Supplementary Data for:

The global repressor FliZ antagonizes gene expression by σ^S-containing RNA polymerase due to overlapping DNA binding specificity

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Supplementary Methods

Generation of strains with chromosomal knockout mutations

Strains used in this study are derivatives of *E. coli* K-12 strain W3110 (1), carrying Δ*lacU169* with the exception of the W3110 wild-type strain used for the primer extension experiment.. The *fliZ::kan* (2) and *mlrA::kan* (3) alleles were described previously. Non-polar in-frame-deletion mutations were obtained by flipping out the insertion cassettes (4). Mutations were transferred by P1 transduction (5).

Construction of plasmids and chromosomal lacZ fusions

The primers used for plasmid constructions are listed in Table S1 (see below). pFliZ was described previously (2). pFliZ and pFliZ-R108A are derivatives of pCAB18 (6), which is a *tac* promoter expression plasmid based on the low copy number vector pACYC184 (7). Replacement of R108 of FliZ by an alanine residue was achieved by a four primer/two-step polymerase chain reaction (PCR) mutagenesis protocol previously described (8). As external primers the ones used for cloning *fliZ* into pCAB18 (2) were used again. The internal mutagenic primers are listed in Table S1.

The chromosomal transcriptional *flhDC::lacZ* fusions (*flhDC1::lacZ, flhDC2::lacZ, flhDC3::lacZ*) were isolated using the fusion vector pCAB6 (6). For construction of these three fusions, *the* primers P*flhDC1-Bam*HI and P*flhDC1-Hind*III, P*flhDC1-Bam*HI and P*flhDC2-Hind*III, and P*flhDC3-Bam*HI and P*flhDC1-Hind*III, respectively, were used. All primers are listed in Table S1. All reporter fusions were transferred to the *att*(λ) location of the chromosome via phage λRS45 or λRS74 (9). Single lysogeny was tested by a PCR approach (10). The following *lacZ* fusions were described earlier: *yciR::lacZ* (3), *flgA::lacZ* (6), *yhjH::lacZ* (2).

For overexpression and purification, FliZ and FliZ-R108A with N-terminal intein tags were expressed from pTYB12 (New England Biolabs). *fliZ* and the gene encoding FliZ-R108A were cloned into pTYB12 using the primers listed in Table S1 and plasmids pFliZ or pFliZ-R108A as templates for PCR reactions.

Generation of mutated mlrA promoter DNA-fragments used for electrophoretic mobility assays

For generation of the mutated *mlrA* promoter variants a derivative of the *lacZ* fusion vector pJL28 containing a DNA fragment comprising the *mlrA* promoter region and part of the translated region (2) was used as a template and pJL28 derivatives carrying mutated *mlrA* promoter DNA mutations were generated by the four primer/two-step PCR mutagenesis protocol (see above) using the pJL-upstream and pJL-downstream external primers and the internal mutagenic primers listed in Table S1 and subsequent digestion of PCR fragments with *Bam*HI and *Hind*III. The resulting plasmids served as templates for the isolation of mutated *mlrA* promoter DNA-fragments used for electrophoretic mobility assays. All primers are listed in Table S1.

RNA preparation and primer extension

RNA preparation and primer extension was performed as described in (11), with alterations. Total RNA was prepared with SV RNA Isolation Kit (Promega) of cells growing in LB medium at 28°C to an OD_{578} of 4. Wildtype W3110 (or its *mlrA::kan* mutant derivative) or W3110 containing a derivative of the *lacZ* fusion vector pJL28 containing a DNA fragment comprising the *mlrA* promoter region and part of the translated region (2), were used for RNA isolation. For the detection of the transcriptional start site by primer extension, the primer listed in Table S1 was used as a primer and the reactions were performed at 45°C for 60 min using 10 μg of total RNA and 200 U SUPERSCRIPT II (Invitrogen) and stopped by a 70°C incubation step for 15 min. The primer was labeled previously with gamma-^{32}P-ATP and T4 PNK (Fermentas). As a reference, sequencing reactions were performed with the same labeled

primer using the CycleReader DNA Sequencing Kit (Fermentas). Reactions were stopped by addition of STOP solution from the kit. After heating to 90°C for 3 min, samples were subjected to electrophoresis on 6% PAA 7M Urea sequencing gels and dried before being autoradiographed using a FLA-2000G Imager (Fuji Photo Film, Japan).

SDS page and immunoblot analysis

Sample preparation for SDS-PAGE and immunoblot analysis were performed as described previously (12). 3 or 6 μg of cellular protein was applied per lane. Polyclonal sera against FliZ, (custom-made by Pineda-Antikörper-Service, Berlin), goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) and a chromogenic substrate (BCIP/NBT; Boehringer Mannheim) were used. For the determination of cellular FliZ-levels, FliZ band intensities were calculated using the quantification software Image Gauge. Defined amounts of purified FliZ were used as a reference to determine the molecules of FliZ per µg total cellular protein, taking into account that during log phase growth in LB, 1 ml of cells of an OD_{578} of 1 corresponds to 107 μg total cellular protein (5). Numbers of molecules per cell were calculated based on measurements of colony forming units (cfu) per cell mass at different OD_{578} along the growth cycle as shown in Fig. S10 (see below).

Overexpression and purification of FliZ and FliZ-R108A

The *fliZ* gene and the mutant gene encoding FliZ-R108A were cloned into pTYB12 (New England Biolabs) with the primers listed in Table S1, the plasmid was transformed into strain ER2566 (New England Biolabs), cells were grown at 28°C in LB medium supplemented with 100 μg/ml ampicillin and overexpression was induced by the addition of 0.5 mM IPTG at an OD_{578} of approximately 0.8. The culture was then transferred to 16°C for overexpression and cells were harvested the next day. Cells were resuspended in buffer A (500 mM NaCl, 20 mM Tris-HCl at pH 8, 0.1 % Triton X 100, DNaseI (Roche)) and lysed using a French Press. Cell debris was removed by centrifugation and the supernatant was incubated with chitin beads (New Englang Biolabs) for 1 hour. After washing with buffer B (500 mM NaCl, 20 mM Tris-HCl at pH 8) and equilibration with buffer C (500 mM NaCl, 20 mM Tris-HCl at pH 8, 50mM DTT) the chitin beads with bound protein were incubated over night at 16-18°C for the self-cleavage reaction releasing the protein. The protein was eluted the next day with buffer B and run over a Superdex 75 (16/60) column (GE Healthcare) using buffer D (500 mM NaCl, 20 mM Tris-HCl at pH 8, 0.2 mM DTT). All purification steps were performed at 4°C and/or on ice.

Protein-DNA interaction assays

Electrophoretic mobility shift assays (EMSA) were performed in 20 µl reaction mixtures including increasing amounts (20-80 nM, only for *flhDC* bandshifts 0-320 nM were used) of purified FliZ or FliZ-R108A proteins, 6 nM DNA (DNA fragment size was 198 - 300 nucleotides; for primers used to amplify DNA fragments and to mutate promoter DNA-fragments, see supplementary table 1), 1 μg poly[d(I-C)] (Roche) as non-specific competitor DNA and reaction buffer containing 10 mM Tris-HCl at pH 7.5, 1 mM EDTA, 5 % glycerol, 10 mM NaCl and 1 mM $MgCl_2$. Reaction mixtures were incubated for 20 min at room temperature and subsequently loaded onto 5 % polyacrylamide gels. Gels were run in 0.5 x TBE buffer (14) and stained with ethidium bromide. The quantification software Image Gauge was used to quantify the photographed polyacrylamide gels.

DNaseI footprint assays were performed as described before (15) after complex formation between DIG-labeled DNA fragments (13 or 26 nM; for primers used to amplify DNA fragments, see supplementary table 1) and increasing amounts of FliZ (0.16-7.45 μM) in 20 μl reaction mixtures as described above. Protected regions were identified by comparison

with a DNA sequence ladder generated with the CycleReader DNA Sequencing Kit (Fermentas) and the same DIG-labeled primer as for generating the DNA fragment by PCR.

Limited proteolysis experiments

FliZ and FliZ-R108A were incubated with 0.2 BAEE units (12.8 ng) TPCK-Trypsin (Thermo-Scientific) (dissolved in water) per µg protein or with 1.875 ng proteinase K (Sigma) (dissolved in 50 mM Tris-HCl at pH 8, 10 mM $CaCl_2$) per µg protein in buffer D (see above) at 25°C for increasing time intervals. Proteolysis reactions were stopped by the addition of SDS-page sample buffer and digestion products were analyzed by SDS-page with subsequent Coomassie staining.

Determination of β-galactosidase activity

β-galactosidase activity was assayed by use of *o*-nitrophenyl- β-D-galactopyranoside (ONPG) as a substrate and is reported as µmol of *o*-nitrophenol per min per mg of cellular protein (5). Experiments showing the expression of *lacZ* fusions along the entire growth cycle were done at least twice, and a representative experiment is shown.

Supplementary Table[1]

I. Primers for generating DNA-fragments used for electrophoretic mobility assays

mlrA, mlrA-C-13G, mlrA-TC-14/-13GG, mlrA-T12A, mlrA-T-7A, mlrA-T-6C, mlrA-AA-22/-21TT, mlrA-C-24T	5´-CGATCACTCAAAATCGCCTGG-3 5´-TAACGCCTCTGCCACGCGCGTAACG-3´
mlrA-TR	5´- ATGGCGCTTTACACAATTGGTG-3´ 5´- CAGGCTATGTAGATTGCCGCTTTGC-3´
yciR, yciR-C-18G	5´- GCGCGCCCGGTCGCGTAATCTCCTTTCACG-3´ 5´- GACTTACATGAAATTAACGGCGGCTAAACGC-3´
rpoS	5´-ACGTTGGTCAGACCTTGCAGGT-3´ 5´-TACTGGTTGATGTACTGCTGA-3´
gadE, gadE-C-13G	5´- CAAGCTGATAACAACCAGG-3´ 5´- CTTTCAACTGCCAAAAGCCCTG-3´
hdeA	5´- CGCGTCTAAGAATGCAGTCG-3´ 5´- GCATTGCTCACAACTGGCAG-3´
yjbJ	5´- GGTTTGCCGCAACGTGACGG-3´ 3´- CGTCATATCATCATCGGTC-3´
csgD	5´- CACCGAAATATTTTTTATATGC-3´ 5´- CAATCTAGCCATTACAAATCTTA-3´
gadB	5´- TATTCGCGTAATATCTCACG-3´ 3´- GTGGAAATAGACTTCGCACC-3´
malE	5´-GGAATTTCGTGATGTTGCTTGC-3´ 5´-GGCGGAAAACATCATCGTCG-3´
gatY	5´-CACGCGCACTTTGCTACGGC-3´ 5´- ATATTGAATGCCGGAACCGC-3´
chaB	5´-CAGAAAGTGTCTGGATATCG-3´

[1] Nucleotides in bold indicate mutations introduced, nucleotides in italics indicate restriction sites.

	5´-CGGTAGAACGTGCTTTACGC-3´
ynhG	5´-GCCGCTGCACTTAGCTAAAC-3´ 5´-GCGGATAATCAACCGCCCAG-3´
malK	5´-GCGCACATAAAATCGCCACG-3´ 5´-ATACCACGACCTCGCCCCAG-3´
flhDC-long	5´-GACTGAGTCAGCCGAGAAG-3´ 5´-GCTGCAATAAGCAGAACCACC-3´
flhDC-short	5´-GACTGAGTCAGCCGAGAAG-3´ 5´-CCTAAATCGACGCAACTGTAC-3´
flgA (classII)	5´-CTGGGATCCGCTTAAATGCCTTTAC-3´ 5´-GCCAAGCTTCGTTTTATTATCAGC-3´
flgM (classIII)	5´-CATGGATCCGGGACAGGTAGTCAGCG-3´ 5´-GAACGAAGCTTACAGGCTTCAGAGG-3´

II. Primers for generating DNA-fragments for DNaseI footprint assays

mlrA	5´-DIG-CGATCACTCAAAATCGCCTGG-3´ 5´-TAACGCCTCTGCCACGCGCGTAACG-3´
yciR	5´-GCGCGCCCGGTCGCGTAATCTCCTTTCACG-3´ 5´-DIG-GACTTACATGAAATTAACGGCGGCTAAACGC-3´
gadE	5´-DIG-GTTCACGAAGGGTAAAGTTC-3´ 5´-CTTTCAACTGCCAAAAGCCCTG-3´
hdeA	5´-DIG-GATGCATCTGTAACTCATTG-3´ 5´-GCATTGCTCACAACTGGCAG-3´
flhDC	5´-TTGTGTGATCTGCATCACGC-3´ 5´-DIG-AGTTGCGATAAGCTGCAATAAGC-3´

III. Primer for determining the 5´-end of *mlrA* mRNA by primer extension:

5´-TAACGCCTCTGCCACGCGCGTAACG-3´

IV. Primers for cloning and mutagenizing the *mlrA, gadE and yciR* promoters on pJL28

pJL-upstream	5´-ACCAGCGTTTCTGGGTGAGC-3´
pJL-downstream	5´-CGCCAGCTGGCGAAAGGG-3´
mlrA-TC-14/-13GG	5´-GCAAAACTGCG**GG**TAAAGTTAAACCGGGACC-3´ 5´-GGTCCCGGTTTAACTTTA**CC**CGCAGTTTTGC-3´
mlrA-T-12A	5´-CTGCGTC**A**AAAGTTAAACCGGGAC-3´ 5´-GTCCCGGTTTAACTTT**T**GACGCAG-3´
mlrA-T-7A	5´-CTGCGTCTAAAG**A**TAAACCGGGACCTC-3´ 5´-GAGGTCCCGGTTTA**T**CTTTAGACGCAG-3´
yciR-C-18G	5´-CGTTTTTCCAAAA**G**TGGATTAC-3´ 5´- GTAATCCA**C**TTTTGGAAAAACG-3´
gadE-C-13G	5´- GAAAAGGATGA**G**ATATTCGAAACG-3´ 5´-CGTTTCGAATAT**C**TCATCCTTTTC-3´

V. Primers for cloning *fliZ* onto pTYB12

P*fliZ-Nde*I	5´-GGTGGT***CATATG***ATGGTGCAGCACCTG-3´

P*fliZ-Eco*RI	5´-TGGT***GAATTC***TCAATATATATCAGAAGAAGGCAGGCTGGAGG-3´

VI. Primers for mutagenizing *fliZ* on pCAB18

P*fliZ*-R108A-f	5´-CCCAGGTACGGTG**GC**TGAATATGTCGTTCG-3´
P*fliZ*-R108A-r	5´-CGAACGACATATTCA**GC**CACCGTACCTGGG-3´

VII. Primers used for generating *lacZ* fusions

P*flhDC1-Bam*HI	5´-CG***GGATCC***CATCCCATTTCGATTATTCC-3´
P*flhDC1-Hind*III	5´-CCC***AAGCTT***GTTTCAGCAACTCGGAGG-3´
P*flhDC2-Hind*III	5´-AGC***AAGCTT***CCTAAATCGACGCAACTGTAC-3´
P*flhDC3-Bam*HI	5´-CG***GGATCC***CGTGTAGTGACGAGTACAGTTGC-3´

Supplementary Figures

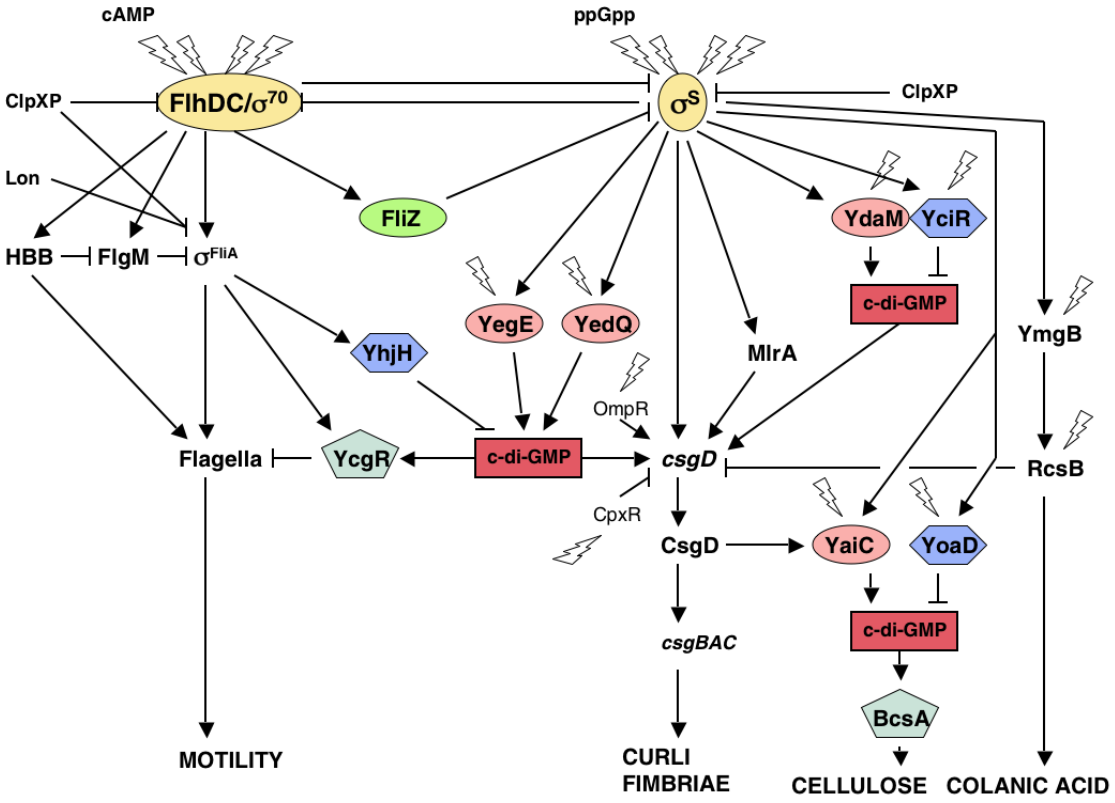

Figure S1. Inverse coordination of the motile and adhesive life-styles in *E. coli* K-12. This coordination is part of the more general transition from the foraging life-style of post-exponentially and therefore slowly growing cells to the stationary phase life-style characterized by maintenance metabolism, stress resistance, high cell density and cellular adherence. The flagellar control cascade (FlhDC/FliA) interferes with the activity of the σ^S/CsgD/curli control cascade at two levels: (i) FliZ, which is expressed from a class 2 gene in the flagellar hierarchy, is an inhibitor of σ^S activity at many σ^S-dependent promoters, including those of *ydaM*, *mlrA* and *csgD*; and (ii) the PDE YhjH, which is expressed from a class 3 gene (under σ^{FliA} control), degrades c-di-GMP and thereby keeps motility going, while not allowing the activation of transcription of *csgD* and therefore curli expression. When the flagellar control cascade (including *yhjH* expression) shuts down in mid-post-exponential phase, the DGCs YegE and YedQ, which are increasingly expressed due to now accumulating σ^S, outbalance the PDE activity of YhjH and c-di-GMP can accumulate. Via YcgR, this c-di-GMP interferes with flagellar activity, and, via an unknown effector, stimulates *csgD* transcription. In essence, this c-di-GMP control module acts as a checkpoint that allows curli expression only *after* flagellar gene expression has ceased. In parallel, a second DGC/PDE system, YdaM/YciR, is expressed under σ^S control. The latter system exclusively acts on *csgD* transcription in a way, which is not additive with the effect of the YegE/YhjH system (but both systems are essential for activation). An additional c-di-GMP control module operates downstream of CsgD expression and affects the expression of cellulose biosynthesis. Note, that only relevant genes or proteins under FlhDC and σ^S control are shown here; overall, FlhDC and σ^S activate more than 60 and 500 genes, respectively. For further details and references, see main text. This figure and legend are modified versions of a figure and legend previously published in (16) that are used here with permission.

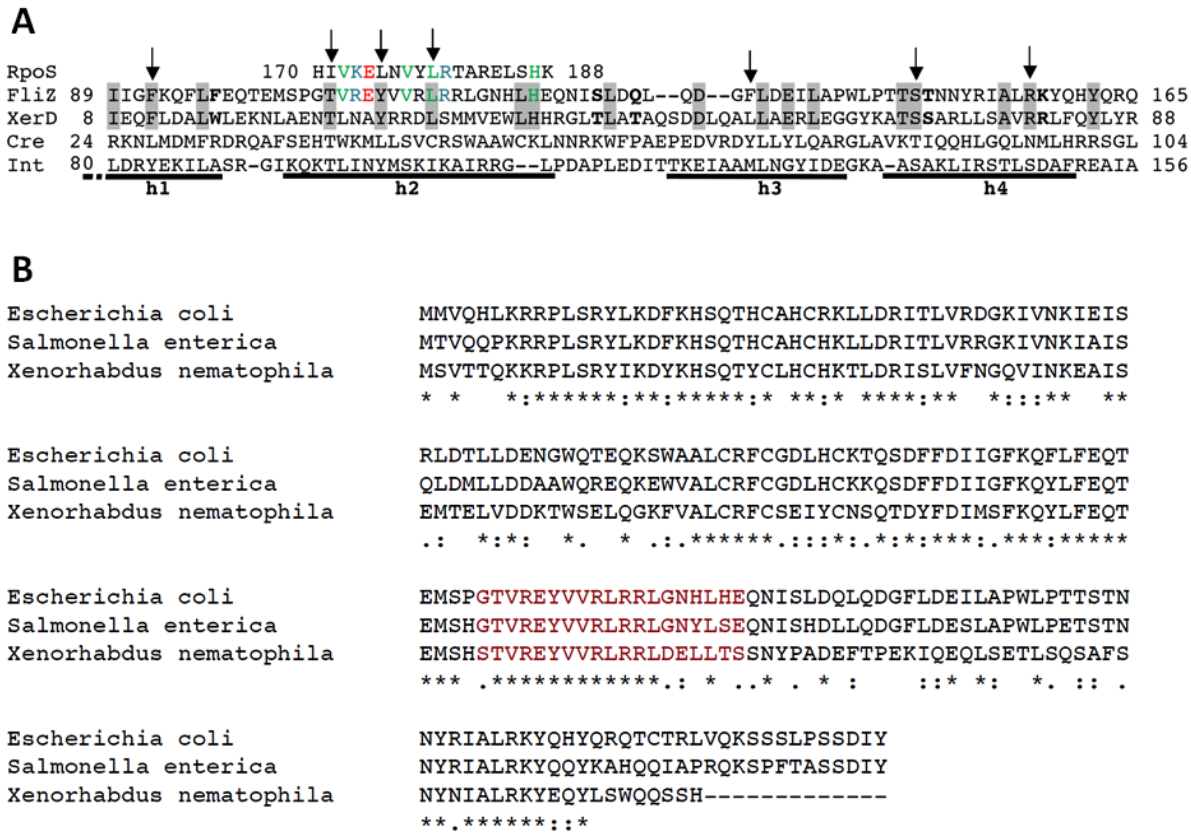

Figure S2. Homologs of FliZ. (A) Alignment of the C-terminal part of FliZ with the core-binding domain of three members of the phage integrase family and with the first alpha helix in domain 3 of σ^S (RpoS). Positions that are positively charged in both FliZ and RpoS are colored in blue, negative charges and other amino acids present in both proteins are printed in red and green, respectively. Identical residues in FliZ and XerD are highlighted by grey background and residues with similar chemical properties present at corresponding positions in both proteins are printed in bold. Arrows indicate strongly conserved residues present throughout the phage integrase family (17), which are also present in FliZ. The alpha helical composition of the core binding domain of XerD from *E. coli*, Cre from P1 phage and lambda phage integrase (Int) is indicated underneath the alignment. The alignment is based on the alignment in (17) with FliZ added after performance of a conserve domain search using the NCBI CD-search interface (18).
(B) Alignment of FliZ proteins from *Escherichia coli*, *Salmonella enterica* and *Xenorhabdus nematophila*. The alignment was performed using ClustalW2 (19). Identical residues in all three sequences are marked with an asterisk, conserved substitutions are marked with two dots and semi-conserved substitutions with one dot. The putative alpha helix, which closely resembles the first alpha helix in domain 3 of σ^S in *E. coli* (see A), and the corresponding sequences in the other two proteins are marked in red.

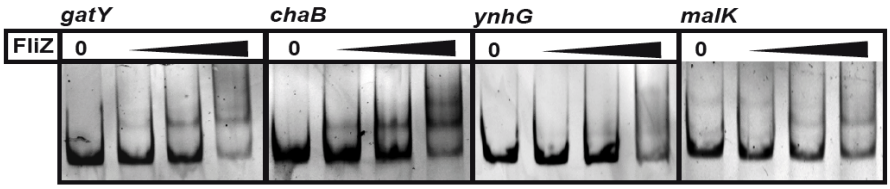

Figure S3. Electrophoretic mobility shift assays using purified FliZ and DNA-fragments containing promoter regions of FliZ-regulated genes identified in whole genome transcription profiling studies (2). *gatY* is the first gene in a σ^S-independent operon containing six genes, three of which (*gatA,B,Z*) had been identified as negatively FliZ-controlled. *chaB* and *ynhG* are positively controlled by FliZ and σ^S, while *malK* is positively FliZ-regulated, but does not require σ^S for expression.

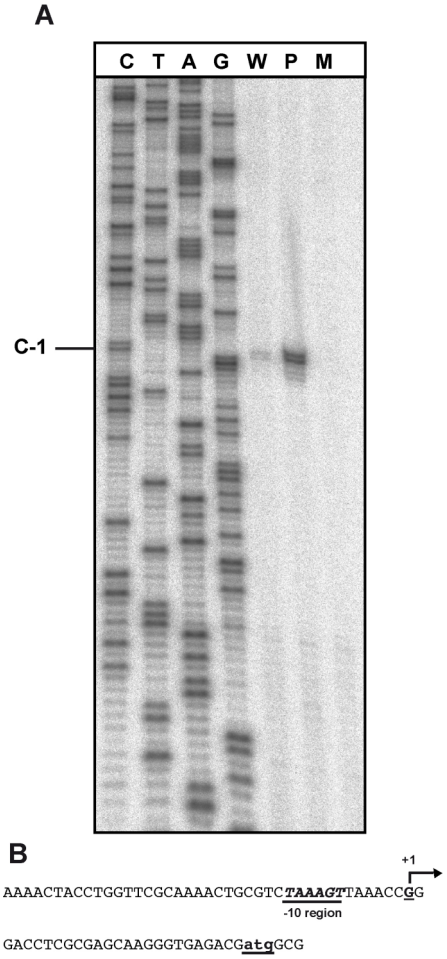

Figure S4. Determination of the 5´-end of *mlrA* mRNA by primer extension. For the primer extension experiment, strain W3110 (W) and its derivative carrying a plasmid with a DNA fragment comprising the *mlrA* promoter region and part of the translated region (P) as well as a W3110 *mlrA::kan* mutant derivative (M) were grown in LB medium at 28°C to an OD_{578} of 4, before RNA samples were taken. Primer extension was performed as detailed below. The experiment was done by F. Mika and A. Possling.

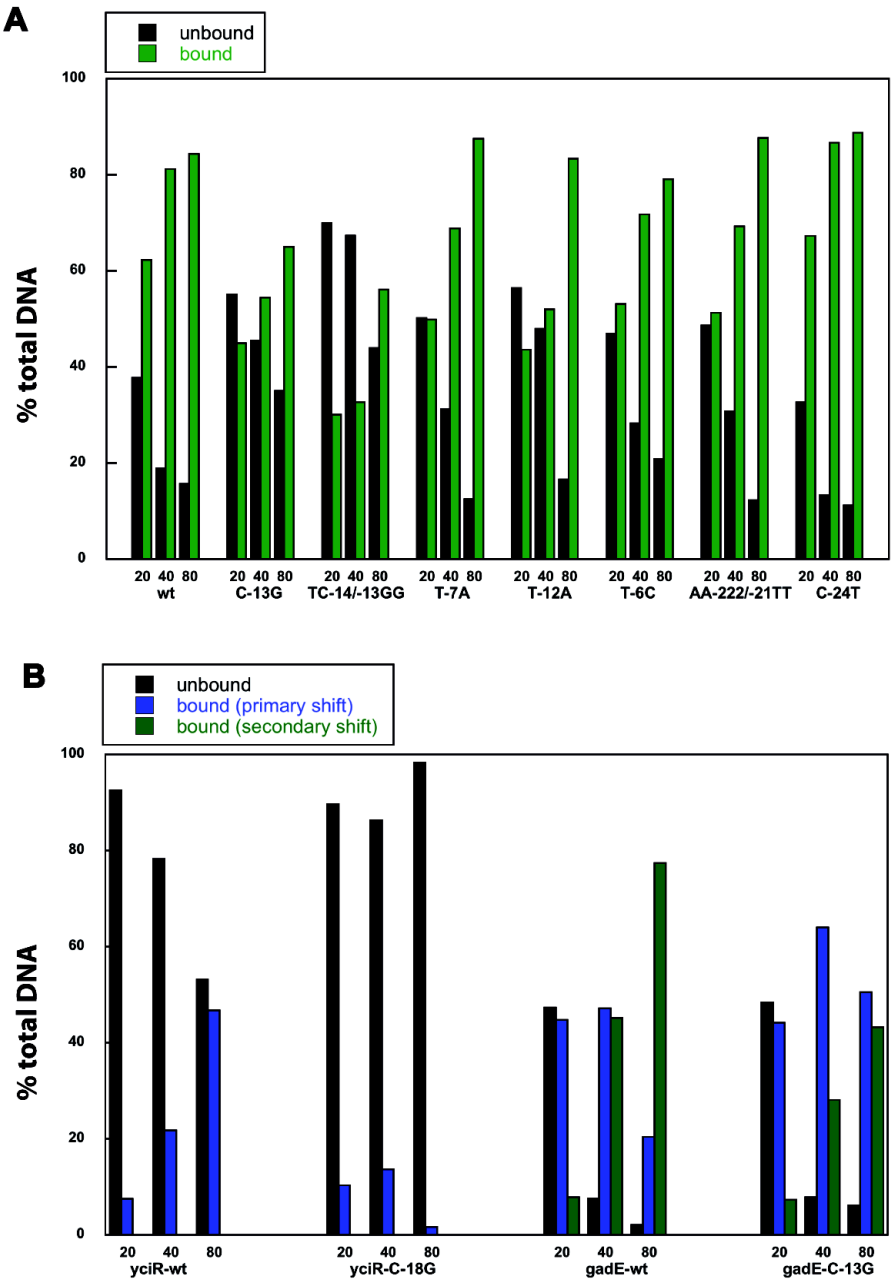

Figure S5. Quantification of electrophoretic mobility shift assays shown in Fig. 3. The photographs shown in Fig. 3B (A) and Fig. 3C (B) were used for quantifying the amount of DNA that was either bound by FliZ or remained unbound in each of the three lanes where FliZ had been added. To this end, the total amount of DNA in each lane was set to 100% and the percentage of bound and unbound DNA was subsequently calculated. Quantification was performed using the quantification software Image Gauge. 20, 40, 80 indicates the respective amount of FliZ (in nM) added to the respective EMSA mixture.

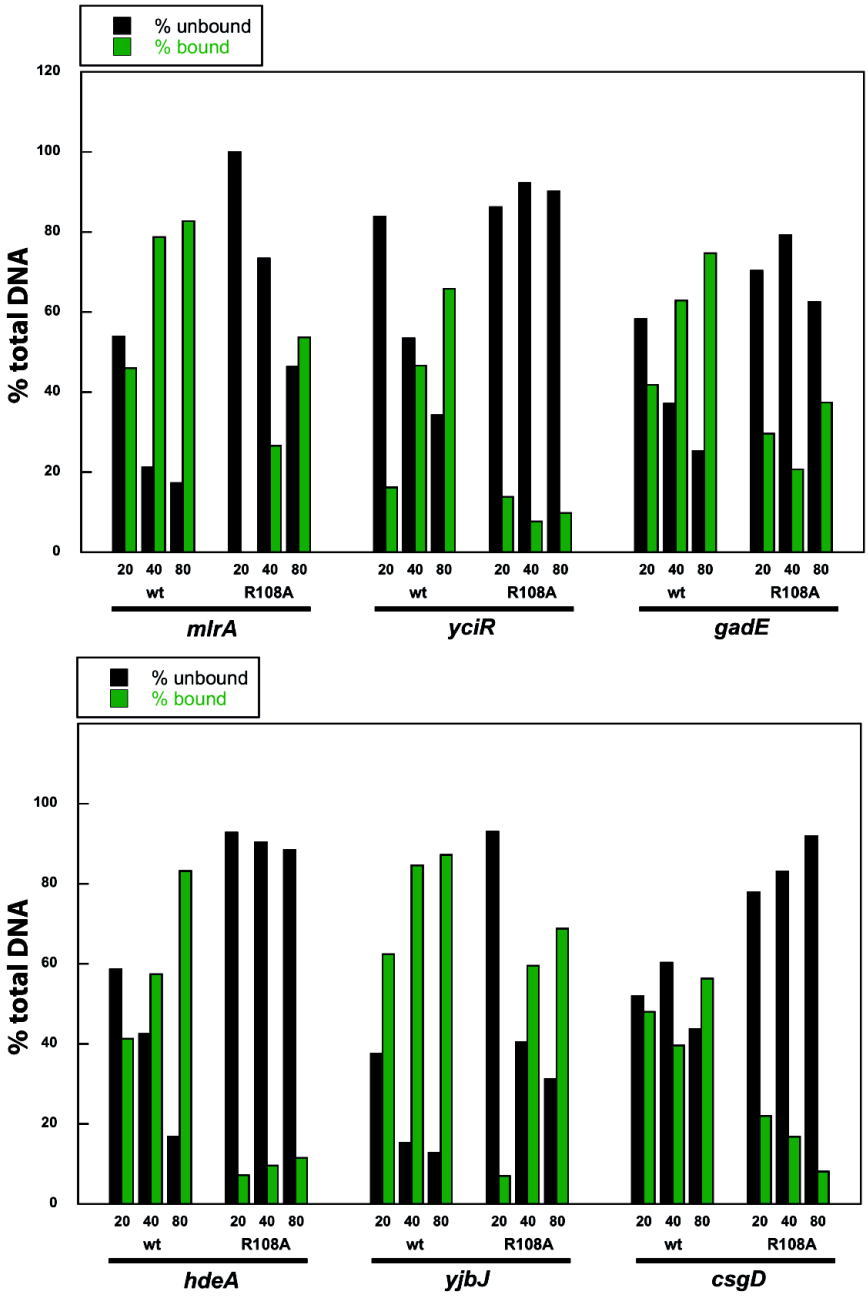

Figure S6. Quantification of electrophoretic mobility shift assays shown in Fig. 4B. The photographs shown in Fig. 4B were used for quantification as described in Fig. S5. Note that for *gadE*, *yjbJ* and *csgD*, which show multiple shifts, the primary and secondary shifts have not been listed separately but are summarized in the bound fraction of DNA. 20, 40, 80 indicates the respective amount of FliZ (in nM) added to the respective EMSA mixture.

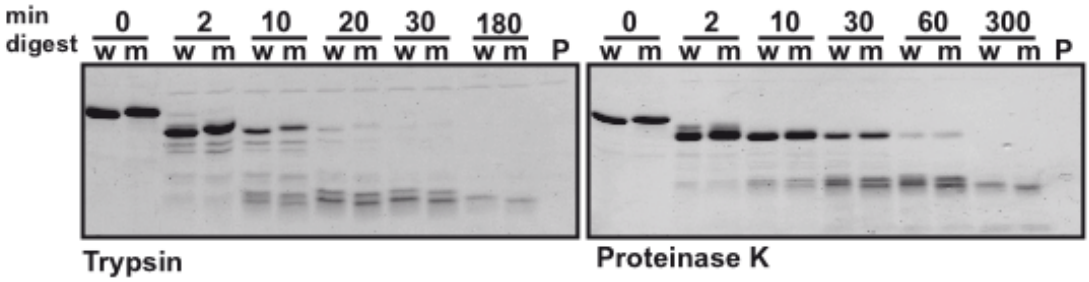

Figure S7. Limited proteolysis of purified wild-type FliZ (w) and FliZ-R108A (m). Purified proteins used in the electrophoretic mobility shift assays shown in Fig. 4B were incubated with either trypsin or proteinase K for increasing time intervals and digestion products were analyzed by SDS-page with subsequent Coomassie staining.

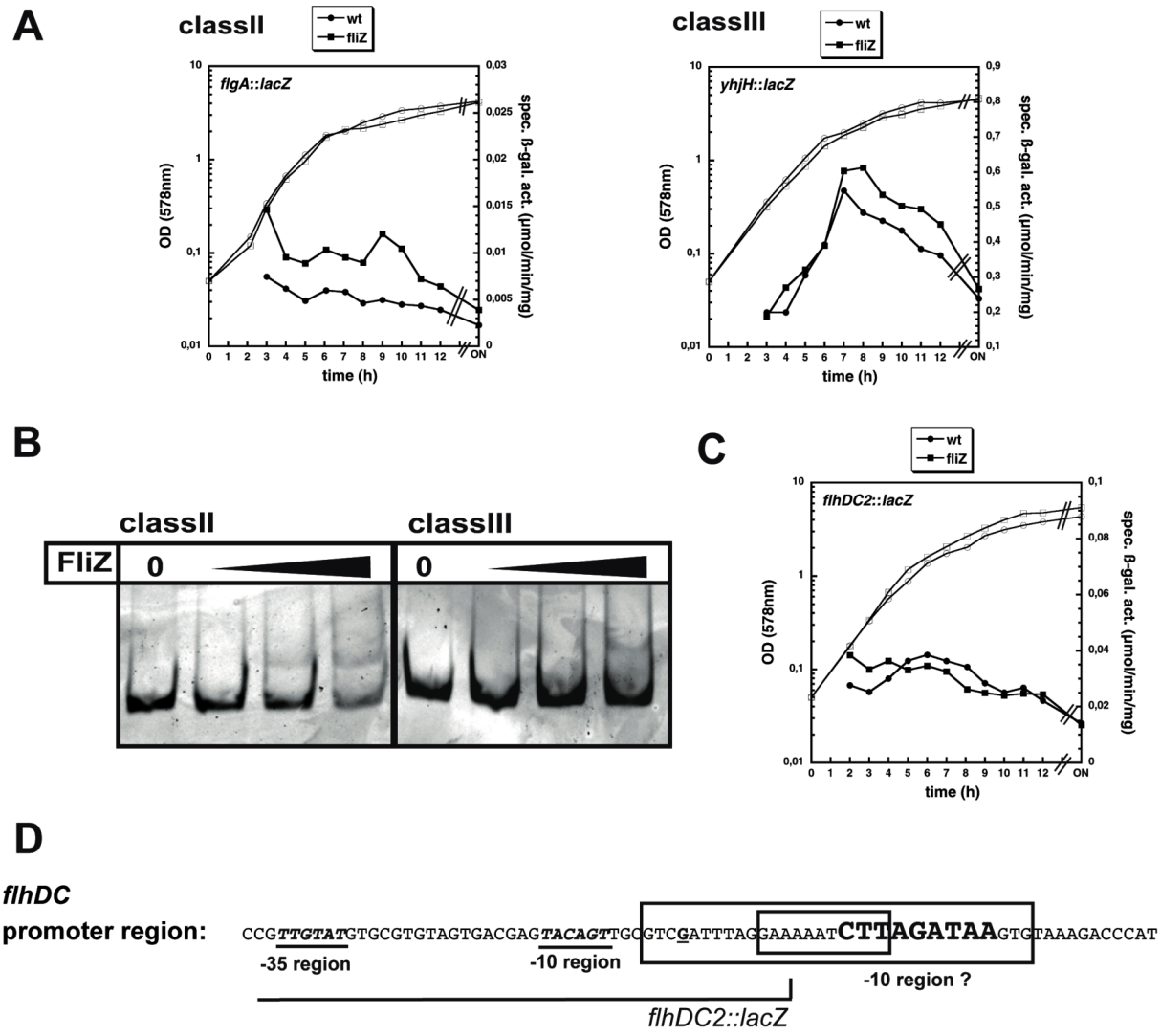

Figure S8. Role of FliZ in flagellar gene expression. (A) Expression of a single-copy transcriptional *lacZ* fusion to the class II promoter of the *flgA* gene and a single-copy translational fusion to the class III *yhjH* gene. FliZ affects expression of class II and III genes but this effect is indirect through regulation of *flhDC* because FliZ does not bind to the

promoters of class II (*flgA*) and III (*flgM*) genes *in vitro* as determined in electrophoretic mobility shift assays (B). (C) Expression of a single-copy transcriptional *lacZ* fusion to the *flhDC* promoter that does not contain the -10 region-like element downstream of the *flhDC* transcription initiation site (*flhDC2::lacZ*) does not show differential regulation in the wild-type (wt) and the Δ*fliZ* mutant. (D) Sequence of the *flhDC* promoter. Promoter elements are indicated as in Fig. 6F and the figure shows the end point of the region used for construction of the *flhDC2::lacZ* fusion.

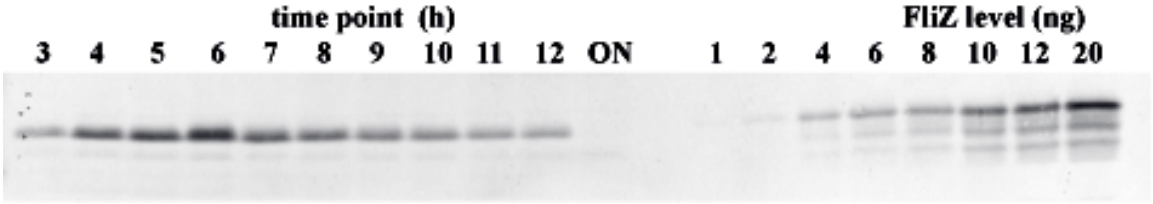

Figure S9. Quantification of cellular FliZ levels during the growth cycle. FliZ levels were determined by immunoblot analysis (the blot shown here is the one also shown in Fig. 7), using defined amounts of purified FliZ (shown on the right side of the blot) as a reference for calculating absolute cellular protein levels (data in molecules per µg total cellular protein are given in Fig. 7). FliZ levels in the overnight sample (ON) were below detection.

OD_{578}	cfu/ml	cfu/ml·OD_{578}
0.1	$2.95 \cdot 10^7$	$3.0 \cdot 10^8$
0.5	$1.48 \cdot 10^8$	$3.0 \cdot 10^8$
1.0	$2.95 \cdot 10^8$	$3.0 \cdot 10^8$
1.5	$5.89 \cdot 10^8$	$3.9 \cdot 10^8$
2.0	$8.82 \cdot 10^8$	$4.4 \cdot 10^8$
2.5	$2.08 \cdot 10^9$	$8.3 \cdot 10^8$
3.0	$3.28 \cdot 10^9$	$1.1 \cdot 10^9$
3.5	$3.93 \cdot 10^9$	$1.1 \cdot 10^9$
4.0	$4.59 \cdot 10^9$	$1.2 \cdot 10^9$
4.5	$6.42 \cdot 10^9$	$1.4 \cdot 10^9$
5.0	$8.26 \cdot 10^9$	$1.6 \cdot 10^9$
o/n (6.5)	$3.80 \cdot 10^9$	$0.6 \cdot 10^9$

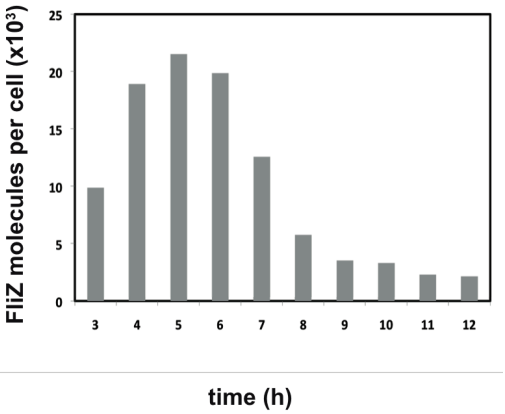

Figure S10. Determination of FliZ molecule numbers per cell during the growth cycle. In order to transform the data for cellular FliZ levels (given in Fig. 7) into numbers of molecules per cell, cell numbers were determined (colony forming units, cfu) during growth in LB at 28°C and normalized to the OD_{578}. The resulting increasing numbers of cells per cell mass reflect the successive miniaturization of *E. coli* cells at an OD_{578} beyond 1.5 until entry into stationary phase. Based on 1 ml of *E. coli* cells of an OD_{578} of 1.0 corresponding to 107 µg of total cellular protein (5), FliZ molecule numbers per cell were calculated (right panel).

Supplementary References

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