SUPPLEMENTAL INFORMATION

Sequential Induction of Fur-Regulated Genes in Response to Iron Limitation in *Bacillus subtilis*

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Materials and methods

Table S1. Strains and plasmids used in this study

Table S2. Primer oligonucleotides

Fig. S1. Expression of FrvA has no effect on intracellular Mn and Co levels.

Fig. S2. Fur-regulated genes are derepressed in response to iron depletion.

Fig. S3. Severe iron deprivation induces some PerR-regulated genes.

Fig. S4. Leaky expression of FrvA induces some genes in the Fur regulon.

Fig. S5. A decrease in mRNA levels of many FsrA-regulated genes is observed at later timepoints in response to iron depletion.

Fig. S6. The chromosomal FLAG-tagged Fur is a functional regulator.

Fig. S7. The stepwise derepression of Fur-regulated genes correlates with protein-DNA binding affinity.

Fig. S8. Fur-DNA binding affinities to different promoter regions.

Fig. S9. Growth advantage of *sfp*$^+$ strains over *sfp*^{0} strains

Fig. S10. Inactivation of bacillibactin uptake affects induction of the Fur-regulated gene.

Fig. S11. Physiological role of Btr on bacillibactin uptake system in both sfp^0 and sfp$^+$ strains.

Materials and methods

Bacterial strains and growth conditions

All strains used in the study are derivatives of *B. subtilis* strain CU1065 (WT) and are listed in Table S1. Cells were grown in LB medium or specified MOPS minimum medium with vigorous shaking or on solid LB agar with appropriate antibiotic selection at 37^0C. The concentrations of antibiotics used are: ampicillin (amp, 100 µg ml^{-1}), spectinomycin (spec, 100 µg ml^{-1}), tetracycline (tet, 5 µg ml^{-1}), chloramphenicol (cm, 10 µg ml^{-1}), kanamycin (kan, 15 µg ml^{-1}), neomycin (neo, 8 µg ml^{-1}), and macrolide lincosoamide-streptogramin B (MLS, 1 µg ml^{-1} erythromycin and 25 µg ml^{-1} lincomycin).

Quantification of intracellular metal ion by ICP-MS

Cells were grown in LB medium amended with 10 µM $FeSO_4$ to an OD_{600} of about 0.25 and 1 mM IPTG was added to induce expression of FrvA where indicated. Aliquots of 4 ml of cell culture were harvested and levels of intracellular metals (Fe, Mn, and Co) were monitored for three hours after IPTG treatment by inductively coupled plasma mass spectrometry (ICP-MS). All samples were washed once with buffer 1 (1X PBS buffer, 0.1 M EDTA) then twice with buffer 2 (1X chelex-treated PBS buffer). Cell pellets were resuspended in 400 µl of buffer 3 (1X chelex-treated PBS buffer, 75 mM NaN_3, 1% Triton X-100) and incubated at 37^0C for 90 min to lyse the cells. Lysed samples were centrifuged and the total protein content was quantified using a Bradford assay. Then, samples were mixed with 600 µl buffer 4 (5% HNO3, 0.1% (v/v) Triton X-100) and heated in a 95^0C sand bath for 30 min. The debris was removed by centrifugation and the total metal ions in the diluted samples were analyzed by Perkin-Elmer ELAN DRC II ICP-MS. Gallium was used as an internal standard. The total intracellular ion levels are expressed as µg ion per gram of protein content (mean ± SD; n=3). Mn, and Co levels were not significantly changed over the course of the experiment. Statistically significant differences between WT and induced P_{spac}-*frvA* cells are determined by two-tailed *t*-test as indicated: *, $P < 0.01$.

Growth curves

Cells were grown overnight in LB medium and subcultured with 1:100 ratio into fresh LB medium amended with 10 µM of $FeSO_4$ and grown to an OD_{600} of 0.25. For IPTG treated cells, 1mM IPTG was added to induce expression of FrvA. Cell Growth (OD_{600}) was monitored every 15 min for 25 h using a Bioscreen growth analyzer (Growth Curves USA, Piscataway, NJ) at 37^0C with continuous shaking. Data shown are representative growth curves and experiments were conducted at least three times with three biological replicates each time.

Microarray analysis

Cells (WT P_{spac}-*frvA*) were grown in LB medium amended with 10 µM $FeSO_4$ to an OD_{600} of ∼0.25 and divided into two 1 L flasks. To induce expression of FrvA, 1 mM IPTG was added into one flask while the other was left untreated as a control. Aliquots of 40 ml of cell culture were harvested from both flasks at different time-points as indicated and total RNA was extracted using an acidic phenol-based method. All RNA samples were treated with Turbo-DNA free™ DNase (Ambion™) and precipitated in ethanol and sodium acetate at -80^{0}C overnight. RNA samples were washed with 70% ethanol and dissolved in RNase-free water then quantified by NanoDrop spectrophotometer. Twenty microgram of total RNA from each sample was used for cDNA synthesis using the SuperScript indirect cDNA labeling system (Thermo Fisher Scientific). cDNA from each uninduced control sample or WT sample was labeled with Alexa Fluor® 647 while cDNA from each induced sample or *fur* mutant was labeled with Alexa Fluor® 555. After labeling, cDNA was quantified by NanoDrop spectrophotometer and aliquots of 250 pmol of cDNA were subject to hybridization onto microarray slides that consist of 4,109 gene-specific antisense oligonucleotides (65-mers; Sigma-Genosys). The transcriptome changes were monitored and compared between the IPTG-induced and uninduced cells at the same timepoints as indicated. Only the fluorescence signals well above the background level are considered. The heat maps were generated by using Cluster 3.0 and Java TreeView. The log_{10}-transformed data was subject to hierarchical clustering using uncentered correlation and complete linkage functions. Genes that are upregulated are shown in red and genes that are downregulated are shown in green. The microarray results have been deposited in the NCBI GEO database under the accession number GSE100668.

RNA extraction and real-time qPCR

To monitor mRNA levels of Fur-regulated genes, cells were grown at 37^{0}C in LB medium overnight and subcultured with 1:100 ratio into fresh LB medium amended with either 10 or 25 µM iron as specified. After OD_{600} reaches ~0.25, 1mM IPTG was added to induce expression of FrvA as indicated. Aliquots of 5 ml of cells were harvested at different time points. To monitor mRNA levels of PerR-regulated genes (Fig. S3B), overnight culture was used to inoculate a fresh culture with 1:100 ratio in LB medium. 1mM IPTG was added to induce expression of FrvA in the beginning of inoculum to create severe iron starvation. Aliquots of 5 ml of cells were harvested at timepoints with different optical density (OD_{600}) as indicated for uninduced cells. Total RNA was extracted using RNeasy Mini Kit following the manufacturer's instructions (Qiagen Sciences, Germantown, MD). All RNA samples were treated with Turbo-DNA free™ DNase (Ambion™) and precipitated with 2-3 volume of ethanol and 0.1 volume of 3M sodium acetate at

-80^{0}C overnight. RNA samples were washed with 70% ethanol and dissolved in nuclease-free water then quantified by NanoDrop spectrophotometer. Two hundred nanogram of total RNA from each sample was subjected to cDNA synthesis using high-capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA). Primers used in this study are listed in Table S2. Quantitative PCR (qPCR) was then conducted using iQ SYBR green supermix in an Applied Biosystems 7300 Real Time PCR System. The housekeeping gene 23S rRNA was used as an internal control.

Construction of chromosomal FLAG-tagged Fur

To construct a C-terminal FLAG-tagged Fur at its native locus, the recombinant plasmid pMUTIN::Fur-FLAG was cloned using Gibson assembly and transformed into *Escherichia coli* DH5α. The correct insertion was confirmed by colony PCR and DNA sequencing. The recombinant plasmid was then transformed into *E. coli* TG1. The resulting transformants were confirmed by colony PCR and the recombinant plasmid was extracted, further verified by DNA sequencing and transformed into *B. subtilis* WT (CU1065) and WT P_{spac}-*frvA*. Colony PCR was conducted to verify the presence of FLAG-tag in the C-terminus of Fur of *B. subtilis*.

Western blot analysis of FLAG-tagged Fur

Cells were grown in LB medium to an OD_{600} of ~0.4 and 10 ml of cell culture was harvested. Cell pellets were washed in PBS buffer, resuspended in a lysis buffer (10 mM Tris pH8.0, 100 mM NaCl, 1 mM EDTA, and 5% glycerol) and lysed by sonication. The total protein content was quantified using a Bradford assay and 20 µg of total protein from whole cell lysate of each sample was loaded to a mini-protein TGX stain-free gel. After electrophoresis, the gel was visualized by a ChemiDoc™ MP imaging system (BioRad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane using a Trans-Blot® Turbo™ transfer system (BioRad, Hercules, CA) and then subjected to western blot using a monoclonal anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s protocol. The Fur-FLAG protein has a molecular mass of ∼19 kDa, in accordance with the signals observed in the blot.

Disk diffusion assay

Cells were grown overnight in LB medium and subcultured at 1% into fresh LB medium to an OD_{600} of 0.4. Cell culture (100 µl) was mixed with 4 ml of 0.75% LB agar and poured onto 1.5% LB agar plates. The plates were dried for 15 min in a laminar flow hood at room temperature. Filter paper disks (6.5 mm in diameter) soaked with 10 µl of 1 M $FeSO_4$ (prepared in 0.1 M HCl) or 5 µl of 5 mg ml^{-1} streptonigrin (SN) were placed on the top of the agar plates, and the plates were incubated at 37^{0}C for 16–18 h.

As shown in Fig. S6C, a purple halo is evident around the inhibition zone in a *fur* null mutant due to derepression of bacillibactin biosynthesis (DhbACEBF) (5); while the FLAG-tagged Fur in both genetic background (WT and P_{spac}-*frvA*) behaves very similarly as WT without FLAG-tagged Fur, indicating the chromosomal FLAG-tagged Fur is a functional regulator. For experiments done in Fig. S11A, overexpression of *L. monocytogenes* FrvA induces derepression of Fur regulon in *B. subtilis*, including *dhbACEBF* operon as reported previously (28). So effects of Btr on BB uptake can be evaluated by comparing the intensity of the purple complex, DHB(G)-Fe^{3+} or BB-Fe^{3+}. For experiments done in Fig. 5, the data are expressed as the diameter (mean ± SD; n=3) of the inhibition zone (mm). Statistically significant differences are determined by two-tailed *t*-test, *, $P < 0.05$.

Quantification of bacillibactin/DHB(G) accumulation in medium

To quantify accumulation of DHB(G) and BB Cells on the plates shown in Fig. S11A, cells were grown overnight in LB medium, subcultured with 1:100 ratio into fresh LB. Cell culture (100 µl of OD_{600} ~ 0.4) was inoculated into MOPS minimum medium, which is amended with 5 µl of $MnCl_2$ and 1 mM $FeSO_4$. For IPTG-treated cells, 1mM IPTG was added to induce expression of FrvA. Accumulation of DHB(G) and BB was quantified using 1 ml of cell-free supernatant after addition of 50 µl of 10 mM $FeCl_3$ (prepared in 100 mM HCl) and neutralization by 100 µl of 1 M Tris-HCl buffer (pH 8.0). The resulting purple DHB(G)-Fe^{3+} or BB-Fe^{3+} complex was measured spectrophotometrically. An optical density at 510 nm (OD_{510}) of 0.5 is equivalent to ~80 µg of DHB(G) or BB per ml (7).

Chromatin immunoprecipitation coupled with quantitative PCR (ChIP-qPCR)

To monitor the occupancy of Fur at its target sites in vivo, we performed chromatin immunoprecipitation (ChIP) coupled with quantitative PCR. Cells were grown in LB medium amended with 25 µM $FeSO_4$ to OD_{600} of ~0.25 and 1 mM IPTG was added to cell culture to induce expression of FrvA as indicated. At different time points, 40 ml aliquots were harvested and the pellets were kept at −80 °C. The pellets were washed and resuspended with buffer CA (10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 137 mM NaCl, and 2.7 mM KCl). The samples were incubated with 1% formaldehyde at room temperature for 10 min for crosslinking and then incubated with 133 mM glycine (pH7.5) at 4 °C for 30 min to quench the crosslinking. Cells were spun down, washed twice with buffer CB (50 mM Tris–HCl pH7.4, 150 mM NaCl and 1 mM EDTA), and then resuspended in 0.5 ml buffer CB followed by sonication for cell lysis and DNA fragmentation. The supernatant was collected after centrifugation and the total protein concentration was quantified using a Bradford assay. Aliquots of 400 µg of total protein were kept at −80 °C. Aliquots of 1% volume of the lysate

were diluted with CB buffer and kept at −80 °C to serve as the input-control (1% of input DNA). For immunoprecipitation, α-FLAG M2 magnetic agarose beads (Sigma, Cat# M8823) were washed and resuspended in 400 µl buffer CB. Aliquots of 400 µg of total protein were diluted, mixed with the washed magnetic beads, and incubated on a rotation mixer overnight in a cold room (4^0C). The bead slurry was recovered by using a magnetic stand and washed twice with 500 µl of buffer CB. The protein–DNA complexes were eluted with 3X FLAG peptide according to the manufacturer's protocol. All samples including 1% input DNA samples were treated at 65^0C for overnight to reverse crosslinking. Co-immunoprecipitated DNA was purified using a PCR purification Kit (Omega Biotek, Norcross, GA), quantified by NanoDrop spectrophotometer, and diluted appropriately followed by quantification using qPCR. Specific primer sets to the promoter regions of the target genes are listed in Table S2. DNA enrichment was calculated based on the input DNA (1% of total DNA used for each ChIP experiment). The housekeeping gene *gyrA* was used as a non-specific negative control.

Electrophoretic mobility shift assays (EMSA)

The promoter region (~160 bp) of each individual Fur-regulated gene tested was amplified by PCR using a specific primer set listed in Table S2. Two hundred nanogram of purified DNA was labelled at the 5'-end with [γ-^{32}P]-ATP using T4 polynucleotide kinase. After labelling, a G10 column (NucAway™ spin columns, Invitrogen) was used to remove the unincorporated (γ-^{32}P) ATP and radioactivity of each probe was quantified by a scintillation counter. The binding reactions were conducted as following: ~1 fmol of labelled DNA probe, 1 mM $MnCl_2$, varied concentration of Fur protein, and 1X binding buffer (10 mM Tris-HCl, pH 8.0, 5% glycerol, 2 µg ml^{-1} salmon testes DNA, 50 mM NaCl, 1 mM DTT, 50 µg ml^{-1} BSA). The reactions were incubated at room temperature for 20 min and then subject to electrophoresis in a 5% polyacrylamide gel using 40 mM TA buffer (pH 8.0, no EDTA). After electrophoresis, the gels were dried using a gel dryer, exposed to a phosphorimager screen overnight, and scanned by a phosphor image analyzer (Typhoon FLA 7000). The band intensity of unbound DNA was quantified using GelQuantNET software. The K_d values, corresponding to the concentration of Fur that leads to 50% half-maximal shifting of the DNA probe, were calculated using GraphPad Prism 5.

Table S1. Strains and plasmids used in this study

Strain	**Genotype**	**Reference**
WT (CU1065)	*trpC2 attSPβ* sfp^0	Lab stock
HB19208	*amyE ::* P_{spac}*- frvA* :: *cm*	(28)
HB17837	*fur* :: *kan*	(5)
HB2168	*fur* :: *kan perR* :: *spc*	(26)
HB19396	pMUTIN *:: fur-FLAG :: spec*	This study
HB19398	*amyE ::* P_{spac}*-frvA*::*cm* pMUTIN *:: fur-FLAG :: spec*	This study
HB8246	*btr* :: *spc*	(14)
HB19401	*btr* :: *spc amyE ::* P_{spac}*- frvA* :: *cm*	This study
HB8248	*btr* :: *spc fur* :: *kan*	(14)
HB5800	sfp^+	(7)
HB19405	sfp^+ *amyE ::* P_{spac}*- frvA* :: *cm*	This study
HB8247	sfp^+ *fur* :: *kan*	(14)
HB8242	sfp^+ *btr* :: *spc*	(14)
HB19409	sfp^+ *btr* :: *spc amyE ::* P_{spac}*- frvA* :: *cm*	This study
HB8249	sfp^+ *btr* :: *spc fur* :: *kan*	(14)
HB7384	*fsrA* :: *mls*	Lab stock
HB19411	*fsrA* :: *mls amyE ::* P_{spac}*- frvA* :: *cm*	This study
HB19403	*dhbA :: mls amyE ::* P_{spac}*- frvA* :: *cm*	This study
Plasmid	**Description**	**Reference**
pPL82	Expression of gene under P_{spac} promoter	Lab stock
pMUTIN	FLAG-tagged Fur in native locus	Lab stock

Table S2 Primer oligonucleotides

Number	Name	Sequence
Primers used for cloning		
5782	pPL82-check-for	AAGAAAGATATCCTAACAGCACA
5783	pPL82-check-rev	ACGATCTTTCAGCCGACTCA
8095	Fur-Flag-fwd	AGCGGATAACAATTAAGCTTCTCCTGAGATCGGTCTCGCTAC
8096	Fur-Flag-rev	CGATCGATAGCGCTGGTACCTTCAGTTTCTTTTCCGTTACAGC
6541	ybbb-UP-F	ATTGGCTTCACTGTTCAACAA
1452	Spec-check-rev	CGTATGTATTCAAATATATCCTCCTCCTCAC
1451	mls-check-rev	GTTTTGGTCGTAGAGCACACGG
8083	dhbA_CHIP_Fwd	TGACGGACCGCATCTATCAATGG
6439	FrvA-seq1	GTGTTCCAATCGATGGATTGA
6440	FrvA-seq2	CTTGAAGGGCTTTGATCGTAC
Primers used for real-time qPCR		
4368	23S-RT-F	AAAGGCACAAGGGAGCTTGACTGC
4369	23S-RT-R	ATGAGCCGACATCGAGGTGCCAAA
6943	FsrA-RT-F	ATAGAGAGAAGCTACTCTCTGTTC
8053	FsrA-RT-RV2	TTCGGATCTTGATCTGATAGAGG
6524	DhbA-RT-F	ACGCTTGCCAGTCAAGGCGCACAT
6525	DhbA-RT-R	AAAGCTTCTGCATGGCGGGCTTCTGCTTT
8058	YclN-RT-F	TATCGGTGTAGAAGATCTGTCGCC
8059	YclN-RT-R	TGCTGATCTGCTGCATAATCAAACC
6553	FeuA-RT-F	AAGGCAAGCGGCACAGCATCTGAGAAGAA
6554	FeuA-RT-R	AAATTGCGCCTTGCGGATGAACGTCAAGCA
8060	YfmC-RT-F	GATTCCAGAGTGATCCATGACGAA
8061	YfmC-RT-R	GCGTGTGCCTACAGATGTGTAATCA
8062	FhuB-RT-F	GAGCAAGGAATGATCCACCGATA
8063	FhuB-RT-R	GTGTTAAGTCTCGGTGACGATCTG
8064	YkuN-RT-F	CCTTGATTACATATGCCAGCATGT
8065	YkuN-RT-R	TAGGTGCCAATCAGTACATAATCAT
8066	YwbL-RT-F	GATATGGATTCGGCACAACAAACGT
8067	YwbL-RT-R	ATGAGACGCAAGCTCTTCAAGCTG
8070	FbpA-RT-F	GCTGATCCAGGAAAACAAAGAGG
8071	FbpA-RT-R	TTGATGCTTGCGGTCGATCC
8072	FbpC-RT-F	GGTGAAGCAAATGACAATGCTGT
8074	FbpC-RT-R	CATCAAACAGATTTATTAGAGATTCC
8075	AhpC-RT-F	CAATGGAGCGTATTCTGCTTCTAC
8076	AhpC-RT-R	TCAGAGCTGTCATGCCAGCCTT
8077	KatA-RT-F	GCTTGAGTGTAGTGATCGTAGTGA
8078	KatA-RT-R	TTATCAGCGTGATGGGCAAATG
8079	PfeT-RT-F	CGAAGGAAGGAATCGAAGAAACA
8080	PfeT-RT-R	TCTCTGCTGCTTTTATTCATCGTGT
8081	MrgA-RT-F	ATACTCTAAGCTCCACCGTTTCC
8082	MrgA-RT-R	TGATAGATGCATGCTCAGTGTATTC
6697	hemA_RT_FW	TTATGCGGTAGTCGACCAGCTT
6698	hemA_RT_RV	ATCACCATAGAATCAAGTCCGCA

Primers used for synthesis of EMSA probes		
8038	FeuA_fwd	CTATCCGGAGATTGTCCATGAT
8039	FeuA_Rev	GCCGTCAGCGCGAGAAGTAAGA
8040	dhbA_fwd	GTCACTGAAATTATATTTGACTG
8041	dhbA_rev	ATCATCAATTCCTTTCTTCGCTC
8042	YkuN_fwd	TGCTGGATCAGGAAAATCCAT
8043	YkuN_rev	TGCTGGCATATGTAATCAAGGC
8044	fhuB_fwd	GGTTGACACGATATTTTTGCAA
8045	fhuB_rv	GATTTCTTCTGATGCAGTCCGT
8046	ywbL_fwd	GACAGCTTTTTTGCTGTCCATCA
8047	ywbL_rv	CCATCAACAAGCTGAATAGAATA
8048	yfmC_fwd	GAGAAAGCAGTAAAAACGCAGCT
8049	yfmC_rv	AACACTCATGATGGCAATCAAC
8050	YclN_EMSA_fw	TGATAAATGACTAGGTTAATATT
8051	YclN_EMSA_rv	CTGCCTCCTTACATCCTTACA
8052	FsrA_EMSA_fwd	GAGCAGGACGGACTGATTTAA
8053	FsrA_EMSA_RV	TTCGGATCTTGATCTGATAGAGG
8054	FbpAB_EMSA_fwd	GGGAAACTTTTTGTCGATTTGTTG
8055	FbpAB_EMSA_rv	TCTGATTTCTGCAGACTGAGGTG
8056	FbpC_EMSA_fwd	GTGTTGTTAAGCGTCAGAATTCG
8057	FbpC_EMSA_rv	ACAGCATTGTCATTTGCTTCACC
Primers used for ChIP-qPCR		
8083	dhbA_CHIP_Fwd	TGACGGACCGCATCTATCAATGG
8084	dhbA_CHIP_rv	AGCTTCGCCTATTCCTTGGGC
8090	YclN_CHIP_fwd	GTAAACAGCCTAACGTTTTGGGATG
8091	YclN_CHIP_rv	CTGCCTCCTTACATCCTTACAGC
8093	YwbL_CHIP_fwd	GACAAAGGACAGGAACTGGCTATG
8094	YwbL_CHIP_rv	CGAGCCATCATGTTCCTCCTATAA
8092	FsrA_CHIP_fwd	CGATTGACATTGATACTGAGAATCA
8089	FsrA_CHIP_rv	GAACAGAGAGTAGCTTCTCTCTAT
8044	fhuB_fwd	GGTTGACACGATATTTTTGCAA
8045	fhuB_rv	GATTTCTTCTGATGCAGTCCGT
8054	FbpAB_EMSA_fwd	GGGAAACTTTTTGTCGATTTGTTG
8055	FbpAB_EMSA_rv	TCTGATTTCTGCAGACTGAGGTG
8056	FbpC_EMSA_fwd	GTGTTGTTAAGCGTCAGAATTCG
8057	FbpC_EMSA_rv	ACAGCATTGTCATTTGCTTCACC
8038	FeuA_fwd	CTATCCGGAGATTGTCCATGAT
8039	FeuA_Rev	GCCGTCAGCGCGAGAAGTAAGA
8042	YkuN_fwd	TGCTGGATCAGGAAAATCCAT
8043	YkuN_rev	TGCTGGCATATGTAATCAAGGC
8048	yfmC_fwd	GAGAAAGCAGTAAAAACGCAGCT
8049	yfmC_rv	AACACTCATGATGGCAATCAAC

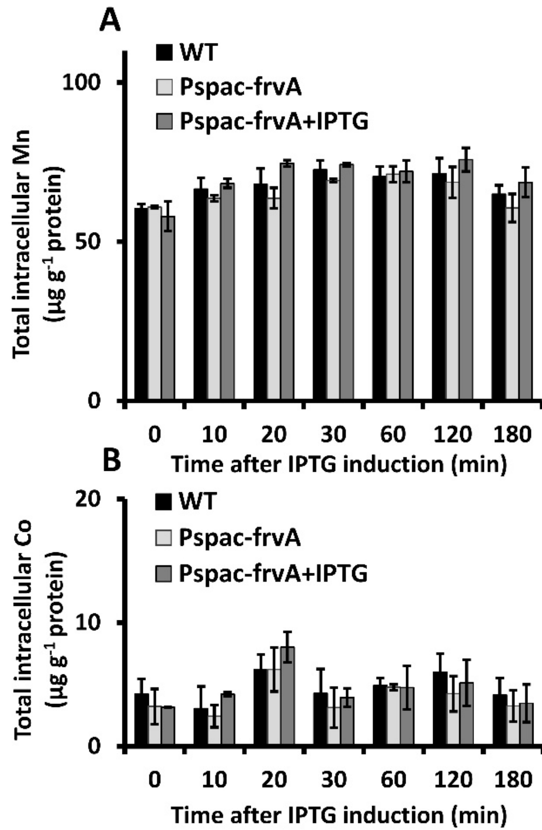


Fig. S1. Expression of FrvA has no effect on intracellular Mn and Co levels.

Levels of intracellular Mn (A) and Co (B) were monitored for three hours after IPTG addition by inductively coupled plasma mass spectrometry (ICP-MS). The total concentration of ions was expressed as µg ion per gram of protein (mean ± SD; n=3). Mn and Co levels were not significantly changed over the course of the experiment.

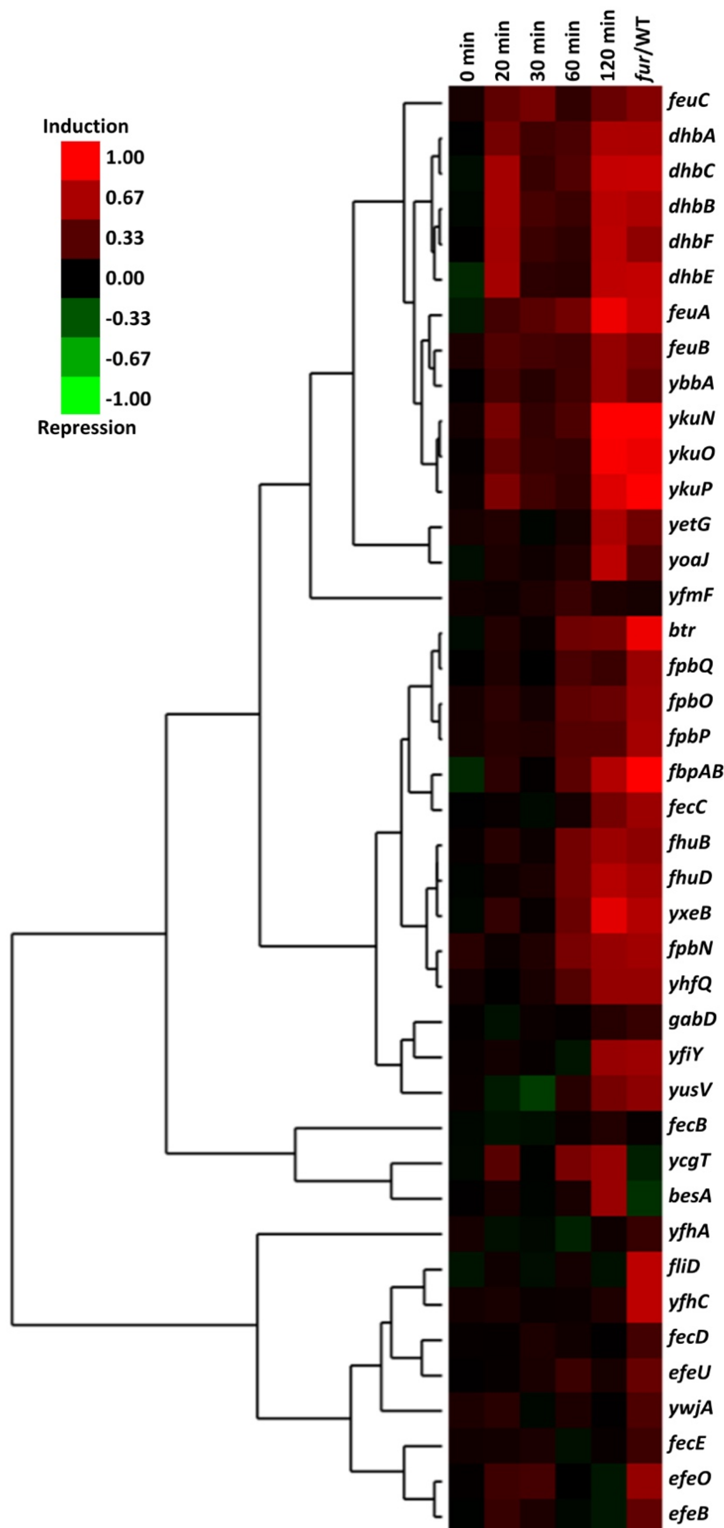


Fig. S2. Fur-regulated genes are derepressed in response to iron depletion. Cells (WT P_{spac}-*frvA*) were grown in LB medium amended with 10 µM $FeSO_4$ to an OD_{600} of ~0.25 and expression of FrvA was induced with 1 mM IPTG. Aliquots of 40 ml of cell culture were harvested at different time-points and total RNA was extracted using an acidic phenol-based method. All RNA samples were treated with Turbo-DNA free™ DNase and 20 µg of total RNA was used for cDNA synthesis followed by cDNA labeling with either Alexa Fluor® 647 (uninduced or WT) or Alexa Fluor® 555 (induced or *fur* null). 250 pmol of cDNA was then subjected to hybridization onto microarray slides. Hierarchical clustering was used to generate a heat map of induction (red) of known Fur-regulated genes at different time points as indicated on the top. Full-derepression of each Fur-regulated gene was shown in the last column where the transcriptome of a *fur* null mutant was compared to that of WT.

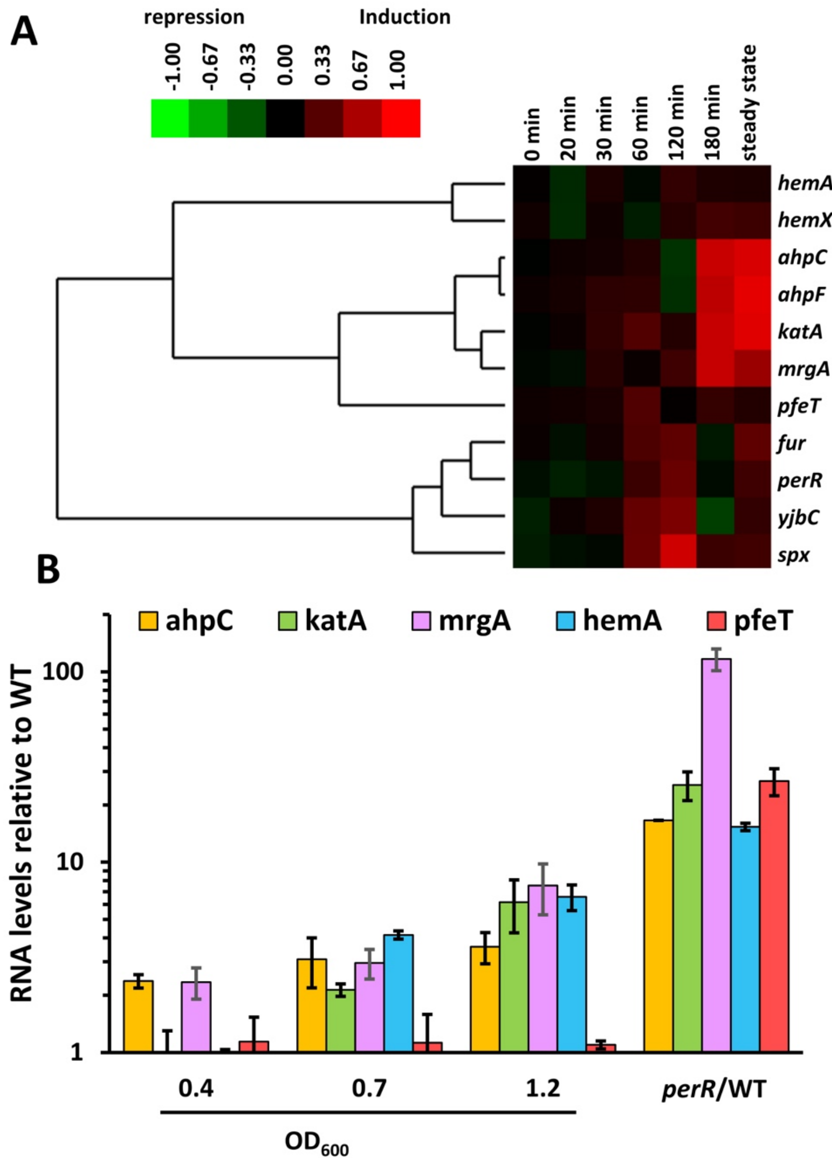


Fig. S3. Severe iron deprivation induces some PerR-regulated genes.

A. Hierarchical clustering was used to generate a heat map of induction (red) of known PerR-regulated genes at different time points as indicated on the top. Evident induction of some genes is shown in the last column, marked as "steady state", where expression of FrvA was induced by 1 mM IPTG from the beginning of the inoculum that leads to severe iron limitation and cells were harvested around OD_{600} ~0.6.

B. Overnight cell culture was inoculated with 1:100 ratio into fresh LB medium in the absence or presence of 1mM IPTG to induce expression of FrvA. Relative expression of each gene at different growth phases was analyzed by comparing gene expression in the IPTG induced cells versus that in wild-type cells. Full-derepression is considered as the mRNA level (in fold change) of each gene in *perR* null mutant compared to WT (shown as *perR*/WT). In the case of *katA*, *perR fur* double mutant was used to evaluate its full derepression since the *perR* single mutant is not genetically stable and often forms spontaneous suppressors with *katA* null mutation (30).

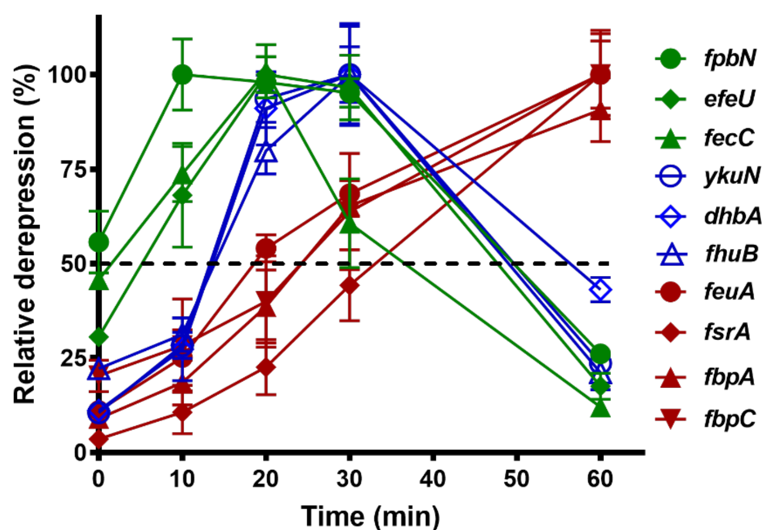


Fig. S4. Leaky expression of FrvA induces some genes in the Fur regulon.

Cells were grown at 37^{0}C in LB medium overnight and subcultured with 1:100 ratio into fresh LB medium amended with 10 μM $FeSO_4$. After OD_{600} reaches ~0.25, 1mM IPTG was added to induce expression of FrvA. Relative expression of each gene tested at different time points was monitored by comparing gene expression in the IPTG induced cells (WT P_{spac}-*frvA*) versus that in wild-type cells (WT). The percentage of derepression was normalized based on the full derepression observed in a *fur* null mutant versus WT cells, which was set as 100% derepression for each gene tested.

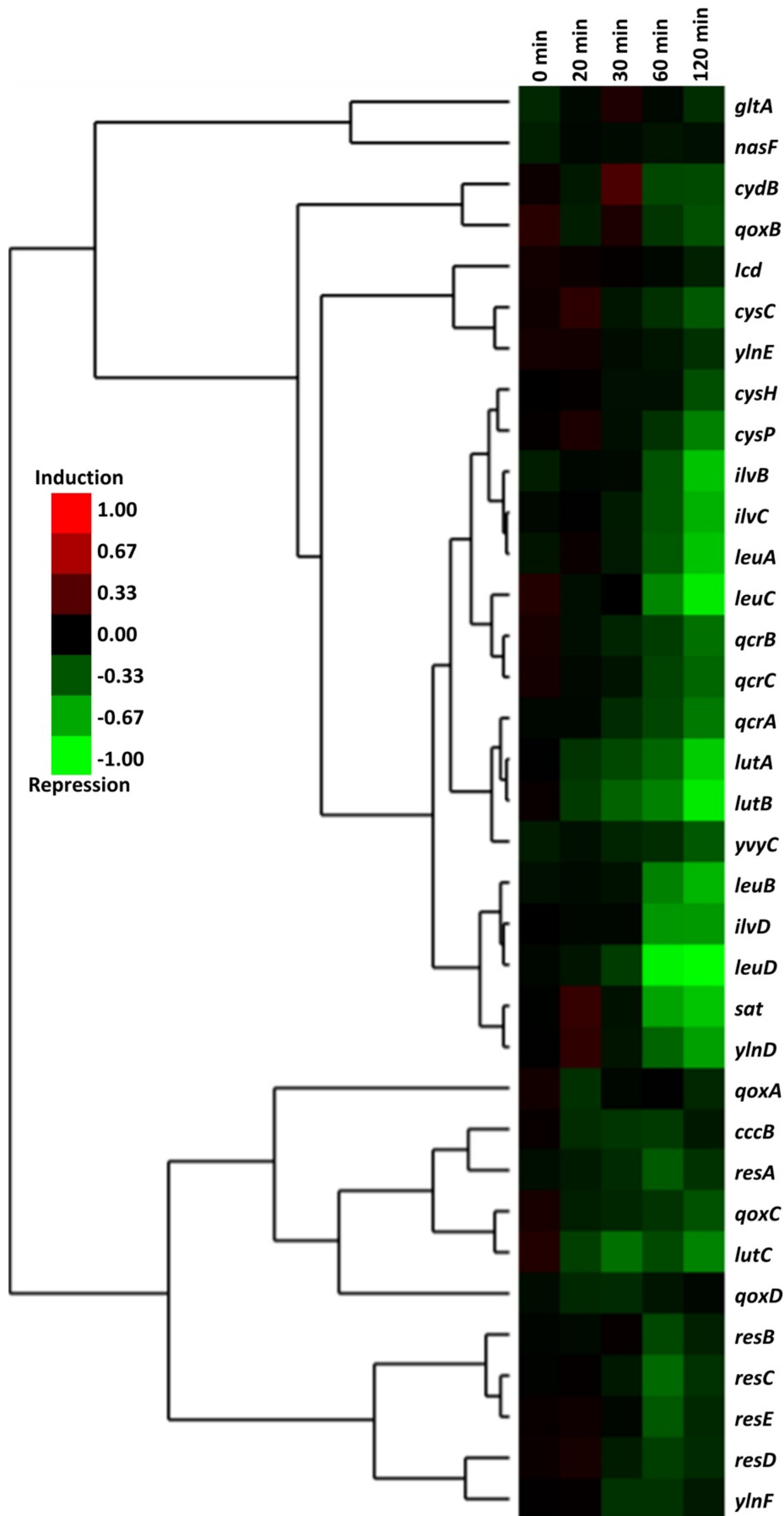


Fig. S5. A decrease in mRNA levels of many FsrA-regulated genes is observed at later timepoints in response to iron depletion.

Hierarchical clustering was used to generate a heat map of down-regulation (green) of known FsrA-regulated genes at different time points as indicated on the top.

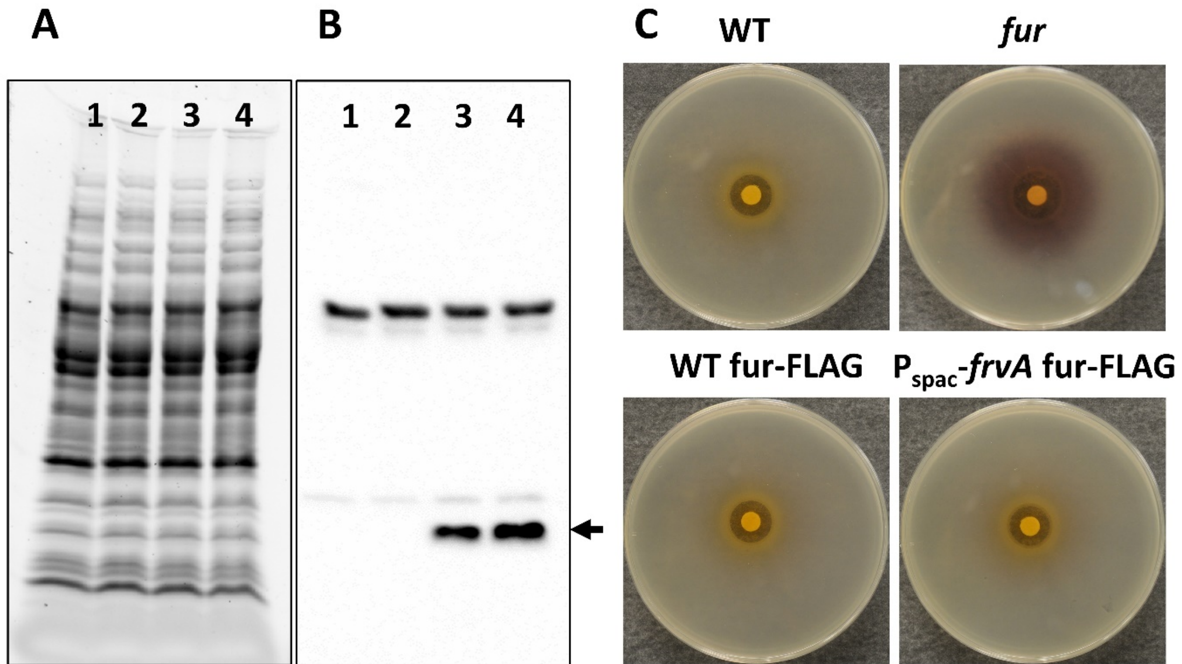


Fig. S6. The chromosomal FLAG-tagged Fur is a functional regulator.

A. 20 µg of total protein from the whole cell lysate of each sample was loaded to a SDS-PAGE gel. Strains tested are: Lane 1, WT; Lane 2, WT P_{spac}-*frvA*; Lane 3, WT::Fur-FLAG; Lane 4, WT P_{spac}-*frvA*::Fur-FLAG.

B. The same SDS-PAGE gel as shown in Fig. S6A was subjected to western blot using monoclonal anti-FLAG antibody. The specific signals are indicated by the arrow.

C. A disk diffusion assay was used to confirm the functionality of the chromosomal FLAG-tagged Fur *in vivo*. 10 µl of 1M $FeSO_4$ was applied onto each disk. A purple halo is evident around the inhibition zone in a *fur* null mutant due to derepression of the siderophore bacillibactin biosynthesis (DhbACEBF), which is under regulation of Fur; whereas the FLAG-tagged Fur in both genetic background (WT and P_{spac}-*frvA*) behaves very similarly as WT without FLAG-tagged Fur, indicating the chromosomal FLAG-tagged Fur is a functional regulator.

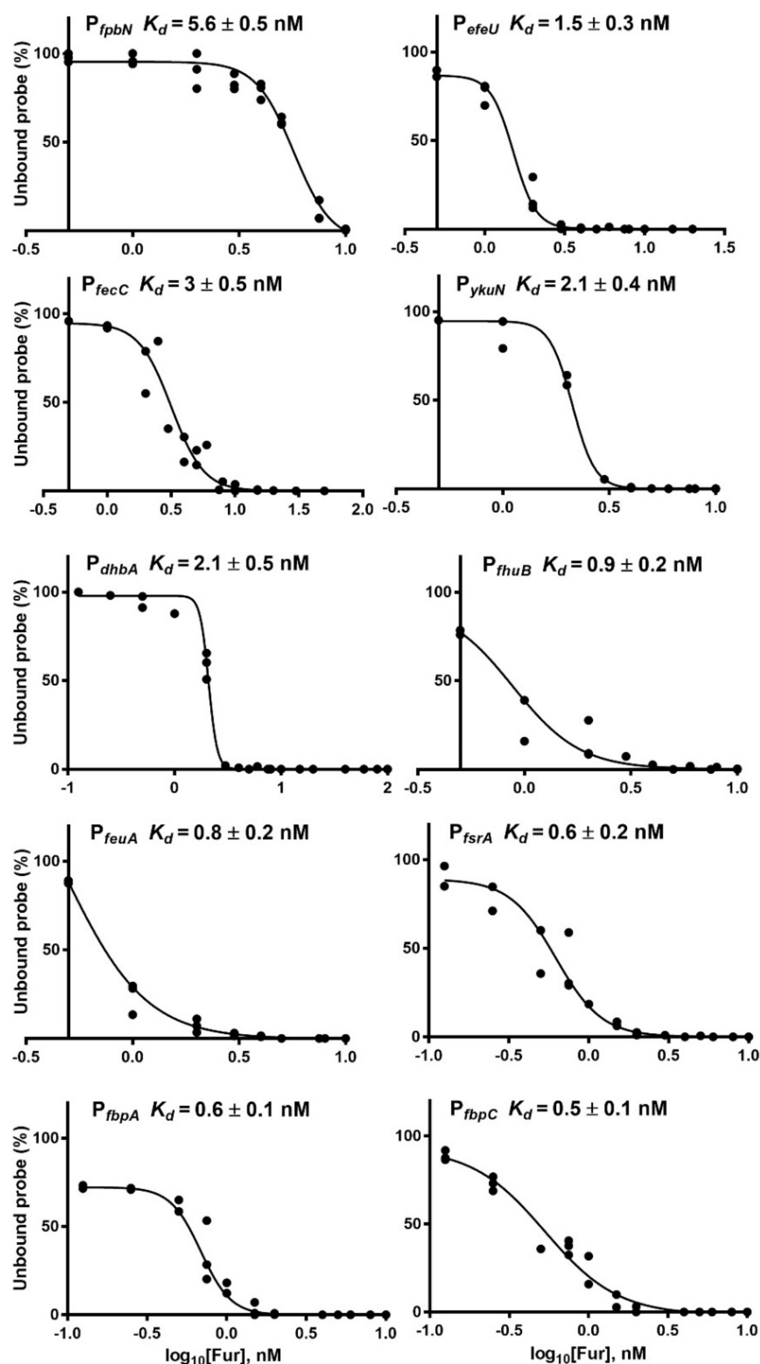


Fig. S7. The stepwise derepression of Fur-regulated genes correlates with protein-DNA binding affinity. Fur-DNA binding affinities to different promoter regions are determined by electrophoretic mobility shift assay (EMSA). The band intensity of unbound DNA probe was quantified using GelQuantNET software. All the data points from three independent experiments were plotted and subjected to K_d determination using GraphPad Prism 5.

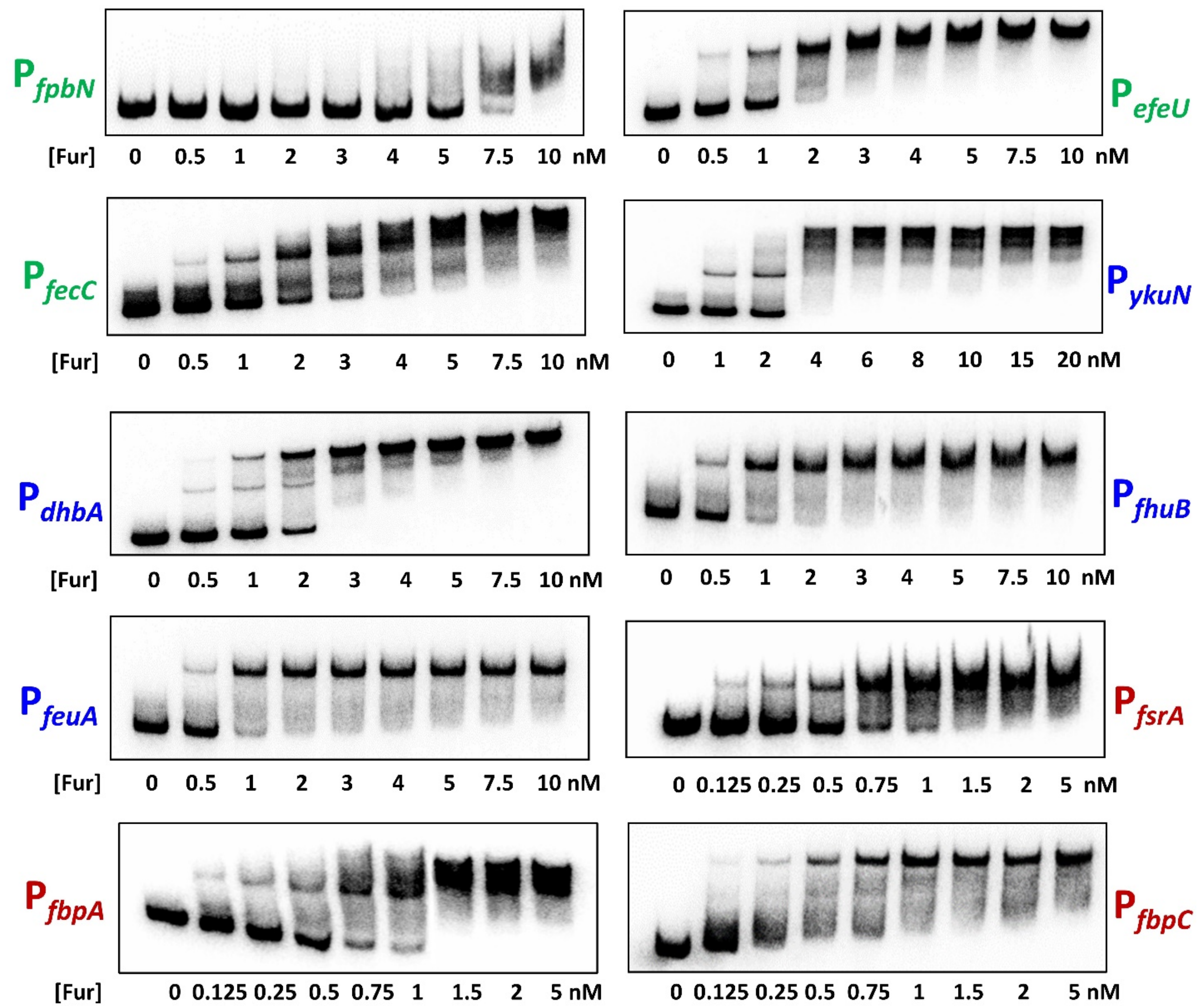


Fig. S8. Fur-DNA binding affinities to different promoter regions.

Representative images to show protein-DNA binding affinities to different promoter regions determined by electrophoretic mobility shift assay (EMSA).

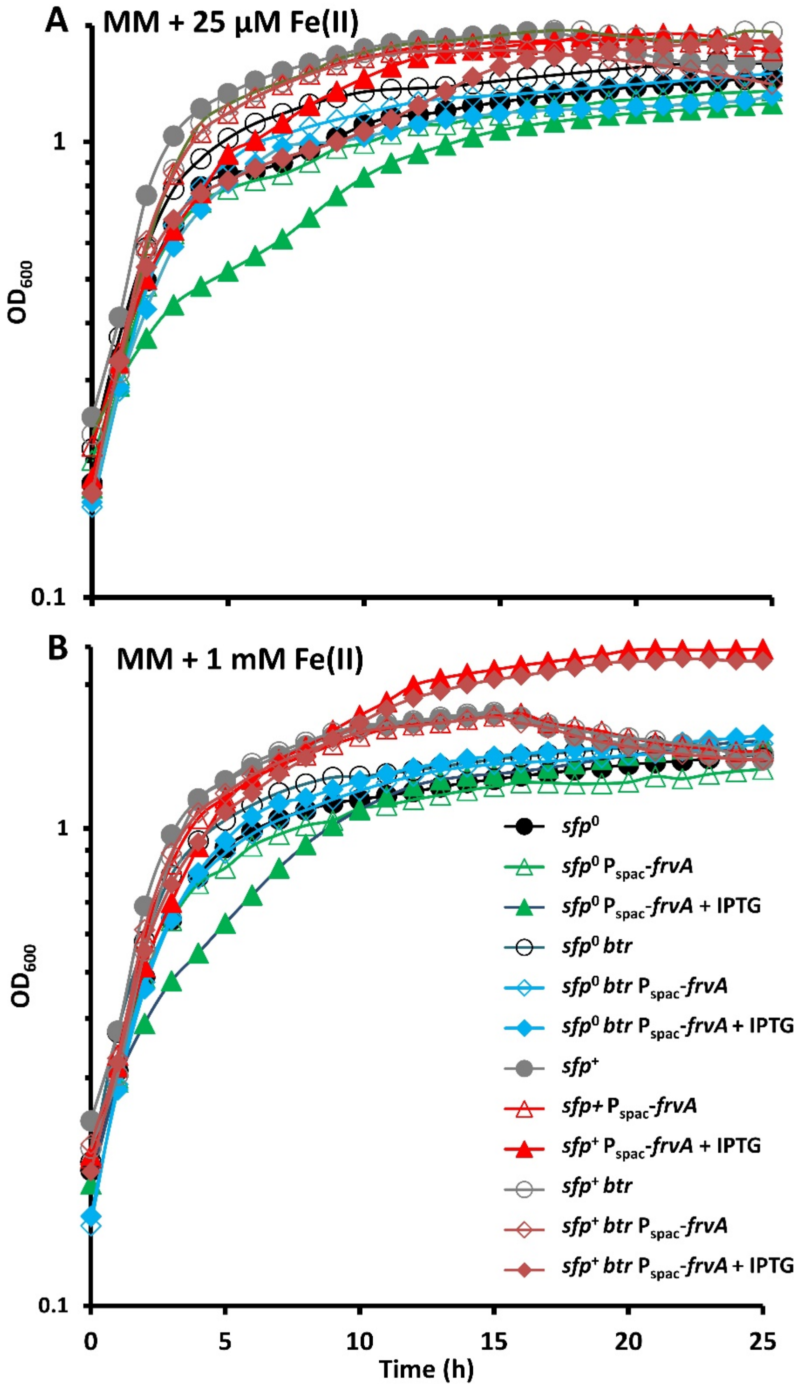


Fig. S9. Growth advantage of *sfp*$^{+}$ strains over *sfp*^{0} strains.

Representative growth curves of *sfp*^{0} and *sfp*$^{+}$ strains in minimum medium (MM) amended with 5 µM of $MnCl_2$ and different concentration of $FeSO_4$, 25 µM (A) or 1 mM (B). Cells were grown at 37^{0}C in LB medium overnight and subcultured at a 1:100 ratio into MM medium. For IPTG treated cells, 1 mM IPTG was added to cell culture when OD_{600} reaches ~0.2. Cell growth were then monitored for 25 h.

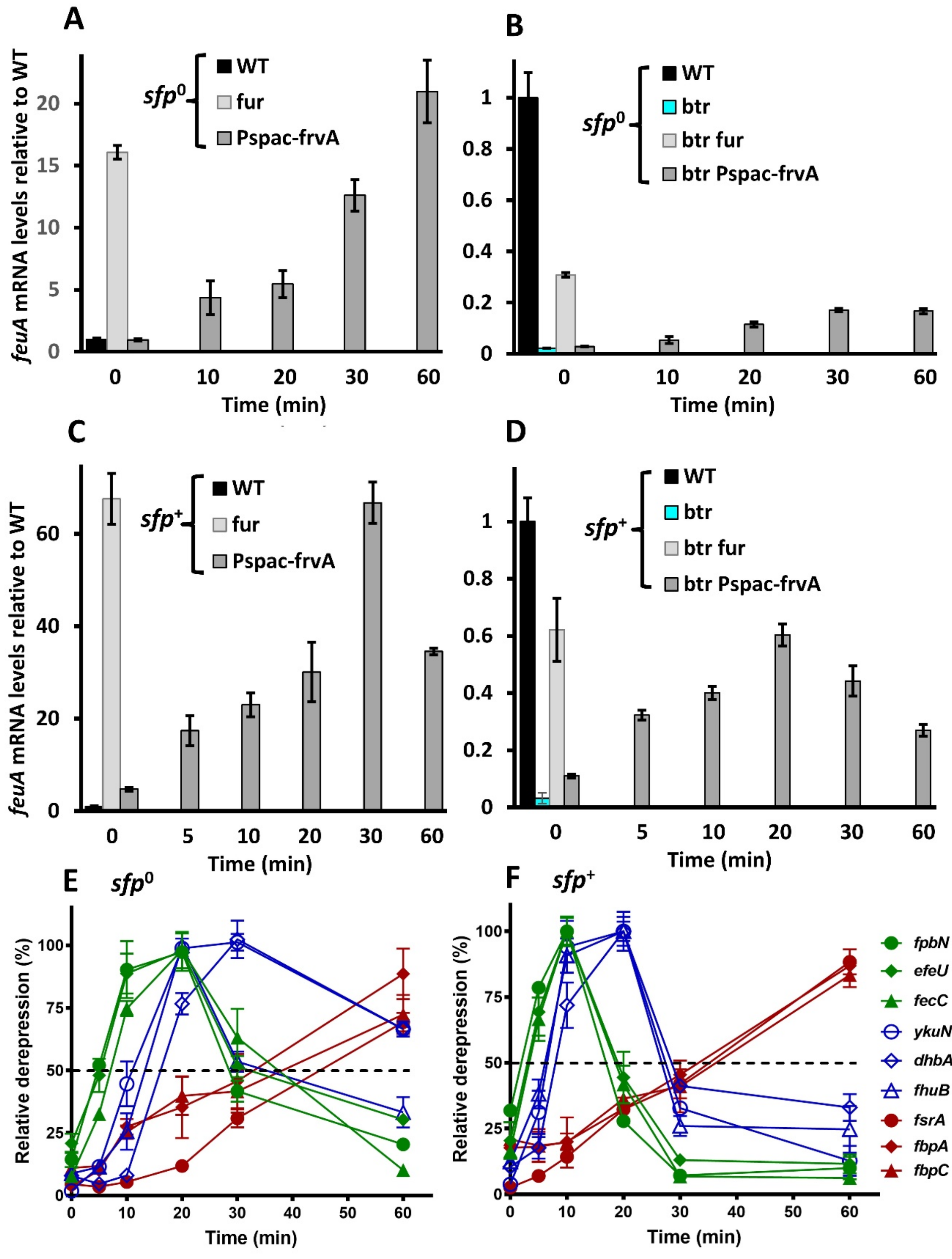

A
B
C
D
E
F
feuA mRNA levels relative to WT
sfp0
sfp+
WT
fur
Pspac-frvA
btr
btr fur
btr Pspac-frvA
Time (min)
Relative derepression (%)
fpbN
efeU
fecC
ykuN
dhbA
fhuB
fsrA
fbpA
fbpC

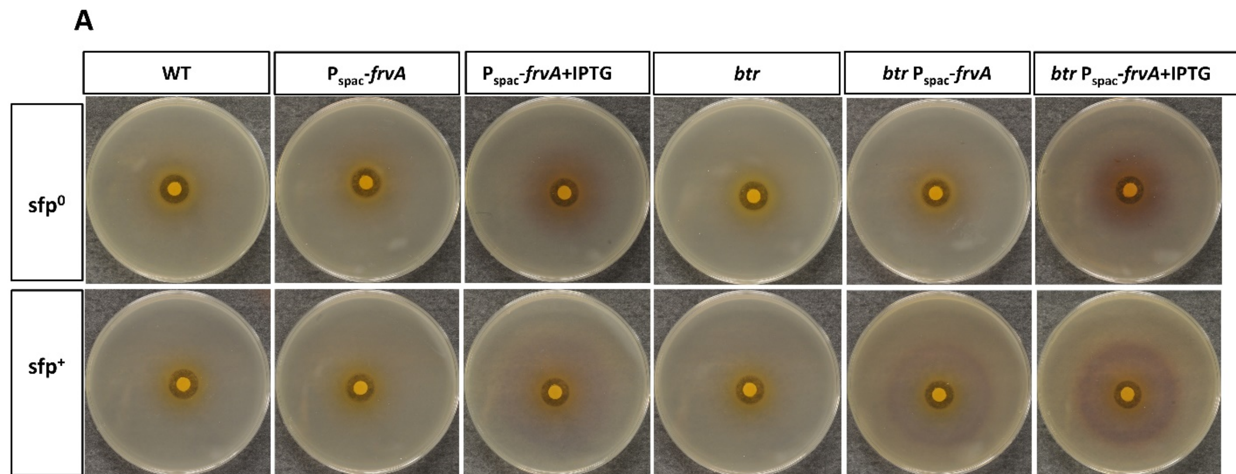
Fig. S10. Inactivation of bacillibactin uptake affects induction of the Fur-regulated genes.

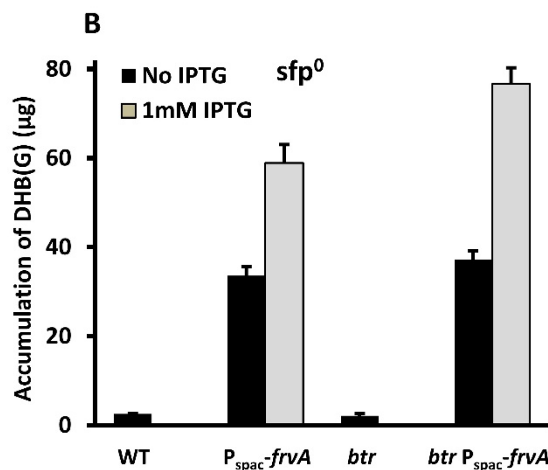
Cells were grown at 37^0C in LB medium overnight and subcultured with 1:100 ratio into fresh LB medium amended with 25 μM $FeSO_4$. After OD_{600} reaches ~0.25, 1mM IPTG was added to induce expression of FrvA. Total RNA was extracted and subjected to cDNA synthesis followed by qPCR.

A, B. The expression level of *feuA* in *sfp*^{0} WT cells is set as 1 in both S10A and S10B. Relative mRNA levels of *feuA* in different strains tested compared to that in wild type (*sfp*^{0}) are presented as fold changes (mean ± SD; n=3);

C, D. The expression level of *feuA* in *sfp*$^+$ wild-type cells is set as 1 in both S10C and S10D. Relative mRNA levels of *feuA* in different strains compared to that in *sfp*$^+$ WT are presented as fold changes (mean ± SD; n=3);

E, F. Relative expression of each gene tested was monitored by comparing gene expression in the IPTG induced cells (*i.e.* E, *sfp*^{0} *btr* P_{spac}-*frvA* + IPTG; F, *sfp*$^+$ *btr* P_{spac}-*frvA* + IPTG) versus that in *btr* null mutants. The percentage of derepression was normalized based on the full-derepression observed in *btr fur* mutant versus a *btr* single mutant, which was set as 100% derepression.





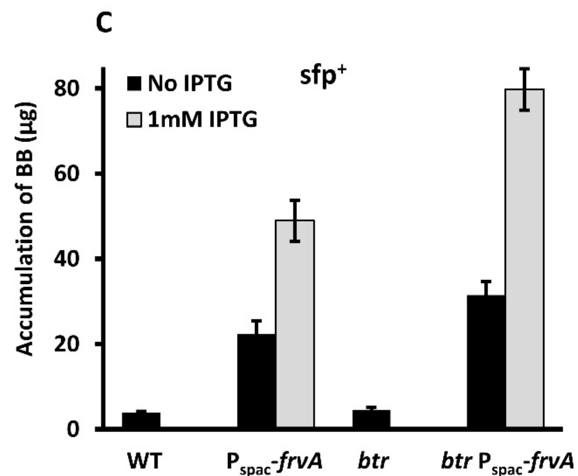


Fig. S11. Physiological role of Btr on bacillibactin uptake system in both sfp^0 and sfp^+ strains.

A. Representative photographs of a disk diffusion assay performed with *sfp*^{0} (top panel) or *sfp*$^+$ (bottom panel) strains. 10 µl of 1M $FeSO_4$ was added onto each filter paper disk. Accumulation of DHB(G) or bacillibactin (BB) in the medium can be evaluated by comparing the intensity of the purple complexes around the zone of inhibition resulting from overexpression of FrvA;

Quantification of DHB(G) production in *sfp*^{0} strains (B) or quantification of BB production in *sfp*$^+$ strains (C) by measuring the optical density (OD_{510}). An optical density at 510 nm (OD_{510}) of 0.5 is equivalent to 80 µg of DHB(G) or BB per ml.