A Comparative Analysis of Perturbations Caused by a Gene Knockout, a Dominant Negative Allele, and a Set of Peptide Aptamers

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Running title: Perturbations by reverse genetics methods
Summary

The study of protein function mostly relies on perturbing regulatory networks by acting upon protein expression levels or using transdominant negative agents. Here, we used the *E.coli* global transcription regulator Fur (ferric uptake regulator) as a case study to compare the perturbations exerted by a gene knockout, the expression of a dominant negative allele of a gene and the expression of peptide aptamers that bind a gene product. These three perturbations caused phenotypes that differed quantitatively and qualitatively from one another. The Fur peptide aptamers inhibited the activity of their target to various extents and reduced the virulence of a pathogenic *E.coli* strain in *Drosophila*. A genome-wide transcriptome analysis revealed that the “penetrance” of a peptide aptamer was comparable to that of a dominant-negative allele but lower than the “penetrance” of the gene knockout. Our work shows that comparative analysis of phenotypic and transcriptome responses to different types of perturbation can help decipher complex regulatory networks that control various biological processes.
Introduction

In those organisms that do not lend themselves to classical genetics, the study of molecular regulatory networks owes a lot to a methodology often referred to as reverse genetics. Reverse genetics encompasses various approaches aimed at inducing three major classes of targeted perturbations within regulatory networks. The first consists of affecting protein expression levels, by overexpressing gene products or by inhibiting their expression, either partially (antisense technology, RNA interference) or totally (gene knockout). The second consists of forming inactive protein complexes by use of dominant negative alleles, which usually titrate interacting proteins away from endogenous wild type proteins (1). The third consists of inhibiting protein function or protein interactions by use of specific ligands such as antibodies (2), nucleic acid aptamers (3) or small molecule inhibitors when available (4).

Peptide aptamers add to this arsenal of protein ligands (5). They are man-made combinatorial protein reagents that bind target proteins and can interfere with their function in living cells and organisms. They consist of conformationally-constrained random sequence peptide loops displayed by a scaffold protein. Peptide aptamers can be selected by yeast two-hybrid methods for their ability to interact with a given protein. They can be selected also for their ability to confer a given phenotype to a cellular model. Typically, they bind target proteins with a high specificity that allows them to discriminate between closely related members within a protein family or even between different allelic variants of a protein. Peptide aptamers have been selected against many proteins and in every case, some aptamers have been shown to interfere with the function of their cognate target when expressed or introduced in cellular models or organisms (reviewed in (6-8)).
While all three above-mentioned classes of perturbation can provide important clues on protein function, they also present limitations that can produce misleading results. Gene knockouts, which often produce either inconspicuous or dramatic phenotypes, do not always deliver direct information on protein function (9). Dominant negative alleles and inhibitory ligands can be prone to limited efficacy and/or lack of specificity.

Here, we set out to compare the perturbations caused by peptide aptamers that bind a given protein, a dominant negative allele of the said protein and the knockout of its coding gene. We reasoned that an E.coli global regulator would offer a convenient model to our endeavor.

The ferric uptake regulator (Fur) protein satisfied many criteria to serve as a good case study. Fur is an iron-dependent global regulator of gene expression in bacteria. In complex with Fe^{2+} that activates the protein through a conformational change (10), Fur represses the transcription of target genes harboring a consensus operator sequence in their promoter (referred to as a Fur box) (11), although it can also positively regulate a smaller number of genes at a post-transcriptional level (12,13). About 100 genes from various E.coli strains have been shown to be regulated by Fur, most being involved in iron homeostasis, others acting in metabolism and oxidative, nitrosative and acidic stress responses (14-17). Since fur deletion mutants of different pathogenic bacteria such as Vibrio cholerae (18) and Pseudomonas aeruginosa (19) show a decreased virulence, Fur is considered as a potential target for novel antibiotic therapies. Finally, Fur has been extensively studied through the use of fur^- strains, reporter genes and fur mutants such as fur90 (H90Y) and fur51 (G51D), which were shown to negatively complement wild-type fur (20). While a total Fur inhibition
achieved by gene knockout is expected to affect the expression of all genes controlled by Fur, the variable levels of Fur inhibition expected from the use of a dominant negative allele or a set of peptide aptamers binding different surfaces of Fur with different affinities should produce finer perturbations.

We selected by a yeast-two-hybrid method a set of four peptide aptamers that bind the Fur protein. We used different in vitro and in vivo phenotypic assays and we performed a whole-genome transcriptome analysis to compare the perturbations caused by the Fur peptide aptamers, a fur dominant negative allele and the fur gene knockout.

**Experimental Procedures**

**Plasmid constructions**

*E.coli expression plasmids*

pBAD-Fur: We amplified the fur gene from *E.coli* genomic DNA using the oligonucleotides 5’-ATATGAATTCATGACTGATAACAATCACGC-3’ and 5’-ATATCTCGAGTTATTTGCCTTCGTGCAGCAT-3’ that contained respectively an EcoRI and a XhoI site. We ligated the PCR product into *EcoRI/XhoI*-cut pBAD24, a plasmid that bears a pBR replication origin, the P*BAD* promoter of the arabinose operon and its regulatory gene araC, and that directs an arabinose-inducible expression of transgenes (21).

pBAD-Fur90 and –Fur51: We performed a site-directed mutagenesis of pBAD-Fur using a “QuickChange" kit (Stratagene) according to the manufacturer’s instructions, using the following oligonucleotides : 5’-GCAACATCACCACGATTACCTGACTGCCTCGACTGC-3’ and 5’-GCAGTCGAGGCAGATCAGGTAATCGTGGTGTGTTGC-3’ for Fur90; 5’-CGATATGGGTGAAGAAATTGATCTGGCTACGGTATATCGCG-3’ and 5’-CGCGATATACCGTAGCCAGATCAATTCTTCACCCATATCG-3’ for Fur51.

pGEX-Fur aptamers and pBAD-Fur aptamers : We ligated *EcoRI/XhoI*-cut pJMX-Fur aptamer inserts into *EcoRI/XhoI*-cut
pBAD24 and/or pGEX4T1 (GE Healthcare), a plasmid that directs the IPTG-inducible expression of GST fusion proteins.

Two-hybrid plasmids

pHA3: We amplified the LexA cDNA of pEG202 using the oligonucleotides 5’-CCAAGCATACAATCAACTCCAAGCTTTGAATTCCATGGGCTCGAGATGAAAGCGTTAACGGCCAGGC-3’ that contained EcoRI, NcoI and XhoI sites, and 5’-CGGAATTAGCTTGGCTGAGGTGCAGTTACAGCCAGTCGCCGTTGCAATAACCCCAACCGCC-3’ that contained a stop codon. We partially digested pEG202 with HindIII and XhoI and we introduced the amplified product into pEG202 by homologous recombination in yeast. The resulting plasmid, pHA3, bears a yeast 2µ replication origin, a HIS3 marker and directs the expression of proteins whose carboxy-termini are fused to LexA.

pJMX: We inserted a XhoI site downstream the trxA gene contained in pJM-1 (5) to create pJMX. For this, we phosphorylated and annealed the oligonucleotides 5’-CTCGAGGCCTAGC-3’ and 5’-AGGCCTCGAGGCT-3’ and we cloned the annealed product into SfiI-cut dephosphorylated pJM-1.

Luciferase reporter plasmid: We started from pRS316, a yeast plasmid that carries a URA3 marker and a CEN/ARS replication origin (22). We amplified the LexAop promoter region of pSH18-34 (23) using the oligonucleotides 5'-TATATACCGCGGCATATCCATATCTAATCTTACC-3’ and 5’-ATTCTGCGGCCGCTATAGTTTTTCTCCTTGACG-3’ that contained a SacII and a NotI site, respectively. We ligated the PCR product into SacII/NotI-cut pRS316 to create pRS316-LexAop. We amplified the firefly luciferase cDNA from pGEMÆ-luc (Promega) using the oligonucleotides 5’-ATAAGAATGCGGCCGCATGGAAGACGCCAAAAACATAAAGAAAGG-3’ and 5’-AAGAAGTCCAAAGCTTTCTCGAGTTACAATTTGGACTTT-3’ by

Downloaded from https://www.mcponline.org
CCGCCCTTCTTG-3’ that contained a NotI and a XhoI site, respectively. We ligated the PCR product into NotI/XhoI-cut pHB1 to create pHB1-luc. To create a 2µ reporter plasmid, we started from Yeplac195, a yeast plasmid that carries a URA3 marker and a 2µ replication origin. We ligated a SacI/KpnI-cut pHB1-luc insert into SacI/KpnI-cut Yeplac195 to create pHB2-luc.

Bait and prey plasmids: We ligated the EcoRI/XhoI-cut pBAD-Fur (wild type and mutant alleles) inserts into EcoRI/XhoI-cut pEG202 and pJG4-5. To construct B112-aptamer fusions, we PCR-amplified the variable regions of F-aptamers from pWP2-C library plasmids using the oligonucleotides 5’- CGGGGCCGATCT CGTCGATTTCGAGTTTCTGGGCAGAGTGGAGCGGTCCG-3’ and 5’- CGTCAGCGATTTCATCCAGAATCGGGCGATCATTTTGCTCGGTCCG-3’. We introduced the amplified product into CpoI-cut pJMX by homologous recombination in yeast. To construct a Fur-LexA fusion, we PCR-amplified the Fur cDNA from pBAD-Fur using the oligonucleotides 5’- ATATGAATTCATGACTGATAACAATACCGCC-3’ and 5’- TTATTTATTCTCGAGTTTTGCCTTCGCGGACGTTCG-3’ that contained respectively a EcoRI and a XhoI site. We ligated the amplified product into EcoRI/XhoI-cut pHA3. We constructed the different Fur truncation bait plasmids by homologous recombination in yeast, co-transforming EcoRI/XhoI-cut pEG202 and PCR products obtained from pEG202-Fur using pairs of the following oligonucleotides: (N1) 5’- ggttggttggtttattccagcaacgcggactggctg GAATTCAATCGACTGATAACAATACCGCC-3’; (N11) 5’- ggttggtgtttatctcagcaacgcggactggctg GAATTCATGACTGATAACAATACCGCC-3’; (N84) 5’- ggttggtgtttatctcagcaacgcggactggctg GAATTCATGACTGATAACAATACCGCC-3’; (C148) 5’- ggttggtgtttatctcagcaacgcggactggctg GAATTCATGACTGATAACAATACCGCC-3’; (C83) 5’- ggttggtgtttatctcagcaacgcggactggctg GAATTCATGACTGATAACAATACCGCC-3’; (C103) 5’- ggttggtgtttatctcagcaacgcggactggctg GAATTCATGACTGATAACAATACCGCC-3’; (C132) 5’- ggttggtgtttatctcagcaacgcggactggctg GAATTCATGACTGATAACAATACCGCC-3’.

Nucleotides in lower case hybridize to pEG202 sequences. EcoRI and XhoI sites are underlined. Bait plasmids were isolated...
from yeast transformants, amplified in *E. coli*, checked, and re-transformed in yeast to perform drop-arrayed two-hybrid mating assays.

**Competitor plasmids:** We ligated the EcoRI/XhoI-cut pBAD-aptamer and Fur inserts into EcoRI/XhoI-cut pBC104 (24).

### Two-hybrid selection of Fur-binding peptide aptamers

Library and strain construction is detailed in the supporting Experimental Procedures. We transformed 700ml of MB210α yeast with 100µg of library to obtain 2.8x10⁷ transformants and we transformed MB226α yeast with pEG202-Fur and pSH18-34. We performed a yeast-two hybrid selection essentially as described (25). We estimated the mating efficiency at 76% and the number of diploid exconjugants at 6x10⁸. We plated 6x10⁷ diploids onto Ura’His”Trp”Leu”Ade” galactose/raffinose plates and incubated for 7 days. We replica-plated onto Ura’His”Trp”X-gal galactose/raffinose plates. We picked 5 clones that grew in absence of leucine and adenine and that displayed a β-galactosidase activity. Library plasmids were recovered and re-transformed into EGY48α. The interaction phenotypes were confirmed by a mating assay with EGY42a transformed with pEG202-Fur and pSH18-34 (23). We sequenced the aptamer genes and observed that we selected 4 different peptide aptamers.

### In vitro binding assays

We transformed BL21(DE3) *E. coli* with pGEX4T1-aptamer plasmids. We induced the expression of the GST fusions with 1mM IPTG for 3h. We collected the bacteria and resuspended them into a lysis buffer (50mM Tris pH8, 0.1M NaCl, 1mM EDTA, 5mM MgCl₂) containing 1mg/ml lysozyme. We froze and thawed the suspensions three times and sonicated on ice. We centrifuged the lysates at 13,000g for 30min, collected the soluble fractions, and analyzed aliquots on SDS-PAGE to verify that the respective amounts of GST-aptamer fusions were similar. We thus immobilized equal amounts of GST-aptamer fusions onto
100 µl glutathion sepharose 4B (GE Healthcare) at room temperature for 1h. We washed the beads with 10ml of lysis buffer and we eluted the bound proteins with lysis buffer containing 1.5M NaCl. We loaded the eluates onto a SDS-PAGE and we performed a Western blot analysis using an anti-Fur rabbit polyclonal antibody (raised against purified dimeric Fur protein, 1/1000 dilution). We revealed the blot using a HRP-linked rabbit antiserum and an ECL kit (Pierce).

Quantitative yeast two-hybrid assay
We co-transformed EGY42a with pHB2-luc and pEG202-Fur. We transformed TB50α with pWP2C plasmids directing the expression of aptamer-B112 fusion proteins or with pJG4-5 plasmids directing the expression of B42-Fur wt or B42-Fur90 fusion proteins. We mated the transformants and selected the diploid exconjugants. We performed the luciferase yeast two-hybrid assays as described§, 24h after addition of galactose.

Drop-arrayed yeast two-hybrid assay
We co-transformed TB50α with pSH18-34 and pJG4-5-Fur or pWP2C aptamer plasmids. We transformed EG42a with the different pEG202 bait plasmids. We mated the transformants on YPD solid medium and we selected the diploid exconjugants by replica plating onto Ura·His·Trp· glucose solid medium. We picked and resuspended the diploids into 50µl sterile water. We deposited 3µl of each diploid onto a very dry Ura·His·Trp·X-gal galactose/raffinose plate.

Yeast two-hybrid competition assay
We co-transformed TB50α with pBC104-aptamer or –Fur expression vectors and with pHB2-luc. We co-transformed TB50α with pEG202-Fur and pJG4-5-Fur. We mated the transformed strains on rich solid medium for 6h and we selected diploid exconjugants

§ : Dupont et al. Meth.Mol.Biol. (in preparation)
by replica-plating onto Ura^-His^-Trp^-Leu^- glucose medium. We performed the luciferase yeast two-hybrid assay as described^, 5h after addition of galactose.

Growth assay on high concentration of Mn^{2+}
We grew XL-1 blue transformants in TN medium containing 0.4% glycerol. We added 0.2% arabinose to half of the culture and grew for another 1 hour. We adjusted the OD_{600} to 0.6 and we performed serial dilutions of the cultures. We spotted 5µl of each dilution onto 0.4% glycerol, 100µg/ml ampicillin, 10mM MnCl_{2}, 2mM MgCl_{2} and 50µM deferroxamine mesylate (desferal) selection medium, with or without 0.2% arabinose, and we observed the plates after 3 days.

Fur transcriptional repression assay
We transformed QC6009 and QC6008 fur^- bacteria with pBAD24 to confer ampicillin resistance. We transformed QC2949 and QC2146 with pBAD plasmids directing the expression of Fur90, aptamer c120, and the Fur aptamers. We selected the transformants on TN medium supplemented with 0.4% glycerol, 2mM MgSO_{4} and 100µg/ml ampicillin and we streaked them onto the same medium. We replica-plated the streaks onto the same medium, supplemented with 1mM Fe(NH₄)₂(SO₄)₂, 1mM X-gal, and with or without 0.2% arabinose. We observed the plates after 15h.

Oxidative stress assay
We grew overnight cultures of E.coli QC2949 or 6009 transformants and we adjusted the OD_{600} at 0.08 in TN medium supplemented with 0.4% glycerol. After 2 hours, we added 0.2% arabinose, and after 30min we added 2mM H₂O₂ to the cultures. We grew the cultures for 15 hours and we measured the OD_{600}.

Drosophila infection
We grew wild type Canton S or Takl^ mutant flies at 25°C. We grew E. coli 1106 transformants to exponential phase (OD_{600}=0.8
to 1) in arabinose-containing medium. We pelleted and resuspended the bacteria into PBS to reach a theoretical OD$_{600}$ of about 200. We pricked 30 adult flies (5-10 days old) into the upper part of the thorax with a thin needle previously dipped into the above-described bacterial suspensions, as described previously (26).

Transcriptome analysis

RNA extraction. We transformed QC6009 (fur-) bacteria with pBAD24 and QC2949 (fur+) bacteria with pBAD24 containing Fur90, cl20 and F1. We grew overnight cultures in TN medium with Ampicillin, which we used to inoculate fresh cultures with a starting OD$_{600}$= 0.02. We grew the bacteria to reach an OD$_{600}$=0.1, added 0.2% arabinose and 1mM (NH$_4$)$_2$Fe(SO$_4$)$_2$·6H$_2$O, and grew again to reach an OD$_{600}$ = 0.3 to 0.5. We extracted the RNA using a Qiagen RNeasy Midi kit (Cat. n° 75142), according to the instructions. We quantified the extracted RNA by UV spectrophotometry and we checked the quality of the samples by agarose gel electrophoresis. We precipitated and resuspended the RNA to reach a concentration of 10$\mu$g/$\mu$l.

RNA labelling and hybridization. Following the instructions of the MWG array application guide, we performed a direct Cy3-dCTP (Amersham Pharmacia Biotech) labelling of single strand cDNA from 50$\mu$g of RNA and we purified the labelled cDNAs using a Qiagen PCR purification kit. We labelled two biological replicates for each experimental condition. We quantified the amounts of cDNAs and the label incorporation rates using a Nanodrop UV spectrophotometer. We then evaporated and resuspended the samples in 300$\mu$l hybridization buffer. We mounted whole genome E. coli K12 V2 OciChips (Ocimum Biosolutions) in Agilent hybridization chambers and we incubated in a rotating oven for 20h at 42 °C. We washed as described in the MWG application guide and we scanned with a GenePix scanner (Axon Instruments, Molecular Devices
Corporation). We measured Cy3 at 570nm, setting laser power at 100% and photomultiplier tube power at 65 to 75%.

**Microarray analysis.** We quantified the TIF images using the Genepix pro 6.0 software (Axon Instruments) and an “adaptative circular feature” quantification method. We normalized the data by the quantile method (“between array” normalization), using LimmaGUI (27) in the R Bioconductor software package (http://www.bioconductor.org). The normalized Cy3 channel intensities were log transformed and a gene expression analysis of variance (GeneANOVA) was performed as previously described (28). We retained those genes that showed a variation of expression of at least two-fold in at least one of the perturbing conditions (fur-, Fur90 and/or F1, as compared to cl20), associated with a P-value < 0.01. We conducted additional statistical analysis (principal component analysis, unsupervised hierarchical classification) using the Anova and the MeV (v.3) softwares (29). We performed hierarchical clustering using R Bioconductor as previously described (30).

**Fur box predictions**

We downloaded the *E.coli* K-12 MG12655 complete genome sequence (U00096.fna) and gene annotation (U00096.ptt) from Genbank. We extracted synonymous gene names from the additional U00096.gbk Genbank file. We identified all sequences matching the 19 bp *E.coli* consensus Fur box (GATAATGATAATCATTATC) (31), with a tolerance of 5, 6, or 7 mismatches, using the pattern search algorithm implemented in the ICM software (Molsoft). We associated Fur box(es) to a gene when this sequence was located between 1 and 250 bp upstream of the transcription start of the gene itself, or of an upstream gene within a same operon. We considered genes to belong to a same operon when all 3 following criteria were satisfied: (i) same strand location; (ii) transcription starts less than 4kb apart; (iii) gene names sharing same first 3 letters. When applicable, we
“manually” attributed Fur box(es) to those few genes (among the 196 retained genes) that did not conform to the above-mentioned criteria but were known to belong to an operon.

To establish the statistical significance of the data shown in Figure 7, we used the hypergeometric distribution to compare the frequency of a category in a group to that expected, knowing the amount of the category in the population. We computed the probability of finding the observed amount or more genes belonging to the considered category in a group. When the probability was less than 0.01, the category was considered highly enriched in the group (32-34).

Supporting Experimental Procedures

Construction of a two-hybrid peptide aptamer library
We first built a synthetic trxA gene that contained codons that are often used in mammals and where both cysteines of the active site were substituted for serines. We also added a second RsrII site (CGGACC), different from the first one (CGGTCCG), to avoid plasmid religation and ligations of multimerized random oligonucleotide duplexes. In this gene, both RsrII sites flank a unique BstEII site. For this construct, we annealed the following 7 sense oligonucleotides

5’- AATTCACCATGAGCGACAAGATCATACCTCCACCTGAGCGACGACGCTTCGACACCAGA-3’
5’- CGTGCTGAGGCGGACGCCCATCTGTGGGACTTTGGTGGCCAGTGGAGCGGT-3’
5’- CCGGTACCCGAGCAAGATGATGCTGTGGGACGCCAAGTCGAGGACGAGGGCGAGC-3’
5’- AGTACCAGGGCAAGCTGACCGTGGCCAAGCTGAACATCGACCAGAACCCCGGCAC-3’
5’- CGCCCCCAAGTACGGCATCAGGGGATTCCTCCACCTGCTGCTGTTCAAGAACGGC-3’
5’- GAGATGGCCGCCACCAAGGTGGGCGCCCTGAGGAAGCAGGGCCACCCAGCTGAGG-3’
5’- TCCTGGAGCCACCTGGCTAGCC-3’

and the following 7 antisense oligonucleotides

5’- TCGAGGCTAGGCCAGGTTGGCGTCAGGCACTCCCTACGCTGGCCCTTGCTCAGG-3’
5’- GCCCGCACATCGGTGGGCGCCACCTCAGCTGGCCCTTGCTCAGG-3’
We performed 5 cycles of PCR reaction on the annealed oligonucleotides. We then PCR amplified the resulting product using the oligonucleotides 5’-GGCACGGAATTACCACATGAGCGACAAG ATCATC-3’ and 5’-GGACCGCTCGAGGCTAGGCCAGGTTGGCGTC-3’ that contained respectively a EcoRI and a XhoI site. We cloned the PCR product into EcoRI/XhoI-cut pJMX to create pWP1-C.

To create pWP2-C, we first constructed pWP2-X, a pJMX derivative that directs the galactose-inducible expression of a NLS-HA-HsTRXss-B112 fusion protein (where HsTRXss is the human TRXA cDNA with both cysteines of the active site substituted into serines). To this end, we first PCR-amplified the HA-HsTRXss coding sequence from pJMX-HsTRXss using the oligonucleotides 5’-AGAAAGGTAGCTGGTTCTGAGTTCCCGGGGTCCTACCCTTATGATGTGCCAGATTATGCCTCTGAATTCGCCATG-3’ and 5’-GGTGATCCCTGGGACTCCTCGAGTGAGACTAATTCATTAATGGTGGC-3’. The former oligonucleotide contained the 3’ terminal sequence of the NLS for homologous recombination. The latter oligonucleotide contained a serine codon, a XhoI site and the 5’ terminal sequence of the B112 activation domain. We also PCR-amplified the B112 activation domain from pJMX-HsTRXss using the oligonucleotides 5’-ATGAATTAGTCTCACTCGAGGAGTTCCCAGGGATCACCTTGCGGATTCAGGAGAC-3’ and 5’-GTCTCGACTCTAGATGGCCAGCTAGGCGCTAGCTTAAAAAAGGTTCTGCTCCGCCTGAGTGACGTTCAGC-3’. The former oligonucleotide contained the 3’ terminal sequence of HsTRXss (without stop codon). The latter oligonucleotide contained a stop codon and the 5’ terminal sequence of the ADHt for homologous recombination. We mixed both PCR products and performed a PCR reaction to obtain a sequence coding for a NLS-HA-HsTRXss-B112 fusion flanked by sequences destined to homologous recombination. We co-transformed yeast with this
PCR product and with *XmaI/XhoI*-cut pJM-X-TRXss, and we selected TRP' transformants. To replace HsTRXss by our codon-optimized *E.coli* trxA, we PCR-amplified its coding sequence from pWP1-C using the oligonucleotides 5'-GGTCTACCTTTAT GATGTGC-3' that hybridized to the HA coding sequence and 5'-GGTGATCCCTGGGAACTCCTCGAGTGAGGCCAGGTTGGCGTCCAGGAAC-3' that substituted the stop codon into a serine and that contained a XhoI site. We digested the PCR product with EcoRI and XhoI and ligated it into EcoRI/XhoI-cut pWP2-X to create pWP2-C, a plasmid that bears a yeast 2µ replication origin, a *TRP1* marker, a GAL1 promoter, and that directs the expression of a NLS-HA-TrxAco-B112 fusion protein (where TrxAco is the product of the gene described above).

To construct the aptamer library, we produced a duplex oligonucleotide using 5'-TGGGCCGAGTGGAGCGGTCCG(NNS)12NNCGGCAGCAAGATGATCGCCC-3', 5'-GGGGCGATCATCTTGCTCGGTCCG-3' and the Klenow DNA polymerase. We digested the duplex with CpoI and subcloned it into RsrII-cut pWP2-C. We transformed the ligation product into *E.coli* DH10B competent bacteria (Invitrogen) and we obtained 2.4x10⁷ transformants.

**Yeast strains**

We constructed MB209 and MB210, two yeast two-hybrid strains bearing respectively 8lexAop::ADE2 2lexAop::LEU2 and 8lexAop::ADE2 6lexAop::LEU2 reporter genes in their genomes. We first PCR-amplified the ADE2 locus from EGY48 genomic DNA using the oligonucleotides 5'-CGTAAAATCGTGATTTGCCATCTCCCTCTTCTTCTAAGTA CATCC-3' and 5'-ATCTCGAGGGACACCTGTAAGCG-3' that contained respectively a BamHI and a XhoI site. We cloned the PCR product into BamHI/XhoI-cut pRS316 to create pRS316::ADE2, an episomal plasmid bearing a URA3 marker. We then PCR-amplified the 8 LexA operators from pSH18-34 using the oligonucleotides 5'-TATATAAGCTGGGACATATCCCATATCTTACC-3' and 5'-TATATAAGCTGGGACATATCCCATATCTTACC-3' that contained respectively a SacII and a NotI site. We cloned the PCR product into
SacII/NotI-cut pRS316::ADE2 to create pRS316::8lexAop-ADE2. We PCR-amplified the 8lexAop-ADE2 cassette using the oligonucleotides 5’- GCCGTATCGTGATTAACGTATTACATAAGTTACAGAATTCATGCTTATGGGTTAGCTATTTCGCCCAATGTGTCCATCTGACATATCCATATCTAATCTTACC-3’ and 5’- ATCTCGAGGGACACCTGTAAGCG-3’. The former oligonucleotide contained the end of YOR129C (the open reading frame upstream of ADE2) and 89 nucleotides from the downstream intergenic region, with a EcoRI site introduced 40 nucleotides downstream from the stop codon of YOR129C. We designed it to address the 8lexAop:ADE2 construct upstream of the ADE2 genomic locus. The latter oligonucleotide contained a XhoI site. We cloned the PCR product into SmaI/XhoI-cut pRS316 to create pRS306::YOR129C::8lexAop::ADE2, an integrative plasmid bearing a URA3 marker. We linearized this plasmid with EcoRI, transformed it into TB50α yeast and selected URA+ transformants. We picked and streaked 3 independent transformants onto 5-fluoroorotic acid-containing solid medium to select recombinants. We co-transformed three recombinants with pEG202-Cdk2 and pJM1-Pep10M (24) or pJM1-M103 (directing the expression of a control aptamer). We selected one clone that exhibited a two-hybrid ADE2 phenotype.

We named this new strain MB205α. We created MB205a by crossing MB205α to TB50α and dissecting spores from the diploid strain. We then crossed MB205a to EGY48α and EGY191α to obtain MB210 (α and α), and MB209 (α and α), respectively.

We created MB226α (a leu2 ade2 ura3 his3 trp1 strain) by crossing MH272-3fa to TB50α and dissecting spores from the diploid strain.

Results

Selection of Fur binding peptide aptamers

We used an optimized yeast two-hybrid method and a new generation library (variable regions of 13 aminoacids) (25) to select peptide aptamers for their ability to bind Fur. Fur
homodimerization offered the opportunity to validate the use of this protein in a yeast two-hybrid assay. We detected a strong interaction phenotype when Fur was expressed both as bait (in fusion to LexA) and as prey (in fusion to the B42 activation domain) (Figure 1A). We selected 4 different Fur-interacting aptamers, named F1 to F4. The sequences of their variable regions did not reveal any similarity with *E. coli* proteins (Table 1). All four aptamers showed a moderate to strong *lacZ* interaction phenotype against LexA–Fur (the bait used for the selection), contrary to cl20 (a control aptamer randomly picked from the library) and Mn1 and Mn2 (two aptamers obtained from a phenotypic selection and that do not interact with Fur — unpublished). In contrast to F1, 2 and 3, F4 bound a Fur-LexA fusion but failed to bind noticeably two dominant negative alleles of Fur (Fur90 and Fur51) (Figure 1A). Interestingly, we detected a strong interaction phenotype between Fur90, Fur 51 and Fur, thus confirming the hypothesis that both mutants form inactive heterodimers with the wild type protein (20).

To confirm that these aptamers bound Fur in *E. coli*, we performed pull-down experiments. We successfully captured the endogenous Fur protein from BL21(DE3) *E. coli* expressing GST–Fur aptamer fusions (Figure 1B).

**Quantification and mapping of interactions**

To compare more accurately the apparent binding affinities of the peptide aptamers, Fur and Fur90 for Fur, we performed yeast two-hybrid assays using a new luciferase reporter gene (*luc*) that allows an easy and precise quantification of two-hybrid phenotypes (8). The *luc* interaction phenotypes measured between Fur and the peptide aptamers revealed that the apparent binding affinities of F1, 2 and 4 are similar to one another and higher than that of F3 (Figure 1C). These results are globally consistent with the *lacZ* interaction phenotypes and with the amounts of Fur protein captured using the GST-
aptamer affinity matrices (Figure 1A and B). The luc interaction phenotype corresponding to Fur homodimerization is comparable to that of aptamers F1, 2 and 4 but lower than the luc interaction phenotype measured between Fur and Fur90 (Figure 1C). The apparent discrepancy with the yeast two-hybrid assay shown in Figure 1A can be explained by the rapid saturation of the blue colorations obtained with a lacZ reporter system, whose dynamic range is much more limited than that of the luc reporter system. These luciferase two-hybrid assays thus indicate that peptide aptamers F1, 2 and 4 bind to Fur with an affinity that is comparable to that of Fur itself, higher than that of aptamer F3, and lower than that of the Fur90 dominant negative protein.

The results shown in Figure 1A suggest that peptide aptamer F4 binds to Fur on a molecular surface that is distinct from the molecular surface(s) bound by aptamers F1, 2 and 3. To map the aptamer binding sites on Fur, we constructed a collection of bait plasmids expressing different truncations of Fur and we performed yeast two-hybrid mating assays. To validate the different bait constructs, we used Fur itself and RG22, a peptide aptamer that interacts with LexA and that usually gives an interaction phenotype with most but not all LexA fusion proteins (25). Every truncated Fur bait construct gave an interaction phenotype with either RG22 or Fur, or both for one of them (Figure 1D). The interaction phenotypes obtained between the different truncations and Fur are consistent with the structural knowledge on Fur homodimerization (10). Peptide aptamer F4 binds a molecular surface that is located on the carboxy-terminal half of Fur, whereas peptide aptamers F1, 2 and 3 probably bind (a) molecular surface(s) formed by the folding of Fur and that involve(s) residues located in the amino- and carboxy-terminal halves of the protein (Figure 1D). This (or these) molecular surface(s) do not exist or are corrupted in the context of a Fur-LexA fusion protein (Figure 1A).
Inhibition of Fur homodimerization and function

Fur exerts its transcriptional repressor activity by binding DNA regulatory sequences as a homodimer. Therefore, an obvious peptide aptamer-mediated Fur inhibitory mechanism would be either to prevent Fur homodimers from binding DNA or to prevent Fur homodimerization. We explored this latter hypothesis by testing the ability of peptide aptamers to inhibit the Fur homodimerization yeast two-hybrid phenotype. We co-expressed HA-NLS-aptamer fusions together with LexA-Fur and NLS-B42-HA-Fur. We validated our competition assay by showing that a HA-NLS-Fur fusion was able to inhibit 80% of the Fur homodimerization two-hybrid phenotype (Figure 2A). All aptamers tested failed to interfere with the Fur homodimerization two-hybrid phenotype except for aptamer F4, which produced a 55% inhibition of the two-hybrid signal (Figure 2A), while not inhibiting an unrelated protein interaction phenotype (not shown). These results suggest that Fur homodimerization is inhibited by peptide aptamer F4 but not by the three other aptamers, which bind to (a) distinct molecular surface(s).

We then examined the capacity of the Fur binding aptamers and the dominant negative allele to phenocopy fur− bacteria in allowing growth on high concentration of Mn2+ (35). We expressed peptide aptamers and Fur90 using pBAD24, a plasmid that bears an arabinose-inducible promoter. Aptamers F1 and 2 were as potent as Fur90 in their ability to allow bacterial growth in these conditions, whereas aptamers F3 and 4 did not allow growth (Figure 2B).

Next, we used a transcriptional repression assay to compare Fur inhibition caused by the gene knockout, the dominant-negative allele and peptide aptamers. We used the bacterial strains QC6008 (fur−) and QC2146 (fur+) (see http://www2.ihm.jussieu.fr/touati/strains.php). Both strains carry an integrated lacZ reporter gene, placed under the
control of the fiu promoter that contains four Fur boxes and is thus strongly repressed by Fur (36). We transformed QC6008 with pBAD24 to obtain a control, devoid of any Fur activity. We transformed QC2146 with pBAD24 plasmids directing the expression of Fur90 and the aptamers to be tested. While aptamer F3 showed no effect, Fur 90 and aptamers F1 and 2 inhibited Fur repression activity to a similar extent. Aptamer F4 caused a slightly weaker inhibition (Figure 2C). We obtained similar results using the bacterial strains QC6009 (fur−) and QC2949 (fur+) containing a lacZ reporter gene controlled by the fhuF promoter, which contains two Fur boxes (not shown). These results show that aptamers F1, 2 and 4 act as Fur inhibitors in this transcriptional repression activity.

Oxidative stress sensitivity

Fur regulates the expression of numerous genes involved in oxidative stress response, among which superoxide dismutases that play a key role in the protection against oxygen toxicity (37). As fur− strains are very sensitive to hydrogen peroxide (H2O2) (14), we wished to determine whether Fur90 or the Fur aptamers would confer sensitivity to H2O2 in a growth assay performed in liquid cultures. While we confirmed the extreme sensitivity of the fur− strain, neither Fur90 nor the tested aptamers conferred any significant sensitivity to H2O2 in this assay (Figure 2D).

Virulence assay in animals

Fur has been shown to play an important role in the virulence of different pathogenic bacteria (18,38). We thus set out to determine whether Fur90 and the Fur-binding peptide aptamers affected the virulence of a pathogenic E. coli strain in an in vivo model system. It is now well established that Drosophila and mammals share conserved immune mechanisms, including the activation of NF-kB-dependent signaling pathways (39). In addition, several bacterial pathogens use similar
virulence mechanisms against mammalian and non-mammalian hosts, including insects (26,40). We infected *Drosophila* flies with the *E.coli* 1106 pathogenic strain, transformed with pBAD plasmids expressing Fur90 and the peptide aptamers to be tested. Since the 1106 strain did not kill wild type flies (*not shown*), we used TAK1 (TGF-ß Activated Kinase 1) immune-deficient *Drosophila* mutants that are strongly impaired for the activation of NF-kB-dependent antimicrobial peptide synthesis (41). In these flies, lethality occurred 3 to 4 days following septic thorax injury. Fur90- and Fur aptamer-expressing bacteria caused a slower mortality than control bacteria (Figure 3). In absence of sustained promoter induction by arabinose, the differential growth between the transformants eventually vanished and all flies died (*not shown*). Thus, the transient expression of Fur90 and of the tested Fur aptamers decreased the virulence of pathogenic *E.coli* in flies 3 to 4 days after infection.

**Genome-wide transcriptome analysis**

To perform a comprehensive comparative analysis of the molecular perturbations exerted by the gene knockout, the expression of a dominant negative allele and the expression of a peptide aptamer, we carried out a global transcriptome study using *E.coli* pan-genomic oligonucleotide microarrays and RNA samples extracted from pBAD24-transformed QC6009 (*fur−*) and from QC2949 (*fur−*) transformed with pBAD24 plasmids directing the expression of Fur90 and peptide aptamers cl20 and F1. We performed two independent experiments and we retained those genes that showed a variation of expression of at least two-fold in at least one of the perturbing conditions (*fur−*, Fur90 and/or F1) as compared to the cl20 condition, and that passed a significance cutoff determined by an ANOVA analysis (see Experimental Procedures). We thus retained 196 genes for further analysis (Figure 4, Table S1). Three genes provided built-in controls for these experiments. *lacZ* (Fur reporter
gene integrated in the genome of both strains), trxA (peptide aptamer scaffold) and fur showed expected expression profiles (Figure 5A), thus confirming the identity of the samples, the proper expression of the transgenes and the strong Fur basal activity under our experimental conditions.

75 of the 196 selected genes have already been identified in previous transcriptome studies as Fur- and/or iron-regulated genes in E. coli (17,42), Helicobacter pylori (38, 43-45), Neisseria meningitidis (46,47), Pseudomonas aeruginosa (48,49), Campylobacter jejuni (50), Shewanella oneidensis (51,52), Pasteurella multicoda (53). Conversely, we unveiled 121 genes that have not been reported by previous studies. The expression of 106 and 78 genes was up- and down-regulated in at least one of the perturbing conditions, respectively (Figure 4A and B). Within the up- and down-regulated gene populations, we defined clusters according to the perturbing condition(s) that caused at least a two-fold deregulation of expression (Figure 4). The vast majority of the up- and down-regulated gene populations belonged either to the fur- only (group 1) or to the fur-/Fur90/F1 (group 2) clusters, whose existence can be easily explained by straightforward biological hypotheses (summarized in Figure 6A). A few typical examples of gene expression profiles from these two groups are shown in Figure 5B (up-regulations) and 5C (down-regulations).

We analyzed the general profiles of the up- and down-regulation values in all three perturbing conditions. The down-regulation values were generally lower than the up-regulation values, especially in the fur- condition. The down-regulation values were comparable in all three perturbing conditions. In contrast, the up-regulation values were comparable in the Fur90 and the F1 conditions, and lower than that observed in the fur- condition (Figure 4, Table S1).

We next investigated whether the predicted sensitivity of a gene to a regulation by Fur determined its belonging to the different groups. The predicted sensitivity of a Fur-
controlled promoter to Fur can be approximated from the number of Fur boxes detected in the promoter sequence. We first applied different Fur box prediction settings to the entire E.coli genome to determine those settings that yielded the highest Fur box enrichment rates within the population of the 196 retained genes (see Experimental Procedures and Figure S1). From these results, we retained the prediction setting that considered a 250bp region upstream of the transcription start of each gene, tolerating up to 5 or 6 mismatches from the Fur box sequence consensus (31). As shown in Figure 7, we observed a striking correlation between the number of predicted Fur boxes and the percentage of fur- only genes. Among the 196 retained genes, the vast majority of those genes harboring two or more Fur predicted boxes (with a tolerance of 5 mismatches) were deregulated by the gene knockout only. Examples of genes deregulated by the gene knockout only include entA-F and fepA-G, which play a key role in iron homeostasis and whose expression is known to be tightly regulated by iron in a fur-dependent manner. Conversely, genes such as exbB and exbD, which present only one predicted Fur box and which are involved in the uptake of various extracellular molecules (including iron), were found deregulated in all three perturbing conditions.

Discussion

Using E.coli Fur as a case study, we have compared the perturbations exerted by gene disruption, expression of a dominant negative allele and expression of a set of peptide aptamers, performing four functional assays and a whole-genome transcriptome study. The four selected aptamers show different apparent binding affinities for Fur and bind to different molecular surfaces. F4 binds to the carboxy-terminal half of Fur, which includes the dimerization domain. This is thus consistent with the ability of F4 to inhibit Fur
homodimerization. F1, 2 and 3 probably bind to (a) molecular surface(s) involving residues located in the amino- and carboxy-terminal halves of the protein. Recent structural studies on Fur homodimers have revealed ionic interactions between carboxylates of the carboxy-terminal part of one subunit and the amino-terminal NH2 of the other subunit (54). The metal-dependent activation mechanism of Fur has been shown to involve the disruption of this ionic interaction together with the folding of the amino-terminal helix (10,54). This suggests that aptamers F1, 2 and 3 bind Fur homodimers and may inhibit their metal-dependent activation mechanism.

F1 and F2, allowed growth on high concentration of Mn2+ and strongly inhibited Fur transcriptional repression activity, thereby phenocopying a fur- strain or a strain expressing Fur90. F4 did not allow growth on high concentration of Mn2+ and inhibited Fur repressor activity to a lesser extent than F1 or F2. None of the tested aptamers was able to confer a detectable sensitivity to H2O2. F3, the weakest binder, failed to induce a detectable perturbation in all phenotypic assays but the virulence test in a Drosophila model of infection. The results of this assay suggest that a sustained expression of Fur peptide aptamers could stably reduce animal mortality caused by an infection with pathogenic bacteria. Hence, they further validate Fur as an interesting target to pursue for antibacterial drug discovery, which could be guided by the use of the Fur peptide aptamers (8).

Altogether, these observations demonstrate that the use of a set of peptide aptamers that bind their target protein on different molecular surfaces with different affinities induces a broad range of perturbations of protein function. As shown recently in another study, peptide aptamers can also activate rather than inhibit the function of their target protein (55).

Our transcriptome study has revealed 121 genes that have not been reported as being regulated by Fur and/or iron in E.coli or other bacteria yet. Although it is possible that our
selection cutoff was not stringent enough, we believe that a high confidence can be attributed to the genes that satisfy either of the following criteria: (i) function showing a conspicuous link to iron metabolism; (ii) co-involvement in a given regulatory pathway (for example, \textit{wzb} and \textit{wzc}, which code for a protein tyrosine phosphatase and kinase, respectively; \textit{nac} and \textit{glnk}, which are both involved in the regulation of nitrogen assimilation; \textit{pyrB} and \textit{pyrI}, which code for the aspartate carbamoyltransferase catalytic and regulatory subunits, respectively); (iii) presence of predicted or experimentally revealed Fur boxes in the promoter (36/121 newly-described genes); (iv) deregulation observed in two or three of the perturbing conditions used in our study. This latter criterion (satisfied by 71/121 newly-described genes) provides a particularly high confidence as it rules out artifactual deregulations that may be caused by compensatory mechanisms (triggered by gene knockout or overexpression) or off-target peptide aptamer effects. In total, 88 of the 121 newly-described genes satisfy at least one of the above-mentioned criteria and can thus be considered with a good confidence as genes regulated directly or indirectly by Fur (Table S1).

Not surprisingly, the perturbation that deregulated the highest number of genes was the gene knockout, which generally induced more pronounced up-regulations than the expression of the dominant negative allele or of the peptide aptamer. In contrast, the down-regulation values, which were generally lower than the up-regulation values, were similar between all three perturbing conditions. The lower down-regulation values (as compared to up-regulation values) could be explained by the fact that Fur can exert a positive control on gene expression through an indirect, small RNA-based mechanism (13). However, Fur box(es) are predicted for 24 out of the 70 genes that were down-regulated at least in the \textit{fur}\textsuperscript{-} condition (Table S1). This suggests that in \textit{E.coli}, Fur might activate
the transcription of a subset of genes also through a direct mechanism, as already described in *H. pylori* (56).

Altogether these results show that the “penetrance” of the dominant-negative allele and of the peptide aptamer is lower than the “penetrance” of the gene knockout. This conclusion is further supported by the observation that the percentage of genes deregulated only by the gene knockout increases with the predicted sensitivity of the gene promoters to Fur. A key determinant of the “penetrance” of an inhibitory ligand (dominant negative protein or peptide aptamer) is the stoichiometry of target molecules in the free state and in complex with the ligand. This stoichiometry is itself determined by the expression level and the binding affinity of the ligands. Here, the lower “penetrance” of the dominant negative allele and of the peptide aptamer could be due to insufficient expression levels and binding affinities.

However, the mRNA expression levels of the peptide aptamers and of Fur90 are very high, and, for the latter at least, largely exceed the expression level of the target (Figure 5A). Moreover, the apparent binding affinity of Fur90 for Fur is very strong and higher than that of Fur itself (Figure 1C). Therefore, while it might be possible to increase the “penetrance” of the peptide aptamer by conducting an in vitro evolution approach and obtaining a higher affinity mutant (24), this “penetrance” will probably not exceed that of Fur90 and will thus remain lower than that of the gene knockout.

Another key determinant of the “penetrance” of an inhibitory ligand lies in its capacity of inhibiting every function of its target protein, when the protein exerts multiple functions. In addition to its transcription regulation activity related to its capacity to bind DNA, Fur has been suspected to exert an iron sequestration function (57). If this hypothesis were correct, the lower “penetrance” of the peptide aptamers and of Fur90 could be also due to the fact that they can only inhibit the DNA binding-dependent
activities of Fur but not its alternative function. More work will be needed to clarify this point.

Relating the phenotypes observed in the functional assays to the results of the transcriptome analysis should be particularly useful to identify Fur-controlled genes that govern various Fur-regulated processes. Those genes whose deregulation allows bacterial growth on high concentration of manganese or inhibits bacterial virulence in flies should be mostly found among the genes that are up- or down-regulated by all three perturbing conditions. The genes that tend to confer protection to oxidative stress should be mostly found down-regulated by fur− only, whereas the genes that tend to confer sensitivity to oxidative stress should be mostly found down-regulated by Fur90 and F1 and up-regulated by fur− only (Figure 6B). Many genes known or predicted to determine oxidative stress sensitivity conform to this prediction. Most genes involved in iron uptake (ent, fep, fec, fhu genes) are upregulated by fur− only. Increased intracellular iron levels are known to confer a high sensitivity to oxidative stress. The group of fur− only, down-regulated genes includes katE (catalase that eliminates H₂O₂), osmC, (an osmotically-induced protein that uses highly reactive cysteine thiol groups to elicit hydroperoxide reduction (58)), yggE (a protein that restores physiological defects caused by oxidative stress (59)), phoB (a positive regulator of polyphosphate accumulation, which confers resistance to oxidative damages (60)), ileS (Isoleucyl-tRNA synthetase, down-regulation of which may cause a partial starvation for isoleucine, which has been shown to cause a repression of genes involved in oxidative stress protection (61)) and of course fur itself, whose gene product may exert a protective role by sequestering ferrous iron (57). The group of genes that are down-regulated by Fur90 and F1 (and slightly up-regulated in the fur− condition) includes codA and codB (cytosine deaminase and
permease respectively), which should increase oxidative mutagenesis and thus sensitivity to oxidative stress (62).

In conclusion, our work establishes that the use of peptide aptamers or of a dominant negative allele induces more limited phenotypic responses than the use of a gene knockout. This can be accounted for by the incomplete "penetrance" of the transdominant agents compared to the gene knockout, as revealed by the transcriptome analysis. Our work also shows that a comparative analysis of the phenotypic and transcriptome responses to different types of perturbation can help identify regulatory network members that govern various biological processes.
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Figure legends

Figure 1: Fur peptide aptamer interaction assays
(A) Yeast two-hybrid mating assay between LexA fusions to the indicated Fur proteins and B112-aptamer or B42-protein fusions. cl20, Mn1 and Mn2 are negative control aptamers. (B) Fur pull-down assays. We ran bacterial protein extracts on the indicated GST-aptamer solid phases. We analyzed the eluates by Western blot using an anti-Fur antibody. (C) Quantitative yeast two-hybrid assay. We quantified yeast two-hybrid phenotypes between LexA-Fur and peptide aptamers, Fur and Fur90 prey proteins using a luciferase reporter gene. We performed two independent experiments. (D) Mapping of peptide aptamer binding sites on Fur. We performed drop-arrayed yeast two-hybrid mating assays between the peptide aptamers and different Fur truncations fused to LexA (depicted linearly). RG22 is a LexA peptide aptamer that interacts with most but not all LexA-fusion constructs.

Figure 2: Fur homodimerization and functional assays
(A) Yeast two-hybrid competition assay. We co-expressed in yeast LexA-Fur, NLS-B42-HA-Fur and different HA-NLS-aptamer or -Fur fusions. We quantified the Fur dimerization two-hybrid phenotypes using a luciferase reporter gene and we normalized the results with the value obtained with aptamer cl20. We performed 3 independent experiments. (B) Bacterial growth on medium containing a high concentration of Mn^{2+}. We spotted serial dilutions of E.coli XL-1 blue transformants expressing peptide aptamers or Fur90 onto TN medium containing 10mM Mn^{2+} and arabinose. (C) Fur transcriptional repression assay. We transformed QC6008 (fur^-) strain with pBAD24 to confer ampicillin resistance and QC2146 (fur^-) strain with pBAD24 expressing peptide aptamers, Fur90 or nothing. We streaked the transformants onto solid medium containing X-gal, with or
without arabinose. (D) Oxidative stress assay. We measured the growth of \textit{E.coli} QC2949 (\textit{fur}⁺) transformants expressing various aptamers or Fur90 and the growth of QC6009 (\textit{fur}⁻) transformed with pBAD24, in presence or in absence of \textit{H₂O₂}. We performed 4 independent experiments.

**Figure 3: Virulence assay**
The percentage of surviving flies following septic injury was determined on 30 infected individuals, 3 and 3.5 days post-infection in the first and second experiment, respectively. cl20 aptamer was tested in the first experiment only.

**Figure 4: RNA expression data**
Heatmaps comparing the normalized log2 of the ratios of Fur⁻, F90 and F1 signals on the cl20 control signal. Clustering was performed using a Manhattan distance and a Ward method of agglomeration. Colors vary from green for the lowest ratios to red for the highest ratios. (A) Up-regulated genes; (B) Down-regulated genes; (C) Unclassifiable and control genes. Gr1: “fur⁻ only” genes; Gr2: fur⁻/F90/F1 genes; Gr3: fur⁻/F90 genes; Gr4: fur⁻/F1 genes; Gr5: F90/F1 genes; Gr6: “F90 only” genes; Gr7: “F1 only” genes; Gr8: unclassifiable genes; Cont: control genes.

**Figure 5: Selected examples of gene expression profiles**
(A) Expression profiles of \textit{lacZ}, \textit{trxA} and \textit{fur} control genes. (B) Typical examples of expression profiles of 3 up-regulated genes. \textit{entC}: involved in enterobactin biosynthesis; \textit{sodA}: manganese superoxide dismutase; \textit{sdhB}: succinate dehydrogenase (iron/sulfur protein). (C) Typical examples of expression profiles of 3 down-regulated genes. \textit{katE}: hydroperoxidase; \textit{ybdR}: putative oxidoreductase; \textit{ftn}: cytoplasmic ferritin.
Figure 6: Global distribution of deregulated genes in the different perturbing conditions

(A) Number of up- and down-regulated genes in the different perturbation groups and corresponding explanations. (B) Venn diagram showing the distribution of up- and down-regulated genes in the different perturbation groups and their expected involvement in the regulation of various biological processes. In this diagram, the 9 unattributed genes, which showed opposite deregulation values according to the perturbing condition, were attributed to the various groups (marked by an asterisk). Hence, some numbers differ from the numbers given in (A).

Figure 7: Occurrence of fur⁻ only genes and expected sensitivity to Fur

From the 196 retained genes, we took into consideration the 165 genes that were found deregulated at least in the fur⁻ condition and we split them into 4 groups according to the number of Fur boxes that were predicted in their promoters. For each group, we calculated the percentage of fur⁻ only genes. Enrichment values labeled with an asterisk are statistically significant (see Experimental Procedures).

Figure S1: Fur box prediction settings

A Fur box prediction was conducted on the entire E. coli genome, looking at 150, 250 or 400 bp upstream each gene transcription start and tolerating 5, 6 or 7 mismatches from the consensus sequence (detailed in Experimental Procedures). The numbers of genes harboring at least 1, 2 and 3 predicted Fur box(es) in each prediction setting are given above the bars. The histogram shows the occurrence of each gene population in the population of the 196 retained genes, which represents 4.5% of the entire E. coli gene population. The threshold bar is set at 4.5%. Contrary to the tolerance of 5 or 6 mismatches, the tolerance of 7 mismatches does not yield
a significant enrichment of predicted Fur boxes in the population of the 196 retained genes.

Table S1: Transcriptome analysis
The 196 retained genes are listed in alphabetical order. The table provides from left to right: gene names, Blattner “B-numbers”, brief descriptions of the function, normalized fold induction or repression values for the three perturbing conditions (compared to the cl20 control), groups of deregulation profile based on the clustering analysis (see Figure 4), number of predicted Fur boxes in each promoter (6 mismatches, 250bp), existence of experimentally-demonstrated Fur boxes, previous transcriptome and proteome studies that identified a Fur- and/or iron-mediated deregulation, and confidence index. This index was determined by attributing one arbitrary unit every time one of the following criteria was satisfied: (i) co-involvement with another gene in a regulatory pathway, (ii) deregulation observed in at least two perturbing conditions, (iii) at least one predicted or experimentally-demonstrated Fur box in the promoter, (iv) gene already found controlled by Fur and/or iron in at least one previous study. Genes down-regulated at least in the fur− condition and showing (a) predicted Fur box(es) are colored in yellow. We used the following web resource to inventory some of the experimentally-demonstrated Fur boxes: http://biocyc.org/ECOLI/NEW-IMAGE?type=ENZYME&object=PD00260 (75).
Table 1: Sequences of peptide aptamer variable regions

| Aptamers | Variable regions |
|----------|-----------------|
| F1       | RLWCRYPHPPLTD   |
| F2       | RQCNICGASLYSY   |
| F3       | ETCKCGSQVWRHS   |
| F4       | CARCGARVNVYKY   |
| c120     | RSLRGRCLSQHQD   |
FIGURE 1
FIGURE 2

A

LexA-Fur X B42-Fur

B

Dilution: $10^{-1}$ $10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$

pBAD24

cl20

F1 F2 F3 F4 F90

C

D

OD 600 nm

$pBAD24$ cl20 F1 F3 F4 Fur90 fur-

$0\text{mM H}_2\text{O}_2$ $2\text{mM H}_2\text{O}_2$
FIGURE 3

Exp 1
Exp 2

% Fly survival

pBAD24  cl20  F1  F3  F4  Fur90

Downloaded from https://www.mcponline.org by guest on May 9, 2020
FIGURE 4

A

B

C

Gr 1

Gr 2

Gr 3

Gr 4

Gr 5

Gr 6

Gr 7

Gr 8

Cont

-3.0 0.0 3.0

Fun - F90 F1

FIGURE 4
FIGURE 5
| Groups          | UP   | DOWN | Demonstrated or tentative explanations |
|-----------------|------|------|----------------------------------------|
| (1) Fur-only    | 32   | 41   | Mostly genes controlled by promoters with high-affinity for Fur -> incomplete penetrance of Fur90 and F1. Compensatory mechanisms caused by Fur eradication. |
| (2) Fur-/Fur90/F1 | 49   | 26   | Mostly genes controlled by promoters with lower-affinity for Fur. Very high-confidence group. |
| (3) Fur-/Fur90  | 12   | 1    | Too modest affinity of F1 for Fur -> incomplete penetrance of F1. High-confidence group. |
| (4) Fur-/F1     | 2    | 2    | Compensatory mechanisms caused by overexpression of a fur dominant negative allele. High-confidence group. |
| (5) Fur90/F1    | 1    | 4    | Compensatory mechanisms caused by Fur eradication. High-confidence group. |
| (6) Fur90       | 2    | 1    | Specific effects caused by overexpression of a fur dominant negative allele. |
| (7) F1          | 8    | 3    | Off-target effects exerted by peptide aptamer F1. Compensatory mechanisms caused by Fur eradication and Fur90 overexpression. |
| (8) Unattributed| *    | 9    | Off-target effects exerted by peptide aptamer F1. Compensatory mechanisms caused by Fur eradication and Fur90 overexpression. |

**FIGURE 6**
FIGURE 7

% "Fur-only" genes

Number of Fur boxes

0 1 2 3/4

Predicted (250bp/5mis.)

Predicted (250bp/6mis.)

*