In vitro transcription profiling of the σ^S subunit of bacterial RNA polymerase: re-definition of the σ^S regulon and identification of σ^S-specific promoter sequence elements

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ABSTRACT
Specific promoter recognition by bacterial RNA polymerase is mediated by σ subunits, which assemble with RNA polymerase core enzyme (E) during transcription initiation. However, σ^70 (the housekeeping σ subunit) and σ^S (an alternative σ subunit mostly active during slow growth) recognize almost identical promoter sequences, thus raising the question of how promoter selectivity is achieved in the bacterial cell. To identify novel sequence determinants for selective promoter recognition, we performed run-off/microarray (ROMA) experiments with RNA polymerase saturated either with σ^70 (Eσ^70) or with σ^S (Eσ^S) using the whole Escherichia coli genome as DNA template. We found that Eσ^70, in the absence of any additional transcription factor, preferentially transcribes genes associated with fast growth (e.g. ribosomal operons). In contrast, Eσ^S efficiently transcribes genes involved in stress responses, secondary metabolism as well as RNAs from intergenic regions with yet-unknown function. Promoter sequence comparison suggests that, in addition to different conservation of the −35 sequence and of the UP element, selective promoter recognition by either form of RNA polymerase can be affected by the A/T content in the −10/+1 region. Indeed, site-directed mutagenesis experiments confirmed that an A/T bias in the −10/+1 region could improve promoter recognition by Eσ^S.

INTRODUCTION
Bacteria must cope with drastic changes in their environment, such as nutritional up- and downshifts, and variations in pH, osmolarity and temperature. Bacterial cells can quickly adapt to such environmental changes by modulating gene expression, at both transcriptional and post-transcriptional levels. At the transcription initiation level, gene expression can be regulated either through accessory transcription factors (activators and repressors), or via assembly of different forms of RNA polymerase. The latter mechanism of gene regulation involves the assembly of RNA polymerase core enzyme (indicated as E) with one of several σ factors that can direct RNA polymerase to specific promoter sequences (1). Typically, in the bacterial cell, one σ factor is devoted to transcription of a large part of the genome, including the essential cellular functions (housekeeping σ factor), while the so-called ‘alternative σ factors’ direct transcription of smaller sets of genes, often linked to specific functions (e.g. response to cellular stresses).

In Escherichia coli, seven σ factors have been identified: σ^70 or σ^D (the housekeeping σ) and six alternative σ factors: σ^E, σ^F, σ^H, σ^I, σ^S and σ^S (2). Most alternative σ factors recognize promoter sequences that strongly diverge from the consensus sequence for σ^70; in contrast, genes under the control of σ^S are characterized by promoter sequences very similar to σ^70-dependent genes (3,4). In line with this observation, in vitro selection of DNA sequences bound with high affinity by RNA polymerase associated with σ^S (Eσ^S) led to the identification of a consensus sequence very similar to the one recognized by σ^70 (5). Some level of overlapping in promoter recognition

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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by $\sigma^S$ and $\sigma^S$ might be consistent with $\sigma^S$ function: indeed, in conditions leading to slow metabolic activity, such as nutrient starvation or oxidative stress, $\sigma^S$ might take over in transcription of genes important for cell survival that are under $\sigma^{32}$ control during faster growth (6). However, in order to switch from the fully active to the slow metabolic state, specific gene expression, and thus specific recognition of $\sigma^{32}$ versus $\sigma^S$-dependent promoters, must take place in the bacterial cell. Some promoter sequence determinants can favour recognition by either $\sigma^{70}$ or $\sigma^S$ (3): for instance, a C nucleotide upstream of the −10 promoter element (−13C) enhances transcription by $\sigma^S$ (7). However, some sequence features favouring promoter recognition by $\sigma^S$ seem to be dependent on specific promoter contexts: for instance, at the $\sigma^{32}$-dependent aidB promoter, $\sigma^S$, but not $\sigma^{70}$, can recognize with equal efficiency either C or T as the first nucleotide in the −10 promoter element (8). However, the percentage of $\sigma^S$-dependent promoters carrying a −12C element is not significantly higher than in $\sigma^{70}$-dependent promoters (9), suggesting that the presence of a −12C might only contribute to specific promoter recognition by $\sigma^S$ at selected promoters.

In addition to sequence determinants, it has been proposed that transcription factors such as CRP, IHF and Lrp can selectively block (or promote) promoter recognition by either $\sigma^{70}$ or $\sigma^S$ (10). A transcription regulator important for the modulation of promoter accessibility to different RNA polymerase holoenzymes is the H-NS protein, which can repress transcription by $\sigma^{70}$, but not by $\sigma^S$, at various promoters (11), a phenomenon known as transcriptional silencing (12). Specific promoter recognition by $\sigma^S$ is also affected by the degree of DNA supercoiling (13). In addition, $\sigma^S$ activity and intracellular concentrations are affected by various factors, such as the presence of an anti-sigma factor for $\sigma^{70}$ (14), and by the accumulation of the signal molecules ppGpp (15) and polyphosphate (3).

Work aimed to the identification of $\sigma^S$-specific promoter elements has mostly been carried out in vivo, comparing relative gene expression in a wild type versus mutants selective for RNA polymerase and promoters under the indirect control of the $\rho$poS gene. In contrast, dependence on $\sigma^S$, as determined by biochemical experiments with purified RNA polymerase, has only been determined for a limited number of promoters [e.g. fic (21), csiD (22) and aidB (23)]. In this work, we have performed in vitro transcription experiments with either $\sigma^{70}$ or $\sigma^S$, using the whole E. coli genome as template, to identify promoter regions selectively recognized by two forms of RNA polymerase. Our results support previous observations that $\sigma^{70}$- and $\sigma^S$-dependent promoters differ in conservation of the UP element and of the −35 sequence, and in the sequence immediately upstream of the −10 promoter element. In addition, we show that differences in the A/T content in the −10/+1 promoter region can favour transcription by either form of RNA polymerase. Finally, our work has led to the identification of novel $\sigma^S$-dependent genes, thus providing further insight on the physiological role of $\sigma^S$.

**MATERIALS AND METHODS**

**Protein purification and RNA polymerase reconstitution**

*Escherichia coli* RNA polymerase core enzyme was purchased from Epicentre (Madison, WI, USA); histidine-tagged $\sigma$ factors were produced and purified as described (8,24). The $\sigma^S$ protein appeared totally pure from contaminants as determined by denaturing protein gel electrophoresis; in contrast, in the $\sigma^{70}$ preparations the presence of faint additional bands, corresponding to the molecular weight of the core RNA polymerase subunits $\alpha$, $\beta$ and $\beta'$, could be detected (data not shown). Weak contamination of $\sigma^S$ preparations by core RNA polymerase subunits is consistent with the high affinity of $\sigma^{70}$ for the core enzyme. For reconstitution of RNA polymerase holoenzymes, the core enzyme was incubated for 10 min at 37°C with either $\sigma^S$ or $\sigma^{70}$ at a 1:10 ratio. For calculation of RNA polymerase concentrations in transcription assays, it was assumed that, after reconstitution, core enzyme would be 100% active and fully saturated by either $\sigma$ factor.

**In vitro transcription on supercoiled plasmids**

Promoter regions of interest were amplified from the genome of E. coli MG1655 (25) and cloned into the pJCD01 plasmid (22) using the BamHI and EcoRI sites, with the exception of the ilvY promoter, which was cloned using the BamHI and SphI sites, due to the presence of an EcoRI site in the ilvY promoter region. Single-round *in vitro* transcription experiments were carried out on supercoiled templates (3 nM) in the presence of 10 nM reconstituted RNA polymerase holoenzyme. Plasmid DNA and reconstituted holoenzyme were incubated for 10 min at 37°C in 18 μl of transcription buffer (40 mM HEPES pH 8.0, 10 mM magnesium chloride, 150 mM potassium glutamate, 2 mM dithiothreitol, 100 μg/ml bovine serum albumin) prior to the addition of 2 μl of a mixture of ribonucleotide triphosphates and heparin to a final concentration of 300 μM each for ATP, CTP, GTP and UTP and 250 μg/ml heparin. Transcription reactions were allowed to proceed for 10 min at 37°C and were stopped by addition of NaCl to a final concentration of 0.5 M followed by incubation at 70°C for 5 min. Samples were extracted with a 1:1 phenol–chloroform mixture, precipitated with ethanol and resuspended in 12 μl TE buffer. A volume of 2.5 μl of resuspended samples were treated with DNaseI (1 U in 10 mM Tris–HCl pH 7.6, 2.5 mM MgCl2, 0.5 mM CaCl2, in a final volume of 20 μl) for 1 h at 37°C, and DNaseI was heat-inactivated at 65°C for 10 min. Transcript amounts were determined by quantitative real-time polymerase chain reaction (PCR): 1 μl of DNaseI-treated samples was retrotranscribed and the cDNAs were amplified in quantitative real-time PCR using the RNA-I as
in vitro transcription reactions performed using RNA polymerase holoenzyme with promoter sequences. Ten independent run-off transcription assays with either Es7 or Es5 were performed, and transcripts were pooled together (~1 μg total RNA). From the pool of the 10 independent-round transcription reactions two distinct hybridizations on microarrays were performed.

For hybridization onto microarrays, we used the Affymetrix GeneChip E. coli Genome 2.0 array, which includes 10 144 probe sets covering all the predicted transcripts from four strains of E. coli: the laboratory strain MG1655, the uropathogenic CFT073 and the enteropathogenic strain OH157:N7, subtypes EDL933 and SAKAI. Due to the high degree of similarity between the E. coli strains, typically a single probe set is tiled to represent the equivalent orthologue in all four strains, and strain-specific probes are only used for genes displaying low levels of conservation, or present solely in one strain. Probe sets match every open reading frame (ORF) in E. coli; in addition, 1427 probe sets targeting 714 E. coli MG1655 intergenic regions, probes for various antibiotic resistance markers, and additional control and reporter genes from the previous generation E. coli arrays are also represented in the Affymetrix GeneChip E. coli Genome 2.0 array.

The RNA samples were processed for microarray hybridization, following the instructions of the GeneChip® Expression Analysis Technical Manual (Chapter 5, 6 and 7, Prokaryotic target Preparation, Hybridization, Washing, Staining and Scanning), except that cDNA synthesis by reverse transcription was performed with 1 μg RNA. cDNA was fragmented with DNase I treatment and labelled with biotin using Terminal Deoxynucleotidyl Transferase. The fragmented and labelled cDNAs were then hybridized for 16 h at 45°C on individual E. coli Genome 2.0 arrays. After hybridization, GeneChips were washed and stained with streptavidin-conjugated phycoerythrin by using the Fluidic Station FS450 (Affymetrix) according to the FS450_0006 Protocol. Fluorescent images of the arrays were acquired using a GeneChip Scanner 3000 7G (Affymetrix). All Chip images and files have been deposited in the GEO (Gene Expression Omnibus) (http://www.ncbi.nlm.nih.gov/geo/) repository (accession number:GSE22207). After quality control of data distribution, the raw data (CEL files) were used to perform normalization and probe set summarization through Robust Multiarray Analysis (RMA) algorithm (by using the Affymetrix Gene Expression Console Software (www.affymetrix.com). Normalized data were also analyzed with OneChannelGUI Software (http://www.bioinformatica.unito.it/oneChannelGUI/) in order to perform an additional expression analysis based on different parameters. OneChannelGUI is an add-on Bioconductor package extending the capability of the affylmGUI package (28); it is a library providing a graphical interface (GUI) for Bioconductor libraries to be used for the complete single-channel microarray analysis.
To perform statistic analysis on the two hybridizations, we decided to apply a simple non-parametric statistical method based on ranks of fold changes to perform a two-class paired differential analysis. To select genes with significantly different transcription levels, we set the following parameters for the Rank Product analysis: 100 permutations and 0.1 cut-off percentage of false positives (pfp) which corresponds to a \( P\)-value < 0.01. Fold differences higher than 1.5-fold were considered indicative of significantly different expression, similar to previous ROMA experiments (29): genes more efficiently transcribed by either \( \sigma^S \) or \( \sigma^{70} \) are listed in Tables 1 and 2, respectively. Determination of start sites on transcripts generated in the \textit{in vitro} transcription assays was carried out by RACE analysis (26).

In order to manage and retrieve microarray data, a genome browser was set up (http://155.253.6.64/cgi-bin/gbrowse/provakappa/#search). The genome browser is based on the Generic Genome Browser (Gbrowse) which is a combination of database and interactive webpage for manipulating and displaying annotations on genomes. This bioinformatic tool allows users to view and navigate through the MG1655 genome (GenBank Accession Number:U00096) with information about the gene annotation, coming from the website http://www-genome.wisc.edu/tools/asap.htm (downloaded as GFF3 file), and all the probe sets contained in the Affymetrix \textit{E. coli} Genome 2.0 Array manually remapped on the genome.

**Determination of \( \textit{rpoS} \)-dependent gene expression in bacterial cells**

Bacterial strains used were MG1655 (wild type) and its \( \textit{rpoS} \) mutant derivative EB1.3 (30). Strains were grown at 30°C in three different media: the complex Luria Bertani (LB) broth, the glucose-based M9Glu/sup medium (31) and LB medium diluted 1:4 (LB1/4). The LB1/4 medium was utilized since it was shown to stimulate expression of \( \textit{rpoS} \)-dependent genes such as the csg operons (32). Thus, growth in LB1/4 medium might positively affect either \( \sigma^S \) concentration or \( \sigma^{70} \) activity. Samples for RNA extraction were taken both in late logarithmic (OD\textsubscript{600} = 0.6–0.7) and in late stationary phase (overnight cultures, OD\textsubscript{600} 1.5). RNA was extracted using the small RNA miRNaseasy Mini Kit (Qiagen), and further reverse transcription and cDNA amplification in quantitative real-time-PCR were performed as described (33). Primer sequences are available upon request. All reactions were performed twice, each time in duplicate, and always showed very similar results. The relative amounts of the transcripts were determined using 16S RNA as the reference gene \([Ct\text{Gene of interest} – Ct\text{16S}] = \Delta Ct\).

**RESULTS**

**ROMA analysis of \( \sigma^S \)- and \( \sigma^{70} \)-dependent promoters**

To identify sequence determinants that can direct selective promoter recognition by either the \( \sigma^{70} \), or the \( \sigma^S \)-associated form of RNA polymerase, we compared \( \text{E}r^S \) and \( \text{E}r^{70} \) in ROMA experiments (29,34). Run-off transcription assays were performed using as DNA template the whole genome of \textit{E. coli} MG1655 after digestion with EcoRI; cDNAs generated from the transcripts were hybridized on microarrays to determine their relative amounts. Unlike genome expression studies performed in living cells, which do not distinguish between direct and indirect effects, such as transcription factor-dependent promoter recognition, the ROMA analysis solely detects genes whose promoter are recognized by either \( \text{E}r^S \) or \( \text{E}r^{70} \) (or both) in the absence of any additional factor. Out of \( \sim 10000 \) probe sets on the microarray, only 173 (~1.7%) showed differences in transcription levels higher than 1.5-fold, considered to be significant (pfp < 0.1, \( P\)-value < 0.01; see ‘Materials and Methods’ section). For several operons, only a portion of the transcriptional unit (or even a single gene) showed significant difference in transcription levels by either \( \text{E}r^S \) or \( \text{E}r^{70} \), in the case of reduced transcription of distal genes within a given operon, this effect might depend either on premature arrest of transcription by RNA polymerase or on the presence of an EcoRI restriction site within the operon, since the EcoRI restriction nuclease was used to digest chromosomal DNA used in ROMA experiments (see ‘Materials and Methods’ section). However, the effects of EcoRI digestion were not so severe as it could be expected, possibly due to the fact that chromosomal DNA was only partially digested by the enzyme in our conditions. For instance, despite the presence of an EcoRI site in the \textit{ydhY} gene, transcription extending into the downstream genes was still detectable (Table 1 and Supplementary Table S1). In the case of distal genes of an operon showing more significant differences in \( \text{E}r^S \)- versus \( \text{E}r^{70} \)-dependent transcription than genes proximal to the promoter, this might be due to either lack of detection of proximal genes in the microarray experiment or the presence of unidentified promoters internal to the operons. We verified that genes within the same operon would not show dependence on different forms of RNA polymerase: out of the several hundred operons in the \textit{E. coli} genome, only in the weakly \( \text{E}r^{70} \)-dependent atpBEFHAGDC and flgBCDEFGHIJ operons could we observe the presence of a single gene showing dependence on \( \text{E}r^S \) in our ROMA experiments (data not shown). This result might suggest the presence of \( \text{E}r^S \)-dependent promoters, or promoter-like sequences, within these operons; however, we decided to focus our investigation on operons consistently showing preferential recognition by either form of RNA polymerase, and the atpBEFHAGDC and flgBCDEFGHIJ operons were not considered further in our study. In Supplementary Table S1, we show the ratios of \( \text{E}r^S \)- versus \( \text{E}r^{70} \)-dependent transcription for operons featuring genes preferentially transcribed in the presence of \( \text{E}r^S \).

Complete results of ROMA experiments are summarized in Tables 1 and 2, listing genes and intergenic regions transcribed more efficiently either by \( \text{E}r^S \) (Table 1) or by \( \text{E}r^{70} \) (Table 2). Out of the 54 genes preferentially transcribed by \( \text{E}r^S \) in ROMA experiments that are annotated in \textit{E. coli} MG1655, 21 (39%) had already been described as \( \textit{rpoS} \)-dependent genes, either from genetic characterization or from microarray experiments comparing wild-type and \( \textit{rpoS} \) mutant strains (underlined in Table 1). In
Table 1. Genes transcribed more efficiently in the presence of EσS

| Genes and promoter regionsa | Gene productb | b numberc | Transcription levels (EσS/EσS ratio)d | Known regulatory factorsa |
|-----------------------------|---------------|-----------|--------------------------------------|---------------------------|
| **Stress response**         |               |           |                                      |                           |
| yieE                        | Chromate reductase | b3713     | 1.50                                 | σS (59); σS (19)          |
| iG1341353_1341620-r         | Intergenic region including omxB promoter | N.A.      | 1.51                                 | σS, ResBA (60)            |
| osmB                        | Osmolarity-inducible lipoprotein | b1283     | 1.51                                 | σS, ResBA (60)            |
| gskA                        | γ-glutamate–cysteine ligase (glutathione biosynthesis) | b2688     | 2.21                                 |                           |
| ahpF                        | Alkyl-peroxidase reductase | b0606     | 1.52                                 | OxyR (61)                 |
| pphA                        | Protein phosphatase | b1838     | 1.54                                 | σH (62,63)                |
| macA                        | Macrolide resistance efflux pump | b0878     | 1.54                                 |                           |
| **DNA and RNA metabolism and modification** |               |           |                                      |                           |
| IG252899_2523146-r         | Intergenic region including xapA promoter | N.A.      | 1.62                                 |                           |
| xapA                        | Xanthosine phosphorylase, nucleotide synthesis/degradation | b2407     | 1.56                                 |                           |
| gyrB                        | B subunit of DNA gyrase | b3699     | 1.65                                 | Fis (65)                  |
| recT                        | Rac prophase, recombinase, in recET-lar-ydaCQ operon | b1349     | 1.93                                 | σS-Dependent in biofilm-growing cells (18) |
| lar                         | Rac prophase, restriction alleviation and modification enhancement, in recET-lar-ydaCQ operon | b1348     | 1.93                                 |                           |
| ydaC                        | Rac prophase, in recET-lar-ydaCQ operon | b1347     | 1.74                                 | as recT                   |
| ydaQ                        | Rac prophase, possible recombinase, in recET-lar-ydaCQ operon | b1346     | 1.66                                 | as recT                   |
| **Polyamine metabolism**    |               |           |                                      |                           |
| puuB                        | γ-glutamyl–putrescine oxidase | b1301     | 1.61                                 | σS (19)                   |
| gabD                        | Succinate semialdehyde dehydrogenase | b2661     | 1.65                                 | σS, Nac (9,66)            |
| speB                        | Agmatinase (in arginine/putrescine degradation pathway) | b2937     | 1.66                                 |                           |
| **Transcription regulation**|               |           |                                      |                           |
| IG582284_582903-f           | Intergenic region including appY promoter | N.A.      | 1.69                                 | H-NS (67)                 |
| appY                        | Regulator of hyaABCDEF operon | b0564     | 1.51                                 | H-NS (67)                 |
| yciT                        | Putative deoR-type transcription regulator | b1284     | 1.56                                 |                           |
| ituY                        | Regulator of ifc operon | b3773     | 1.75                                 |                           |
| rhaS                        | Regulator of rhamnose transport | b3905     | 1.81                                 | CRP (29); RhaS (68)       |
| **Sugar metabolism**        |               |           |                                      |                           |
| treF                        | Trehalase | b3519     | 1.53                                 | σS (9,19)                 |
| ygfT                        | Putative galactose ABC transporter | b4230     | 1.55                                 | σS (19)                   |
| atsB                        | Trehalose-6-phosphate phosphatase | b1897     | 1.56                                 | σS (7)                    |
| araF                        | Arabinose transporter, component of an ABC transport system | b1901     | 1.57                                 | CRP, AraC (69); σS (19)   |
| glgP                        | Glycogen phosphorylase, part of glgCAP operon | b3428     | 1.60                                 | CRP (70); σS controls glgCAP operon (19) |
| gpmM                        | Putative 2,3-bisphosphoglycerate mutase | b3612     | 1.61                                 |                           |
| glvC                        | Sugar transport phosphotransferase | b3683     | 1.79                                 |                           |
| **Other metabolic functions** |               |           |                                      |                           |
| IG1030936_1031361-f         | Intergenic region including hyaABCDEF promoter | N.A.      | 2.02                                 |                           |
| hyaA                        | Hydrogenase small subunit; in hyaABCDEF operon | b0972     | 1.68                                 | σS, AppY, anaerobic regulation by ArcA and NarP; NarL (20,71,72) |
| hyaB                        | Hydrogenase large subunit; in hyaABCDEF operon | b0973     | 1.66                                 | as hyaA                   |
| hyaF                        | Hydrogenase subunit (nickel-binding protein); in hyaABCDEF operon | b0977     | 1.50                                 | as hyaA                   |
| syd                         | SecY-interacting protein | b2793     | 1.59                                 |                           |
| murP                        | Acetyl-muramic acid permease | b2429     | 1.59                                 | CRP, MurR (73)            |
| murR                        | Transcriptional repressor of murQP operon | b2427     | 1.62                                 |                           |
| tam                         | Trans-acetoinate methyltransferase | b1519     | 1.60                                 | σS (74)                   |
| ydhY                        | Predicted oxidoreductase, Fe-S protein; in ydhYFWXUT operon | b1674     | 1.80                                 | FNR, NarL (75)            |
| ydhV                        | Predicted oxidoreductase, Fe-S protein; in ydhYFWXUT operon | b1673     | 1.62                                 | FNR, NarL (75). σS-dependent in biofilm-growing cells (18) |

(continued)
### Table 1. Continued

| Genes and promoter regions<sup>a</sup> | Gene product<sup>b</sup> | b number<sup>c</sup> | Transcription levels (Es/E<sub>S</sub>/Es<sub>70</sub> ratio)<sup>d</sup> | Known regulatory factors<sup>e</sup> |
|-------------------------------------|--------------------------|---------------------|-------------------------------------------------|----------------------------------|
| **Unknown and miscellaneous functions** |                          |                     |                                                 |                                  |
| yceT                               | Unknown                  | b0964               | 1.51                                            | Induced in stationary phase (76) |
| yffP                               | Predicted protein, prophage | b2447               | 1.52                                            |                                  |
| yidK                               | Putative membrane transporter | b3679               | 1.53                                            |                                  |
| ynfD                               | Predicted protein         | b1586               | 1.54                                            |                                  |
| yflL                               | Putative lipoprotein      | b2602               | 1.55                                            |                                  |
| yflNa                              | Unknown, in CP4-6 prophage sequence | b0255               | 1.56                                            |                                  |
| yflL                               | Unknown, possible prophage gene | b2625               | 1.57                                            | σ<sup>5</sup> (9)                |
| yagL                               | Unknown, prophage protein | b0278               | 1.59                                            | σ<sup>5</sup>-dependent in biofilm-growing cells (18) |
| eutA                               | Reactivating factor for ethanolamine ammonia lyase | b2451               | 1.59                                            | eutH, in eutHA operon, is σ<sup>e</sup>-dependent (9) |
| yhfG                               | Predicted outer membrane protein | b3524               | 1.61                                            |                                  |
| ybeH                               | Hypothetical protein      | b0625               | 1.66                                            |                                  |
| yedS                               | Unknown                   | b1964               | 1.69                                            |                                  |
| yfgJ                               | Unknown, mutant affecting swarming motility | b2510               | 1.74                                            |                                  |
| G7353                              | Phantom gene              | b2596               | 1.79                                            | Upstream of a ribosome modulation factor induced in stationary phase (yfjA) |
| yflG                               | Unknown, interrupted by IS element | b3046               | 1.81                                            |                                  |
| IG1006824_1007066-r                | Intergenic region including ycbX promoter | N.A.               | 2.26                                            |                                  |
| ycbX                               | Unknown                   | b0947               | 2.05                                            |                                  |
| yflH                               | Unknown                   | b0974               | 2.01                                            |                                  |
| IG2755422_2755664-r                | Intergenic region downstream of yflH | N.A.               | 2.01                                            |                                  |
| ycbS                               | Unknown                   | b1228               | 3.21                                            |                                  |
| **Non-coding RNAs**                |                          |                     |                                                 |                                  |
| ryeE (cyaR)                        | Small RNA, promotes degradation of ompX and nadE RNA | b4438               | 1.50                                            | CRP, σ<sup>5</sup> (77,78)      |
| sgrS/sgrT                          | Small RNA, inhibits ptsG translation/SgrT protein | b4577               | 1.51                                            | SgrR protein                     |
| ECs3934                            | sibD/sibE non coding RNA-ibsD/ibsE toxic peptides | b4447               | 1.62                                            | Complex locus including two non coding RNAs overlapped by two small ORFs encoding a putative toxin/antitoxin system |
| micA                               | micA small RNA (downregulates ompA expression) | b4442               | 2.00                                            |                                  |
| **Intergenic regions, genes not annotated in MG1655** |                          |                     |                                                 |                                  |
| e3878                              | Unknown, annotated in CFT073 | N.A.               | 1.50                                            |                                  |
| IG2922538_2922756-f                | Intergenic region between yidSl (predicted protein) and crsB (ncRNA), antisense to malM | N.A.               | 1.50                                            |                                  |
| e5008                              | Unknown, annotated in CFT073, downstream of malM | N.A.               | 1.50                                            |                                  |
| IG2438141_2438404-f                | Between fabB and ycfJ, antisense to malM | N.A.               | 1.50                                            |                                  |
| EGS5375                            | Unknown, annotated in O157:H7 SAKAI | N.A.               | 1.50                                            |                                  |
| IG2885243_2885600-r                | Intergenic region, upstream of predicted helicase ygcB | N.A.               | 1.51                                            |                                  |
| e4656                              | Unknown, annotated in CFT073 (antisense of atpC) | N.A.               | 1.52                                            |                                  |
| IG3665211_3665420-f                | Intergenic region upstream of gadA, antisense to malM | N.A.               | 1.52                                            |                                  |
| e110                               | Unknown, annotated in CFT073, upstream of aqpZ | N.A.               | 1.52                                            |                                  |
| e0723                              | Unknown, annotated in CFT073, upstream of aqpZ | N.A.               | 1.52                                            |                                  |
| IG2481360_2481774-r                | Intergenic region upstream of emrK operon (transcribed in opposite direction) | N.A.               | 1.54                                            |                                  |
| IG2228406_2228643-f                | Intergenic region upstream of yohJK (inner membrane proteins) | N.A.               | 1.54                                            |                                  |
| IG2201932_2202549-r                | Intergenic region between yhel-yehK, antisense to malM | N.A.               | 1.54                                            |                                  |
| e2806                              | Unknown, annotated in CFT073 (antisense of menB) | N.A.               | 1.55                                            |                                  |
| e3010                              | Unknown, annotated in CFT073, upstream of perM gene, transcribed in opposite direction | N.A.               | 1.55                                            |                                  |
| IG3669525_3669971-r                | Intergenic region, upstream of yhfB | N.A.               | 1.58                                            |                                  |
| IG2898371_2898613-f                | Intergenic region upstream of yaeE | N.A.               | 1.60                                            |                                  |
| e5221                              | Unknown, annotated in CFT073 (antisense of aspA) | N.A.               | 1.63                                            |                                  |
| IG223409_223770-r                  | Intergenic region upstream of rrsH ribosomal operon (transcribed in opposite direction) | N.A.               | 1.64                                            |                                  |

<sup>a</sup> Genes and promoter regions associated with unannotated regions on the E. coli chromosome.

<sup>b</sup> Gene product, abbreviated.

<sup>c</sup> b number, accession number.

<sup>d</sup> Transcription levels (Es/E<sub>S</sub>/Es<sub>70</sub> ratio).

<sup>e</sup> Known regulatory factors.
Table 1. Continued

| Genes and promoter regions | Gene product | b number | Transcription levels (EσS/Eσ70 ratio) | Known regulatory factors |
|---------------------------|-------------|----------|--------------------------------------|-------------------------|
| IG3420831_3421058-r       | Intergenic region, downstream of rff ribosomal operon | N.A.     | 1.66                                  |                         |
| e492                      | Unknown, annotated in CFT073 (antisense of rplL) | N.A.     | 1.67                                  |                         |
| IG2428784_2429041-f       | Intergenic region, upstream of cypA gene, transcribed in opposite direction | N.A.     | 1.71                                  |                         |
| c1908                     | Unknown, annotated in CFT073 (antisense of yddM) | N.A.     | 1.71                                  |                         |
| c0703                     | Unknown, annotated in CFT073 (antisense of citF) | N.A.     | 1.74                                  |                         |
| ECs5165                   | yfjO (biomembrane-related protein) in O157:H7 SAKAI | N.A.     | 1.86                                  |                         |
| IG1903284_1903567-r       | Intergenic region downstream of yobD, transcribed in opposite direction | N.A.     | 1.87                                  |                         |
| IG3538642_3358810-f       | Intergenic region downstream of gldD | N.A.     | 1.89                                  |                         |
| IG2190243_2190534-r       | Intergenic region downstream of yehE | N.A.     | 1.94                                  |                         |
| c3113                     | Unknown, annotated in CFT073 (upstream of rrsg ribosomal operon, transcribed in opposite direction) | N.A.     | 1.95                                  |                         |
| c2317                     | Unknown, annotated in CFT073 (antisense of azuC) | N.A.     | 1.99                                  | Complex locus including small RNA tarB |
| IG2755422_2755664-r       | Intergenic region downstream yfjH | N.A.     | 2.01                                  |                         |
| IG2519349_2519612-f       | Intergenic region downstream of xapR | N.A.     | 2.05                                  |                         |
| IG330721_331594-r         | Intergenic region upstream of yabA, transcribed in opposite direction | N.A.     | 2.07                                  |                         |

aFor known genes, we used the nomenclature reported in the NCBI database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi); intergenic region are indicated using the nomenclature by Affymetrix, which indicates the start and the end of the intergenic region (IG) covered by the various probe sets; -f or -r indicate if the probe sets are in forward or reverse orientation relative to the (+) strand of the E. coli chromosome; genes already described as rpoS-dependent in vivo are underlined.

bGene product (or predicted product).

cRelative location of the various ORFs on the E. coli MG1655 chromosome.

dDetermined by microarray analysis as described in ‘Materials and Methods’ section.

eWhen not otherwise stated, the information is taken from http://ecocyc.org/.

In contrast, no known rpoS-dependent gene was found to be preferentially transcribed by Er70 (Table 2). However, several genes clearly identified by previous works as rpoS-dependent (e.g. katE, dps and gadA) only showed slight (<1.5-fold) preferential recognition by Er70 in our experimental conditions (Supplementary Table S2). This would suggest that, for these rpoS-dependent genes, promoter recognition by Er70 might be mediated by regulatory proteins, or facilitated by additional factors such as DNA supercoiling or effector molecules (e.g. ppGpp), missing in ROMA experiments. Alternatively, in ROMA experiments, Er70 might recognize promoters which might not be accessible to this form of RNA polymerase in vivo, due to selective negative recognition by regulatory proteins such as H-NS (11,12).

A significant fraction of genes more efficiently transcribed by Er70 in vivo -dependent transcript correspond to non-coding RNAs, intergenic regions and ORFs of unknown function only annotated in pathogenic E. coli strains. This last result was surprising, since the DNA template used in the in vitro transcription experiments came from E. coli MG1655; however, probe set analysis and sequence comparison performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that these ORFs are also present in MG1655, although they are not annotated in this strain. Several of these unlisted ORFs overlap known genes in an antisense direction, as indicated in Table 1. Interestingly, one of such ORFs (c3113, annotated in the uropathogenic strain CFT073), as well as one intergenic region (IG223409_223770-r), is located in the promoter regions of ribosomal operons rrsg and rrsH in an antisense direction, possibly suggesting that they may play a role in Er70-dependent control of ribosomal operon transcription.

In contrast, Er70 appeared to transcribe with higher-efficiency genes encoding ribosomal proteins and other protein synthesis-related genes, such as rRNA- and tRNA-encoding genes and prefB, encoding for release factor 2 (Table 2). Several of the protein synthesis-related genes listed in Table 2 are essential, as are the yjeE and the amiB genes, part of a multifunctional operon also preferentially transcribed by Er70. Interestingly, regulatory genes directly affecting σ70 activity, such as relA, responsible for biosynthesis of the ppGpp alarmone in response to amino acid starvation (35), and ssrS, encoding a 6S
Table 2. Genes transcribed more efficiently in the presence of Es70

| Genes and promoter regionsa | Gene productb | b numberc | Transcription levels (Es70/Es5 ratio)d | Known regulatory factors e |
|----------------------------|---------------|-----------|----------------------------------------|--------------------------|
| **Ribosomal genes and protein synthesis** | | | | |
| IG1286552_1286760-r | Intergenic region including tyrT promoter | N.A. | 2.97 | FIS (80) |
| rttR | Non-coding RNA, part of tyrT transcript | b4425 | 1.97 | FIS (80) |
| tpr | Small protamine-like protein part of tyrT transcript | b1229 | 2.03 | |
| serT | Serine tRNAs gene | b0971 | 1.89 | FIS (79); upregulated in rpoS mutant of MG1655 (17) |
| rpmI | Ribosomal protein L35 | b1717 | 1.91 | |
| pefB | Release Factor RF2 | b2891 | 1.91 | Upregulated in a biofilm-growing rpoS mutant derivative of MG1655 (18) |
| vrnP | Ribosomal maturation protein | b3170 | 2.01 | |
| rpsF | Ribosomal protein S6 | b4200 | 2.04 | |
| rpmG | Ribosomal protein L33 | b3636 | 2.10 | |
| relA | ppGpp alarmone biosynthetic enzyme | b2784 | 2.35 | |
| rplU | Ribosomal protein L21, in rplU-rpmA operon | b3186 | 2.50 | |
| rpmA | Ribosomal protein L27, in rplU-rpmA operon | b3185 | 2.58 | |
| rpsU | Ribosomal protein S21 | b3065 | 2.78 | Upregulated in a biofilm-growing rpoS mutant derivative of MG1655 (18) |
| IG3426400_3426656-r | Intergenic region including rrnH promoter | N.A. | 2.87 | |
| sulA | SOS response inhibitor of cell division | b0958 | 2.04 | LexA (81) |
| dinI | AP endonuclease, SOS response | b1061 | 2.18 | LexA (82); upregulated in an rpoS mutant derivative of OH157:H7 EDL 933 (19) |
| **Multifunctional operons** | | | | |
| cvpA | Colicin V production; in cvpA-purF-ubiX operon | b2313 | 1.85 | PurR (83); upregulated in an rpoS mutant derivative of OH157:H7 EDL 933 (19) as cvpA |
| purF | Amidophosphoribosyl transferase (ribonucleotide metabolism); in cvpA-purF-ubiX operon | b2312 | 2.12 | |
| ubiX | 3-octaprenyl-4-hydroxybenzoate decarboxylase (ubiquinone biosynthesis); in cvpA-purF-ubiX operon | b2311 | 1.90 | as cvpA |
| c2854 | Unknown, annotated in CFT073 as part of the cvpA-purF-ubiX operon | N.A. | 2.00 | |
| yjeF | Putative carbohydrate kinase; in yjeFE-amiB-mutL operon | b4167 | 1.88 | |
| yjeE | Essential protein with weak ATPase activity; in yjeFE-amiB-mutL operon | b4168 | 1.95 | |
| amiB | N-acetylmuramyl-l-alanine amidase needed for septum formation during cell division; in yjeFE-amiB-mutL operon. | b4169 | 2.02 | |
| mutL | Methyl-directed mismatch repair, subunit in MutHLS complex; in yjeFE-amiB-mutL operon. | b4170 | 2.06 | |
| yhbE | Inner membrane protein; in yhbE-objE operon | b3184 | 2.36 | |
| objE | GTP-binding protein, involved in ppGpp turnover; in yhbE-objE operon. | b3183 | 1.91 | |
| **Transcription regulation** | | | | |
| IG3717398_3717677-f | Intergenic region including cspA promoter | N.A. | 1.85 | Upregulated in an rpoS mutant derivative of OH157:H7 EDL 933 (19) |
| cspA | Cold shock protein A | b3556 | 2.34 | |
| alpA | CP4-57 prophage gene, regulator of tmRNAs | b2624 | 2.31 | |
| **Metabolic functions** | | | | |
| fhaF | Iron reductase | b4367 | 1.83 | Fur, OxyR (84) |
| ydhR | Putative monoxygenase | b1667 | 1.91 | |
| **Unknown and miscellaneous functions** | | | | |
| ycgY | Unknown | b1196 | 1.83 | |

(continued)
RNA able to modulate Eσ\textsuperscript{70}-dependent at several promoters (36), showed dependence on Eσ\textsuperscript{70} in ROMA experiments (Table 2). For ssrS, our data confirm literature data showing that the main ssrS promoter (ssrS P1) is strictly Eσ\textsuperscript{70}-dependent in vitro (37). Interestingly, several genes preferentially transcribed by Eσ\textsuperscript{70} in ROMA experiments are upregulated in rpoS mutant strains (underlined in Table 2); it has been proposed that negative regulation by σ\textsuperscript{S} can depend on competition with σ\textsuperscript{70} for a limiting amount of RNA polymerase core enzyme, which results in lower intracellular Eσ\textsuperscript{70} concentrations and, in turn, in impaired transcription initiation at strictly σ\textsuperscript{70}-dependent promoters (38).

### Table 2. Continued

| Genes and promoter regions\textsuperscript{a} | Gene product\textsuperscript{b} | b number\textsuperscript{c} | Transcription levels (Eσ\textsuperscript{70}/Eσ\textsuperscript{S} ratio)\textsuperscript{d} | Known regulatory factors\textsuperscript{e} |
|-----------------------------------------------|---------------------------------|-------------------------------|-----------------------------------------------|-----------------------------------------------|
| yefM                                          | Antitoxin in yefM-yoeB toxin-antitoxin system | b2017 | 1.85 |                      |
| yehL                                          | Unknown, possible component of ABC transport system | b2119 | 1.92 |                      |
| yehN                                          | Unknown, putative membrane protein | b1821 | 2.06 |                      |
| ydiE                                          | Putative lipoprotein | b1705 | 2.10 |                      |
| **Non-coding RNAs**                           |                                  |     |     |                      |
| isrB                                          | Small RNA                        | b4434 | 2.25 | Upregulated in a rpoS mutant derivative of MG1655 (20) |
| sspf                                          | Small RNA, regulates DNA polymerase I activity | b3864 | 2.72 |                      |
| ssrS                                          | 6S RNA, modulates Eσ\textsuperscript{70} activity | b2911 | 3.74 |                      |
| **Intergenic regions, genes not annotated in MG1655** |                              |     |     |                      |
| c1714                                         | Unknown, annotated in CFT073, upstream of ets, transcribed in opposite direction | N.A. | 1.83 |                      |
| IG330721_331594-f                             | Intergenic region including yehA promoter region | N.A. | 1.85 |                      |
| EC41613                                        | Unknown, annotated in O157:H7 SAKAI, possible prophage gene (renD) | N.A. | 1.86 |                      |
| IG2428909_2425028-r                           | Intergenic region between argT and hisJ | N.A. | 1.92 |                      |
| IG1120179_1120464-r                           | Intergenic region upstream bssS (regulator of biofilm formation) | N.A. | 1.92 |                      |
| c2481                                         | Unknown, annotated in CFT073, upstream of cobU | N.A. | 1.94 |                      |
| IG127588_127911-f                             | Intergenic region upstream of lpd | N.A. | 1.96 |                      |
| IG2404662_2405580-r                           | Intergenic region upstream of lerA | N.A. | 1.98 |                      |
| Z0043                                         | Annotated in O157:H7 OH157:H7 EDL 933, caiC | N.A. | 2.05 |                      |
| c2568                                         | Annotated in CFT073, wcaM | N.A. | 2.13 |                      |
| Z5055                                         | Annotated in O157:H7 OH157:H7 EDL 933, rfaG | N.A. | 2.16 |                      |
| c4052                                         | Unknown, annotated in CFT073 | N.A. | 2.27 |                      |
| IG2815526_2815805-5                           | Intergenic region upstream yqbaA | N.A. | 2.31 |                      |
| IG583654_583902-r                             | Intergenic region downstream of ompT | N.A. | 2.35 |                      |
| c2230                                         | Unknown, annotated in CFT073 (antisense of cspC) | N.A. | 2.42 |                      |
| IG1905616_1906284-f                           | Intergenic region, between yebF and yebO | N.A. | 2.44 |                      |
| Z3239                                         | Annotated in O157:H7 OH157:H7 EDL 933 (antisense of yegI) | N.A. | 2.49 |                      |
| c4719                                         | Annotated in CFT073 homologous to askA | N.A. | 2.54 |                      |
| e4352                                         | Annotated in CFT073 as dppC | N.A. | 2.99 |                      |
| IG2428784_2429041-f                           | Intergenic region, between yohD and yehN | N.A. | 3.10 |                      |
| c1434                                         | Unknown, annotated in CFT073, ydhR | N.A. | 3.45 |                      |
| Z5945                                         | Annotated in O157:H7 OH157:H7 EDL 933 (toxic peptide) | N.A. | 4.02 |                      |
| Z5868                                         | Annotated in O157:H7 OH157:H7 EDL 933; yfpm. putative acetyltransferase | N.A. | 4.44 |                      |

\textsuperscript{a}For known genes, we used the nomenclature reported in the NCBI database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi); intergenic region are indicated using the nomenclature by Affymetrix, which indicates the start and the end of the intergenic region (IG) covered by the various probe sets; \textsuperscript{b} For or \textsuperscript{r} indicate if the probe sets are in forward or reverse orientation relative to the (+) strand of the E. coli chromosome; genes previously described as being upregulated in an rpoS mutant strain are double underlined.

\textsuperscript{b}Gene product (or predicted product).

\textsuperscript{c}Relative location of the various ORFs on the E. coli MG1655 chromosome.

\textsuperscript{d}Determined by microarray analysis as described in ‘Materials and Methods’ section.

\textsuperscript{e}When not otherwise stated, the information is taken from http://ecocyc.org/.
transcribed by $\sigma^S$, comparing their transcript levels in MG1655 and in its $rpoS$ mutant derivative EB1.3. Transcript levels were determined by quantitative real-time PCR. Different genetic backgrounds (MG1655, MC4100 and OH157:H7 EDL933 strains) and different growth conditions (LB medium, glucose-based medium and biofilm growth) can strongly affect $rpoS$-dependent gene expression (9,17–20), suggesting that most genes belonging to the $\sigma^S$ regulon are also subject to additional forms of gene expression regulation. Thus, in vivo gene expression was determined in three different growth media: the peptone-based LB medium, either full strength or diluted 1:4, and the glucose-based M9Glu/sup medium (Table 3). In addition, we took samples from cultures both in late exponential phase and in late stationary phase of growth; indeed, although $\sigma^S$-mediated gene expression is typically associated with stationary phase, several $rpoS$-dependent genes are maximally expressed at the transition between exponential and stationary phase or even in mid-exponential phase (19,39; Landini, P., unpublished data).

For five genes (recT, hyaA, gabD, punB and treF), dependence on a functional $rpoS$ gene has been described (Table 1); however, for the recT gene, part of the recET-lar-ydaCQ operon, both positive and negative control by $rpoS$ was reported (17,18). As a positive control, we tested the expression of $dps$ that, although showing only weak dependence on $\sigma^S$ in our ROMA experiments (Supplementary Table S2), has consistently been described as $rpoS$-dependent in vivo in several reports (9,16,17); in agreement with the literature data, $dps$ expression showed strong $rpoS$-dependence in all conditions tested (Table 3). We also tested the expression of cspA, which was transcribed with higher efficiency by $\sigma^S$ compared to $\sigma^70$ in ROMA experiments (Table 2); consistent with this result, in vivo gene expression studies show that a functional $rpoS$ allele is not required for cspA expression, which is in fact upregulated in the $rpoS$ mutant when grown in peptone-based media (Table 3), in agreement with previous observations (20). For in vivo gene expression experiments, we considered as significant a fold difference $\geq 2.5$ in WT versus $rpoS$ mutant relative expression ratio.

As shown in Table 3, growth conditions strongly affected gene expression: in LB medium, expression of only three genes ($hyaA$, $gabD$, $punB$ and $treF$) was dependent on a functional $rpoS$ allele, while seven genes were in fact upregulated in the $rpoS$ mutant, suggesting negative control by $\sigma^S$. In contrast, 12 genes were expressed in an $rpoS$-dependent fashion when bacteria were grown in 1:4 diluted LB, while 15 genes showed dependence on $rpoS$ in M9Glu/sup medium (Table 3). Of all genes tested, only the $bsmA$ gene did not show relative expression values $\geq 2.5$ in any growth condition, while recT and ycbX only displayed weak dependence on a functional $rpoS$ allele (2.5- and 2.6-fold increase in one growth condition). All other genes showed either strong dependence on the $rpoS$ gene (up to 86.3-fold for $speB$ in LB1/4 medium) or were affected by the $rpoS$ mutation in more than one growth condition (e.g. $rhaS$, induced 2.7-fold in the wild-type strain both in M9Glu/sup and in LB1/4 growth media). Finally, in vivo gene expression experiments demonstrated that the $c3113$ gene, tested as a representative of ORFs only annotated in the uropathogenic E. coli CFT073, is indeed expressed in MG1655, and it shows dependence on the $rpoS$ gene when bacteria are grown in M9Glu/sup (Table 3).

### Table 3. Gene expression in bacterial cells

| Gene | LB Exp | Stat | M9Glu/sup Exp | Stat | LB1/4 Exp | Stat |
|------|--------|------|---------------|------|-----------|------|
| ycbS | 2.4    | 0.1  | 6.7           | 1.7  | 2.0       |
| gshA | 1.0    | 1.5  | 1.0           | 0.9  | 2.6       |
| ycbX | 1.1    | 0.5  | 0.7           | 2.0  | 1.9       |
| recT | 0.9    | 0.4  | 2.5           | 1.8  | 3.0       |
| rhaS | 1.8    | 0.3  | 1.5           | 2.7  | 2.6       |
| cybR | 1.0    | 1.7  | 10.0          | 0.8  | 2.6       |
| ilvY | 1.7    | 0.3  | 1.0           | 2.5  | 1.9       |
| hyaA | 1.2    | 5.6  | 2.1           | 2.9  | 17.5      |
| xapA | 1.5    | 1.5  | 2.7           | 1.2  | 68.3      |
| speB | 1.4    | 0.8  | 0.9           | 2.0  | 3.1       |
| gabD | 4.8    | 0.7  | 12.1          | 5.1  | 69.0      |
| ydhY | 1.5    | 0.3  | 19.2          | 1.1  | 2.1       |
| appY | 1.2    | 0.7  | 3.4           | 2.0  | 11.4      |
| punB | 1.7    | 0.2  | 0.6           | 3.5  | 3.0       |
| treF | 1.5    | 3.0  | 7.2           | 2.1  | 52.6      |
| bsmA | 1.1    | 1.0  | 2.3           | 0.9  | 2.1       |
| xapA | 2.4    | 0.2  | 0.8           | 4.3  | 2.7       |
| c3113| 1.0    | 1.4  | 1.3           | 8.1  | 2.3       |
| $dps$| 0.9    | 10.7 | 10.7          | 0.3  | 14.2      |
| $cspA$| 1.3    | 0.2  | 1.1           | 0.8  | 0.7       |

Relative expression is indicated as WT/$rpoS$ ratio. Values higher than 2.5-fold were considered significant and are shown in boldface type. Values are the average of two independent experiments performed in duplicate.
As shown in Figure 1, *in vitro* transcription experiments performed on plasmids showed that the *ilvY, speB, xapA* and *ydhY* are transcribed more efficiently in the presence of Eσ^S^. Extent of dependence on Eσ^S^ was higher than in ROMA experiments, ranging from a 3.4-fold difference for *speB* to a 10.2-fold difference for *xapA*, thus suggesting that, at least at these promoters, DNA supercoiling does not negatively affect promoter recognition by Eσ^S^.

In contrast, the *ssrS* P1 promoter showed clear dependence on Eσ^70^, in agreement with previous observations (37). Thus, results of *in vitro* transcription experiments performed on single promoters were fully consistent with ROMA experiments.

**Sequence elements involved in specific Eσ^S^ - versus Eσ^70^ recognition of promoter regions**

The genes identified in ROMA experiments define, at least partially, what can be considered as the ‘core σ^S^ regulon’, i.e. a set of genes whose transcription is directly controlled by Eσ^S^. The core σ^S^ regulon can be opposed to the ‘expanded σ^S^ regulon’, i.e. genes dependent on a functional rpoS allele *in vivo*, whose promoters are, however, not necessarily recognized by Eσ^S^. In order to identify sequence features important for specific promoter recognition by Eσ^S^, it can be very informative to compare the sequences of Eσ^S^-dependent promoters; however, it is important to limit this comparison to the promoters that are exclusively, or at least preferentially, under Eσ^S^ control.

Thus, we performed a sequence alignment on the promoter sequences of genes preferentially transcribed by either Eσ^S^ or Eσ^70^. Known promoter sequences were retrieved from the Ecocyc database (http://ecocyc.org/); only promoters whose transcription initiation start site had been experimentally determined were considered for sequence alignment. However, since many rpoS-dependent genes are controlled by multiple promoters *in vivo*, suggesting complex regulation that might involve different sigma factors, we verified that their transcription start sites in the *in vitro* transcription reactions did indeed correspond to the transcription sites reported in the literature.

Thus, using RACE analysis, we determined transcription start sites on *in vitro* transcription assays carried out with Eσ^S^ and performed as in the ROMA experiment (data not shown): we were able to identify precisely the *in vitro* transcription start sites at 31 promoter regions controlling 29 different genes, as listed in Supplementary File S1. For most genes with already known promoter regions, we could confirm the transcription start site observed *in vivo*, although for several genes reported to be controlled by multiple promoters, only one promoter was found to be recognized by Eσ^S^ *in vitro* (e.g. *osmB, otsB, glgC* and *murQ*).

For the *gbd* gene, two of the three promoters described as functional in the bacterial cell were also recognized by Eσ^S^ in the *in vitro* transcription assays. In contrast, for the *araF* gene, we identified a second promoter additional to the one already described in the literature. Finally, we determined the transcription start sites for eight genes (or operons) preferentially transcribed by Eσ^S^ in ROMA experiments with yet-unknown transcription start sites, namely *puuCBE*, *tam*, *treF*, *ycI*, *yfOP*, *ylgG*, *yi91a* and *ygiG*: their transcription start site and putative promoter elements are listed in Supplementary File S1. For Eσ^70^-dependent promoters, we verified the transcription start sites for the *ssrS* transcript obtained in ROMA experiments performed with Eσ^70^: the transcription start site matched the known transcription site for the *ssrS* P1 promoter previously identified (37).

For promoter sequence comparison, we considered DNA sequences extending from −100 to +2 bp relative to the transcription start site, i.e. an area that includes all promoter elements described for Eσ^70^.

For sequence analysis, we divided the −100 to +2 promoter sequence in...
three parts: the −17 to +2 region, carrying the −10 element and the transcription start site; the −60 to −18 region, containing the UP element and the −35 sequence; and the −100 to −61 region. Alignment of the −17 to +2 regions was centred on the first nucleotide of the −10 element (conventionally referred to as the −12 position, Figure 2B). For the −60 to −18 regions, the alignment was centred either on the first nucleotide of the −35 element (when present) or on the nucleotide located 22 bp upstream of the −10 element, which would correspond to the first nucleotide of a hypothetical −35 element placed at the optimal 17 bp distance from the −10 sequence (Figure 2A). In total,

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Conserved sequence features in promoters of genes showing preferential transcription by either Eσ\(^{70}\) or Eσ\(^{5}\), shown as a sequence logo derived from multiple sequence alignments. (A) Comparison of alignments in the −60 to −18 promoter regions. (B) Comparison of alignments in the −17 to +2 promoter regions. Note that different y-axis scales were used in the two panels to account for the different levels of sequence conservation. Multiple alignment included 31 promoters controlling genes preferentially transcribed by Eσ\(^{5}\) in ROMA experiments (listed in Supplementary File S1) and 29 promoters preferentially recognized by Eσ\(^{70}\) (listed in Supplementary File S2).
31 promoters showing preferential recognition by Eσ^S (listed in Supplementary File S1) and 30 promoters preferentially recognized by Eσ^70 (Supplementary File S2) were selected for sequence analysis. Consensus sequence conservation within promoters preferentially recognized by either Eσ^S or Eσ^70 was displayed as sequence logos using the Weblog 2.8.2 application (40) (http://weblogo.berkeley.edu/). Results of sequence analysis showed that, as expected, the most conserved sequences corresponded to the known promoter elements and were comprised between −60 and +2 (Figure 2). Comparison of the sequences located further upstream (−100 to −61) is shown in Supplementary Figure S2.

For promoters more efficiently recognized by Eσ^70, the −35 and −10 sequences, i.e. the most important Eσ^70-dependent promoter elements, are clearly recognizable in the sequence logos (Figure 2). In addition, the −60/−40 region is characterized by a high occurrence of A and T residues (Figure 2A), consistent with strong conservation of the UP element, i.e. a binding site for the σ subunit of RNA polymerase (41). Other than the already known promoter features for σ^70, only the transcription start site (−3/+1) showed a moderately conserved sequence (CCCG, Figure 2B).

Several differences in conserved regions were detectable in the promoter set for genes more efficiently transcribed by Eσ^70. The −10 sequence is clearly the main conserved promoter element in this set (Figure 2B). No conserved sequences were detectable in the −35 region (Figure 2A), in agreement with previous works reporting that the −35 element does not play an important role in promoter recognition by Eσ^S (42,43), while a weakly conserved sequence similar to a −35 element seems to be located at around −30 (Figure 2A). Another difference between the two promoter sets resides in the lack of an A/T rich region between −60 and −40 (Figure 2A). In contrast, however, promoters more efficiently transcribed by Eσ^S seem to possess an increased occurrence of T residues in the −90 to −70 region (Supplementary Figure S2). Finally, the discriminator, i.e. the sequence located between the −10 element and the transcription start site, appears to be biased towards a high A/T content in promoters transcribed more efficiently by Eσ^S. In particular, T residues appear to be conserved at positions −6, −2 and −1. Rather than being associated to specific nucleotide position, however, the bias towards an A/T-rich discriminator seems to be a common feature in promoters of genes preferentially transcribed by Eσ^S. Indeed, while the average length of the discriminator is identical for both Eσ^70- and Eσ^S-dependent promoters (6.1 bp, Supplementary Table S3), the GC content is significantly higher in Eσ^70-dependent promoters (0.58 versus 0.41, Supplementary Table S3).

Finally, an additional deviation between Eσ^70- and Eσ^S-specific promoter elements could be observed immediately upstream of the −10 hexamer: the Eσ^70-dependent promoters showed some conservation of a TGG motif upstream of the −10 [the ‘extended −10′ (44)], which was replaced by a TNCG motif, which includes the characteristic −13C element, in Eσ^S-dependent promoters.

Mutations in the discriminator region affect selective promoter recognition at the Eσ^70-dependent promoter ssrS P1

It might be inferred that differences in the specific elements of the promoter regions might account for different transcription efficiency by either Eσ^S or Eσ^70 in ROMA experiments. In particular, we investigated whether the presence of an A/T rich region immediately downstream of the −10 element, conserved among promoters preferentially recognized by Eσ^S in ROMA experiment (Figure 2B), could indeed be a determinant for selective promoter recognition by Eσ^S. To test this possibility, we targeted for mutagenesis the ssrS P1 promoter, which shows preferential recognition by Eσ^70 in our in vitro assays (Table 1 and Figure 1) and whose dependence on Eσ^70 had already been reported in the literature (37). We changed the GC nucleotides at positions −3/−2 of the ssrS P1 promoter to TA (Figure 3), thus making the −6/+1 region of the promoter A/T-rich. In vitro transcription experiments on supercoiled templates in the presence of either Eσ^S or Eσ^70 showed that CG to TA substitutions at positions −3/−2 of the ssrS P1 promoter resulted in loss of specific recognition by Eσ^70 from 3.5- to 1.6-fold (Figure 3), suggesting that the A/T content in the −6/+1 region can indeed play a role in modulating transcription efficiency by either Eσ^S or Eσ^70.

DISCUSSION

In this work, we have attempted to identify bona fide Eσ^S-dependent promoters through in vitro transcription experiments using the whole E. coli genome as template, followed by identification of Eσ^S-dependent transcripts.
by microarray analysis (ROMA experiments). The in vitro transcription experiments have been performed on linear DNA, in the absence of any additional factor or molecule able to affect transcription initiation by either ErS or ErS70 (i.e. DNA supercoiling, transcription regulators, histone-like proteins, ppGpp), and thus they represent a direct measurement of sequence-specific interactions between promoters and RNA polymerase. Our results indicate that selective promoter utilization by ErS70 and ErS is mediated by specific sequence features, and confirm the role of σS-specific promoter elements previously identified. Promoters more efficiently recognized by ErS70 in ROMA experiments are characterized by a high occurrence of the UP-like element, i.e. an A/T-rich region located immediately upstream of the −35 sequence and acting as a binding site for the α subunit of RNA polymerase (41), suggesting that, at least in vitro, the UP element might favour promoter recognition by ErS70. It is worth mentioning that, in Bacillus subtilis, UP elements are highly conserved among σA-dependent promoters (45), thus suggesting that UP elements might favour promoter recognition by the housekeeping σ factor in different bacteria. In contrast, UP-like elements flanking the −35 sequence are less conserved in promoters better recognized by ErS, where, however, A/T-rich elements seem to be scattered in the region spanning 70–90 nt upstream of the transcription start (Supplementary Figure S2). It might be speculated that, similar to the UP element for ErS70-dependent promoters, AT-rich sequences located in the −70 to −90 region might also be involved in interaction with RNA polymerase α subunit at σS-dependent promoters. It is conceivable that the α subunit might contact alternative upstream promoter elements when assembled in different forms of RNA polymerase holoenzyme; indeed, differential ability to interact with UP elements has already been described for ErS70 and ErS (46).

A sequence element showing strong differences between the two promoter sets is the discriminator, i.e. the region spanning between −10 and +1. This region shows a high T/A content for promoters more efficiently recognized by ErS; in contrast, ErS70-dependent promoters identified in ROMA experiments are biased towards a CG-rich region in the −3/+1 residues (CCCCG, Figure 2). Mutations in the discriminator region of the ErS70-dependent srrS P1 promoter increasing its A/T content result in partial loss of preferential recognition by ErS70 (Figure 3), providing further confirmation for a role of the −10/+1 region in specific recognition by either form of RNA polymerase. GC versus AT content in the discriminator region can directly affect promoter melting, and GC-rich discriminators are a common feature among promoters subject to negative regulation by ppGpp (47,48), which, indeed, inhibits ErS70-dependent transcription while promoting transcription by alternative forms of RNA polymerase (49).

The sequence features indicated by our promoter analysis are consistent with previous observations based on sequence analysis of rpoS-dependent promoters in the bacterial cell (4); this also includes the fairly strong conservation of a C nucleotide immediately upstream of the −10 hexamer (the −13C element) among σS-dependent promoters. Indeed, the −13C element occurs in almost half of the promoters transcribed more efficiently by ErS in ROMA experiments (14 out of 31 promoters; Supplementary File S1), opposed to a much lower frequency in ErS70-dependent promoters (4 out of 29, Supplementary File S2). This result is not particularly surprising: indeed, a large amount of data has clearly shown that the presence of a C residue immediately upstream of the −10 nt favours promoter recognition by ErS70 (7,8,50). The −13C element might play a similar role as the TG motif at ErS70-dependent promoters lacking a −35 region (44). It is noteworthy that an in vivo analysis suggests that the −13C element occurs in more than 70% of putative rpoS-dependent promoters (4); this observation would suggest that the −13C element might be needed to improve ErS-dependent promoter interaction in the bacterial cell, possibly to overcome the negative effects of DNA-binding proteins, such as H-NS, that can modulate RNA polymerase–promoter interaction.

The results of the ROMA approach have also expanded our knowledge of the σS regulon: the σS protein is considered a central element of the so-called ‘general stress response’ (51). Intracellular σS concentration and expression of σS-dependent genes respond to reduction in growth rate (52); thus, any cellular stress affecting growth rate is likely to induce σS accumulation, which in turn plays a direct role in oxidative, acid and osmotic stress through activation of specific genes. In addition, σS activates metabolic genes associated to stationary-phase metabolism, in particular carbon storage genes involved in glycogen (19,20) and trehalose metabolism (9,20); consistent with these observations, stress response and carbon metabolism genes are highly represented among genes preferentially transcribed by ErS in ROMA experiments (Table 1). In addition, our results underline the importance of σS for the expression of genes involved in polyamine metabolism. Polyamines, in particular putrescine, play an important role in various cellular processes and can affect intracellular concentrations of the regulatory proteins σA, Cra and H-NS, thus impacting global gene expression (53). As shown in Figure 4, putrescine, the most abundant polyamine in bacterial cells (54), is a product of arginine degradation. Putrescine accumulates at the transition between exponential and stationary phase (55), and it can be subsequently converted into other polyamines or degraded to succinate, which can be shunted into the tricarboxylic acid (TCA) cycle, in a NADPH-generating process (Figure 4). Interestingly, mutants unable to synthesize polyamines are more sensitive to oxidative stress, since they cannot induce ahpC, katE and katG genes, encoding three different peroxidase, suggesting that polyamine accumulation might control expression of rpoS-dependent genes involved in the response to oxidative stress (54). In turn, our results indicate that genes encoding enzymes involved in both accumulation and degradation of putrescine, one of the main polyamines found in the bacterial cell, are rpoS-dependent. Indeed, the speB gene, encoding the putrescine-biosynthetic enzyme agmatinase, is preferentially transcribed by ErS70 in vitro (Table 1 and Figure 1) and is
The gabDTP operon, as well as the puuA and puuD genes, also involved in putrescine degradation, have already been reported to be rpoS-dependent in vivo (20,55,56). Thus, σS could control every step in putrescine metabolism by regulating genes responsible for both its biosynthesis and its degradation, as shown in Figure 4. Activation of putrescine biosynthesis rather than degradation might respond to different growth conditions and environmental cues, as also suggested by the very different levels of rpoS-dependent regulation of putrescine-related genes (i.e. speB, gabD and puuB) in different growth media (Table 3).

rpoS-dependent control of intracellular polyamines concentrations represents an important mechanism of indirect gene regulation by the σS protein, since polyamines act as signal molecules able to impact global transcription pattern in the bacterial cell (53). Indirect control of gene expression by σS can also occur through activation of regulatory proteins and of regulatory RNAs. Indeed, ROMA experiments would suggest that EsσS directly controls at least four genes encoding regulatory proteins (appY, ilvY, rhaS and yciT) and four genetic loci encoding regulatory RNAs (cyrR, micA, sgrS/sgrT and ECs3934) (Table 1). Interestingly, the sgrS/sgrT locus, encoding both a non-coding RNA (SgrS) and a small regulatory protein (SgrT), negatively affects expression of the ptsG glucose uptake system (57), which is overexpressed in rpoS mutant strains of E. coli (20,58). However, the extent of σS-dependent indirect regulation of gene expression through non-coding RNA might not be limited to these four loci. Indeed, a significant number of intergenic regions, often located immediately upstream of known ORFs in antisense direction, was efficiently transcribed in the bacterial cell and function as cis-acting regulatory RNAs. Similarly, several ORFs only annotated in pathogenic E. coli strains were detectable as transcripts in the ROMA experiment, although MG1655 genomic DNA had been used in the experiments. Sequence comparison allowed us to determine that these ORFs are indeed present in MG1655, but they are not accounted for in the available databases. As observed for intergenic regions efficiently transcribed by EsσS, several of these ORFs also overlap known genes in an antisense direction, suggesting that at least some of them might be involved in modulating gene expression. One ORF, c3113, partially overlaps the promoter region of the rrsG ribosomal operon, and quantitative real-time PCR experiments suggest that it is transcribed in a stationary-phase-dependent and rpoS-dependent manner in bacterial cells grown in glucose-based medium (Table 3). Similar to c3113, an intergenic region showing preferential transcription by EsσS (IG223409_223770-r) also partially overlaps the ribosomal operon rrsH, but in an antisense direction. Thus, our observations would suggest that small proteins or non-coding RNAs might play an important role in rpoS-dependent negative regulation of genes associated with fast growth, such as ribosomal operons and the ptsG glucose uptake system.
SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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