Promoter Strength Properties of the Complete Sigma E Regulon of *Escherichia coli* and *Salmonella enterica*[†]

Vivek K. Mutalik,¹ Gen Nonaka,¹ Sarah E. Ades,³ Virgil A. Rhodius,^{1*} and Carol A. Gross^{1,2*}

Department of Microbiology and Immunology¹ and Department of Cell and Tissue Biology,² University of California at San Francisco, San Francisco, California 94158, and Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802³

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The σ^E -directed envelope stress response maintains outer membrane homeostasis and is an important virulence determinant upon host infection in *Escherichia coli* and related bacteria. σ^E is activated by at least two distinct mechanisms: accumulation of outer membrane porin precursors and an increase in the alarmone ppGpp upon transition to stationary phase. Expression of the σ^E regulon is driven from a suite of approximately 60 σ^E -dependent promoters. Using green fluorescent protein fusions to each of these promoters, we dissected promoter contributions to the output of the regulon under a variety of in vivo conditions. We found that the σ^E promoters exhibit a large dynamic range, with a few strong and many weak promoters. Interestingly, the strongest promoters control either transcriptional regulators or functions related to porin homeostasis, the very functions conserved among *E. coli* and its close relatives. We found that (i) the strength of most promoters is significantly affected by the presence of the upstream (–35 to –65) region of the promoter, which encompasses the UP element, a binding site for the C-terminal domain of the α -subunit of RNA polymerase; (ii) ppGpp generally activates σ^E promoters, and (iii) σ^E promoters are responsive to changing σ^E holoenzyme levels under physiological conditions, reinforcing the idea that the σ^E regulon is extremely dynamic, enabling cellular adaptation to a constantly changing environment.

In bacteria, a repertoire of σ s directs RNA polymerase to discrete sets of promoters. The housekeeping σ directs transcription from promoters of the thousands of genes encoding functions required generally for growth, whereas the alternative σ s orchestrate specialized responses to cellular, developmental, and environmental signals (19, 23). Most alternative σ s transcribe on the order of 50 to 100 genes and use few auxiliary regulatory factors. These characteristics make it feasible to examine the behaviors of an entire set of promoters transcribed by a particular σ , thereby determining how the promoters themselves contribute to the transcriptional output of the response. In this report, we analyze the behaviors of promoters recognized by σ^E , an alternative σ factor in *Escherichia coli*.

The σ^E -directed envelope stress response maintains outer membrane homeostasis and is an important virulence determinant upon host infection in *E. coli* and related bacteria (22, 41). Studies across several organisms have revealed that a small core group of promoters is conserved across these organisms, whereas a larger group varies between organisms

(38). The two most conserved promoters drive regulatory circuits: for the transcription of σ^E itself and for σ^{32} , responsible for the cytoplasmic heat shock response. A second core conserved group of promoters drives genes with a coherent function primarily directed to the synthesis and/or assembly primarily of porins and to a lesser extent lipopolysaccharide (LPS), and together these constitute the two unique constituents of the outer membrane (32). This group includes the two small RNAs (sRNAs) that downregulate the expression of several porins (5, 25, 34, 48, 49), all four members of the recently discovered machine that inserts β -barrel proteins into the outer membrane (44, 53), and the chaperones that deliver porin precursors to the β -barrel insertion machine. Indeed, upon inactivation of σ^E in *E. coli*, cells lyse and bleb, indicating its key role in envelope integrity (22). The more variable members of the regulon are enriched for functions important in pathogenesis, suggesting that this response may be an early warning system to bacteria entering a host environment (38).

Three different signals are believed to alter the activity of σ^E . First, σ^E activity is sensitive to the assembly status of porins (1, 29). This fairly well-characterized signal transduction pathway involves destruction of RseA, the membrane-spanning anti-sigma factor that inactivates σ^E (1–2). RseA binding to σ^E holds it in an inactive state during normal growth (13, 17, 30). Upon accumulation of unfolded porins, DegS cleavage of RseA initiates a protease cascade that degrades RseA, thereby increasing the pool of σ^E able to form holoenzyme and initiate transcription. σ^E holoenzyme transcribes both itself and its negative regulator. Therefore, as long as the signal persists, σ^E increases but its negative regulator is rapidly degraded (3, 9, 16, 26). The DegS protease itself is the sensor of the porin signal (51). The protease domain of DegS is held in an inactive

* Corresponding author. Mailing address: 600 16th Street, Genentech Hall, S372E, University of California at San Francisco, San Francisco, CA 94158-2517. Phone: (415) 476-4161. Fax: (415) 514-4080. E-mail for C. A. Gross: cgross@cgl.ucsf.edu. E-mail for V. A. Rhodius: virgil.rhodius@ucsf.edu.

[†] Supplemental material for this article may be found at <http://jb.asm.org/>.

[‡] Present address: Joint Bioenergy Institute, Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720.

[§] Present address: Ajinomoto Co., Inc., 15-1, Kyobashi 1-chome, Chuo-ku, Tokyo 104-8315, Japan.

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state by binding to its PDZ domain (45, 46). Inhibition is relieved when the DegS PDZ domain binds a YXF motif located in the C terminals of most porins and several other outer membrane proteins. As porin C termini are buried in the trimer interface of assembled porins, accumulation of exposed YXF residues signals a block in porin assembly. Thus, this signal ties activation of σ^E to porin status in the cell. Second, it has been proposed that σ^E activity is sensitive to the status of LPS, although a molecular mechanism has not been elucidated (47). A plausible scenario is that this pathway is the second arm of the stress pathway just described. LPS or some derivative of it could bind to the N-terminal domain of RseB, which is structurally very similar to the lipoprotein binding domain of LolB (28, 52). RseB is a periplasmic protein that binds tightly to RseA and inhibits cleavage by DegS (8). Binding of an LPS-related substrate to RseB could weaken its interaction with RseA so that DegS cleavage could ensue. These two signals together could constitute a “stress” arm of the response, enabling the response to stress-induced imbalances in the outer membrane of the cell.

A third mode of regulation, independent of the RseA and RseB pathway, activates σ^E in response to intracellular signals related to growth phase and nutrient availability (10, 11). σ^E activity is significantly upregulated as cells enter stationary phase. ppGpp and its coactivator DksA mediate this response in two different ways: (i) directly, by facilitating transcription by σ^E holoenzyme, as demonstrated in *in vitro* transcription studies (11); (ii) indirectly, by redistributing the σ^{70} holoenzyme away from rRNA operons (responsible for >50% of the transcription during exponential phase [6]), thereby increasing the availability of RNA polymerase for σ^E -dependent transcription. Indirect effects are possible because it is believed that there are more σ s than core RNA polymerase in the cell, resulting in competition among σ s for binding to core RNA polymerase (18). When RNA polymerase is relieved from rRNA transcription, more RNA polymerase is either free or nonspecifically bound to DNA and able to freely exchange its σ s, thereby increasing the ability of σ s to bind to core RNA polymerase and mediate transcription. Additionally, ppGpp may increase the ability of alternative σ s to compete for RNA polymerase (reviewed in reference 33). Taken together, it is clear that the σ^E holoenzyme is under complex control in the bacterial cell.

Thus far, there have been only a few quantitative *in vivo* dissections of promoter contributions to a regulon, and most of these examined variations in transcription factor binding sites. For example, different configurations of PhoP binding sites have been correlated with promoter strength (31, 35, 36), and differential responses of Cpx-regulated promoters have been examined (37). Only a single study has focused on the strength of promoters driven by alternative σ s. Expression driven by the regions upstream of putative σ^S genes has been examined, but this analysis used 500-bp regions and did not dissect contributions of particular promoters (43).

In the present work, we explore the contributions of all previously validated σ^E -dependent promoters to the complex σ^E holoenzyme response. This includes unique σ^E -dependent promoters from two *E. coli* strains (K-12 MG1655 and CFT073) and *Salmonella typhimurium* (38). We show that the strongest promoters control either transcriptional regulators or

functions related to porin homeostasis, both core functions of the regulon, that the strength of most promoters is significantly affected by the presence of the upstream (−35 to −65) region of the promoter, the region encompassing the UP element, a binding site for the C-terminal domain of the α -subunit of RNA polymerase, that ppGpp generally activates promoters, an inference that had thus far been derived from only a few promoters, and that promoter output is responsive to physiologically relevant variations in the amount of σ^E .

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. All strains were grown in Luria-Bertani (LB) broth or M9 complete minimal medium supplemented with appropriate antibiotics at 30°C with shaking. M9 complete minimal medium was prepared as described previously (42) and supplemented with 0.2% glucose, 1 mM MgSO₄, vitamins, and all amino acids (40 μ g/ml). Medium was supplemented with 30 μ g/ml kanamycin, 100 μ g/ml ampicillin, 10 μ g/ml tetracycline, and/or 20 μ g/ml chloramphenicol as required.

Construction of assay strains. Assay strains and promoters are listed in Table S1 of the supplemental material. All assay strains are derivatives of *E. coli* K-12 strains MG1655 and MC1061. Transformations were performed using standard methods; P1 transductions were performed using P1 *vir*, and transductants were selected on LB plates with appropriate antibiotics. Assay strains are derivatives of strains CAG22216, CAG45113, CAG55907, and CAG58200, transformed with derivatives of the green fluorescent protein (GFP) expression vector, pUA66, carrying the long and short σ^E promoter libraries (see Table S1 in the supplemental material). Transformants were grown overnight in LB medium supplemented with appropriate antibiotics in deep 96-well plates and stored as glycerol stocks. Assays in the absence of σ^E , or with basal levels of σ^E , were performed with derivatives of CAG22216 (MC1061 Δ lacX74 [ϕ lppHP3::lacZ] *rpoE*:: Ω Cm) or CAG45113 (MG1655), respectively. Assays with overexpression of σ^E were performed with derivatives of CAG58200 (MG1655 Δ lacX74 [ϕ lppHP3::lacZ]) carrying the plasmid pLC245 expressing *rpoE* from the isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *P*_{trc} promoter. Assays with basal levels of σ^E in ppGpp⁰ strains were performed with derivatives of CAG55907 (MG1655 *lacX74* [ϕ lppHP3::lacZ] Δ relA Δ spoT::cam).

The ppGpp⁰ strain CAG55907 was made as follows. First, strain CAG55906 was constructed by transducing the Δ relA::kan allele from the Keio collection (4) into CAG45114 (MG1655 *lacX74* [ϕ lppHP3::lacZ]) and removing the kan marker by transforming with pCP20, screening for Kan sensitivity, and subsequently screening for loss of pCP20 (12). CAG55907 was then constructed by transducing CAG55906 with SEA2010, selecting for the Δ spoT207::cam allele. Alleles were confirmed by PCR, and the ppGpp⁰ phenotype was confirmed as an amino acid auxotroph (inability to grow on M9 minimal medium lacking amino acids [54]). All assays were repeated using at least two independent transductants.

Promoter library plasmid construction. All σ^E promoter constructs were carried on the low-copy-number vector pUA66, driving the expression of GFP from the reporter gene *gfpmut2*. Promoter sequences are displayed in Table S1 of the supplemental material. The long σ^E promoter library (plasmids pUA E1 to E60) was constructed by PCR amplifying genomic sequences from −65 to +20 with respect to the transcription start point from *E. coli* K-12 MG1655, *E. coli* CFT073, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 for each promoter, using forward primers containing an XhoI site and reverse primers containing a BamHI site. The PCR fragments were cloned into the XhoI-BamHI sites of pUA66, and final constructs were verified by sequencing. The short σ^E promoter library (plasmids pUA E1 to pUA E60) was constructed by PCR amplifying sequences from the −35 motif to +20 downstream of the transcription start point for each promoter, using the long promoter plasmid library as templates and forward primers containing an XhoI site and reverse primers containing a BamHI site. The −35 motif was identified by searching for the overrepresented GGAAGTT motif in aligned promoter sequences as described by Rhodius et al. (38). As before, the PCR fragments were cloned into the XhoI-BamHI sites of pUA66, and final constructs were verified by sequencing.

In vivo promoter assays. Assay strains were stored as glycerol stocks in deep 96-well plates (1 ml). Cultures were grown in 96-well plates containing 150 μ l of M9 complete or LB medium with appropriate antibiotics, inoculating from glycerol stocks using a sterilized 96-pin metal pinner. Cultures were grown overnight (~16 h) in 96-well U-bottom tissue culture plates covered with sterile breathable

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Source, reference, or P1 donor
<i>E. coli</i> strains		
MG1655 (CAG45113)	K-12 (MG1655) <i>rph-1</i>	<i>E. coli</i> Genetic Stock Center (20, 24)
MC1061	K-12 <i>araD</i> Δ (<i>ara-leu</i>)7697 Δ (<i>codB-lacI</i>) <i>galK16 galE15 mcrA0 relA1 rpsL150 spoT1 mcrB9999 hsdR2</i>	<i>E. coli</i> Genetic Stock Center (7)
CAG 22216	MC1061 Δ <i>lacX74</i> [ϕ l <i>rpoH</i> P3::lacZ] <i>rpoE</i> :: Ω Cm	14
CAG45114 (CAG25196)	MG1655 Δ <i>lacX74</i> [ϕ l <i>rpoH</i> P3::lacZ]	38
CAG58200 (CAG25197)	CAG45114 pLC245	38
CAG55701 to -55760	CAG58200 pUA E1 to E60 (long σ^E promoter library; 60 strains) ^a	This work
CAG58301 to -58360	CAG58200 pUA Et1 to Et60 (short σ^E promoter library; 60 strains) ^a	This work
CAG55601 to -55660	CAG22216 pUA E1 to E60 (long σ^E promoter library; 60 strains) ^a	This work
CAG58140 to -58197, -58138 and -9	CAG22216 pUA Et1 to Et60 (short σ^E promoter library; 60 strains) ^a	This work
CAG55801 to -55860	CAG45113 pUA E1 to E60 (long σ^E promoter library; 60 strains) ^a	This work
CAG58201 to -58260	CAG45113 pUA Et1 to Et60 (short σ^E promoter library; 60 strains) ^a	This work
CAG55761	CAG58200 pUA66	This work
CAG55661	CAG22216 pUA66	This work
CAG55861	CAG45113 pUA66	This work
CAG55906	CAG45114 Δ <i>relA</i>	This work, P1 Δ <i>relA</i> :: <i>kan</i> (Keio collection [4])
CAG55907	CAG55906 Δ <i>spoT</i> :: <i>cam</i>	This work, P1 SEA2010 (11)
CAG55912 to -55921	CAG55907 pUA E1 to E60 (select long σ^E promoters; 10 strains) ^a	This work
CAG55934 to -55943	CAG55907 pUA Et1 to Et60 (select short σ^E promoters; 10 strains) ^a	This work
CAG55922	CAG55907 pUA66	This work
Δ <i>relA</i> :: <i>kan</i> (Keio collection)	BW25113 (K-12 <i>rrnB3</i> Δ <i>lacZ4787 hsdR514</i> Δ (<i>araBAD</i>)567 Δ (<i>rhaBAD</i>)568 <i>rph-1</i>) Δ <i>relA</i> :: <i>kan</i>	Keio collection (4)
SEA2010	MG1655 Δ <i>lacX74</i> [ϕ l <i>rpoH</i> P3::lacZ] Δ <i>relA251</i> :: <i>kan</i> Δ <i>spoT207</i> :: <i>cam</i>	11
Plasmids		
pUA66	Vector, SC101 ori, Kan ^r GFP reporter plasmid carrying <i>gfpmut2</i>	55
pUA E1-E60	Long σ^E promoter library, used to measure activity of σ^E -dependent long promoter fragments (−65 to +20) cloned in XhoI-BamHI sites upstream of <i>gfpmut2</i> in pUA66 (60 constructs) ^a	This work
pUA Et1-Et60	Short σ^E promoter library, used to measure activity of σ^E -dependent short promoter fragments (−35 to +20) cloned in XhoI-BamHI sites upstream of <i>gfpmut2</i> in pUA66 (60 constructs) ^a	This work
pTrc99A	Vector, pBR322 ori, Ap ^r , expression vector containing an IPTG-inducible <i>trc</i> promoter	38
pLC245	<i>rpoE</i> cloned in pTrc99A downstream of the IPTG-inducible <i>trc</i> promoter, Ap ^r	38

^a See Table S1 in the supplemental material.

sealing film (sterile AeraSeal; E&K Scientific) at 30°C with shaking at 850 rpm on Elmi shakers. The following day the overnight cultures were diluted 1:200 into a final volume of 150 μ l of fresh medium with appropriate antibiotics in clear-bottom black plates, covered with a clear lid, and incubated in a multimode microplate reader-incubator-shaker (Varioskan; Thermo Fisher Scientific). For strains overexpressing σ^E (derivatives of CAG58200) the fresh medium also contained 100 μ M IPTG to induce *rpoE* expression. Cultures were grown for 8 h with orbital shaking at 480 rpm at 6-mm diameter. Repeated measurements of the optical density at 450 nm (OD₄₅₀) for M9 complete (or OD₆₀₀ for LB) and fluorescence (relative fluorescence units [RFU]; excitation at 481 nm and emission at 507 nm) were performed every 15 min. All experiments were repeated at least three times, with less than 5% standard error between replicate experiments. Differences in growth and expression profiles of randomly chosen strains between shake flask batch cultures and microplate reader plates were found to be negligible. This was also confirmed by changing the well positions of strains in the microplates to avoid any local effects.

Data analysis. Culture background fluorescence was determined using a promoterless pUA66 vector transformed into the parent strains of each promoter library. This pUA66 vector strain was grown and assayed in triplicate with each promoter library. The triplicate data points were averaged and used to generate a standard curve for OD against fluorescence (RFU). This standard curve was then used to subtract background fluorescence from the reporter strain fluorescence value at the same OD (not necessarily at the same time point), generating a background-subtracted OD versus RFU differential rate plot for each reporter strain. The slope of the linear portion of each differential rate plot corresponds to the promoter activity of the specific promoter-GFP fusion in that reporter

strain. Background fluorescence slopes (OD versus RFU) were between −2 and −0.8 for OD₆₀₀ values between 0.7 and 1.4 and were ~0 for an OD₆₀₀ values between 1.5 and 2.7.

A standard curve was generated to compare OD values for cultures measured either as 150- μ l samples in 96-well plates by the Varioskan or in a 1-ml path length cuvette by a regular spectrophotometer, in either M9 complete medium or LB broth. The conversion factors were 3.8 at OD₄₅₀ for cultures grown on M9 complete medium and 3.4 at OD₆₀₀ for cultures grown in LB broth. Using this conversion factor, the growth profiles of reporter strains were divided into exponential and stationary phases to compare with reported growth phase regulation of σ^E activity (11). The slopes of linear portions of the OD versus RFU differential plots at exponential and stationary phases of growth were then used to obtain the promoter activities at respective growth phases. The reported promoter activities are averages from at least three independent experiments.

Preliminary data analysis was performed using the SkanIT software for the Varioskan. Subsequent data analysis was performed automatically using Matlab software (Mathworks) with in-house-developed scripts (available upon request).

RESULTS AND DISCUSSION

Methodology of the experiments. To quantitatively determine expression of all σ^E -dependent promoters in vivo, we created a series of GFP reporter strains, each carrying a derivative of the low-copy-number plasmid pUA66 with a unique

FIG. 1. Methodology for measuring the strength of σ^E -dependent promoters. For 60 σ^E -dependent promoters, short fragments containing the core $-35/-10$ elements (-35 to $+20$) and long fragments that also included the UP element (-65 to $+20$) were cloned into the GFP expression vector pUA66. These promoter plasmid libraries were transformed into assay strains that either overexpressed σ^E (derivatives of CAG58200), contained basal levels of σ^E (derivatives of CAG45113 and CAG55907), or with σ^E deleted (derivatives of CAG22216 [contains a suppressor of σ^E essentiality]). Strains were grown in 96-well plates at 32°C with aeration (shaking) in a multimode Varioskan. Cell growth (optical density) and GFP fluorescence (RFU) were measured every 15 min. For each strain, differential plots were constructed of GFP fluorescence versus cell growth. Promoter strength was calculated from the slope of the linear portions of the plot, corresponding to the exponential or stationary growth phase. All calculated promoter strengths were subtracted for background culture and medium fluorescence by using control assay strains carrying the promoterless GFP vector pUA66. See Materials and Methods for further details.

σ^E promoter driving expression of a rapidly folding GFP. This promoter library contains two versions of each of the 60 validated σ^E promoters: a “short” promoter, extending from -35 to $+20$ relative to the start site of transcription, and a “long” promoter, extending from -65 to $+20$. Short promoters contain only the core promoter (-10 and -35 motifs), whereas long promoters include the upstream UP element sequences (39). Cultures were grown in 96-well plates in the multimode Varioskan, which allows aerated growth under controlled temperature conditions and automatically monitors both cell growth (OD) and GFP fluorescence at timed intervals. We measured the change in GFP fluorescence as a function of cell growth (differential plot). Promoter strength is taken as the slope of this line, and this parameter is plotted as a bar graph in all subsequent figures (Fig. 1). Control experiments by us (data not shown) and the Alon laboratory (27) indicate that plasmid copy number does not change under any of the conditions tested. The entire methodology is described in detail in Materials and Methods. We note that the σ^E -dependent *rpoH* promoter has a closely overlapping σ^{70} promoter that is present in our constructs (15). The commonly utilized σ^E -dependent *rpoHP3::lacZ* reporter does not contain this additional promoter (29).

Activities of σ^E promoters under “stress activation” conditions. During stress, the anti-sigma RseA is degraded rapidly,

freeing σ^E to carry out transcription (1); additionally, as σ^E transcribes its own gene (*rpoE*) (40), a positive feedback loop increases the amount of σ^E until stress subsides and degradation of RseA slows. To mimic these conditions, we overexpressed σ^E in cells growing in LB medium and examined transcription from the “long” and “short” derivatives of each of the validated σ^E promoters both during exponential-phase growth (OD_{600} , 0.7 to 1.4) (Fig. 2) and during the transition to stationary phase (OD_{600} , 1.5 to 2.7) (Fig. 3). In both figures, promoters are listed in ranked order from most to least active “long” promoter variant. We observed that in almost all cases, the “long” promoter variants were more active than the “short” constructs, suggesting that “UP element” binding by the α -C-terminal domain contributes to promoter strength. However, the magnitude of the contribution varied, with most promoters exhibiting ~ 1.5 - to 3.0-fold increases in activity (e.g., *rpoE* and *clpX*) but with some exhibiting as much as a 5- to 7-fold increase in activity (e.g., *rybB* and *micA*). Similar results were obtained with cells grown in M9 minimal medium supplemented with amino acids (data not shown). We note that our cloned promoter fragments end at $+20$. We chose this endpoint because we wanted to include the initial transcribed sequences, which can affect promoter escape. As a result, the stability and translation of GFP mRNA could differ in vivo. However, we have found a similar hierarchy of promoter ac-

FIG. 2. Exponential-phase strengths of long and short σ^E -dependent promoters after σ^E overexpression. Promoter strengths are shown for the long and short σ^E promoter plasmid libraries in derivatives of the σ^E overexpression strain CAG58200, carrying the *rpoE* overexpression plasmid pLC245 during exponential-phase growth (OD₆₀₀ of 0.7 to 1.4) at 32°C in LB. σ^E overexpression was induced by growth in 100 μ M IPTG. The bars represent the averages of three independent experiments, and error bars represent 1 standard deviation.

tivities using in vitro transcription assays (V. A. Rhodius, data not shown), indicating that the promoter-*gfp* reporter system provides a reasonable measure of promoter strength and that any influences on stability and translation of *gfp* mRNA are minimal.

We tested whether any of the promoters had significant σ^E -independent activity by assaying them in a strain lacking σ^E but with a mutation that suppresses σ^E essentiality (14) (Fig. 4). σ^E -independent activity was detected from only nine promoters. For three of these (*plsB*, *yjiO*, and *rpoH*), both the “long” and “short” forms of the promoters were more active in the presence of σ^E , suggesting that they are predominantly driven by σ^E . The six remaining promoters (*yhjJ*, *yieE*, *ycbR*, *ybjW*, *yecI*, and *ychH*) had approximately similar activities with and without σ^E , for either or both the long and short forms. However, in vitro transcriptions assays identified σ^E -dependent transcripts from four of these promoters (*yhjJ*, *yieE*, *ycbR*,

and *ychH*) (data not shown), suggesting that these sequences are still recognized by σ^E and, in addition, are also regulated by other σ s. We have previously mapped the transcript start sites of four of the nine promoter regions (*plsB*, *rpoH*, *yhjJ*, and *yieE*) using 5' random amplification of cDNA ends (38). All four regions show σ^E -dependent starts as well as adjacent σ^E -independent starts that are absent or much weaker in the presence of σ^E , indicating nearby promoters that are outcompeted in the presence of σ^E . In toto, these data show that most σ^E promoters are completely σ^E dependent with little direct overlap of promoters regulated by other σ s.

Examination of promoter strength data from the exponential phase of growth revealed that about 40 promoters have significant activity, and their promoter strength varies widely (~100-fold). Interestingly, the most highly active “long” promoters are predominantly comprised of those carrying out conserved functions of σ^E : regulatory transcription of σ^E and

FIG. 3. Stationary-phase strengths of long and short σ^E -dependent promoters after σ^E overexpression. Promoter strengths are shown for the long and short σ^E promoter plasmid libraries in derivatives of the σ^E overexpression strain CAG58200, carrying the *rpoE* overexpression plasmid pLC245, during stationary-phase growth (OD₆₀₀ of 1.5 to 2.7) at 32°C in LB. σ^E overexpression was induced by growth in 100 μ M IPTG. The bars represent the averages of three independent experiments, and error bars represent 1 standard deviation.

FIG. 4. σ^E -independent strengths of the long and short promoters. The graph compares long and short promoter strengths in σ^E overexpressing (o/exp; derivatives of CAG58200 carrying the *rpoE* overexpression plasmid pLC245) and no- σ^E (delta sE; derivatives of CAG22216) strains. Only nine promoters gave significant σ^E -independent activities. All promoter strengths were determined from exponential-phase cultures (OD_{600} of 0.7 to 1.4) grown at 32°C in LB. σ^E overexpression was induced by growth in 100 μ M IPTG. The bars represent the averages of three independent experiments, and error bars represent 1 standard deviation.

its anti-sigma factor (*rpoE* and *rseA*), as well as σ^{32} (*rpoH*), and functions related to porin homeostasis, the two sRNAs responsible for downregulating porins (*rybB* and *micA*) and portions of the machinery required for insertion of proteins into the outer membrane (*yaeT*, *smgA*, and *yfgL*) and the *degP* chaperone, implicated in porin transport. Importantly, almost all of the 10 most active “long” promoters are also the most active “short” promoters, indicating that core promoter strength contributes heavily to expression. The promoter driving *rybB* is an interesting exception, as its “UP” element boosts strength by about sevenfold, making the strength of this promoter heavily dependent on its UP element.

Comparison of promoter strength data between exponential and stationary phases of growth revealed that most promoters increase in strength around threefold with remarkably little deviation from this, apart from promoters that remain inactive under both conditions (Fig. 5). Consequently, the hierarchy of promoter strength remains relatively constant (compare Fig. 2 and 3). For example, 8 of the 10 most active promoters in exponential phase are among the 10 most active in stationary phase, and those promoters that do switch positions in the hierarchy generally show only small changes (e.g., *rpoE* is the most active promoter in exponential phase and the second most active promoter in stationary phase). Increased activity of σ^E promoters during stationary phase has been carefully studied for the *rpoH* promoter, and it has been concluded that this effect derives from direct and indirect effects of increased ppGpp during this growth phase (10, 11). The results presented here (see Fig. 7 and other findings, below) indicate that most σ^E -controlled promoters are subject to this effect and that promoters both with and without an UP element are activated during this growth phase. These data suggest that under these conditions most promoters increase their activity by similar amounts upon entry into stationary phase.

The activity of σ^E promoters under normal growth conditions. The experiments performed thus far used σ^E overex-

FIG. 5. Comparison of stationary-phase and exponential-phase promoter strengths. Scatter plots comparing long promoter strengths (A) and short promoter strengths (B) during stationary and exponential phase after σ^E overexpression. Experimental data are from Fig. 2 and 3.

pression conditions to examine behavior of promoters during stress. However, during normal cell growth, the amount of active σ^E in the cell is much lower. It is of great interest to compare promoter behavior under σ^E -limiting (basal) and σ^E excess (overexpression) conditions. The high autofluorescence of *E. coli* cells limits reliable detection (promoter activity is ≥ 2 -fold autofluorescence) to only the 10 most active promoters (Fig. 6). Six of these promoters have some σ^E -independent activity (Fig. 4), making it important to consider only the σ^E -dependent contribution to activity. Indeed, four promoters exhibited greater activity in exponential phase in the absence of σ^E (e.g., *yecI*, *plsB*, *ychH*, and *rpoH*) than in its presence, even though in vitro transcription data indicate that they are σ^E -dependent (data not shown). A plausible explanation is that these promoter fragments have overlapping σ^{70} promoters (50), a scenario known to be true for *PrpoH* (15). The activity of these additional competing promoters may be decreased in the presence of σ^E , due to occupancy and increased activity of the σ^E promoter by $E\sigma^E$. As it is not possible to quantify this accurately, we made the assumption here that the σ^E -independent activity of these promoters remains unchanged whether or not σ^E is present. Therefore, the quantitative arguments presented below are based on the 4 promoters that have no σ^E -independent promoter activity (*micA*, *rpoE*, *rybB*, and *rseA*), although all 10 promoters exhibit qualitatively similar behaviors. These results reveal two very significant differences in promoter behavior during σ^E -limiting and excess conditions: behavior upon entry to stationary phase and the magnitude of the UP element effect (Fig. 6A and B; Table 2).

There was a much greater increase in promoter activity upon entry into stationary phase when σ^E was limiting than when it was in excess. Whereas long promoters increased an average of 20-fold and short promoters increased an average of about 7-fold under σ^E -limiting conditions, these same promoters increased only about 3- to 4-fold under excess σ^E conditions (Table 2). Importantly, these same average values are obtained whether one considers only the four completely σ^E -dependent promoters or whether the increase is computed over the entire data set, indicating that this is a σ^E -dependent property. We showed that ppGpp is responsible for these large increases in promoter activity. Upon transition into stationary phase under σ^E -limiting conditions, cells lacking ppGpp had little or no

FIG. 6. Strengths of long and short σ^E -dependent promoters under basal levels of σ^E expression. The graphs show promoter strengths of the top 10 most active long and short σ^E -dependent promoters in strains expressing basal levels of σ^E (basal; derivatives of CAG45113) compared with promoter strengths in strains without σ^E (delta σ^E ; derivatives of CAG22216). Promoter strengths were measured during the exponential phase (A) or stationary phase (B) of growth at 32°C in LB. The bars represent the averages of three independent experiments, and error bars represent 1 standard deviation. Note the delta σ^E activities of the long *yecI* and *plsB* promoters in exponential phase are represented numerically.

increase in activity of these promoters (Fig. 7), as was shown previously for *rpoH* and *rybB* (10, 11, 48).

Under σ^E -limiting conditions, the UP element also has a proportionately greater effect on promoter strength than under σ^E excess conditions. This parameter can be reliably quantified only in stationary-phase cultures, where activities of both the long promoters (with UP element) and the short promoters are relatively high (Fig. 6; see also Table S2 in the supplemental material). The presence of an UP element increased expression 15-fold under σ^E -limiting conditions but only 3.6-fold under σ^E excess conditions for the four promoters completely dependent on σ^E , indicating that this is a σ^E -dependent property. Similar effects were seen for the whole data set (Table 2). Thus, the UP element plays a major role in determining σ^E promoter strength under limiting σ^E conditions.

Our data suggest that when σ^E is limiting, the strength of σ^E -dependent promoters is very sensitive to conditions, i.e.,

exponential versus stationary phase and the presence/absence of UP sequences. Interestingly, UP elements at σ^{70} -dependent promoters predominantly enhance promoter binding (K_B) (21). If a similar mechanism is true at σ^E -dependent promoters, then this explains why UP elements have little effect under σ^E excess conditions, where promoters are mostly saturated with σ^E holoenzyme, but contribute so greatly to promoter strength under σ^E -limiting conditions where σ^E holoenzyme binding proficiency determines strength. In this regard, it is interesting that during exponential phase under basal σ^E levels, UP elements have little effect (Table 2), although these data were close to background and may not be reliable. Nonetheless, they suggest the interesting hypothesis that under basal conditions, there is too little available σ^E holoenzyme for UP elements to enhance promoter activity. Consequently, this line of reasoning suggests that the role of UP elements in σ^E promoters is to enable the promoter to dramatically change its strength in response to physiologically relevant changes in σ^E holoenzyme levels from exponential to stationary phase (Table 2; compare long and short promoter activities under basal σ^E levels during growth phase).

Conclusions and future prospects. This is the first study that analyzes the complete target promoter set of a σ factor. We found that the σ^E promoters exhibit a large dynamic range and that promoter strength is asymmetrically distributed. There are just a few very strong promoters, somewhat more of the moderate-strength promoters, and many weak promoters. Very interestingly, promoter strength is strongly correlated with the function of the genes. The three strongest promoters all carry out regulatory roles in the σ^E response: the strongest promoter transcribes σ^E itself and its negative regulators, and the next two strongest transcribe the two sRNAs that downregulate porin production, thereby reducing the signal that generates the stress response in the first place. These promoter regions are highly conserved across the various *E. coli* strains and their close relatives, suggesting that their promoter strength is likely to be conserved across these organisms. This finding is somewhat reminiscent of a recent study of the PhoP regulon across multiple genomes, which found that the only targets directly regulated by PhoP in all species were the *phoPQ* operon itself and its negative regulator *slyB* (36). The moderately active promoters all carry out functions of the regulon that are conserved across many bacteria, and most of the weak promoters

TABLE 2. Comparison of promoter properties between basal and high (overexpression) levels of σ^E

Promoter ^c	Fold change in promoter strength							
	Growth phase ^a				UP effect ^b			
	Long (stat/exp)		Short (stat/exp)		Basal (long/short)		o/Exp (long/short)	
	Basal	o/exp	Basal	o/exp	Exp	Stat	Exp	Stat
<i>micA</i>	27.6	4.8	14.3	6.3	13.7	26.4	3.7	2.8
<i>rpoE</i>	18.7	2.7	10.6	2.9	2.5	4.5	1.7	1.6
<i>rseA</i>	29.8	3.3	0.9	3.2	0.4	13.7	3.5	3.7
<i>rybB</i>	10.5	2.0	2.3	2.3	3.9	17.9	7.2	6.3
<i>plsB</i> *	7.4	3.9	6.9	5.0	14.3	15.4	2.9	2.2
<i>rpoH</i> *	11.4	2.3	6.0	2.2	0.5	1.0	0.8	0.9
<i>ybcR</i> *	68.4	2.7	9.3	2.3	0.3	2.0	1.4	1.7
<i>ychH</i> *	16.1	4.0	5.3	3.4	13.6	41.4	3.4	4.0
<i>yecI</i> *	3.0	5.0	3.4	5.3	3.2	2.8	4.3	4.1
<i>yfjO</i> *	8.6	3.7	6.8	3.6	4.9	6.2	1.7	1.8
Avg	20.1	3.4	6.6	3.6	5.7	13.1	3.1	2.9

^a Comparing change in promoter activity over growth phase (stationary/exponential) for long and short promoters under either basal or overexpressed (o/exp) levels of σ^E . Values are ratios of stationary/exponential promoter activities.
^b Comparing the UP effect of long and short promoter activities for basal or overexpressed (o/exp) levels of σ^E under either the exponential (exp) or stationary (stat) growth phase. Values are ratios of long and short promoter activities.
^c *, promoters exhibiting significant σ^E -independent activity (Fig. 6).

FIG. 7. The requirement for ppGpp in the strength of σ^E -dependent promoters. The graphs show promoter strengths of the top 10 active long (A) and short (B) promoters under basal levels of σ^E expression during exponential (expo) or stationary (stat) phases of growth in either wild-type (derivatives of CAG45113) or ppGpp⁰ (derivatives of CAG55907; $\Delta relA \Delta spoT::cam$) strains. The bars represent the averages of three independent experiments, and error bars represent 1 standard deviation.

carry out less-conserved functions. That less-conserved functions of the regulon are generally driven by weak σ^E promoters is interesting and may reflect the fact that they have recently been incorporated into the regulon, either following horizontal transfer from a different organism or mutational alteration in the upstream region to permit some σ^E -mediated expression. The large number of low-activity promoters driving nonconserved functions underlines the tremendous plasticity of bacterial regulons.

Most of the promoters are completely σ^E dependent and are regulated during growth phases by ppGpp. In addition, most σ^E promoters require sequences upstream of their core motifs for full activity. In our previous work we identified AT-rich upstream sequences within the -65 to -35 regions of σ^E promoters that are candidates for α -binding UP elements. This work supports the idea that these are functional UP elements. Our ongoing in vitro transcription analysis of these promoters indicates that these sequences enhance promoter strength in the absence of additional transcription factors, further arguing that they encode UP element sequences (data not shown). This work also indicates that the σ^E promoters are extremely responsive to changing σ^E holoenzyme levels under physiological conditions, reinforcing the idea that the σ^E regulon is extremely dynamic, enabling cellular adaptation to a constantly changing environment.

The range of promoter strengths and their dynamics exhibited in vivo illustrate that in order to fully understand a response, promoter identification and knowledge of promoter strength is crucial. However, successful prediction of promoters and their strength from just sequence data remains a challenging problem. We are using these libraries of promoters to examine the correlation between promoter sequence and promoter strength to further improve predictive promoter models (V. A. Rhodius and V. Mutalik, unpublished data). Our findings thus far suggest that the degree of conservation of select motifs is moderately predictive of promoter strength and that higher-order relationships between motifs may be important for promoter function.

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