putative transcriptional regulator     | 4361287                  | -             |                                                  |
| 4437000    | 4437349  | ytfJ                     | -             | 4437309 unknown, periplasmic protein           |                          |               | 16,19                                           |

### Table 2. Location of putative En^5 binding sites in intergenic regions between divergent genes. *Genes for which regulation by En^5 has already been shown (see last column) are indicated in boldface type; genes with promoter DNA regions that were studied in vitro are underlined.

| peak start | peak end | nearest gene* (= strand) | Gene function | experimentally validated TSS inside the peak | nearest gene* (= strand) | Gene function | References showing gene regulation by σ^S or by other alternative σ factors |
|------------|----------|--------------------------|---------------|-----------------------------------------------|--------------------------|---------------|--------------------------------------------------|
| 1257750    | 1258199  | pth                      | peptidyl-tRNA hydrolase | 1257765 (pth) 1257961 (ychH)      | ychH unknown, oxidative stress | 1258199      |                                                  |
| 1288250    | 1288399  | ychH                     | unknown        | 1288401 (ychH) 1288329 (rsA)         | rsA unknown               | 1288399      |                                                  |
| 1438800    | 1439049  | ydbK                     | pyruvate flavodoxin oxidoreductase, involved in oxidative stress | 1439053 (ydbK)           | ydbK unknown               | 1439049      |                                                  |
| 1488650    | 1488949  | (gacP_C1)                | glyceraldehyde 3-phosphate dehydrogenase (pseudogene) | 1488960 (gacP_C1)        | cybB cytochrome b561       | 1488949      |                                                  |
| 1820250    | 1820399  | osmE                     | osmotically inducible lipoprotein | 1820307(osmE) 1820326 (nadE) | nadE NAD synthetase, NH3-dependent | 1820399      |                                                  |
| 2022850    | 2023149  | dsrB                     | unknown        | 2023150 (dsrB)                           | yodD involved in oxidative and acid stress | 2022850      |                                                  |
| 2493450    | 2493549  | yfdY                     | biofilm-dependent membrane protein | 2493550 (yfdY)           | lpxP palmitoyleoyl acyltransferase (LPS biosynthesis) | 2493450      |                                                  |
| 2627100    | 2627399  | yfgG                     | C-di-GMP phosphodiesterase | 2627275 (yfgG)           | yfgG unknown               | 2627100      |                                                  |
| 2903350    | 2903649  | queE                     | conserved protein | 2903650 (queE)            | ygcG small protein involved in cell envelope stress | 2903350      |                                                  |
| 3851100    | 3851399  | istR-1/istR-2            | regulatory small RNA for istR | 3851215-3851280 (istR) 3851360 (istR) | tisB toxic peptide         | 3851100      |                                                  |
| 4124850    | 4125049  | priA                     | DNA replication restart factor | 4124931 (rpmE)           | rpmE L31 ribosomal protein | 4124850      |                                                  |
| 4414650    | 4414899  | bamA                     | biofilm-dependent protein involved in oxidative stress | 4414899 (bamA)           | ypp esterase               | 4414650      |                                                  |
| 4434400    | 4434749  | cpdB                     | 2’3’ cyclic nucleotide phosphodiesterase and nucleotidase | 4434652 (cpdB)           | cysQ adenosine 3’-5’ bisphosphate (PAP) nucleotidase | 4434400      |                                                  |

Altogether, the peaks identified in the ChIP-seq experiment overlapped with the promoters of 36 genes that had been shown to be at least partially rpoS-dependent (highlighted in Tables 1 and 2). Stress-related genes defined the most represented functional category in our ChIP-seq analysis (see Tables 1–2), in agreement with the role of σ^S as master regulator of the general stress response. Interestingly, binding sites for En^5 were also found upstream of several genes involved in cell envelope structure (eufK, lpp, ynhG) and lipopolysaccharide (LPS) biosynthesis (lpxC, wbbH, wbbI), suggesting that En^5 might be important for the expression of cell surface-related genes in response to growth cessation.

The majority of the intergenic regions not linked to rpoS-dependent genes included known or putative promoters recognized by En^50, in agreement with previous results indicating extensive cross-recognition between En^5 and Eσ^70 regulons7,9. Interestingly, however, several promoters are also recognized by other alternative σ factors, namely σ^H (ytfJ and lpxP) and σ^H (hepA, sdaA, raiA and rpmE) (Tables 1–2).

**In vivo expression of genes identified by ChIP-seq analysis.** The results of our ChIP-seq experiments seem to indicate that a large percentage of En^5-binding sites are associated with promoters directing transcription of rpoS-independent genes. Alternatively, regulation of these genes by En^5 might have been overlooked in previous investigations of the rpoS regulon, mostly carried out as whole genome
transcription analysis comparing an rpoS mutant to its parental strain\(^ {14-19}\). In order to elucidate the functional role of the E\(\sigma^S\)-binding sites, we measured relative expression of 10 genes whose promoters, according to our ChiP-seq results, are recognized by E\(\sigma^S\), by performing qRT-PCR experiments comparing *E. coli* MG1655 to its otherwise isogenic rpoS mutant. As control genes in the qRT-PCR experiment, we chose 4 genes previously proposed to be rpoS-dependent: *dps*, *ycgB*, *rssA* and *bsmA*\(^ {15,16,20}\). The remaining 6 genes, never previously shown to be rpoS-dependent, were selected based either on their function or on promoter features: *ipp* encodes Braun lipoprotein, which bridges the outer membrane to peptidoglycan and is extremely abundant in *E. coli*\(^ {21}\); *ssrA* is a transfer-messenger RNA (tmRNA)-encoding gene; *uxaB* is involved in galacturonate metabolism; *ybiI* is a gene of unknown function whose promoter had been indicated as putative E\(\sigma^S\)-dependent through bioinformatics prediction\(^ {22}\); *ydbK* is an oxidative stress-related gene\(^ {23}\); *ygjR*, like *ybiI*, is an unknown function gene with a known transcription start site\(^ {24}\), whose putative – 10 region shows some features typical of E\(\sigma^S\)-dependent promoters, such as the –13C.

Results of the qRT-PCR experiments (Fig. 3) could demonstrate rpoS-dependent gene expression for *dps*, *ycgB*, *ybiI* and *ydbK*, suggesting that the latter two are yet unidentified members of the rpoS regulon. In contrast, the expression of the remaining genes was not affected by the lack of a functional rpoS gene, at least in the conditions tested. To further investigate whether these genes showed any kind of dependence on \(\sigma^S\), we tested their expression levels in a rpoS-overexpressing strain (MG1655/pBADrpoS) grown to early stationary phase in LB medium supplemented with 0.1% arabinose. Although intracellular \(\sigma^S\) amounts were almost 10-fold higher in the pBADrpoS-bearing strains compared to MG1655, no significant changes in relative expression levels were detected for any of the genes tested (data not shown).

**In vitro E\(\sigma^S\)-promoter interactions.** Results of the ChiP-seq and qRT-PCR experiments failed to show strong correlation between E\(\sigma^S\) promoter binding and E\(\sigma^S\)-dependent transcription, even for genes previously described as rpoS-dependent, such as *rssA* and *bsmA* (Fig. 3). In order to confirm the ChiP-seq results, we studied \(\sigma^S\)-promoter interactions in *vitro*, by comparing E\(\sigma^S\) and E\(\sigma^{70}\) for their ability to bind and to promote open complex formation at a subset of the promoters studied in qRT-PCR experiments. We selected the promoter regions of the two newly identified rpoS-dependent genes, *ybiI* and *ydbK*, together with the promoters of the known rpoS-dependent *dps* and *bsmA* genes, which, however, showed different behaviour in our qRT-PCR experiments. Firstly, we performed GMSA with either E\(\sigma^S\) or E\(\sigma^{70}\), in the presence of heparin to select for open complexes, on regulatory DNA fragments (extending from 250 bp upstream to 30 bp downstream of the start codon). E\(\sigma^S\) was clearly more efficient than E\(\sigma^{70}\) in promoting open complex formation at the *ybiI*, *ydbK* and *bsmA* promoters (compare amounts of unbound DNA probes, Fig. 4A), while both forms of RNA polymerase showed similar proficiency in open complex formation at the *dps* promoter, despite its strong E\(\sigma^{70}\)-dependence *in vivo* (Fig. 3; 8,16). As a negative control for binding by E\(\sigma^S\), we performed GMSA experiments on the strictly E\(\sigma^{70}\)-dependent *ctrl* promoter, which clearly showed preferential binding by E\(\sigma^{70}\) (Supplementary Fig. S1).

To further investigate promoter DNA-RNA polymerase interaction, and to map the exact location of the -10 promoter elements for *ybiI*, *ydbK* and *bsmA*, we performed KMnO\(_4\) reactivity assays (Fig. 4B). Treatment with permanganate oxidizes thymidine residues in single-stranded DNA, allowing us to identify precisely the location of open complexes. As expected, no open complex formation by E\(\sigma^S\) was detected at the E\(\sigma^{70}\)-dependent *ctrl* promoter (Supplementary Fig. 1). In contrast, open complex formation at the *bsmA* promoter was only observed in the presence of E\(\sigma^S\), consistent with GMSA results.
and confirming specific recognition by $\sigma^S$ at this promoter. Similarly, at the $ybil$ promoter, binding by $\sigma^S$ resulted in much stronger reactivity than $\sigma^{70}$, indicating more efficient open complex formation. A more complex picture emerged from KMnO$_4$ experiments at the $ydbK$ promoter, which showed that both $\sigma^S$ and $\sigma^{70}$ can recognize a promoter located, in agreement with bioinformatics predictions, at a region 70 nucleotides upstream of the $ydbK$ ORF. However, subtle changes can be observed in the pattern of KMnO$_4$ reactivity induced by the two RNA polymerase-promoter complexes, with binding by $\sigma^S$ resulting in higher reactivity in the T residues at positions $-4$ to $-2$ (marked by an arrow in Fig. 4B).

Taken together with GMSA results, this observation suggests that, at the $ydbK$ promoter, $\sigma^S$ might trigger formation of an open complex more resistant to heparin challenge and possibly more proficient in transcription initiation. Finally, at the $dps$ promoter, both $\sigma^S$ and $\sigma^{70}$ induced open complex formation with equal efficiency, indicating lack of preferential recognition by either form of RNA polymerase in vitro.

**Regulation of non-coding RNAs by $\sigma^S$.** Results of ChIP-seq analysis indicate that three $\sigma^S$ binding sites are positioned in the proximity of genes encoding regulatory RNAs. A putative $\sigma^S$ binding site was identified upstream of the 88 nt-long regulatory RNA omrA, which controls expression of genes involved in flagellar motility, iron uptake, adhesion factors and various outer membrane proteins. The omrA gene lies next to omrB, which codes for a highly similar small RNA and also regulates some of the targets for omrA. The other two $\sigma^S$ binding sites were found in proximity of two complex loci: the ryeA/ryeB locus, which includes two small RNAs overlapping in antisense directions, and the sibC/sibC locus, in which a non-coding RNA (sibC) overlaps a small ORF, sibC, reading in the opposite direction, and encoding a toxic peptide. The location and extension of the three ChIP-seq peaks suggest that $\sigma^S$
might bind the promoter regions of omrA (but not omrB), and of ryeB and sibC, rather than ryeA and ibsC (Fig. 2B), consistent with recent observations that omrA and ryeB are rpoS-dependent in Salmonella enterica29,30. To confirm this result, we performed northern blots comparing small RNA levels in the wild type versus the rpoS mutant strain of E. coli (Fig. 5). In addition to standard growth conditions (LB medium at 37°C), we also carried out northern blot experiments at 28°C, since low growth temperature favors σS accumulation and positively affects stability of some small RNA31. Due to difficulties in obtaining a clean result with a probe for RyeB, we measured the relative amounts of RyeA, which upon pairing with RyeB, is degraded in an RNaseIII-dependent fashion and shows therefore transcript levels inversely proportional to ryeB27,29. Inactivation of the rpoS gene almost abolished omrA transcription, while strongly increasing RyeA transcript levels (Fig. 5A), consistent with rpoS-dependence of transcription of the omrA and ryeB genes. Interestingly, the OmrA and RyeA transcripts also displayed opposite temperature-dependence, with OmrA being more expressed at 28°C and RyeA at 37°C. As further confirmation that rpoS-dependent regulation specifically targets omrA, but not omrB, we performed gfp reporter assays. Reporter genes experiments clearly showed very different effects of rpoS inactivation

Figure 5. Regulation of small non-coding RNAs by σS. A. Northern blot hybridization. RNA were extracted at the onset of stationary phase (OD600nm of 3) from bacteria grown in LB at either 28°C or 37°C and probed for SibC, OmrA, and RyeA transcript levels (left to right). Numbers on the right side of each panel indicate the size of the respective ncRNA. The gels were probed for the genes of interest, then the probe was removed by washing and the gels were re-probed for SS RNA, which was used as internal control. B. Relative fluorescence of transcriptional fusions of the omrA and omrB promoters to the GFP reporter gene. The promoter activity (solid line) is expressed as ratio between the fluorescence and the absorbance of the culture (dashed line) after background correction (RFU/OD600nm). C. Effects of the substitution of the −12C to a T nucleotide in the omrA promoter region. Data were taken from overnight cultures and are the average of four independent experiments.
on transcription of the two genes, with omrA showing almost complete rpoS-dependence, while omrB expression was actually slightly increased in the rpoS mutant background (Fig. 5B). Interestingly, the first nucleotide of the −10 region of omrA is a −12C (Supplementary Table S3), a feature favouring specific promoter opening by EσS but not by Eσ70, while at the omrB promoter, such a selective determinant is replaced by a canonical −12T for Eσ70 and might explain lack of preferential binding by EσS. Substitution of the −12C nucleotide by a −12T in the omrA−10 promoter element increases promoter strength by more than 10-fold and almost completely overcomes its dependence on rpoS (Fig. 5C), suggesting that the −12C act as a determinant for EσS specificity in the omrA promoter. A more complex picture emerged from analysis of the SibC transcript, which, like RyeA, showed increased expression at 37 °C than at 28 °C. At the latter temperature, SibC was transcribed in an rpoS-dependent manner; however, the effect of the rpoS mutation was reversed at 37 °C, possibly suggesting additional regulatory mechanism affecting SibC expression at this temperature (Fig. 5A). The complexity of SibC regulation is also suggested by the presence of two transcripts, either due to the presence of multiple promoters or to RNA processing as already described28.

Sequence analysis of σS-bound promoters. In order to assess the importance of σS-specific promoter determinants for binding by σS, we analyzed the sequences of the experimentally determined promoters controlling genes identified in the ChIP-seq experiments (30 promoters, listed in Supplementary Table S3). The promoters were divided in two subsets: the ones directing transcription of genes reported to show some level of dependence on σS (21 promoters) and those controlling genes whose expression is not affected by lack of a functional rpoS gene (9 promoters). In good agreement with the previously proposed consensus for σS4,8,10,16, −10 region alignment of σS-dependent genes (from −20 to +1, Fig. 6) suggests that their consensus sequence in the −17 to −6 region would be TNTGCYAAACTT, where N is any nucleotide and Y is a pyrimidine and W is either A or T (Fig. 6); in addition, promoters of σS-independent genes lack conservation of the C residues at positions −13, −12, and −8, reduced frequency of a T at position −6, and display a discriminator region richer in G/C (Fig. 6). Alignment of the −35 regions of σS-bound promoters (listed in Supplementary Table S4) highlighted some conservation of the σ70 consensus sequence, TTGACA, in the promoters of genes whose expression is independent of σS; in contrast, in the promoters of σS-dependent genes, the −35 region showed a weakly conserved sequence, GCTGACAAA, with some resemblance to the −35 promoter element for σ70 (Supplementary Fig. S2). It remains to be understood whether this sequence might play any role in σS–promoter interactions.

Discussion
In this work, we used a ChIP-seq approach in order to identify promoters bound by EσS during the early stationary phase, in which σS concentrations surge in the bacterial cell (Fig. 1A). The experimental conditions used in this work were chosen in order to identify genes directly regulated by σS that are induced in response to transition into stationary-phase. Indeed, we only detected 63 promoter regions bound by EσS (Tables 1–2); this number only represents a fraction of the σS-bound promoters previously identified either by microarray or by ChIP-on-chip analysis14,19,33, which, however, were performed under a variety...
of different growth conditions and include genes subject to complex regulation and only indirectly regulated by $\sigma^5$. Out of the 63 promoters identified in our study, 38 (60%) control transcription of genes regulated by the $\sigma^5$-encoding rpoS gene (Tables 1–2 and references within). Two of these, ybi and ydbK, had not yet been identified as part of the rpoS regulon, and we confirmed their preferential recognition by E$\sigma^5$ via in vitro binding and open complex formation experiments (Fig. 4). However, a large percentage of $\sigma^5$-bound promoters control genes whose expression is not affected by the presence of this factor (see Tables 1–2, Fig. 3), suggesting that these promoters are recognized with similar efficiency by $\sigma^5$ and other $\sigma$ factors, mostly $\sigma^{32}$.

This result is consistent with the notion that $\sigma^5$ does not only serve to promote expression of its own regulon, but it can also contribute to transcription of constitutively expressed genes. Promoter sequence comparison between bona fide $\sigma^5$-dependent genes and those not showing altered expression in an rpoS mutant highlighted the importance of the promoter elements associated with selective recognition by $\sigma^5$ (Fig. 6). At least some $\sigma^5$-specific determinants might be more important for preventing recognition by $\sigma^{70}$ in vivo rather than increasing binding affinity or promoter opening by $\sigma^5$, such as the presence of a C rather than a T as first nucleotide of the $-10$ hexamer, as is the case at the omrA promoter (Fig. 5C). Although the mechanisms of regulation by $\sigma^5$ appear to be well conserved in Enterobacteria, some of the $\sigma^5$-independent genes found in our ChIP-seq analysis (e.g., tomb, sdaA, bsmA) appear to be rpoS-dependent in Salmonella Typhimurium36, possibly suggesting more efficient promoter recognition by E$\sigma^5$ in this bacterium.

Promoter cross-recognition with $\sigma^5$ also seems to extend to the alternative factors $\sigma^6$ and $\sigma^{34}$ (Tables 1–2), in line with previous results showing similar functions of the rpoE and rpoS regulons and some promoter overlap between the two $\sigma$ factors in vitro30,34. Indeed, our results confirm a strong interplay between $\sigma^5$ and $\sigma^{34}$, as the rpoH promoter is directly recognized by E$\sigma^5$ (Table 1), in agreement with its rpoS-dependent expression31. Our results would be consistent with recent reports showing co-regulation of the rpoE, rpoH and rpoS regulons in response to osmotic stress in enteropathogenic E. coli O157:H736, and an extensive analysis of the $\sigma$ factor network in E. coli, showing extensive overlap in promoter recognition by alternative $\sigma$'s33.

At least 10 of the rpoS-dependent genes identified in the ChIP-seq experiments encode small proteins involved in resistance to oxidative stress (bsmA, dps, uspB, yaiA, ychH, ydbK, ygcG, yge, yobB and yodD; Tables 1–2), while two more are linked to osmotic stress (osmB and osmE). Our results would support the notion that, rather than being part of an adaptive response triggered by exposure to specific environmental stresses, the rpo$S$ gene activates, in response to redox in vivo, a variety of stress-related genes, thus allowing the bacterial cells to “brace themselves” for any stressful conditions that might arise. However, promoter binding by E$\sigma^5$ does not necessarily translate in increased transcription levels for E$\sigma^5$-dependent genes, suggesting that, upon binding, E$\sigma^5$ might be unable to initiate transcription efficiently at some promoters. For the bsmA promoter, this hypothesis would fit with the results of in vitro promoter interaction studies (Fig. 4) and with our previous results, showing E$\sigma^5$-dependent transcription of the bsmA gene in vitro10, but not in the bacterial cell. Since bsmA is induced in biofilm growth37, it is possible that its transcription is repressed in planktonic cells, and triggered during biofilm growth. Thus, our results suggest that E$\sigma^5$ might be poised at various promoters waiting for additional signals (e.g., leading to removal of a repressor protein) in order to form a complex proficient in transcription initiation.

While stress responses are well known examples of gene functions associated with the rpoS regulon, our results suggest direct involvement of $\sigma^5$ in the expression of genes involved in biogenesis and structure of the LPS and outer membrane proteins (Tables 1–2). Indeed, changes in cell surface structure and composition are known to take place in stationary phase38. According to our ChIP-seq results, in addition to LPS genes, E$\sigma^5$ also binds to the promoter of lpp, encoding Lpp or Braun lipoprotein, which links the outer membrane to peptidoglycan and is the most abundant outer membrane-associated lipoprotein in E. coli21. Although lpp gene expression does not depend on the rpoS gene (Fig. 3), a connection of the rpoS gene with the function of Braun lipoprotein is further suggested by the identification of two more binding sites for E$\sigma^5$ upstream of the erfK and ynhG genes, encoding two of the four alternative transpeptidases that crosslink Lpp to peptidoglycan. Both the erfK and ynhG genes had already been described as rpoS-dependent15,16. Thus, it appears that, upon entry in the stationary phase of growth, rpoS might be required for maintenance of Lpp-transpeptidase activity in the periplasmic space.

Finally, our results point to a direct role of E$\sigma^5$ in the finely tuned regulation of non-coding RNAs: for instance, E$\sigma^5$ promotes transcription of omrA, but not of the flanking gene, omrB (Fig. 5). Both genes encode very similar non-coding RNAs which target the same genes. It appears possible that different dependence on E$\sigma^5$ by the two promoters might have evolved so to allow differential expression of the OmrA and OmrB non-coding RNAs in response to different signals, with OmrA induced as part of the rpoS regulon. The results of mutagenesis at the -12 position of the omrA promoter strongly reinforce the notion that the -12C nucleotide can favourably bias transcription initiation by E$\sigma^5$ at several promoters39. Since both the OmrA and OmrB RNAs affect translation of several outer membrane proteins and extracellular structures such as curli and flagella40, their selective regulation might mediate the impact of E$\sigma^5$ on these structures, contributing to a general reorganization of the bacterial cell surface in response to stationary phase.
Methods
Strain construction. The *E. coli* MG1655 His6-rpoS strain (from now on MG1655-rpoSHis6), carrying an rpoS gene in which a 6-histidine tag is added to an otherwise wild type allele, was constructed following the genetic procedures described for allele replacement\(^4\). Linear DNA fragments containing a kanamycin resistance gene and the ccdB gene under the control of a rhamnose inducible promoter were amplified by PCR from the pKD45 plasmid. The first 45 nucleotides of either primer used for amplification (primers rpoS_OF and rpoS_OR, Supplementary Table S1) correspond to the DNA regions immediately upstream and downstream of rpoS, targeting the gene for mutagenesis. After PCR amplification, the resulting DNA fragment including the kanR-ccdB cassette was used to transform the DY330 strain; the rpoS knockout was then P1-transduced into MG1655, selecting for kanamycin resistance. The ΔrpoS:kanR-ccdB cassette was then replaced by an otherwise wild type rpoS sequence to which an additional sequence coding for a 6-histidine tag (6xHis-tag) had been added by PCR amplification, using the rpoS_IF and rpoS_IR primers (Supplementary Table S1). To this aim, DY330 cells carrying the rpoS knockout were transformed by electroporation with a linear DNA fragment encoding for the rpoSHis6 gene, carrying the His-tag at the 3′ end. Transformant selection was performed on M9 minimal medium agar plates containing 0.2% rhamnose and 0.01% biotin: due to the toxicity of the ccdB gene in the presence of rhamnose, only the cells in which an allele replacement has taken place are able to grow on this medium. The rpoSHis6 allele was P1-transduced into MG1655 carrying the rpoS::kan-ccdB knockout, again selecting for loss of the ccdB gene by plating on M9 minimal medium agar plates containing 0.2% rhamnose and 0.01% biotin. The stability and functionality of the RpoS protein was verified by Western blot and measurement of HPII catalase activity.

σ^5^-His6 immunoprecipitation. For immunoprecipitation of the σ^5^-protein carrying a 6xHis-tag at its C-terminal end (σ^5^-His6), the MG1655-rpoSHis6 strain was grown in 50 ml LB medium at 37 °C with vigorous shaking to an OD\(_{600}\) of 3.0. In order to enrich the amount of RNA polymerase bound to promoters, cells were treated with rifampicin, which inhibits transcription initiation blocking RNA polymerase at transcription start site, following the protocol described\(^43\). To obtain protein-DNA crosslinking, formaldehyde was added at a final concentration of 1% for 5 minutes at room temperature. The crosslinking reaction was stopped by addition of 0.25 M glycerol followed by 20 minute incubation at 4 °C with gentle shaking. The cells were washed, resuspended and treated with 100 μg/ml lysozyme for 30 minutes at 37 °C. The lysate was sonicated in order to fragment chromosomal DNA to a size between 100-400bp, and treated with RNaseI (100 μg/ml) for 15 minutes at 37 °C. Cells debris was removed by centrifugation (10 minutes at 10000xg). A 250 μl-fraction of the sample was treated with 100 μg/ml Proteinase K and 5 mM CaCl\(_2\) for two hours at 42 °C, and then at 65 °C overnight, to remove proteins non specifically bound to DNA. DNA was recovered by phenol-chloroform extraction and analyzed on a 2% agarose gel.

DNA from untreated MG1655-rpoSHis6 was sonicated and 200 μl were taken to be used as a control in sequencing reactions (Input=non-immunoprecipitated DNA). The Input and immunoprecipitated DNA samples were analyzed with the Agilent Bioanalyzer using the High Sensitivity DNA kit (Agilent Technologies). Five IP samples were pooled on the same DNA purification column (minElute, QIAGEN) to reach 5 ng of total DNA, which is the minimum amount for sequencing library preparation. Two pools of IP DNAs were produced. Prior to sequencing libraries construction, quantitative Real Time reverse transcriptase-PCR (qRT-PCR) was carried out to assess the enrichment of the promoter region of the rpoS-dependent dps gene in the immunoprecipitated samples in comparison to the Input sample. The sequences of the primers used for qRT-PCR are listed in Supplementary Table S1.

Library preparation and sequencing procedure. Illumina libraries were prepared either from 5 ng of each of the two pools of immunoprecipitated-DNA (RpoS-IP) or from 5 ng of the two control DNA (Input) following the Illumina TruSeq ChiP-seq DNA sample preparation kit; then each library was sequenced in a lane of a single strand 51 bp Illumina run on a GAIIx sequencer. Raw data are publicly available at Sequence Reads Archive under accession number BioProject SRP041323; BioSample SRSS595203; Experiment SRX523029; Run1 SRR1265068; Run2 SRR1271103.

Statistical and bioinformatic data analysis. Raw reads were mapped against the *Escherichia coli* MG1655 genome using Bowtie\(^45\) with zero mismatches. The resulting BAM files were processed using SAMtools\(^46\) and BEDTools\(^47\). The quality of each sequenced sample was checked using cross-correlation analysis implemented in spp R package\(^48\). ChiP-seq peak calling was performed using CiSgenome\(^12\) by imposing default parameters. Input data (control DNA) was used to model the background noise.

Determination of rpoS-dependent gene expression *in vivo*. For all gene expression experiments, bacterial strains were grown in LB medium to OD\(_{600}\)=3.0. For qRT-PCR, RNA was extracted and
experiments performed as previously described, using 16S RNA as reference. Primers used in qRT-PCR experiments are listed in Supplementary Table S1. For northern blots, total RNA was extracted using a hot-phenol procedure, so to maintain small RNA molecules. 5 to 20 µg of RNA were separated onto a 6% denaturing acrylamide gel prior to their electro-transfer onto a nylon membrane. As gene specific probes, 5'-Biotinylated oligomers (Supplementary Table S1) were used at 1 nM in combination with 20 pM of the 5S RNA probe as internal control. Saturation and hybridization were performed with the ULTRAhyb®-Oligo buffer (Ambion) at 45°C and signals were detected using a Chemi nucleic acid detect module (Thermo Scientific Pierce). GFP reporter assays were performed as previously described.

RNA polymerase in vitro assays. RNA polymerase reconstitution, gel mobility shift and KMnO₄ reactivity assays were performed as previously described. ³²P-labeled DNA was produced by PCR after 5'-phosphorylation of the primer complementary to the coding strand (see Supplementary Table S1) in order to generate linear DNA pieces of about 250 bp, typically encompassing the first 10 codons of the gene and 220 bp of the upstream DNA, including the promoter region. For gel mobility shift assays (GMSA), complexes between reconstituted RNA polymerase (18 to 150 nM) and DNA (1 nM) were allowed to form for 15 min at 37°C in K-glut100 buffer (40 mM HEPES, pH 8.0, 10 mM magnesium chloride, 100 mM potassium glutamate, 4 mM dithiothreitol (DTT), and 500 µg/ml bovine serum albumin), in a final reaction volume of 10 µl. The reaction mixture was loaded onto a 5% native polyacrylamide gel after addition of 2.5 µl of heparin-supplemented loading buffer and gel electrophoresis was carried out in 0.5×TBE buffer at 120 V. Experiments were performed at least twice and gave very similar results.

For KMnO₄ reactivity assays, 50 nM of either form of RNA polymerase (Eσ and Eσ₇₀) were incubated with about 3 nM of labeled promoter DNA for 20 min at 37°C in K-glut100 buffer without DTT for complex formation. KMnO₄ was added to a final concentration of 10 mM and the reaction was stopped after 30 seconds by adding 2 mM DTT. Samples were phenol-extracted and precipitated, treated with 1 mM piperidine, resuspended in pure formamide blue before being loaded onto a 7% polyacrylamide denaturing gel. A DNA ladder was generated for each labeled DNA fragment by partial G/A sequencing using formic acid and piperidine.

Other methods. Determination of HPII catalase activity and Western blot experiments were carried out as previously described. Mutagenesis of the omrA promoter was carried out by generation of PCR products with mutagenic primers carried the desired substitutions, as previously described.

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Author Contributions
The experiments were conceived and designed by C.P., T.E., G.D.B., S.L., P.L. and performed by C.P., J.W., J.D., E.R. and S.L. Data analysis was carried out by E.R., L.P. and J.G. The paper was written by P.L. with contributions from C.P., T.E. and S.L. All authors reviewed the manuscript.

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