Supporting Information

Pimentel et al. 10.1073/pnas.1308241111

SI Materials and Methods

Oligonucleotides. Oligonucleotides used in this work are described in Table S2.

Plasmids. DNA vectors used in this work and their characteristics are listed in Table S3. Mini-R1 plasmids bearing *kis-kid* (mR1KK) or its toxin-dead (*kid18*) control variant (mR1Ctrl) are the same as mR1wt and mR118, as described by Pimentel et al. (1). Mini-R1 plasmids bearing *hok-sok* (mR1hs) were made by introducing the *hoksok* toxin-antitoxin (TA) pair from R1 into mR1Ctrl. For this, we first amplified the *hoksok* operon from plasmid R1*drd19* (2) by PCR, using oligonucleotides EKX-*hoksok* and *hoksok*-NH, and the resulting product was digested with XhoI and NotI. Additionally, a dsDNA bearing the *rrnB* transcription terminator, flanked by 3′-overhangs complementary to digested NotI and HindIII sites, was produced by annealing oligonucleotides N-*rrnBTr*-H sense (ss) ss and N-*rrnBTr*-H antisense (as) as. The two DNAs above were joined to each other through their common NotI site, and the resulting fragment was inserted between the XhoI and HindIII sites of plasmid mR1Ctrl, which rendered mR1hs. To make arabinose-inducible pBAD22-*copA*, antisense (as) RNA *copA* was amplified from mR1Ctrl using oligonucleotides S-*copA* and *copA*-E, and the resulting PCR product was cloned between NheI and EcoRI in plasmid pBAD22 (3). To make mR1tetO_{240}, the NsiI-XhoI DNA fragment from mR1KK containing the basic replicon of plasmid R1 was ligated to the NsiI-XhoI DNA fragment containing a gentamicin resistance gene and 240 *tetO* sites from plasmid pLAU44 (4). Expression of TetR-YFP and LacI-CFP, required to visualize mR1-, *oriC*- and *ter*-foci in ILO strains (see next section), was carried out using plasmid pWX6, as described by Lau et al., (4).

In addition to the mini-R1 plasmids above, expression of Kid was induced using vectors with different replicon types, antibiotic resistance genes, and promoter types in our experiments. Thermoinducible pPrTs$_{LWC}$Kid and pPrTs$_{HC}$Kis plasmids (and their parental vectors, pPrTs$_{LWC}$ and pPrTs$_{HC}$) were described previously (1). To make pPrTs$_{MC}$Kid and its control vector pPrTs$_{MC}$, plasmids pPrTs$_{LWC}$Kid and pPrTs$_{LWC}$ were digested with EcoRI, blunt-ended with Klenow (New England Biolabs), and digested again with PstI. DNA products containing the thermosensitive λ-repressor (cI^{Ts}), its regulated promoter (*Pr*λ), and (in the case of pPrTs$_{LWC}$Kid) *kid* were inserted into pACYC177 (5) digested with BamHI and processed with Klenow, and subsequently cleaved with PstI. Arabinose-inducible vectors expressing Kid (or its RNase dead mutant Kid18) were also used. To make pBAD22Kid and its control, pBAD22Kid18, we amplified *kid* and *kid18* from plasmids mR1wt and mR118 (1), respectively, using oligonucleotides N-*kid* and *kid*-S. Resulting PCR products were digested with NcoI and SphI, and cloned between the same sites of plasmid pBAD22 (3). To make p177Pr$_{ara}$Kid and p177Pr$_{ara}$Kid18, arabinose-responsive transcriptional cassettes encoding *kid* and *kid18* were excised from pBAD22Kid and pBAD22Kid18 with NsiI and ScaI, and inserted between the PstI and HincII sites of plasmid pACYC177. Tetracycline-inducible plasmid pTetKid was made in several steps. First, a dsDNA containing a ribosome binding site (RBS) followed by an initiation codon and a six-histidine track was produced, annealing oligonucleotides X-RBS-S-6H-Nss and X-RBS-S-6H-Nas. Insertion of this DNA between XbaI and NheI in plasmid pASK-IBA4 (Institut für Bioanalytik, GmbH) produced pTet-HS. Then, a second dsDNA fragment containing three Flag epitopes followed by a suitable multicloning site was produced by joining annealed oligonucleotides pairs K-3F-PBNEH ss1/as1 and ss3/as3 with each other. Introduction of the resulting product between the KasI and HindIII sites in pTet-HS generated pTet-HS3F. Finally, *kid* was amplified from mR1KK by PCR, using oligonucleotides X-RBS-*kid* and *kid*-S, and the resulting product was cloned between XbaI and SphI of pTet-HS3F, which generated pTetKid.

We also made tetracycline-inducible vectors to control, independently and sequentially, the expression of Kis, EGFP-RepA, or DnaB in cells already producing Kid from arabinose-inducible vectors. To make pTet-HS3FKis, oligonucleotides B-*kis* and *kis*-E were used to amplify *kis* by PCR from plasmid pPrTs$_{HC}$Kis, and the resulting DNA product was cloned between BamHI and EcoRI of plasmid pTet-HS3F. To make pTet-H-EGFP-RepA^r, we used oligonucleotides N-*egfp* and *egfp*$_{nostop}$-P to amplify a stop codon-less EGFP DNA from plasmid pEGFP-C2 (Clontech) by PCR. The resulting product was digested with NheI and PmlI, and inserted between the same sites in pTet-HS, which produced precursor plasmid pTet-HS-EGFPC2. Oligonucleotides B-*repA* and *repA*-E were then used to amplify the *repA* gene from mR1Ctrl by PCR, and the resulting product was introduced between the BamHI and EcoRI sites of the latter plasmid to generate pTet-H-EGFP-RepA^r, encoding UUACU-less *egfp-repA*. To produce pTet-H-EGFP-RepA^s, oligonucleotides P-TTACTx2-B ss and P-TTACTx2-B as were annealed with each other, and the resulting DNA product was exchanged with that between the PmlI and BamHI sites in pTet-H-EGFP-RepA^r. Finally, to make pTet-H-DnaB^r and pTet-H-DnaB^s, oligonucleotides N-SfiI-B ss and N-SfiI-B as were annealed with each other and the resulting DNA was cloned between NheI and BamHI of pTet-HS3F, producing pTetHS3F(SfiI). In parallel, the two TTACT sites in *dnaB* were mutated (silently) to TTACC using oligonucleotides *dnaB*$_{mutTTACC5'}$ ss, *dnaB*$_{mutTTACC5'}$ as, *dnaB*$_{mutTTACC3'}$ ss, and *dnaB*$_{mutTTACC3'}$ as, and plasmid pGADT7-*dnaB* (1) as a template. The *dnaB* gene from the resulting plasmid (*dnaB^r*) and from its parental vector (*dnaB^s*) was excised using SfiI and BamHI, and subcloned between the same sites of pTetHS3F(SfiI) to produce pTet-H-DnaB^r and pTet-H-DnaB^s, respectively.

We also made plasmids required for the construction of new *Escherichia coli* strains (see next section). For instance, *kid* was excised from mR1wt with EcoNI and EcoRI, and the resulting DNA product was processed with Klenow and recircularized to make mR1Kis, used in the construction of strain GCM2. Construction of p6G-*egfp-cat* required several steps. First, oligonucleotides K-6Gly-X ss and K-6Gly-X as were inserted between KpnI and XmaI in pUC18NotI (a pUC18 derivative with NotI sites flanking its multicloning site), which produced pUC18NotI-6Gly. Then, a *cat* operon flanked by FRT sites was amplified from plasmid pKD3 (6) by PCR, using oligonucleotides HEX-CTTAA-P2 and P1-PE. This PCR product was digested with EagI and PstI and ligated to an *egfp*-encoding fragment excised from pEGFP-N1 (Clontech) with AgeI and NotI, and the resulting DNA was subcloned between XmaI (compatible with AgeI) and PstI in pUC18NotI-6Gly to generate p6G-*egfp-cat* (Table S3). The latter was used to generate plasmids p*ftsZ*-6G-*egfp-cat* and p*zapA*-6G-*egfp-cat*, used in the construction of strains DH4FZGFP and DH4ZAGFP. To make these plasmids, PCR products spanning 150 bp immediately upstream and downstream of the stop codons of *ftsZ* and *zapA* were produced using oligonucleotide pairs E-*ftsZ*$_{upstop}$/*ftsZ*$_{upstop}$-K, E-*zapA*$_{upstop}$/*zapA*$_{upstop}$-K, S-*ftsZ*$_{dwnstop}$/*ftsZ*$_{dwnstop}$-H, and S-*zapA*$_{dwnstop}$/ *zapA*$_{dwnstop}$-H, and

genomic DNA as a template. These PCR products were digested with EcoRI and KpnI (for upstream fragments) or with SphI and HindIII (for downstream fragments) and subcloned sequentially between the same sites in p6G-*egfp-cat* to generate final vectors p*ftsZ*-6G-*egfp-cat* and p*zapA*-6G-*egfp-cat* (Table S3).

Strains. Strains in this work are listed in Table S4. DH10B (7) was used in most experiments. ILO1 and ILO6 cells, carrying long tracks of *tetO* and/or *lacO* sites in their chromosomes, were described by Wang et al. (8). Strain GCM2 expresses low levels of Kis from the chromosome, avoiding growth problems due to leaky production of Kid from noninduced pTetKid in our experiments (Fig. 3*B*). To make this strain, a DNA fragment spanning Pr_{parD}-*kis*-FRT*kan*^{r}FRT was amplified from mR1Kis by PCR, using oligonucleotides *chpBK*$_{as}$FRT*kan*^{r} and $Pr_{chpBss}Pr_{parD}$. The resulting PCR product was exchanged by the chromosomal *chpB* locus in DHB4 *E. coli* cells by homologous recombination. Subsequent removal of the FRT*kan*^{r}FRT cassette in this inserted DNA using FLP recombinase generated strain GCM2. To make strains DH4FZGFP and DH4ZAGFP, plasmids p*ftsZ*-6G-*egfp-cat* and p*zapA*-6G-*egfp-cat* were digested with NotI, and the resulting *ftsZ*- and *zapA*-containing fragments were integrated in the genome of DHB4 cells by homologous recombination, which produced *ftsZ-egfp* and *zapA-egfp* chromosomal fusions. DNA integrations and removals required to make GCM2, DH4FZGFP, and DH4ZAGFP cells were carried out as described elsewhere (6).

Toxin-Antitoxin Activation and Cell Viability. DH10B cells carrying mR1KK, mR1hs, or mR1Ctrl plus either pBAD22 or pBAD22-*copA* were grown exponentially in M9 medium supplemented with all amino acids minus methionine (M9aa – Met), 0.2% glucose (to repress *copA* expression), ampicillin (100 mg/L), and kanamycin (50 mg/L) at 37 °C. To start the experiments, cells were diluted to an OD_{600} of 0.05 in 10 mL of M9aa – Met plus 0.5% glycerol, 0.2% arabinose (to induce *copA* expression), and 100 mg/L ampicillin. To avoid counterselection of cells losing the mR1 derivatives, no kanamycin was added at this stage. Culture growth was followed, monitoring OD_{600} every 2 h and, when required, cells were diluted in fresh medium to keep them growing exponentially. Quantification of dead cells in our samples was performed using a BD LSRII flow cytometer (Becton Dickinson), after staining cultures with the LIVE/DEAD BacLight Bacterial Viability Kit as instructed by the supplier (Invitrogen). To determine the relative number of mR1KK-containing cells that resumed growth upon cessation of *copA* production in our experiments above, serial dilutions of mR1KK/pBAD22*copA* and mR1Ctrl/pBAD22*copA* samples were plated on LB supplemented with 0.2% glucose (to stop further *copA* synthesis), 100 mg/L ampicillin, and 50 mg/L kanamycin. Cells were seeded before and 4, 8, and 12 h after induction of *copA* synthesis, and plates were incubated at 30 °C for 24 h before counting colonies growing on each of them. Experiments were repeated three times, and plating was done in duplicate for every time point and dilution. Only plates producing 200–400 colonies were used for counting, correcting for dilution factors afterward, and the percentage ratio of mR1KK-containing cells growing on these plates relative to mR1Ctrl-containing cells was calculated for every time point. Finally, to analyze the potential cross-talk between Kid and Hok, *E. coli* DH10B cells cotransformed with pBAD22 and either mR1Ctrl or mR1hs, or with both mR1hs and pBAD22Kid were grown exponentially at 37 °C in M9aa – Met and 0.2% glucose (to keep Kid expression repressed), plus ampicillin (100 mg/L) and kanamycin (50 mg/L). To start the experiments, cells were diluted to an OD_{600} of 0.05 in 10 mL of M9aa – Met supplemented with 0.5% glycerol and 0.02% arabinose (to induce Kid expression) and kept growing at 37 °C. Dead (i.e., propidium iodide-permeable) cells, both before and 1 and 2 h after inducing Kid expression, were quantified as above, using the LIVE/DEAD BacLight Bacterial Viability Kit and a BD LSRII flow cytometer, and values obtained were normalized against those in the mR1Ctrl/pBAD22 sample. Exponentially growing DH10B cells in 70% (vol/vol) isopropanol were used as a positive control for cell death.

Induction of Expression Vectors. All experiments were performed similarly, with minor modifications depending on the type and combination of expression vector(s) used. Precultures were always started from a single colony of cells and grown in M9aa – Met medium supplemented with 0.2% glucose and appropriate antibiotics at 30 °C for 12 h. When working with thermosensitive expression vectors (pPrTs; Figs. 2, 4, and 5 and Fig. S2), cells were diluted to an OD_{600} of 0.05 in the same medium and grown for 1 h at 30 °C before induction. In other cases (i.e., when working with arabinose- and anhydrotetracycline (A-Tet)–inducible vectors; Fig. 3 and Figs. S1 and S3), cells were diluted in M9aa – Met supplemented with 0.5% glycerol and grown for 1 h at 37 °C before induction. Expression of Kid was induced after that time, either shifting the temperature to 42 °C (Figs. 2, 4, and 5 and Fig. S2) or adding A-Tet (0.2 μg/mL; Fig. 3*B*) or arabinose (0.02%; Fig. 3 *A*, *C*, and *D* and Figs. S1 and S3). Induction of Kid expression with arabinose was used when sequential production of a second protein from a tetracycline expression vector was required. In those cases, 0.2 μg/mL A-Tet was added to the medium, either 3 h (Kis in Fig. 3*A* and Fig. S1) or 1 h (EGFP-RepA variants in Fig. 3*C* and DnaB variants in Fig. 3*D*) after expression of Kid had started. Antibiotic selection was maintained in all cases during the induction of protein synthesis to counterselect any cell in our cultures that could lose the corresponding expression vector.

Analysis of Protein Synthesis. To examine protein synthesis, 5 μCi of [^{35}S]methionine was added to 1 mL of cultures at the indicated times, and these were incubated at 42 °C (Fig. 2 *B* and *C*) or 37 °C (Fig. 3) for 2 min before stopping the reaction as follows. For scintillation counting (Fig. 2*B*), 10 μL from these samples was spotted onto Whatman 3 MM filters preblocked with 0.1% nonradioactive methionine, allowed to dry, and then placed in cold 10% (vol/vol) trichloroacetic acid (TCA) for 20 min. After that, filters were transferred to a boiling solution of 5% (vol/vol) TCA for 15 min, washed once with 5% (vol/vol) ice-cold TCA and once with 95% (vol/vol) ethanol, air-dried, and transferred to vials containing scintillation mixture (Ultima Gold; PerkinElmer). The amount of incorporated radioactivity measured this way was corrected to the specific OD_{600} of the corresponding sample. For analysis on SDS/PAGE gels (Figs. 2*C* and 3), 50 μg/mL nonradioactive methionine and 100 μL of 100% (wt/vol) ice-cold TCA were added directly to our cells after labeling, and the mixture was incubated on ice for 2 h. Proteins were then collected by centrifugation at 16,800 × *g* and 4 °C for 40 min, and washed twice with 95% (vol/vol) ethanol. For conventional gels, samples were resuspended in loading buffer and boiled before loading. For 2D gel electrophoresis, they were solubilized in 8 M urea, 4% (wt/vol) CHAPS, 40 mM Tris, 50 mM DTT, and traces of bromophenol blue. Immobilized pH gradient (IPG) Immobiline DryStrip gels (18 cm, pH 3–10) were rehydrated overnight in 200 μL of each sample plus 200 μL of Rehydration Solution (Amersham), 0.5% (vol/vol) IPG buffer (pH 3–10), and bromophenol blue, and then inserted into a Multiphor II flatbed electrophoresis unit (Amersham Biosciences) as instructed by the manufacturer. Isoelectric focusing was carried out for 105 min in a 0- to 350-V gradient, for another 105 min in a 350- to 3,500-V gradient, and for 17 h at 3,500 V. For the subsequent SDS/PAGE, the IPG strips were equilibrated in 50 mM Tris·HCl (pH 8.8), 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, 2% DTT, and bromophenol blue for 10 min, and for another 10 min

with 4% iodoacetamide. Strips were sealed with 1% agarose onto conventional gradient 6–20% SDS polyacrylamide gels. Following electrophoresis, gels were dried and the resolved proteins were detected by autoradiography. Nonradioactive samples were also processed in parallel, either to cut specific proteins from 2D gels and analyze them by MALDI TOF (Fig. 2*C*) or to examine them by immunoblotting (Fig. 3 *A* and *C*). In the latter case, cells (1 mL) were collected by centrifugation and proteins were extracted with Bugbuster (Novagen) and analyzed by Western blot (ECL; GE Healthcare) with polyclonal antibodies raised against Kid and Kis (Fig. 3*A*) or with monoclonal anti-EGFP antibody JL-8 (Clontech) (Fig. 3*C*).

Cell Microscopy. In all these experiments, production of Kid and Kis was induced from thermosensitive vectors, using the strain-plasmid(s) combinations listed in Tables S3 and S4. When ILO strains were used, production of TetR-YFP and LacI-CFP from pWX6 was regulated by adding 0.5 mM isopropyl-β-D-thio-galactopyranoside and 40 ng/mL anhydrotetracycline to the growth medium. For snapshots, samples induced at 42 °C for the indicated times were transferred to a slide covered with a thin layer of 1% agarose in PBS and analyzed with a 100× objective in a Nikon Eclipse TE2000-U microscope, equipped with a Photometrics Cool-SNAP HQ CCD camera. At least 600 cells were analyzed per time point and experiment. To calculate the average number of mR1TetO$_{240}$, *oriC*, and *ter* foci per cell, the total number of foci of each type was divided by the total number of cells counted per sample and time point. For time-lapse experiments, cells were deposited in a thin layer of 1% agarose in M9aa − Met plus 0.2% glucose, and they were imaged at regular intervals using a Zeiss LSM 510 Meta(inverted) confocal microscope equipped with a live cell imaging chamber heated at 42 °C. In some cases, 0.5 μg/mL FM4-64 dye (Invitrogen) was used to stain the cell membrane. For EM, 1.5 mL of each sample was collected 2 h after shifting the incubation temperature to 42 °C, and cells were fixed in 0.4% glutaraldehyde at 4 °C for 3 h before rinsing them with 0.1 M Pipes buffer. Scanning EM and transmission EM image acquisition and processing were performed at the Multi-Imaging Centre in Cambridge, United Kingdom.

Analysis of mRNA Cleavage. Primer extension and sequencing reactions were carried out using the Primer Extension System AMV Reverse Transcriptase kit (Promega) and the Sequenase 2.0 (USB) kit, as described by Pimentel et al. (1). Briefly, total RNA was purified from strains DH4FZGFP and DH4ZAGFP carrying pPrTsMCKid (Kid) or pPrTsMC (control) and cultured at 42 °C for 30 min. Fifty micrograms of these RNA samples was analyzed by primer extension using oligonucleotides ftsZ-primext and zapA-primext. A pUC18 vector containing inserts spanning the regions analyzed in *ftsZ* and *zapA* was used as a template for sequencing reactions, using the same oligonucleotides.

1. Pimentel B, Madine MA, de la Cueva-Méndez G (2005) Kid cleaves specific mRNAs at UUACU sites to rescue the copy number of plasmid R1. *EMBO J* 24(19):3459–3469.
2. Ghigo JM (2001) Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412(6845):442–445.
3. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177(14):4121–4130.
4. Lau IF, et al. (2003) Spatial and temporal organization of replicating Escherichia coli chromosomes. *Mol Microbiol* 49(3):731–743.
5. Chang AC, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* 134(3):1141–1156.
6. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
7. Durfee T, et al. (2008) The complete genome sequence of Escherichia coli DH10B: Insights into the biology of a laboratory workhorse. *J Bacteriol* 190(7):2597–2606.
8. Wang X, Possoz C, Sherratt DJ. (2005) Dancing around the divisome: Asymmetric chromosome segregation in Escherichia coli. *Genes Dev* 19:2367–2377.

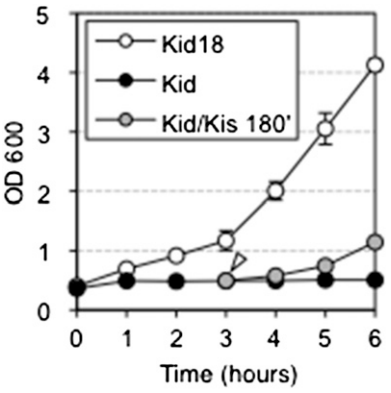

Fig. S1. Kis expression reverts the growth arrest imposed by Kid in *E. coli* cells. Growth curves of DH10B cells carrying expression vector pTet-HS3FKis plus either p177Pr$_{ara}$Kid18 or p177Pr$_{ara}$Kid are shown. At the start of our experiments, cultures were induced to produce Kid (black circles) or Kid18 (white circles) using arabinose, and OD$_{600}$ was measured at 1-h time intervals. At the 3-h time point (white arrowhead), cultures expressing Kid were split into two halves and expression of Kis was induced in one of them using A-Tet (gray circles; Kid/Kis 180′). OD$_{600}$ in these samples was measured with the same periodicity as before until the end of the experiment. $n = 6$; bars represent SEM.

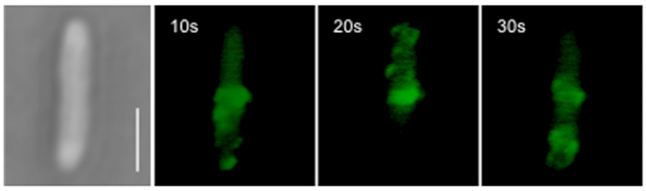

Fig. S2. Bright-field (left-most) and fluorescent (other) images of one DH4FZGFP *E. coli* cell carrying pPrTs$_{MC}$Kid and induced to express the toxin in our time-lapse experiments shown in Fig. 5C. These images were taken at 10-s intervals to highlight the rapid pole-to-pole oscillation of FtsZ-EGFP in these cells. (Scale bar: 2 μm.)

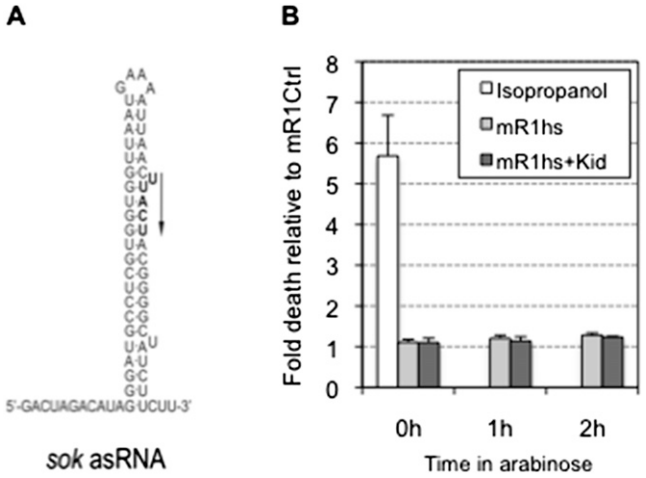

Fig. S3. Expression of Kid does not induce premature activation of the *hok-sok* toxin-antitoxin (TA) pair in cells carrying mR1hs. (*A*) Sequence and structure of *hok* mRNA, showing the location of its single UUACU site in a double-stranded structure that is essential for the correct functioning of this antitoxin. (*B*) Fold change in propidium iodide-permeable cells in DH10B cultures carrying mR1hs and either pBAD22Kid (mR1hs + Kid) or empty control plasmid pBAD22 (mR1hs) and grown in the presence of arabinose (to induce Kid expression) for the indicated times. Values are relative to those observed in cultures of cells carrying mR1Ctrl and pBAD22 and grown in the same conditions. DH10B cultures treated with 70% (vol/vol) isopropanol were used as positive controls for cell death. $n = 3$; bars represent SEM.

Table S1. Numbers of UAA, UAC, UAU, and UUACU sites in mRNAs spanning the coding sequence of *E. coli* proteins relevant to this work

Protein	Gene	UAA	UAC	UAU	UUACU
Ribosomal S1	*rpsA*	23	11	16	0
Ribosomal L4	*rplD*	6	5	4	0
EF-Ts	*tsf*	10	5	7	0
LacI	*lacI*	9	7	11	0
Rho	*rho*	20	16	13	0
Enolase	*eno*	15	14	14	0
DnaK	*dnaK*	24	18	25	0
SAM decarboxylase	*sped*	13	14	24	0
SlmA	*ttk*	6	4	6	1
B-subunit DNA pol III	*dnaN*	6	15	12	1
Subtotal		132	109	132	2
Kis	*kis*	2	3	3	0
Kid	*kid*	2	4	3	0
RepA	*repA*	5	7	7	0
EGFP	*egfp*	2	10	2	0
Subtotal		11	24	15	0
Total		143	133	147	2

Top ten genes/proteins were identified by MS in our 2D gels in Fig. 2C. SAM, S-adenosylmethionine.

Table S2. Oligonucleotides used in this work

Name	Description	Sequence (5′ to 3′)
EKX-*hoksok*	EcoRI-KpnI-XhoI-*hoksok*	CCGAATTCGGTACCCTCGAGAACAAACTCCGGGAGGCAGCGTGAT
hoksok-NH	*hoksok*-NotI-HindIII	TTCAAGCTTGCGGCCGCACAACATCAGCAAGGAGAAAGGGGCTA
N-*rnnBTr*-H ss	NotI-*rnnBTr*-HindIII ss	AGCTTGAAGGCCATCCTGACGGATGGCCTTTTTTAAGCTTGC
N-rnnB*Tr*-H as	NotI-*rnnBTr*-HindIII as	GGCCGCAAGCTTAAAAAAGGCCATCCGTCAGGATGGCCTTCA
S-*copA*	SpeI-*copA*	GGACTAGTCATAGCTGAATTGTTGGCTATAC
copA-E	*copA*-EcoRI	GCGAATTCGCCAGAAAAGCAAAAACCCCGA
X-RBS-S-6H-N ss	XbaI-*RBS*-SphI-*M6His*-NheI ss	CTAGATAACGAGGGCAAAGCATGCACCATCATCATCATCATTCTTCTGGTG
X-RBS-S-6H-N as	XbaI-*RBS*-SphI-*M6His*-NheI as	CTAGCACCAGAAGAATGATGATGATGATGGTGCATGCTTTGCCCTCGTTAT
K-3F-PBNEH ss1	KasI-*3FLAG*-PmlI-BamHI-NcoI-EcoRI-HindIII ss1	GCGCCGGAGGAGACTACAAAGACCATGACGGTGACTATAAAGATCAT GACATCGACTATAA
K-3F-PBNEH as1	KasI-*3FLAG*-PmlI-BamHI-NcoI-EcoRI-HindIII as1	ATCCTTATAGTCGATGTCATGATCTTTATAGTCACCGTCATGGTCTTTGTAG TCTCCTCCG
K-3F-PBNEH ss3	KasI-*3FLAG*-PmlI-BamHI-NcoI-EcoRI-HindIII ss3	GGATGACGATGACAAGCACGTGGTGACTGGAGTGACTGGATCCGCCATGG GGGGAATTCA
K-3F-PBNEH as3	KasI-*3FLAG*-PmlI-BamHI-NcoI-EcoRI-HindIII as3	AGCTTGAATTCCCCCCATGGCGGATCCAGTCACTCCAGTCACCACGTGCTTG TCATCGTC
X-RBS-*kid*	XbaI-*RBS-kid*	GCTCTAGATAACGAGGGCAAAAAATGGAAAGAGGGGAAATCTGGCTT
kid-S	*kid*-SphI	ACATGCATGCTAATTCCTACCAATGCTCAAGTCAGAATAGTGGACAGGC GGCCAA
N-*kid*	NcoI-*kid*	GAATTCCCATGGAAAGAGGGGAAATCTGCTCTAGAGCATGCTCAAGTC AGAATAGT
B-*kis*	BamHI-*kis*	TTTGGATCCATGCATACCACCCGACTGAAGA
kis-E	*kis*-EcoRI	TTTGAATTCTCAGATTTCCTCCTGACCAGTC
N-*egfp*	NheI-*egfp*	CCCTAGCTAGCATGGTGAGCAAGGGCGAGGA
$egfp_{nostop}$-P	$egfp_{no\ stop}$-PmlI	AAACACGTGCTTGTACAGCTCGTCCATG
B-*repA*	BamHI-*repA*	CGGGATCCATGACTGATCTTCACCAAACGTA
repA-E	*repA*-EcoRI	GGAATTCTCAGGGAGAAGCTGTGGCCA
P-TTACTx2-B ss	PmlI-TTACTx2 linker-BamHI ss	GTGGTTATCGGAGTTACTG
P-TTACTx2-B as	PmlI-TTACTx2 linker-BamHI as	GATCCAGTAACTCCGATAACCAC
N-SfiI-B ss	NheI-SfiI(GGCCAAAAAGGCC)-BamHI ss	CTAGCGGCCAAAAAGGCCG
N-SfiI-B as	NheI-SfiI(GGCCAAAAAGGCC)-BamHI as	GATCCGGCCTTTTTGGCCG
$dnaB_{mutTTACC5'}$ ss	Mutation of the upstream TTACTss	GCCCACACCGTCATATCTTTACCGAAATGGCGCGTTTGCAGGAAA
$dnaB_{mutTTACC5'}$ as	Mutation of the upstream TTACTas	TTTCCTGCAAACGCGCCATTTCGGTAAAGATATGACGGTGTGGGC
$dnaB_{mutTTACC3'}$ ss	Mutation of the downstream TTACTss	GTAGCCCTATCGATCTGATTACCCTTGCGGAATCGCTGGAACGCC
$dnaB_{mutTTACC3'}$ as	Mutation of the downstream TTACTas	GGCGTTCCAGCGATTCCGCAAGGGTAATCAGATCGATAGGGCTAC
$chpBK_{as}$FRTkan^r		GGGCTTCGGTTAGTAAGGGTTTTTTTATGCCCGCGATAAATAAACACACCG TGTAGGCTGGAGCTGCTTC
$Pr_{chpBss}Pr_{parD}$		CCATATGCATACGCCACCTTCGGGTGGCGTTGTTTTTTGCGAGACGACTCAA GCAACCACGCTGGGTTTAC
K-6Gly-X ss	KpnI-6Gly linker-XmaI ss	CGGAGGAGGAGGAGGAGGC
K-6Gly-X as	KpnI-6Gly linker-XmaI as	CCGGGCCTCCTCCTCCTCCTCCGGTAC
HEX-CTTAA-P2	HincII-EagI-XhoI-CTTAA-P2 from pKD3	ACGCGTCGACCGGCCGGAGCTCGAGCTTAACATATGAATATCCTCCTTAGTT
P1-PE	P1 from pKD3-PstI-EcoRI	CGGAATTCCTGCAGGTGTAGGCTGGAGCTGCTTC
E-$ftsZ_{upstop}$	EcoRI-*ftsZ* (150 bp upstream of *ftsZ* stop)	CGGAATTCGTGATGGATCGCTACCAGCAG
$ftsZ_{upstop}$-K	*ftsZ*-KpnI (immediately upstream of *ftsZ* stop)	GGGGTACCTTAATCAGCTTGCTTACGCAG
E-$zapA_{upstop}$	EcoRI-*zapA* (150 bp upstream of *zapA* stop)	CGGAATTCATCAGCTATGAGTTAGCGCAAGA
$zapA_{upstop}$-K	*zapA*-KpnI (immediately upstream of *zapA* stop)	GGGGTACCTCATTCAAAGTTTTGGTTAGTTTTTT
S-$ftsZ_{dwnstop}$	SphI-*ftsZ* (immediately downstream of *ftsZ* stop)	ACATGCATGCGAATTGACTGGAATTTGGGTTTC
$ftsZ_{dwnstop}$-H	*ftsZ*-HindIII (150 bp downstream of *ftsZ* stop)	CCCAAGCTTCCGACACCCGTCGCCTGAA
S-$zapA_{dwnstop}$	SphI-*zapA* (immediately downstream of *zapA* stop)	ACATGCATGCCACTTTTCGGTTTACTGTGGTAG
$zapA_{dwnstop}$-H	*zapA*-HindIII (150 bp downstream of *zapA* stop)	CCCAAGCTTCAACCGCGGAGCGCCACA
ftsZ primext	Antisense 50 bp downstream of TTACT in *ftsZ*	CCATCATTGCGTAGCCCATCTCA
zapA primext	Antisense 50 bp downstream of TTACT in *zapA*	TCGCCCTTGCTCACCATGGT

as, antisense; RBS, ribosome binding site; ss, sense; Tr, terminator.

Table S3. Plasmids used in this work, in order of appearance

Plasmid	Description	Relevant information	Figs.
mR1KK	R1 replicon + *kiskid*, kan^r	This plasmid is identical to mR1wt in Pimentel et al. (1)	1 *A–C*
mR1Ctrl	R1 replicon + *kiskid18*, kan^r	This plasmid is identical to mR118 in Pimentel et al. (1)	1 *A–C*
mR1hs	R1 replicon + *kiskid18* + *hoksok*, kan^r	This plasmid is mR1Ctrl-bearing also in the *hoksok* TA pair	1 *A* and *C*, S4
pBAD22*copA*	pMB1 ori, amp^r, Pr_{ara}-*copA*	Expression of copA induced by arabinose and repressed by glucose	1 *A–C*
$pPrTs_{LWC}$	pSC101 ori, $chlr^r$, cI^{Ts} + Prλ	Thermosensitive (empty) expression vector; promoter induced at ≥40 °C	2, 4, 5*A*
$pPrTs_{LWC}$Kid	pSC101 ori, $chlr^r$, cI^{Ts} + Prλ-*kid*	Thermosensitive Kid expression vector; promoter induced at ≥40 °C	2, 4, 5*A*
p177Pr_{ara}Kid	p15A ori, kan^r, Pr_{ara}-*kid*	Kid expression induced by arabinose and repressed by glucose	3 *A*, *C*, and *D*; S1
p177Pr_{ara}Kid18	p15A ori, kan^r, Pr_{ara}-*kid18* (RNase dead Kid)	Kid18 expression induced by arabinose and repressed by glucose	3*C*, S1
pTet-HS3FKis	pUC ori, amp^r, Pr_{Tet}-HS3F*kis*	His_6-Strep tag-3x(Flag)-Kis expression induced by tetracycline	3*A*, S1
pTetKid	pUC ori, amp^r, Pr_{Tet}-*kid*	Kid expression induced by tetracycline	3*B*
pTet-H-EGFP-$RepA^r$	pUC ori, amp^r, Pr_{Tet}-EGFP-$repA^r$(UUACU$^-$)	His_6-EGFP-RepA (UUACU$^-$) expression induced by tetracycline	3*C*
pTet-H-EGFP-$RepA^s$	pUC ori, amp^r, Pr_{Tet}-EGFP-$RepA^s$ (UUACU$^+$)	His_6-EGFP-RepA (UUACU$^+$) expression induced by tetracycline	3*C*
pTet-H-$DnaB^s$	pUC ori, amp^r, Pr_{Tet}-H-$dnaB^s$ (UUACU$^+$)	His_6-$DnaB^s$ (UUACU$^+$) expression induced by tetracycline	3*D*
pTet-H-$DnaB^r$	pUC ori, amp^r, Pr_{Tet}-H-$dnaB^r$ (UUACU$^-$)	His_6-$DnaB^r$ (UUACU$^-$) expression induced by tetracycline	3*D*
pBAD22Kid	pMB1 ori, amp^r, Pr_{ara}-*kid*	Kid expression induced by arabinose and repressed by glucose	S4
pBAD22Kid18	pMB1 ori, amp^r, Pr_{ara}-*kid18*	Kid18 expression induced by arabinose and repressed by glucose	S4
pWX6	pBR322 ori, amp, Pr_{ftsK}-*lacI-cfp* + *tetR-yfp*	Constitutive LacI-CFP and TetR-YFP coexpression from Pr_{ftsK}	4 *A–C*
$mR1tetO_{240}$	R1 replicon, gem^r, 240 tetO sites	Bound by TetR-YFP; allows its in vivo tracking in cells also carrying pWX6	4*A*
$pPrTs_{HC}$	pBR322 ori, amp^r, cI^{Ts} + Prλ	Thermosensitive (empty) expression vector; promoter induced at ≥40 °C	5*A*
$pPrTs_{HC}$Kis	pBR322 ori, amp^r, cI^{Ts} + Prλ-*kis*	Thermosensitive Kis expression vector; promoter induced at ≥40 °C	5*A*
$pPrTs_{MC}$	p15A ori, kan^r, cI^{Ts} + Prλ	Thermosensitive (empty) expression vector; promoter induced at ≥40 °C	5 *B–D*
$pPrTs_{MC}$Kid	p15A ori, kan^r, cI^{Ts} + Prλ-*kid*	Thermosensitive Kid expression vector; promoter induced at ≥40 °C	5 *B–D*
mR1Kis	R1 replicon + Pr_{pard}-*kis*, kan^r	mR1KK derivative lacking Kid; used as PCR template to make strain GCM2	
p6G-*egfp-cat*	pUCori, amp^r/*cat*, MCS_1-Gly_6-*egfp* + *cat*-MCS_2	Used to produce C-terminal fusions of chromosomal genes to Gly_6-*egfp*	
p*ftsZ*-6G-*egfp-cat*	pUC ori, $amp^r/^{cat}$, *ftsZ*-Gly_6-*egfp* + *cat-ftsZ*	Used to produce *E. coli* strain DH4FZGFP	
p*zapA*-6G-*egfp-cat*	pUC ori, $amp^r/^{cat}$, *zapA*-Gly_6-*egfp* + *cat-zapA*	Used to produce *E. coli* strain DH4ZAGFP	

amp_r, ampicillin resistance gene; $chlr_r$, chloramphenicol resistance gene; cI_{ts}, thermosensitive λ-repressor; gem_r, gentamicin resistance gene; kan_r, kanamycin resistance gene; MCS, multicloning site; ori, replication origin; Pr, promoter.

Table S4. Strains used in this work

Strain	Genotype/description	Relevant information	Figs.
DH10B	F–*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*)7697 *gal*U *gal*K *rps*L *nup*G λ–		1; 2; 3 *A*, *C*, and *D*; 5*A*; S1; S4
DH4B	F′ *lac–pro lacIQ*/[*ara–leu*]7697 *araD139 lacX74 galE galK rpsL phoR* (*phoA*) *Pvu*II *malF3 thi*		
GCM2	DHB4 [Δ*chpB::PrparD-kis*]	*kis* and its promoter substitute the *chpB* locus in DHB4	3*B*
ILO1	AB1157 [lacO240-K_m]3908	Insertion of 240 lacO sites 15 kb from *oriC* in strain AB1157	4*A*
ILO6	AB1157 [lacO240-Gm]3908 + [tetO240-K_m]1644	Insertion of 240 lacO and 240 tetO sites 15 kb from *oriC* and 50 kb from dif (ter3), respectively, in strain AB1157	4 *B* and *C*
DH4FZGFP	DHB4 Φ(*ftsZ-egfp*) *cat*	In-frame insertion of *egfp* immediately upstream of the *ftsZ* stop codon in DHB4; produces FtsZ-EGFP hybrid protein	5 *B* and *C*, S3
DH4ZAGFP	DHB4 Φ(*zapA-egfp*) *cat*	In-frame insertion of *egfp* inmediately upstream of the *zapA* stop codon in DHB4; produces ZapA-EGFP hybrid protein	5 *B* and *D*