Published online 6 December 2009

Nucleic Acids Research, 2010, Vol. 38, No. 4 ***1273–1283***
doi:10.1093/nar/gkp1123

Promoter and regulon analysis of nitrogen assimilation factor, σ^{54}, reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis

Kai Zhao[1], Mingzhu Liu[2] and Richard R. Burgess[1,*]

[1]McArdle Laboratory for Cancer Research, [2]Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI 53706, USA

Received July 10, 2009; Revised November 10, 2009; Accepted November 15, 2009

ABSTRACT

Bacteria core RNA polymerase (RNAP) must associate with a σ factor to recognize promoter sequences. Promoters recognized by the σ^{54} (or σ^{N}) associated RNA polymerase are unique in having conserved positions around −24 and −12 nucleotides upstream from the transcriptional start site. Using DNA microarrays representing the entire *Escherichia coli* genome and promoter validation approaches, we identify 40 *in vivo* targets of σ^{54}, the nitrogen assimilation σ factor, and estimate that there are 70 σ^{54} promoters in total. Immunoprecipitation assays have been performed to further evaluate the efficiency of our approaches. In addition, promoter consensus binding search and primer extension assay helped us to identify a new σ^{54} promoter carried by insB-5 in the upstream of flhDC operon. The involvement of σ^{54} in flagellar biosynthesis in sequenced *E. coli* strain MG1655 indicates a fluid gene regulation phenomenon carried by some mobile elements in bacteria genome.

INTRODUCTION

The upstream regulatory region of all bacterial genes or operons contains one or more promoter(s). This is a special DNA sequence that can be specifically recognized by the RNA polymerase sigma subunit to allow binding and initiation of transcription. A major mode of gene regulation occurs via the binding of sigma factors to these specific DNA sequences. Sigma factors are identified by their ability to bind to core RNA polymerase (RNAP) and by their ability to direct promoter-specific transcription.

The *Escherichia coli* housekeeping σ factor, σ^{70}, was the first prokaryotic σ factor to be purified and characterized (1). Since then, numerous sigma factors have been found and characterized in *E. coli* and other prokaryotic organisms (2–6). The seven known *E. coli* sigma factors (σ^{70}, σ^{54}, σ^{32}, σ^{S}, σ^{F}, σ^{E} and σ^{FecI}) have been categorized into two families. The σ^{70} family contains σ^{70}, σ^{32}, σ^{S}, σ^{F}, σ^{E} and σ^{fecI}, whereas σ^{54}, because of differences in sequence, promoter architecture, and function, is placed in its own separate family (7,8). The intracellular levels of each individual σ factor change in response to growth transitions and environmental conditions (9,10) that play important roles in the regulation of gene expression.

σ^{54} (σ^{N}) was identified as a sigma factor involved in the transcription of genes involved in the cellular assimilation of ammonia and glutamate under conditions of nitrogen limitation (11). σ^{54} is structurally and functionally distinct from the other *E. coli* σ factors and shares very little if any sequence similarity with the primary σ factors. The three major differences that separate σ^{54} from the σ^{70} family of the other σ factors are: (i) unlike members of σ^{70} family, σ^{54} is able to bind promoter DNA in the absence of core RNA polymerase (7); (ii) regulatory proteins like NtrB and NtrC activate σ^{54} holoenzyme (12,13); (iii) σ^{54} recognizes promoter sequences with conserved GG and GC elements located −24 to −12 nucleotides upstream of the transcription start site (3,7). Although some bioinformatics approaches have been applied to search σ^{54} consensus binding site in different bacteria species (14–17), no large-scale experimental effort has been undertaken to unravel in detail the σ^{54} regulon in *E. coli*. Here, we present an updated list of σ^{54}-dependent promoters in *E. coli*. Computer programs, such as BioProspector and HMMer, have been utilized together to search and present the derivation of an extended consensus sequence for σ^{54} binding. Different from previous computational methods

*To whom correspondence should be addressed. Tel: +1 608 263 2635; Fax: +1 608 262 2824; Email: burgess@oncology.wisc.edu
[i]Present address:
Kai Zhao, Department of Microbiology, Tufts University, 136 Harrison Avenue, Boston, MA 02111, USA.

that only focused on the upstream intergenic sequences extracted from the genomes of several bacterial species, we found 18% of σ^{54}-promoters are located within the coding region of known genes or between convergently transcribed genes. This suggests a previously uncharacterized regulatory function of σ^{54}. We also compare σ^{54}-dependent genes identified in this study with σ^{70}-dependent genes identified in a separate study (our unpublished data). We found that 14% of σ^{54}-dependent genes can be directly transcribed by σ^{70} *in vitro*. This might indicate that bacteria use different promoter organizations to produce different regulatory outcomes in different environments. In addition, we also found a new σ^{54}-dependent promoter upstream of the flhDC operon in the sequenced strain MG1655 and provide an alternative explanation for the high motility of this sequenced strain compared with its closely related *E. coli* strains.

EXPERIMENTAL PROCEDURES

Reagents, strains and plasmids

All reagents were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise indicated. A 10X MOPS minimal media was prepared as described in Neidhardt *et al.* (18). The media were filter sterilized through a 0.2 μm filter and stored at 4°C. The defined media for log-phase cell growth contained 1× MOPS minimal media, 0.1% glucose, 0.66 mM K_2HPO_4. Neidhardt's MOPS-based defined media are now available commercially from Teknova, Inc.

Because the *E. coli* Genechip probe set is based on the sequenced *E. coli* K-12 strain MG1655 (λ^- F^- $ilvG^-$ rfb^-50 rph-1, prototroph) (19), we chose this bacterial strain for use in our study. In order to disrupt the expression of σ^{54} in *E. coli*, we used a simple and highly efficient method (20,21) to prepared in-frame deletion strains for σ^{54} as described (22,23). For controllable induction of individual regulators *in vivo*, we used the P_{Ltet} promoter which is controlled by the repressor TetR to construct these overexpression vectors as described previously (23). A downstream gene can be induced in the presence of the inducer anhydrotetracycline (aTc). All strains used in this study were derivatives of *E. coli* K12 MG1655.

Growth conditions, preparation of cell lysates

All cultures were grown in a New Brunswick Gyrotory water bath shaker (model G76) with vigorous aeration unless otherwise indicated. For cultures of cells carrying antibiotic resistance markers, the media were supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), or kanamycin (50 μg/ml) where appropriate. For induction of σ^{54} under the control of the anhydrotetracycline (aTc)-regulated promoter, aTc was added at a concentration of 100 ng/ml as described previously (22,23).

Escherichia coli MG1655 WT strain as well as derived deletion mutant strains were grown overnight in MOPS minimal media at 37°C in an air shaker with vigorous aeration (225 r.p.m.). Two microliters of the overnight culture was used to inoculate 100 ml of fresh MOPS minimal medium. When the culture density reached OD_{600} 0.2, a 1000 μl portion of culture was harvested into a prechilled 1.5 ml Eppendoff tube and then immediately put on ice for 1 min before being centrifuged at 10 000*g* (12 000 r.p.m. for BECKMAN Microfuge^R) for 10 min at 4°C. The supernatant was removed and the cell pellet resuspended immediately in 40 μl lysis buffer (1× SDS) and heated at 75°C for 5 min to quickly lyse the cells and prevent changes in the intracellular levels of the sigma factors being measured. We confirmed the absence of σ^{54} in the *rpoN* deletion strain by Western blot analysis using a monoclonal antibody (6RN3) (24).

Instead of using a σ^{32}/σ^F-inducible strain as shown in previous σ^{32}/σ^F regulon studies (22,23), we used strains carrying a plasmid with an aTc-inducible σ^{54} gene in this work. The same experimental procedures for induction, collection and treatment of sample were performed as described below and in more detail in our σ^{32}/σ^F regulon papers (22,23).

RNA isolation, cDNA synthesis, labeling and hybridization for microarray experiments

For preparing the total RNA for microarray experiments, *E. coli* strains were grown overnight in MOPS minimal media at 37°C in an air shaker with vigorous aeration (225 r.p.m.). Two microliters of the overnight culture was used to inoculate 100 ml of fresh MOPS minimal medium. A total of 15 ml samples of culture (corresponding to 7.5×10^9 cells) were taken for wild-type and mutant strains when the culture density OD_{600} value reached 0.2 and the same amount of culture was taken before and 5 min after induction in σ^{54}-overexpression strains. RNA was stabilized immediately by mixing with a double volume of RNAprotect Bacterial Reagent (Qiagen) and incubated at room temperature for 10 min. Cells were centrifuged at 5800*g* for 20 min and cell pellets were stored at −80°C prior to RNA extraction.

Total nucleic acid was isolated using MasterPure kits (Epicentre) as described by the manufacturer. DNase I (Epicentre) was used to remove genomic DNA contamination. Total RNA was purified, precipitated and resuspended in diethylpyrocarbonate (DEPC)-treated water. The quality and integrity of the isolated RNA was checked by visualizing the 23S and 16S rRNA bands on a 2% agarose gel. A 10 μg of total RNA was mixed with 500 ng random hexamers and then was reverse transcribed for first strand cDNA by using the Superscript II system (Invitrogen). RNA was removed by using RNase H (Life Technologies) and RNase A (Epicentre). cDNA was purified by using Qiaquick PCR purification kit (Qiagen) and followed by partial DNase I digestion to fragment cDNA to an average length of 50–100 bp. The fragmented cDNA was 3′-end-labeled by using terminal transferase (New England Biolabs) and biotin-N6-ddATP (PerkinElmer) and was added to hybridization solution to load on Affymetrix GeneChip^R *E. coli* Antisense Genome Arrays. Hybridization was carried out at 45°C for 16 h. The arrays were then washed and subsequently stained with streptavidin,

biotin-bound anti-streptavidin antibody and streptavidin-phycoerythrin (Molecular Probes) to enhance the signal. Arrays were scanned at 570 nm with 3 μm resolution using a confocal laser scanner.

Data analysis

Image analysis was carried out by Affymetrix® Microarry Suite 5.0 software. Cell intensity files were first generated from the image data files. An absolute expression analysis then computes the detection call, detection *P*-value and signal (background-subtracted and adjusted for noise) for each gene. Genes were considered up-regulated relative to the 0 time point (before induction)/wild-type strain sample if they had a 2-fold or greater increase in signal intensity and the signal intensity in the experiment had a $\log_2$ value of at least 8.0 and a detect level equal one; the higher $\log_2$ intensity values were used to limit the analysis to those genes for which we have a high degree of confidence in their level of expression.

Array design

The GeneChip® *E. coli* Antisense Genome Array was purchased from Affymetrix (catalog number: 900381). It contains *in situ* synthesized probe sets to detect the antisense strand of more than 4200 known open reading frames and over 1350 intergenic regions. A given gene is represented by 15 different 25-mer oligonucleotides that are designed to be complementary to the target sequence (25–27). Sequence information for probes on the array corresponds to the M54 version of the *E. coli* Genome Project database at the University of Wisconsin. Complete array information, including the location for each feature on the array, can be found at www.affymetrix.com.

Purification and fluorescence labeling of proteins and MAbs for immunoblot assay

Purified core RNA polymerase was made from *E. coli* MG1655 according to the method of Thompson *et al.* (28). Purified sigma factors and monoclonal antibodies (MAbs) were made as described in Anthony *et al.* (24). Purified core RNA polymerase and sigma factors were used in *in vitro* transcription assays. Mouse MAbs used in this experiment were anti-β′ (NT73) and anti-σ^{54} (6RN3) for measuring the intracellular level changes of σ^{54}. Both MAbs are available from Neoclone (Madison, WI, USA). Fluorescent dye, IC5-OSu (Dojindo), was used to label the primary antibodies according to previously described methods (29). The IC5-labeled MAbs, at final stored concentrations of 1 mg/ml, were diluted 1: 2000 for use in this experiment. Electrophoresis and immunoblot assays were performed as described in a previous paper (22,23). Signal intensities of the bands obtained with the Molecular Dynamics Typhoon system were quantified using the ImageQuant program.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assay is usually used to crosslink proteins to adjacent DNA by adding formaldehyde to an *in vivo* culture. Recently, the Aseem Ansari group at UW-Madison has developed the current ChIP protocol (30,31) for *E. coli* studies. To map promoters in bacteria, they sought a way to force RNAP to reside only at promoters so that identifying DNA fragments bound to a given sigma factor associated RNAP *in vivo* would report promoter locations. A variety of small-molecule inhibitors of RNA polymerase were evaluated for the immobilization of RNAP, and rifampin was found to work best (31). The antibiotic rifampin inhibits bacterial growth by binding the ß-subunit of RNAP near the active site, blocking the synthesis of RNAs longer than 2–3 nt (32). Rifampin has no effect on RNAP promoter binding (33) and has no effect on RNAP *in vitro* when added after elongating RNAP has cleared the promoter (34,35).

The general procedures for the confirmation of σ^{54}-dependent genes are: cross-linked chromatin is isolated from rifampin-treated σ^{54} overexpression and deletion cells, cells were then resuspended in 500 ml lysis buffer [10 mM Tris–HCl (pH 8.0), 50 mM NaCl] with 3 mg/ml lysozyme and incubated for 30 min at 37°C. A 500 ml of 2 × IP buffer [200 mM Tris–HCl (pH 8.0), 600 mM NaCl, 4% Triton X-100] was added and the DNA was sheared by sonication. Cell debris was removed by centrifugation, and the supernatant was transferred to new tubes. The samples were immunoprecipitated with monoclonal antibodies against σ^{54} (6RN3) (NeoClone, Madison, WI, USA). The immunoprecipitated protein-DNA crosslinks are then reversed at 65°C overnight. The ChIP DNA served as template for amplifying σ^{54}-dependent promoters by PCR.

Primer extension assays

The total RNA was prepared as described in microarray experiments above. The primer (5′-GTTGCGATAAGCT GCAA) was 5′-end-labeled using T4 polynucleotide kinase (New England Biolabs) and 50 μCi of [γ-^{32}P]-ATP (PerkinElmer). Approximately 1.5 pmol of ^{32}P-end-labeled oligonucleotide was added to the reaction. To denature nucleic acids, the reactions were heated at 95°C for 3 min and quenched on wet ice. An incubation step at 50°C for 15 min was done to promote annealing of the oligonucleotide to the RNA template. Two units of AMV Reverse Transcriptase (Promega) and final concentrations of 1 × AMV Reverse Transcriptase buffer [50 mM Tris–HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol (Promega)], and 0.2 mM each of the four deoxynucleoside triphosphates were added to each reaction tube. Reaction mixtures were incubated at 42°C for 90 min. Primer extension products were electrophoresed on a 5% denaturing polyacrylamide gel. The gel was dried, exposed in a phosphorimaging cassette, and scanned by using a Molecular Dynamics Typhoon (Model 8600).

RESULTS

The level of σ^{54} in *E. coli* MG1655 derivative deletion and overexpression strains

Escherichia coli σ^{54} in-frame deletion strains as well as σ^{54}-overexpression strains were constructed as described in Zhao *et al.* (22,23) and in Experimental procedures section. A fast and reliable improved western blot assay was used for quantitative analysis of the intracellular level of σ^{54} in the in-frame deletion and overexpression strains. The β′-subunit of core RNA polymerase was also examined to serve as an internal control because its intracellular levels remain constant under various conditions (9,36). The signal intensities of the proteins were immunodetected by corresponding IC5-labeled monoclonal antibodies. Our results (Figure 1A) show that the σ^{54} protein was not expressed (Figure 1A) in the *rpoN* deletion strain, confirming inactivation of this gene. The σ^{54} protein level, which is normalized to the β′ subunit of RNA polymerase, rapidly increased 5 min after induction with an ~2.9-fold change. Using the same overexpression system, previous data (22,23) showed σ^{32} and σ^{F} protein levels after 5 minutes induction increased almost 7.4- and 2.3-fold, respectively. The discrepancy of the fold changes, for different σ factors under the same P_{Ltet} promoter control, is mainly due to the fact that the experiments were performed at log-phase (OD_{600} = 0.2) in minimum medium, in which different σ factors have different initial protein levels.

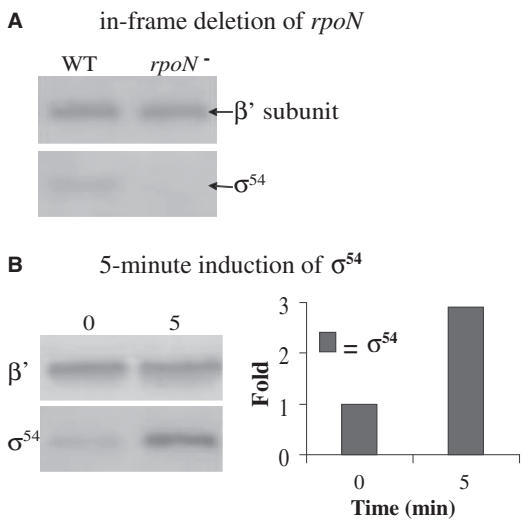


Figure 1. Determination the σ^{54} protein level in the *rpoN* deletion (KZ30) and σ^{54} overexpression (KZ7) strains, respectively. (**A**) Western blot analysis of β′ and σ^{54} protein expression in the wild-type MG1655 as well as in the *rpoN* deletion strains. Expression of β′ subunit of core RNA polymerase, which served as internal controls, can be detected in both strains. Expression of σ^{54} can only be detected in wild-type strain, but the expression of *rpoN* gene is absent in the respective mutant strain. (**B**) Left: western blot of β′ and σ^{54} expression before and 5 min after induction. Right: quantification of western blot. The σ^{54} protein level increases ~2.9-fold after 5 min of induction. Signal intensities are determined using ImageQuant version 5.2 software.

Known σ^{54}-dependent promoters

To characterize the effect of the decreasing and increasing σ^{54} protein level *in vivo* on gene expression, global RNA transcript abundance was monitored in the deletion mutant strain and the overexpression strain 5 min after σ^{54} induction with cells grown in log-phase (OD_{600} = 0.2) in MOPS minimal medium at 37°C. Transcription profiles were obtained as described in 'Experimental procedures' section. The sample for the wild-type strain and for the overexpression strain at time zero before induction was used as the reference to identify genes whose transcript abundance had significantly changed in the *rpoN* deletion mutant strain or the induced σ^{54}-overexpression strain, respectively.

DNA microarray results showed the transcriptional level of all well-characterized genes belonging to the σ^{54} regulon are downregulated/upregulated in the *rpoN* deletion strain/σ^{54} overexpression strain (Table 1). These results are consistent with our previous hypothesis that a change of the intracellular level of a given sigma factor will cause a change of the transcriptional level of genes dependent on this sigma factor. Jishage (9) reported that the intracellular level of σ^{54} is maintained at 16% or 6% the level of σ^{70} during log and stationary phase growth, respectively, in two different strains. Loss of σ^{54} in cells will decrease the transcription of σ^{54}-dependent genes. Induction of σ^{54} will show an increase in the transcriptional level of σ^{54}-dependent genes.

There are 18 known promoters (controlling 52 genes) under the control of σ^{54} in *E. coli*. Using a stringent cut off (2-fold decrease/increase to reduce the potential noise caused by array signal variation), 15 out of these 18 promoters significantly downregulated its operon genes in the *rpoN* deletion strain and 13 out of these 18 significantly upregulated their controlled genes in the σ^{54} overexpression strain. This indicates our microarray experiments can detect most known σ^{54}-dependent promoters in our assays.

New candidate genes for σ^{54} regulon

Expression profiling of transcripts corresponding to the complete set of ORFs in the *E. coli* genome revealed that the response to the changes of σ^{54} *in vivo* was quite broad. In addition to identifying the known σ^{54}-dependent genes, our microarray data allowed us to assign many additional new candidate genes to the σ^{54} regulon. Comparative analysis of the microarray data from the set of genes whose transcription is downregulated in the *rpoN* deletion strain (decrease of σ^{54}) and the set of genes with increased transcription at 5 minutes after σ^{54} induction (increase of σ^{54}) allows us to narrow down to 22 new candidate genes with high confidence in the σ^{54} regulon (Table 2). Results from promoter region consensus analysis using the algorithms MEME (37) and BioProspector (38) revealed the upstream regulatory sequences of most newly identified genes have a good match with the previously known σ^{54} consensus binding site (Table 2).

To further confirm new genes in the σ^{54} regulon, we performed ChIP assays to test the binding of σ^{54}-associated

Table 1. 18 Known σ^{54}-dependent genes with their promoter sequence

b#[a]	Gene[b]	Product	Function	Over[c]	Del[d]	Start[e]	End	promoter_sequence[f]
b0450	glnK[g]	regulatory protein (P-II 2) for nitrogen assimilation, regulates GlnL (NRII), GlnE (ATase), and AmtB (ammonium transporter)	Central intermediary metabolism	1.5	−18.8	−68	−51	tttcTGGCACACCGCTTGCAATacct
b3870	glnA[g]	glutamine synthetase	Amino acid biosynthesis: Glutamine	1.2	−11.4	−99	−82	aagtTGGCACAGATTTCGCTTTatct
b0811	glnH[g]	high-affinity glutamine transport protein	Transport of small molecules	1.1	−8.3	−70	−53	aaacTGGCACGATTTTTTCATAtatg
b1988	nac	transcriptional repressor of histidine utilization/ nitrogen assimilation (LysR family)	Central intermediary metabolism	1.3	−4.2	−71	−54	aaacTGGCAAGCATCTTGCAATctgg
b1304	pspA[g]	phage shock protein; negative regulatory gene for the psp operon	Phage-related functions and prophages	2.1	−3.9	−67	−50	aaatTGGCACGCAAATTGTATTaaca
b3227	dcuD	putative C4-dicarboxylate transport protein (DcuC family)	Not classified	12.5	−3.3	−313	−296	aaaaTGGCAGGGTTTTCTCTTTgtgc
b4002	zraP	periplasmic Zn-binding protein, zinc resistance-associated	Unknown	4.1	−3.1	−51	−34	tcgtTGGCACGGAAGATGCAATaccc
b0331	prpB	putative carboxyphosphonoenolpyruvate mutase	Not classified	2.2	−2.8	−62	−45	attgTGGCACACCCCTTGCTTTgtct
b4003	zraS	sensory histidine kinase in two-component regulatory system with ZraR, regulates zraP expression, senses Zn	Energy metabolism, carbon: Fermentation	3.5	−2.3	−47	−30	aagaTGGCATGATTTCTGCTGTcaga
b2725	hycA[g]	regulatory protein for HycE (part of the FHL complex)	Energy metabolism, carbon: Fermentation	13.0	−2.3	−52	−35	aagtTGGCACAAAAAATGCTTAaagc
b2221	atoD[g]	acetyl-CoA:acetoacetyl-CoA transferase, alpha subunit	Degradation of small molecules: Fatty acids	12.6	−2.3	−61	−44	attcTGGCACTCCCCTTGCTATtgcc
b4079	fdhF	formate dehydrogenase H, selenopolypeptide subunit	Energy metabolism, carbon: Anaerobic respiration	3.0	−2.2	−67	−50	aatgTGGCATAAAAGATGCATActgt
b3686	ibpB[g]	small heat shock protein	Adaptations, atypical conditions	4.4	−2.1	−185	−168	aaccTGGTAAATGGTTTGCTGTatat
b1748	astC[g]	succinylornithine transaminase, also has acetylornitine transaminase activity, PLP-dependent	Amino acid biosynthesis: Arginine	3.1	−2.1	−88	−71	tggcTGGCACGAACCCTGCAATctac
b3421	rtcB[g]	conserved hypothetical protein	Unknown	2.1	−2.1	−54	−37	tttcTGGCACGACGGTTGCAATtatc
b2713	hydN[g]	electron transport protein (formate to hydrogen), Fe-S center	Energy metabolism, carbon: Anaerobic respiration	1.4	−1.7	−53	−36	aaacTGGCATGATTTGTGAATGtatc
b2726	hypA[g]	guanine-nucleotide-binding protein in formate-hydrogenlyase system, functions as nickel donor for HycE of hydrogenlyase 3	Energy metabolism, carbon: Anaerobic respiration	2.4	−1.3	−46	−29	acacTGGCACAATTATTGCTTGtagc
b3073	ygjG	putative acetylornithine aminotransferase, PLP-dependent	Amino acid biosynthesis: Arginine	7.1	−1.2	−35	−18	ggagTGGCGCAATCCCTGCAATactt

[a]b no. indicates Blattner number.
[b]It is possible that one gene has several different gene names.
[c]Numbers indicate fold increase relative to pre-σ^N induction.
[d]Numbers indicate fold decrease relative to wild-type strain.
[e]Numbers indicate the distance from the gene's translation start site.
[f]Potential σ^N-related promoter
[g]The first gene in a known or predicted multicistronic operon.

Table 2. 22 New genes for the σ^{54} regulon

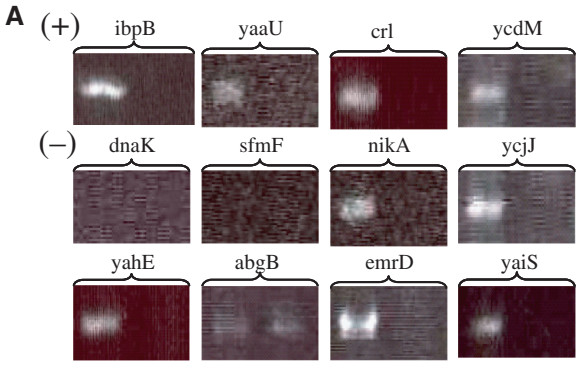
b#[a]	Gene[b]	Product	Function	Over[c]	Del[d]	Start[e]	End	promoter_sequence[f]
b0319	yahE[g]	conserved protein	Unknown	2.6	−5.4	−215	−198	ctacTGGAAGCGATTGTGCTTAatga
b0045	yaaU	putative transport protein (MFS family)	Not classified	5.7	−4.9	−244	−227	aaacAGGCGCTGGAGCTGCTGGtgaa
b0534	sfmF	putative fimbrial-like protein	Not classified	3.2	−3.4	−347	−330	ggccGGGTAATCGACCTGCTGGtgtc
b1337	abgB[g]	putative amidohydrolase (aminoacylase), p-aminobenzoyl-glutamate utilization	Unknown	7.5	−3.1	−169	−152	atgaTGGCCCGCGTGCAGCAACatca
b0240	crl	transcriptional regulator of cryptic genes for curli formation and fibronectin binding	Surface structures	2	−2.9	−34	−17	aattTGGTAAAACAGTTGCATCacaa
b3476	nikA[g]	nickel transport protein (ABC superfamily, peri_bind)	Transport of small molecules: Cations	3.3	−2.9	−257	−240	cgccTGGCAAATCGTCAGCGTAgaca
b3673	emrD	multidrug transport protein (MFS family)	Drug/analog sensitivity	2.2	−2.7	−100	−83	ttccTGGCGTATATCTGGCTAAcatt
b1012	ycdM[g]	putative enzyme	Unknown	2.6	−2.7	−44	−27	aaacTGGCATCCGCTTTGCAAAcaag
b1296	ycjJ	putative transport protein (APC family)	Not classified	5.8	−2.6	−161	−144	gttaTGGAGCGCGGGCGGCAACgggc
b3521	yhjC	putative transcriptional regulator (LysR family)	Not classified	2.5	−2.5	−234	−217	ttggTGGTTAGTACGCATGCAATtaa
b0364	yaiS	conserved hypothetical protein	Unknown	7.6	−2.5	−49	−32	gctgTGGCGCATCGCTTGCTCGtctt
b2878	ygfK[g]	"putative oxidoreductase, Fe-S subunit"	Not classified	2.1	−2.4	−97	−80	aaccTGGCAAGAGTGGTGCGATtgtt
b3800	aslB	putative transcriptional activator of acrylsulfatase synthesis	Not classified	3.4	−2.4	−206	−189	agctTGGTAGCGCAACTGGTTTggga
b2710	b2710[g]	flavorubredoxin (FIRd) with NO-binding non-heme diiron center	Not classified	9.8	−2.3	−63	−46	aaacTGGCACGCAATCTGCAATtagc
b3383	yhfZ	unknown CDS	Unknown	2.6	−2.3	−91	−74	ccgtTGGCCTGACGCAGGCCGCgttg
b4067	yjcG	putative transport protein (SSS family)	Not classified	2.1	−2.2	−62	−45	catcTGGCGGGCGAACGGCGAAttcg
b2184	yejH	putative ATP-dependent helicase	Not classified	2.8	−2.1	−39	−22	tccaTGGCATACTATTAGCAGAataa
b1488	ddpX[g]	D-Ala-D-Ala dipeptidase, Zn-dependent	Unknown	2.7	−2.1	−60	−43	ggcaTGGCATGAGATCTGCATAagcg
b0473	htpG	chaperone Hsp90, heat shock protein C 62.5	Chaperones	3.7	−2.1	333	316	cgtcTGGAACAGCGTCTGGCAGAggaa
b2866	xdhA[g]	putative xanthine dehydrogenase subunit, molybdenum cofactor-binding domain	Unknown	4.9	−2.1	−110	−93	tttcTGGCGTAAATCTTGCCTGctta
b3902	rhaD	rhamnulose-1-phosphate aldolase	Degradation of small molecules	3.6	−2	−143	−126	tatcAGGCCTACAGGTCGGCAATagtt
b2470	acrD	aminoglycoside/multidrug efflux pump (RND family)	Drug/analog sensitivity	2	−2	−74	−57	cgatTGGCTCGTACCTTGCCGCtaca

[a]b no. indicates Blattner number.
[b]It is possible that one gene has several different gene names.
[c]Numbers indicate fold increase relative to pre-σ^N induction.
[d]Numbers indicate fold decrease relative to wild-type strain.
[e]Numbers indicate the distance from the gene's translation start site.
[f]Potential σ^N-related promoter
[g]The first gene in a known or predicted multicistronic operon except *yahE* and *abgB*.

holoenzyme to promoter regions of these genes *in vivo*. We used the *ibpB* gene as positive control for the ChIP assay because this gene is known to be under the control of σ^{54} (39). The upstream sequence of the *dnaK* gene was chosen as a negative control because transcription of this gene is regulated by σ^{70} and σ^{32} but is not σ^{54}-dependent (40).

Using specific monoclonal antibodies (mAb) against σ^{54}, immunoprecipitation (ChIP) assays show the *ibpB* gene's promoter DNA sequence can be pulled down by anti-σ^{54} mAb in σ^{54} overexpression strain, but not in σ^{54} deletion strain due to lack of functional σ^{54}. For the negative control, the promoter DNA sequence of *dnaK* cannot be pulled down in either the σ^{54} overexpression or the σ^{54} deletion strain by anti-σ^{54} mAb *in vivo* because the gene lacks a σ^{54} promoter in its regulatory region. In addition, our ChIP assays show that 8 out of 10 of the newly identified genes' promoter DNA fragments (as shown in Figure 2) can be directly pulled-down in the σ^{54}-overexpression strain and not in the σ^{54}-deletion strain. This indicates that most genes identified by our microarray experiments are genuine.

In a separate study, we identified σ^{70} targets comprehensively across the *E. coli* genome. We compare those σ^{70} target genes with the σ^{54}-dependent genes identified in this study. We found 14% of the σ^{54}-dependent genes can be transcribed by σ^{70}-associated RNAP (unpublished data), indicating bacteria use different promoter organizations to produce different regulatory outcomes under the appropriate environment conditions (41).



B

yahE:	ctacTGGAAGCGATTGTGCTTAatga
yaaU:	aaacAGGCGCTGGAGCTGCTGGtgaa
sfmF:	ggccGGGTAATCGACCTGCTGGtgtc
abgB:	atgaTGGCCCGCGTGCAGCAACatca
crl:	aatttGGTAAAACAGTTGCATCacaa
nikA:	cgccTGGCAAATCGTCAGCGTAgaca
emrD:	ttccTGGCGTATATCTGGCTAAcatt
ycdM:	aaacTGGCATCCGCTTTGCAAAcaag
ycjJ:	gttaTGGAGCGCGGGCGGCAACgggc
yaiS:	gctgTGGCGCATCGCTTGCTCGtctt
Consensus	tGGca--N7---tGC(t/a)(t/a)

Figure 2. Chromatin immunoprecipitation assays to test the DNA fragments carrying putative σ^{54} promoter element(s). (**A**) *ibpB* served as a positive control and *dnaK* is serve as negative control. ChIPs assays are performed by using specific monoclonal antibody against σ^{54} (6RN3). The experiments are performed in both σ^{54} overexpression (left) and σ^{54} deletion (right) strains, respectively. The samples of DNA fragments containing σ^{54}-dependent promoters were immunoprecipitated by monoclonal antibodies against σ^{54}. (**B**) Potential σ^{54} consensus binding sites of each gene are predicted and aligned by computer program. The previously known σ^{54} two-block promoter element consensus is shown below.

Computer prediction of σ^{54}-related promoter elements

A computer program was used to examine upstream DNA sequence of upregulated genes in our microarray data to look for regulatory sequence motifs. As prokaryotic promoter motifs often occur in two blocks with a gap of variable length, BioProspector (38), a C program which is capable of modeling motifs with two blocks and uses a Gibbs sampling strategy, was used to find the −12 and −24 consensus regions for σ^{54} binding. Upstream sequences (400 bases from the first genes in transcription units that contain 2-fold up-regulated/down-regulated genes in our microarray data) were extracted as input sequences. A number of overall highest scoring motifs as position- specific probability matrices were reported. According to the reported highest scoring motif and its site locations on the input sequence, a graphical display of the results was generated using SEQUENCE LOGO (42) (Figure 3). The resulting consensus is represented as a TGGca-$(N)_{(4–5)}$-ttGCaa, where lower case indicates a less highly conserved site. This consensus agrees well with previously reported Eσ^{54} consensus which was aligned to maximize alignment (TGGcacg-$(N)_{4–5}$-tGCtat) in the −24 and −12 regions of several published Eσ^{54} promoters (3,43–45), indicating the conservation of σ^{54} promoter across these genes.

An alternative strategy for *E. coli* MG1655 flagellar synthesis

In Gram-negative bacteria, the hierarchy of the flagellar regulatory system has been well characterized in micro-organisms with peritrichous flagella, such as *E. coli* and *Salmonella typhimurium* (46,47) and polar flagellated micro-organisms, such as *Caulobacter crescentus* (48). Recently, the regulatory cascade components of bacteria with one polar flagellum, such as *Vibrio cholerae*, *Pseudomonas aeruginosa* and *P. fluorescens,*

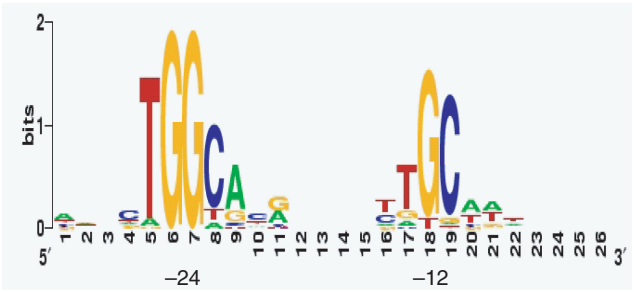


Figure 3. Determination of the σ^{54} consensus binding site. σ^{54}-related two-block promoter element is aligned using Bioprospector (38) from the upstream sequence of genes in σ^{54} regulon identified in our assays and displayed using SEQUENCE LOGO (42). The height of each column reflects the non-random bias of particular residues at that position, the size of each residue letter reflecting its frequency at that position.

have been characterized (49–51). The regulators at the top of the polar flagellar hierarchy belong to the NtrC family of σ^{54}-associated transcription activators. This regulator, together with σ^{54}, activates the expression of genes in polar flagellar system. In addition to its role in flagellar gene expression in these bacteria, this alternative sigma factor is known to participate in transcription of genes in nitrogen assimilation in *E. coli* and *S. typhimurium* (11,52) and in pilin synthesis in *P. aeruginosa* and *Neisseria gonorrhoeae* (53). No experiments have been performed and reported to test whether or not σ^{54} is involved in *E. coli* flagellar biosynthesis.

To investigate this hypothesis, we first compared the motility of wild-type strain and *rpoN* in-frame deletion strain by growing them on the swarm plate. We found the *rpoN* null mutant significantly reduces its motility compared with its derivative wild-type strain (Figure 4). Our microarray data also showed a significantly down-regulation of the transcription of flagellar genes in *rpoN* in-frame deletion strain (Supplementary Table S2). Because the expression of the FlhDC operon can be regulated by multiple positive and negative regulators (Supplementary Figure S1) (54–57), we checked whether σ^{54} affects the expression of these regulators and thus indirectly affects flhDC operon expression. Our microarray data show the expression of most negative regulators is downregulated and the expression of most positive regulators is upregulated (Supplementary Table S3) in σ^{54} deletion strain. Therefore, the downshifting of expression of the flhDC gene in the σ^{54} deletion strain is not primarily due to the level changes of those regulators in the same strain.

The Matsumura group found the sequenced *E. coli* strain MG1655 is extremely motile compared with other *E. coli* strains (58). Sequence analysis shows that there are mobile elements, insA-5 and insB-5, inserted into the upstream regulatory region of flhDC operon. They proposed that the high motility of MG1655 is due to insertion element insB-5, which prevents binding of the negative regulator OmpR, and thus increase the expression of flhDC operon for flagellar synthesis (Supplementary Figure S1). We think we can provide an alternative explanation for at least some of this high motility phenomenon; a σ^{54}-dependent promoter has been brought in by this insertion element.

A bioinformatics approach (38) has been used to search the σ^{54} consensus binding site in the upstream regulatory region of *flhDC* operon. A good match of a σ^{54}–dependent promoter has been found in this insB-5 insertion element (Figure 5A). RT-PCR as described in previous paper (22,23) (PrimerUp: 5′-GCATGACAAAGTCATC GG, PrimerDown: 5′-GTTGCGATAAGCTGCAA) has been performed and showed that there is an additional transcript from the upstream region of the known σ^{70}-dependent promoter on this operon (Figure 5B). Primer extension assays showed this transcript present in the σ^{54}-overexpression strain but not in the σ^{54}-deletion strain (as shown in Figure 5C), indicating it was transcribed from a σ^{54}-dependent promoter that was brought in by the insB-5 insertion element. The biological significance of this is discussed below.

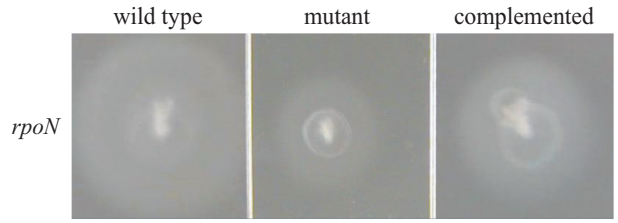


Figure 4. Motility in *E. coli* wild-type and the *rpoN* deletion strains. Compared with wild-type strain, disruption of *rpoN* causes impaired movement on a swarm plate. The motility can be complemented from this mutant strain by *in vivo* expression of σ^{54} from a plasmid-borne *rpoN* gene.

DISCUSSION

Transcription is a key control point for regulation of numerous cellular activities. Bacteria regulate levels of gene expression by using transcription factors that modulate the recruitment of RNAP to promoter elements in the DNA. Because σ factors are required to initiate gene transcription in *E. coli*, and there are different σ factors required to regulate different sets of genes or regulons to adapt to changes in external environment, determination of σ regulons will provide valuable information for classifying genes into different functional groups and will be the first essential step to understanding global gene regulation under different growth conditions.

The aim of the present work is to identify promoters under control of $E\sigma^{54}$ *in vivo*. Under defined, steady-state growth conditions, we used two different genetic approaches to alter σ^{54} concentration in cells: (i) moderately expressing σ^{54} from a plasmid-borne *rpoN* gene controlled by anhydrotetracycline (aTc)-inducible and Tet repressor-controlled P_{Ltet} promoter; (ii) disrupting the expression of σ^{54} in *rpoN* deletion mutant strains. These combined methodologies used to determine $E\sigma^{54}$ promoters proved highly effective as nearly all known σ^{54}-dependent promoters were identified. Our analysis has identified 40 targets of $E\sigma^{54}$, including 22 previously undescribed targets. This is far more $E\sigma^{54}$ promoters than expected. We chose a stringent cutoff (less than 2-fold change) when analyzing the microarray data to ensure that almost all called targets are genuine targets of $E\sigma^{54}$. Our site validation and ChIP assays confirms that most, if not all, are genuine. We repeated our analysis using a less stringent cutoff ($\geq$1.5-fold). In this case, we identified an additional 30 target regions (Supplementary Table S1) with strong σ^{54}-like promoter in its upstream regulation region. On the basis of this analysis, we estimate that there are 70 σ^{54} promoters in *E. coli*, almost four times the number of previously identified promoters. Around 20% of the newly identified genes are hypothetical and the role of these genes remains to be elucidated. To obtain a better understanding of consensus binding sites controlled by σ^{54}, we carried out an *in silico* analysis on the presence of $-24/-12$ type promoters in the upstream region of these genes. Under the condition of our experiment, we think the expression of some of the identified

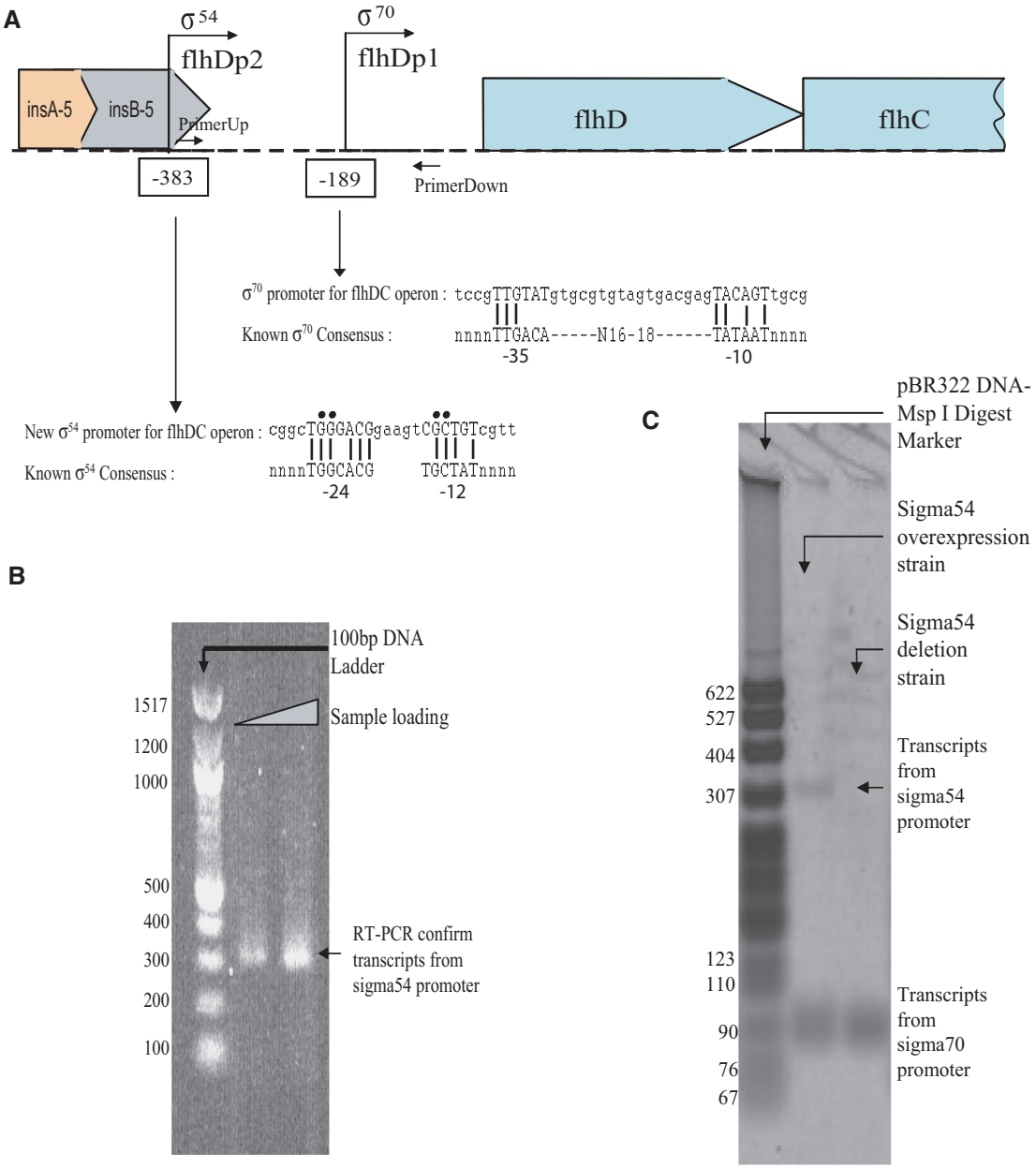


Figure 5. Evidence indicating potential new σ^{54} promoter in the upstream of flhDC operon. (**A**) Schematic show the positions of insertion elements (insA-5 and insB-5) and the σ^{70}, σ^{54} promoters in the upstream of flhDC operon. Two-block binding site for these two promoters have been selected to compare with respective known consensus promoter binding sites as shown below. (**B**) Total RNA was prepared and reverse transcribed to cDNA. The primer pair as shown in (A) was used to amplify the transcripts starting from upstream of the σ^{70}-dependent promoter in the regulative region of the *flhDC* operon. (**C**) Primer extension assays have been performed in the σ^{54}-overxpression strain and deletion strain. A σ^{54}-dependent transcript is present in the σ^{54} overexpression strain and is not in the σ^{54} deletion strain. The σ^{70}-dependent transcript can be detected in both lanes.

σ^{54}-depenedent targets might be just a small fraction of the maximum due to strict dependence on AAA-family activators (59–62) some of whose activities might be limiting in our experiments. Likewise, it is possible that a few σ^{54}-dependent targets will be missed in our experiments due to very low or no activities of certain AAA-family activators.

We characterized an alternative strategy for sequenced *E. coli* strain MG1655 flagellar biosynthesis. In this case, σ^{54}-dependent promoter is brought by an insertion element into the upstream regulatory region of *flhDC* operon which encodes FlhDC, the master regulator for flagellar biosynthesis. Primer extension assays show there is a transcript transcribed from this new σ^{54}-dependent promoter *in vivo*. This also provides additional explanation for the high motility of this strain compared with other related bacteria. Lateral gene transfer is a major factor in the evolution of bacteria. Bacteria genomes contain a significant number of mobile elements—DNA that can move around on chromosomes, among organisms and even between species. It has been reported that mobile DNA can carry genes for virulence and drug resistance, as well as benign genes (63). The discovery of a σ^{54}-dependent promoter in a motile element in our work might

provide another paradigm for the horizontal exchange of genetic information in prokaryotes. This additional fluidity allows bacteria to turn on different genes in various conditions to enhance their ability to survival or cause disease. The implication of σ^{54} involvement in bacterial motility regulation is widespread in Gram-negative bacteria. Indeed, the elements of flagellar regulatory cascades including σ^{54}-associated regulators have also been identified in micro-organisms such as *C. crescentus* (48), *Rhodobacter sphaeroides* (64–66), *Helicobacter pylori* (67) and *Campylobacter jejuni* (68). Although environmental and genetic factors that control dissemination of these mobile elements remain to be determined, the σ^{54} involvement in *E. coli* MG1655 flagellar biosynthesis might indicate a remarkable rearrangement/improvement in the functional organization of regulatory mechanisms that has existed in its other close-related bacteria. This might also indicate an evolutionary conservation among Gram-negative bacteria.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr Shaun Brinsmade for critical reading of the article and Burgess group for many useful discussions. We also thank Sandra Splinter-BonDurant, Wayne Davis, John Leucke at the Gene Expression Center for excellent technical assistance.

FUNDING

NIGMS (GM28575 to R.R.B.).

Conflict of interest statement. RRB acknowledges financial interest in NeoClone, LLC., (Madison, WI) which markets mAbs (NT73 and 6RN3) used in this study.

REFERENCES

1. Burgess,R.R., Travers,A.A., Dunn,J.J. and Bautz,E.K. (1969) Factor stimulating transcription by RNA polymerase. *Nature*, **221**, 43–46.
2. Haldenwang,W.G. (1995) The sigma factors of Bacillus subtilis. *Microbiol. Rev.*, **59**, 1–30.
3. Wosten,M.M. (1998) Eubacterial sigma-factors. *FEMS Microbiol. Rev.*, **22**, 127–150.
4. Gomez,J.E., Chen,J.M. and Bishai,W.R. (1997) Sigma factors of Mycobacterium tuberculosis. *Tuber. Lung Dis.*, **78**, 175–183.
5. Sauer,U., Santangelo,J.D., Treuner,A., Buchholz,M. and Durre,P. (1995) Sigma factor and sporulation genes in Clostridium. *FEMS Microbiol. Rev.*, **17**, 331–340.
6. Potvin,E., Sanschagrin,F. and Levesque,R.C. (2008) Sigma factors in Pseudomonas aeruginosa. *FEMS Microbiol. Rev.*, **32**, 38–55.
7. Cannon,W., Claverie-Martin,F., Austin,S. and Buck,M. (1993) Core RNA polymerase assists binding of the transcription factor sigma 54 to promoter DNA. *Mol. Microbiol.*, **8**, 287–298.
8. Buck,M. and Cannon,W. (1992) Specific binding of the transcription factor sigma-54 to promoter DNA. *Nature*, **358**, 422–424.
9. Jishage,M., Iwata,A., Ueda,S. and Ishihama,A. (1996) Regulation of RNA polymerase sigma subunit synthesis in Escherichia coli: intracellular levels of four species of sigma subunit under various growth conditions. *J. Bacteriol.*, **178**, 5447–5451.
10. Jishage,M. and Ishihama,A. (1995) Regulation of RNA polymerase sigma subunit synthesis in Escherichia coli: intracellular levels of sigma 70 and sigma 38. *J. Bacteriol.*, **177**, 6832–6835.
11. Hirschman,J., Wong,P.K., Sei,K., Keener,J. and Kustu,S. (1985) Products of nitrogen regulatory genes ntrA and ntrC of enteric bacteria activate glnA transcription in vitro: evidence that the ntrA product is a sigma factor. *Proc. Natl Acad. Sci. USA*, **82**, 7525–7529.
12. Klose,K.E., Weiss,D.S. and Kustu,S. (1993) Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. *J. Mol. Biol.*, **232**, 67–78.
13. Rombel,I., North,A., Hwang,I., Wyman,C. and Kustu,S. (1998) The bacterial enhancer-binding protein NtrC as a molecular machine. *Cold Spring Harb. Symp. Quant. Biol.*, **63**, 157–166.
14. Studholme,D.J., Buck,M. and Nixon,T. (2000) Identification of potential sigma(N)-dependent promoters in bacterial genomes. *Microbiology*, **146(Pt 12)**, 3021–3023.
15. Salgado,H., Gama-Castro,S., Martinez-Antonio,A., Diaz-Peredo,E., Sanchez-Solano,F., Peralta-Gil,M., Garcia-Alonso,D., Jimenez-Jacinto,V., Santos-Zavaleta,A., Bonavides-Martinez,C. *et al.* (2004) RegulonDB (version 4.0): transcriptional regulation, operon organization and growth conditions in Escherichia coli K-12. *Nucleic Acids Res.*, **32**, D303–D306.
16. Dombrecht,B., Marchal,K., Vanderleyden,J. and Michiels,J. (2002) Prediction and overview of the RpoN-regulon in closely related species of the Rhizobiales. *Genome Biol.*, **3**, RESEARCH0076.
17. Cases,I., Ussery,D.W. and de Lorenzo,V. (2003) The sigma54 regulon (sigmulon) of Pseudomonas putida. *Environ. Microbiol.*, **5**, 1281–1293.
18. Neidhardt,F.C., Bloch,P.L. and Smith,D.F. (1974) Culture medium for enterobacteria. *J. Bacteriol.*, **119**, 736–747.
19. Blattner,F.R., Plunkett,G. III, Bloch,C.A., Perna,N.T., Burland,V., Riley,M., Collado-Vides,J., Glasner,J.D., Rode,C.K., Mayhew,G.F. *et al.* (1997) The complete genome sequence of Escherichia coli K-12. *Science*, **277**, 1453–1474.
20. Datsenko,K.A. and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl Acad. Sci. USA*, **97**, 6640–6645.
21. Kang,Y., Durfee,T., Glasner,J.D., Qiu,Y., Frisch,D., Winterberg,K.M. and Blattner,F.R. (2004) Systematic mutagenesis of the Escherichia coli genome. *J. Bacteriol.*, **186**, 4921–4930.
22. Zhao,K., Liu,M. and Burgess,R.R. (2007) Adaptation in bacterial flagellar and motility systems: from regulon members to 'foraging'-like behavior in E. coli. *Nucleic Acids Res.*, **35**, 4441–4452.
23. Zhao,K., Liu,M. and Burgess,R.R. (2005) The global transcriptional response of Escherichia coli to induced sigma32 protein involves sigma32 regulon activation followed by inactivation and degradation of sigma32 in vivo. *J. Biol. Chem.*, **280**, 17758–17768.
24. Anthony,L.C., Foley,K.M., Thompson,N.E. and Burgess,R.R. (2003) Expression, purification of, and monoclonal antibodies to sigma factors from Escherichia coli. *Methods Enzymol.*, **370**, 181–192.
25. Wilson,K.H., Wilson,W.J., Radosevich,J.L., DeSantis,T.Z., Viswanathan,V.S., Kuczmarski,T.A. and Andersen,G.L. (2002) High-density microarray of small-subunit ribosomal DNA probes. *Appl. Environ. Microbiol.*, **68**, 2535–2541.
26. Liu,G., Loraine,A.E., Shigeta,R., Cline,M., Cheng,J., Valmeekam,V., Sun,S., Kulp,D. and Siani-Rose,M.A. (2003) NetAffx: Affymetrix probesets and annotations. *Nucleic Acids Res.*, **31**, 82–86.
27. Bockhorst,J., Qiu,Y., Glasner,J., Liu,M., Blattner,F. and Craven,M. (2003) Predicting bacterial transcription units using sequence and expression data. *Bioinformatics*, **19(Suppl 1)**, i34–i43.
28. Thompson,N.E., Hager,D.A. and Burgess,R.R. (1992) Isolation and characterization of a polyol-responsive monoclonal antibody

useful for gentle purification of Escherichia coli RNA polymerase. *Biochemistry*, **31**, 7003–7008.
29. Bergendahl,V., Glaser,B.T. and Burgess,R.R. (2003) A fast western blot procedure improved for quantitative analysis by direct fluorescence labeling of primary antibodies. *J. Immunol. Methods*, **277**, 117–125.
30. Herring,C.D., Raffaelle,M., Allen,T.E., Kanin,E.I., Landick,R., Ansari,A.Z. and Palsson,B.O. (2005) Immobilization of Escherichia coli RNA polymerase and location of binding sites by use of chromatin immunoprecipitation and microarrays. *J. Bacteriol.*, **187**, 6166–6174.
31. Raffaelle,M., Kanin,E.I., Vogt,J., Burgess,R.R. and Ansari,A.Z. (2005) Holoenzyme switching and stochastic release of sigma factors from RNA polymerase in vivo. *Mol. Cell*, **20**, 357–366.
32. Campbell,E.A., Korzheva,N., Mustaev,A., Murakami,K., Nair,S., Goldfarb,A. and Darst,S.A. (2001) Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell*, **104**, 901–912.
33. McClure,W.R. and Cech,C.L. (1978) On the mechanism of rifampicin inhibition of RNA synthesis. *J. Biol. Chem.*, **253**, 8949–8956.
34. Carpousis,A.J. and Gralla,J.D. (1985) Interaction of RNA polymerase with lacUV5 promoter DNA during mRNA initiation and elongation. Footprinting, methylation, and rifampicin-sensitivity changes accompanying transcription initiation. *J. Mol. Biol.*, **183**, 165–177.
35. Sippel,A. and Hartmann,G. (1968) Mode of action of rafamycin on the RNA polymerase reaction. *Biochim. Biophys. Acta*, **157**, 218–219.
36. Ishihama,A. (2000) Functional modulation of Escherichia coli RNA polymerase. *Annu. Rev. Microbiol.*, **54**, 499–518.
37. Bailey,T.L. and Gribskov,M. (1998) Methods and statistics for combining motif match scores. *J. Comput. Biol.*, **5**, 211–221.
38. Liu,X., Brutlag,D.L. and Liu,J.S. (2001) BioProspector: discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes. *Pac. Symp. Biocomput.*, 127–138.
39. Kuczynska-Wisnik,D., Laskowska,E. and Taylor,A. (2001) Transcription of the ibpB heat-shock gene is under control of sigma(32)- and sigma(54)-promoters, a third regulon of heat-shock response. *Biochem. Biophys. Res. Commun.*, **284**, 57–64.
40. Liu,X. and Matsumura,P. (1996) Differential regulation of multiple overlapping promoters in flagellar class II operons in Escherichia coli. *Mol. Microbiol.*, **21**, 613–620.
41. Wade,J.T., Roa,D.C., Grainger,D.C., Hurd,D., Busby,S.J., Struhl,K. and Nudler,E. (2006) Extensive functional overlap between sigma factors in Escherichia coli. *Nat. Struct. Mol. Biol.*, **13**, 806–814.
42. Schneider,T.D. and Stephens,R.M. (1990) Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res.*, **18**, 6097–6100.
43. Gross,C.A. (1996) *Function and Regulation of Heat Shock Proteins*. ASM Press, Washington, DC.
44. Yura,T., Nagai,H. and Mori,H. (1993) Regulation of the heat-shock response in bacteria. *Annu. Rev. Microbiol.*, **47**, 321–350.
45. Takashi Yura,M.K. and Morita,M.T. (2000) *The Heat Shock Response: Regulation and Function*. ASM Press, Washington, DC.
46. Kutsukake,K., Ohya,Y. and Iino,T. (1990) Transcriptional analysis of the flagellar regulon of Salmonella typhimurium. *J. Bacteriol.*, **172**, 741–747.
47. Komeda,Y. (1986) Transcriptional control of flagellar genes in Escherichia coli K-12. *J. Bacteriol.*, **168**, 1315–1318.
48. Brun,Y.V. and Shapiro,L. (1992) A temporally controlled sigma-factor is required for polar morphogenesis and normal cell division in Caulobacter. *Genes Dev.*, **6**, 2395–2408.
49. Klose,K.E. and Mekalanos,J.J. (1998) Distinct roles of an alternative sigma factor during both free-swimming and colonizing phases of the Vibrio cholerae pathogenic cycle. *Mol. Microbiol.*, **28**, 501–520.
50. Arora,S.K., Ritchings,B.W., Almira,E.C., Lory,S. and Ramphal,R. (1997) A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in Pseudomonas aeruginosa in a cascade manner. *J. Bacteriol.*, **179**, 5574–5581.
51. Ritchings,B.W., Almira,E.C., Lory,S. and Ramphal,R. (1995) Cloning and phenotypic characterization of fleS and fleR, new response regulators of Pseudomonas aeruginosa which regulate motility and adhesion to mucin. *Infect. Immun.*, **63**, 4868–4876.
52. Reitzer,L.J. and Magasanik,B. (1986) Transcription of glnA in E. coli is stimulated by activator bound to sites far from the promoter. *Cell*, **45**, 785–792.
53. Ishimoto,K.S. and Lory,S. (1989) Formation of pilin in Pseudomonas aeruginosa requires the alternative sigma factor (RpoN) of RNA polymerase. *Proc. Natl Acad. Sci. USA*, **86**, 1954–1957.
54. Ko,M. and Park,C. (2000) Two novel flagellar components and H-NS are involved in the motor function of Escherichia coli. *J. Mol. Biol.*, **303**, 371–382.
55. Shin,S. and Park,C. (1995) Modulation of flagellar expression in Escherichia coli by acetyl phosphate and the osmoregulator OmpR. *J. Bacteriol.*, **177**, 4696–4702.
56. Stojiljkovic,I., Baumler,A.J. and Hantke,K. (1994) Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated Escherichia coli genes by a fur titration assay. *J. Mol. Biol.*, **236**, 531–545.
57. Lehnen,D., Blumer,C., Polen,T., Wackwitz,B., Wendisch,V.F. and Unden,G. (2002) LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in Escherichia coli. *Mol. Microbiol.*, **45**, 521–532.
58. Barker,C.S., Pruss,B.M. and Matsumura,P. (2004) Increased motility of Escherichia coli by insertion sequence element integration into the regulatory region of the flhD operon. *J. Bacteriol.*, **186**, 7529–7537.
59. Buck,M., Gallegos,M.T., Studholme,D.J., Guo,Y. and Gralla,J.D. (2000) The bacterial enhancer-dependent sigma(54) (sigma(N)) transcription factor. *J. Bacteriol.*, **182**, 4129–4136.
60. Elderkin,S., Jones,S., Schumacher,J., Studholme,D. and Buck,M. (2002) Mechanism of action of the Escherichia coli phage shock protein PspA in repression of the AAA family transcription factor PspF. *J. Mol. Biol.*, **320**, 23–37.
61. Cannon,W., Bordes,P., Wigneshweraraj,S.R. and Buck,M. (2003) Nucleotide-dependent triggering of RNA polymerase-DNA interactions by an AAA regulator of transcription. *J. Biol. Chem.*, **278**, 19815–19825.
62. Joly,N., Schumacher,J. and Buck,M. (2006) Heterogeneous nucleotide occupancy stimulates functionality of phage shock protein F, an AAA + transcriptional activator. *J. Biol. Chem.*, **281**, 34997–35007.
63. Paulsen,I.T., Banerjei,L., Myers,G.S., Nelson,K.E., Seshadri,R., Read,T.D., Fouts,D.E., Eisen,J.A., Gill,S.R., Heidelberg,J.F. *et al.* (2003) Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. *Science*, **299**, 2071–2074.
64. Ballado,T., Camarena,L., Gonzalez-Pedrajo,B., Silva-Herzog,E. and Dreyfus,G. (2001) The hook gene (flgE) is expressed from the flgBCDEF operon in Rhodobacter sphaeroides: study of an flgE mutant. *J. Bacteriol.*, **183**, 1680–1687.
65. Garcia,N., Campos,A., Osorio,A., Poggio,S., Gonzalez-Pedrajo,B., Camarena,L. and Dreyfus,G. (1998) The flagellar switch genes fliM and fliN of Rhodobacter sphaeroides are contained in a large flagellar gene cluster. *J. Bacteriol.*, **180**, 3978–3982.
66. Poggio,S., Aguilar,C., Osorio,A., Gonzalez-Pedrajo,B., Dreyfus,G. and Camarena,L. (2000) sigma(54) Promoters control expression of genes encoding the hook and basal body complex in Rhodobacter sphaeroides. *J. Bacteriol.*, **182**, 5787–5792.
67. Spohn,G. and Scarlato,V. (1999) Motility of Helicobacter pylori is coordinately regulated by the transcriptional activator FlgR, an NtrC homolog. *J. Bacteriol.*, **181**, 593–599.
68. Jagannathan,A., Constantinidou,C. and Penn,C.W. (2001) Roles of rpoN, fliA, and flgR in expression of flagella in Campylobacter jejuni. *J. Bacteriol.*, **183**, 2937–2942.