Supplementary Materials and Methods

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 FlhDC/σ^F under the control of the anhydrotetracycline (aTc)-regulated promoter, aTc was added at a final concentration of 100 ng/ml as described previously (1).

E. coli MG1655 WT strain as well as deletion mutant strains were grown overnight in MOPS minimal media at 37°C in an air shaker with vigorous aeration (225 rpm). 2 mL 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 pre-chilled 1.5 mL Eppendoff tube and then immediately put on ice for 1 minute before being centrifuged at 10,000 g (12,000 rpm for BECKMAN Microfuge^R) for 10 minutes at 4°C. The supernatant was removed and the cell pellet resuspended immediately in 40 μl lysis buffer (1XSDS) and heated at 75°C for 5 minutes to quickly lyse the cells and prevent changes in the intracellular levels of the sigma factors being measured. The *fliA* deletion strain was confirmed to have lost expression of σ^F compared to the expression of σ^F in wild-type MG1655 by Western blot analysis using a monoclonal antibody against σ^F (2).

Instead of using a σ^{32}-inducible strain as shown in previous σ^{32} regulon studies (1), we used strains carrying a plasmid with either an aTc-inducible FlhDC or σ^F 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} regulon paper.

RNA isolation, cDNA synthesis, labeling and hybridization

For preparing the total RNA for microarray experiments, 15 mL samples of culture (corresponding to 7.5x 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 minutes after induction in FlhDC or σ^F 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 5,800 g for 20 minutes 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. 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 50-100bp. 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 (Hewlett-Packard).

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 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 4,200 known open reading frames and over 1,350 intergenic regions. A given gene is represented by 15 different 25-mer oligonucleotides that are designed to be complementary to the target sequence (3-5). 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.

Reverse transcriptase (RT)-PCR

Total RNA was isolated using the Epicentre MasterPure RNA Purification kits. cDNA was reverse transcribed from a mixture containing 5 µg total RNA, 250 ng random hexamers and Invitrogen Superscript II enzyme system at a volume of 30 µl (see RNA isolation, cDNA synthesis, labeling and hybridization). Reverse transcription was carried out at 42ºC for 90 minutes.

PCR protocol. Each reaction was done in a final volume of 25 µl containing 0.1 µl of cDNA, 2.5 µl of Taq 10x buffer, 0.5 µl of dNTP (10 mM), 25 pmol/primer, 0.1 µl of Taq polymerase (5 U). The following cycles were applied: 5 min at 95°C; 24 cycles (for semi-

quantitative PCRs) or 35 cycles (for all other PCRs) of: 30 s at 92°C; 30 s at 56°C; 60 s at 72°C. Ten microliter PCR mixtures were loaded on a 1.2% agarose gel for electrophoresis and analysis.

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. (6). Purified sigma factors and monoclonal antibodies (MAbs) were made as described in Anthony et al (2). 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^F (1RF18) for measuring the intracellular level changes of σ^F in cells. Fluorescent dye, IC5-OSu (Dojindo), was used to label the primary antibodies according to previously described methods (7). 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 (1). Signal intensities of the bands were quantified using the ImageQuant program.

Supplemental Figure Legends

Figure S1: Construction of *E. coli* in-frame deletion strains. In order to disrupt the expression of target gene(s) in *E. coli*, we used a simple and highly efficient method (8,9) to prepared in-frame deletion strains. In this procedure, we generated PCR products by using primers with 60- to 70-nt extensions that are homologous to regions adjacent target gene(s) and a template plasmid carrying kanamycin-antibiotic resistance genes. The PCR generated linear DNA was then electroporated into competent cells harboring the pKD46 plasmid that are induced with arabinose to activate expression of the λRed system (γ, β, and *exo*) for homologous recombination in *E. coli*. Deletion mutants were isolated as kanamycin antibiotic-resistant colonies after introducing the respective PCR products into bacteria genome. The temperature-sensitive replication allows easily curing of the pKD46 plasmid from the resultant mutants.

Figure S2: Purification CRP protein. CRP overexpression strain was constructed using plasmid pET28b from Novagen. After overexpression, CRP protein (as shown in lane B10, B11 and B12) was purified from the soluble fraction (as shown in lane B4) by Ni-NTA chromatography. CRP protein (shown in lane C5, C6 and C7 as output) was further purified from the eluted protein fractions of the Ni-NTA column (shown in Lane C4 as input) by chromatography on a HiLoad 16/60 superdex 200 size exclusion column (Amersham Bioscience).

Figure S3: Transcriptional level changes of CRP-dependent genes in the cyaA deficient strain in response with different carbon sources. The transcript abundance of CRP-dependent genes, *cstA*, *cpdB* and *flhD*, show no significant changes in cyaA deficient cells grown on different carbon sources. CyaA encodes adenylate cyclase that is required to catalyze the intramolecular transfer of the adenylyl group of ATP from pyrophosphate to the 3'-hydroxyl group to form cyclic AMP (10).

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Table SI: Transcriptional levels of all 53 known flagellar genes are downregulated in FlhDC deletion strain

b no. [a]	Gene	Product	Function	*flhDC* deletion
b1881	cheZ [b*]	chemotactic response, CheY protein phosphatase	Chemotaxis and mobility	-181.6 [c]
b1887	cheW*	purine-binding chemotaxis protein; regulation	Chemotaxis and mobility	-108.8
b1889	motB*	enables flagellar motor rotation, linking torque machinery to cell wall	Chemotaxis and mobility	-82.1
b1890	motA*	proton conductor component of motor, torque generator	Chemotaxis and mobility	-40.5
b1886	tar*	methyl-accepting chemotaxis protein II, aspartate sensor receptor	Chemotaxis and mobility	-31.8
b1888	cheA*	chemotactic sensory histidine kinase (soluble) in two-component regulatory system with CheB and CheY, senses chemotactic signal	Chemotaxis and mobility	-29.8
b1883	cheB*	chemotactic response regulator; methylesterase, in two-component regulatory system with CheA, regulates chemotactic response	Chemotaxis and mobility	-20.5
b1882	cheY*	chemotactic response regulator in two-component regulatory system with CheA, transmits signals to FliM flagelllar motor component	Chemotaxis and mobility	-15.2
b1884	cheR*	chemotactic response, glutamate methyltransferase	Chemotaxis and mobility	-14.5
b1885	tap*	methyl-accepting chemotaxis protein IV, peptide sensor receptor	Chemotaxis and mobility	-8.9
b4355	tsr*	methyl-accepting chemotaxis protein I, serine sensor receptor	Chemotaxis and mobility	-7.8
b1421	trg*	methyl-accepting chemotaxis protein III, ribose and galactose sensor receptor	Chemotaxis and mobility	-2.0
b3072	aer*	aerotaxis sensor receptor, senses cellular redox state or proton motive force	Degradation of small molecules	-2.7
b1880	flhB	putative part of export apparatus for flagellar proteins	Not classified	-4.5
b1920	fliY	cysteine transport protein (ABC superfamily)	Not classified	-2.6
b1944	fliL	flagellar biosynthesis	Surface structures	-200.9
b1080	flgI	putative flagella basal body protein	Surface structures	-186.8
b1073	flgB	flagellar biosynthesis; cell-proximal portion of basal-body rod	Surface structures	-181.6
b1923	fliC*	flagellar biosynthesis; flagellin, filament structural protein	Surface structures	-152.2
b1074	flgC	flagellar biosynthesis; cell-proximal portion of basal-body rod	Surface structures	-143.5
b1076	flgE	flagellar biosynthesis; hook protein	Surface structures	-134.8
b1083	flgL*	flagellar biosynthesis; hook-filament junction protein	Surface structures	-110.3
b1922	fliA	sigma F (sigma 28) factor of RNA polymerase, transcription of late flagellar genes (class 3a and 3b operons)	Surface structures	-67.9
b1949	fliQ	flagellar biosynthesis	Surface structures	-65.8
b1924	fliD*	flagellar biosynthesis; filament capping protein, enables filament assembly	Surface structures	-56.9
b1948	fliP	flagellar biosynthesis	Surface structures	-55.7
b1945	fliM	flagellar biosynthesis; component of motor switch and energizing	Surface structures	-36.4
b1077	flgF	flagellar biosynthesis; cell-proximal portion of basal-body rod	Surface structures	-32.8
b1075	flgD	flagellar biosynthesis; initiation of hook assembly	Surface structures	-31.3
b1070	flgN*	flagellar biosynthesis; believed to be export chaperone for FlgK, FlgL	Surface structures	-29.9
b1078	flgG	flagellar biosynthesis; cell-distal portion of basal-body rod	Surface structures	-28.6

b1079	flgH	flagellar biosynthesis; basal-body outer-membrane L (lipopolysaccharide layer) ring protein	Surface structures	-28.4
b1071	flgM*	anti-FliA (anti-sigma) factor; also known as RflB protein	Surface structures	-26.5
b1942	fliJ	flagellar fliJ protein	Surface structures	-26.4
b1082	flgK*	flagellar biosynthesis; hook-filament junction protein 1	Surface structures	-26.3
b1879	flhA	putative export protein for flagellar biosynthesis	Surface structures	-23.3
b1947	fliO	flagellar biosynthesis	Surface structures	-22.2
b1946	fliN	flagellar biosynthesis; component of motor switch and energizing	Surface structures	-21.6
b1925	fliS*	flagellar biosynthesis; repressor of class 3a and 3b operons (RflA activity)	Surface structures	-19.4
b1941	fliI	flagellum-specific ATP synthase	Surface structures	-13.5
b1072	flgA	flagellar biosynthesis; assembly of basal-body periplasmic P ring	Surface structures	-13.2
b1950	fliR	putative flagellar biosynthetic protein, putative regulator	Surface structures	-12.3
b1878	flhE	flagellar protein	Surface structures	-11.9
b1937	fliE	flagellar biosynthesis; basal-body component	Surface structures	-11.5
b1938	fliF	flagellar biosynthesis; basal-body MS(membrane and supramembrane)-ring and collar protein	Surface structures	-10.7
b1943	fliK	flagellar hook-length control protein	Surface structures	-10.4
b1891	flhC	transcriptional activator of flagellar class II biosynthesis, tetramer with FlhD	Surface structures	-8.7
b1939	fliG	flagellar biosynthesis; component of motor switching and energizing	Surface structures	-7.8
b1926	fliT*	flagellar biosynthesis; putative export chaperone for FliD	Surface structures	-7.5
b1081	flgJ	flagellar biosynthesis	Surface structures	-6.0
b1892	flhD	transcriptional activator of flagellar class II biosynthesis, tetramer with FlhC	Surface structures	-5.7
b1940	fliH	flagellar biosynthesis; putative export of flagellar proteins	Surface structures	-4.4
b1921	fliZ	putative regulator of FliA	Unknown	-52.5

[a] b no. indicates Blattner number
[b] It is possible that one gene has several different gene names
[c] Numbers indicate fold change relative to wild-type strain
* Known to be class III genes, transcribed by σ^F

Table SII: Additional candidate genes for FlhDC regulon[d]

b no.[a]	Gene	Product	Function	*flhDC* deletion
b1904	yecR[b]	conserved hypothetical protein	Unknown	-25.1[c]
b1044	ymdA	conserved hypothetical protein	Unknown	-14.6
b0306	ykgE	putative oxidoreductase	Not classified	-5.3
b3435	gntU_2	split gene, low-affinity gluconate transport permease protein in GNT I system, fragment 2	Transport of small molecules	-5.2
b3666	uhpT	hexose phosphate transport protein (MFS family)	Transport of small molecules	-5.1
b0625	ybeH	unknown	Unknown	-4.5
b3543	dppB	dipeptide transport protein 1 (ABC superfamily, membrane)	Protein, peptide secretion	-4.2
b0971	serT	serine tRNA 1	tRNA	-4.2
b3436	gntU_1	split gene, low-affinity gluconate transport permease protein in GNT I system, fragment 1	Transport of small molecules	-4.0
b4077	gltP	glutamate:aspartate symporter (DAACS family)	Transport of small molecules	-3.4
b1722	ydiY	conserved hypothetical protein	Unknown	-3.3
b1084	rne	RNase E: endoribonuclease for rRNA processing and mRNA degradation	Degradation of RNA	-3.2
b0974	hyaC	putative Ni/Fe-hydrogenase, 1 b-type cytochrome subunit	Energy metabolism, carbon: Aerobic respiration	-3.2

[a] b no. indicates Blattner number
[b] It is possible that one gene has several different gene names.
[c] Numbers indicate fold change relative to wild-type strain.
[d] Gene downregulated >=3 fold

Figure S1:

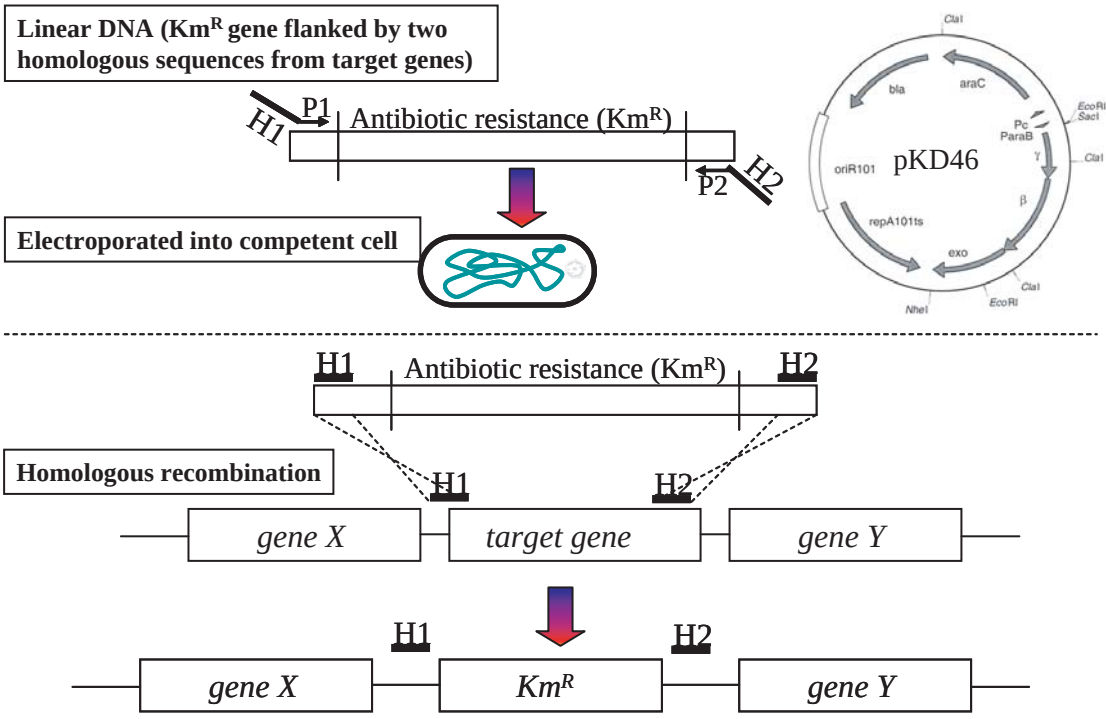
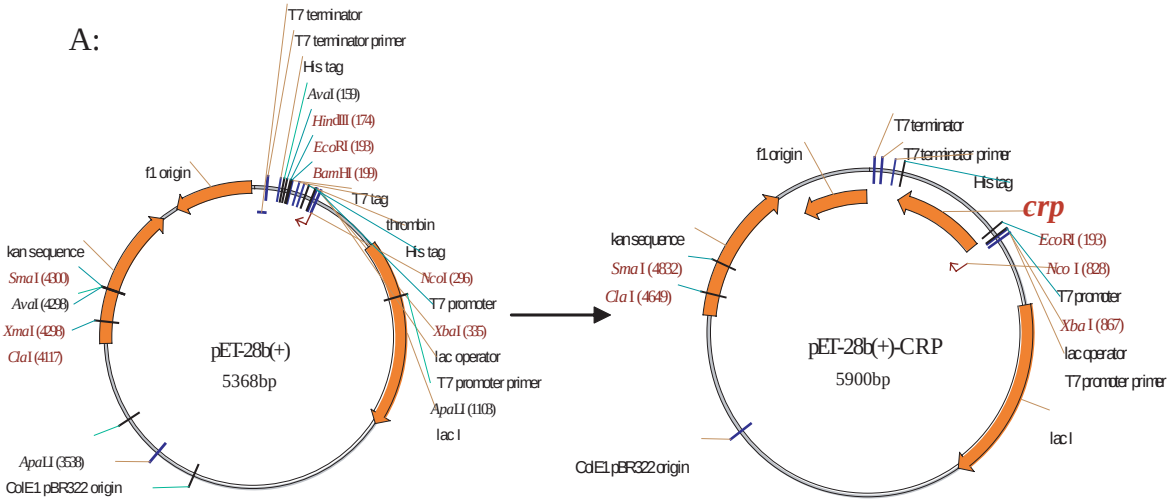

Linear DNA (Km^R gene flanked by two homologous sequences from target genes)
P1
H1
Antibiotic resistance (Km^R)
P2
H2
Electroporated into competent cell
ClaI
bla
araC
EcoRI SacI
Pc ParaB
oriR101
pKD46
ClaI
repA101ts
exo
ClaI
NheI
EcoRI
H1
Antibiotic resistance (Km^R)
H2
Homologous recombination
H1
H2
gene X
target gene
gene Y
H1
H2
gene X
Km^R
gene Y

Figure S2:

A:



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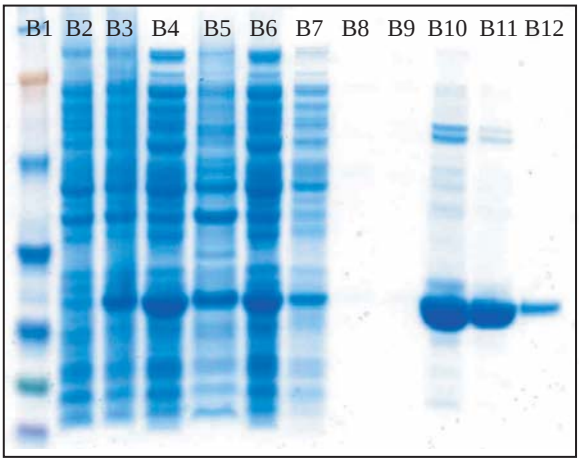

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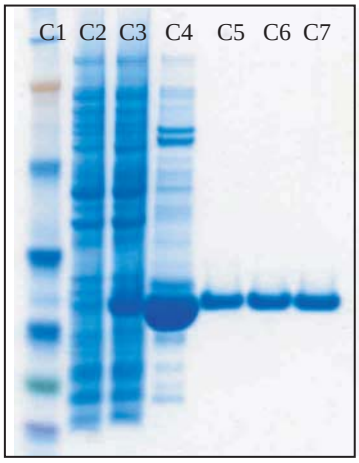
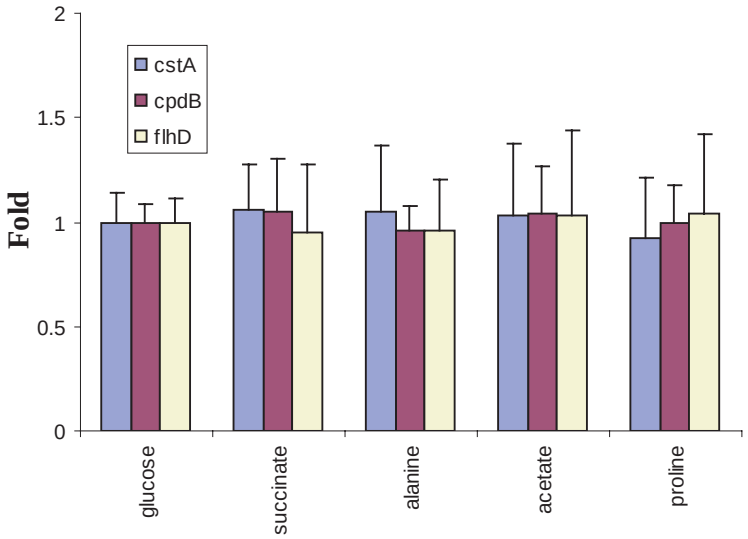


Figure S3:


2
1.5
1
0.5
0
Fold
cstA
cpdB
flhD
glucose
succinate
alanine
acetate
proline