Global transcriptional response of *Caulobacter crescentus* to iron availability

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**Abstract**

**Background:** In the alpha subclass of proteobacteria iron homeostasis is controlled by diverse iron responsive regulators. *Caulobacter crescentus*, an important freshwater α-proteobacterium, uses the ferric uptake repressor (Fur) for such purpose. However, the impact of the iron availability on the *C. crescentus* transcriptome and an overall perspective of the regulatory networks involved remain unknown.

**Results:** In this work we report the identification of iron-responsive and Fur-regulated genes in *C. crescentus* using microarray-based global transcriptional analyses. We identified 42 genes that were strongly upregulated both by mutation of *fur* and by iron limitation condition. Among them, there are genes involved in iron uptake (four TonB-dependent receptor gene clusters, and *feoAB*), riboflavin biosynthesis and genes encoding hypothetical proteins. Most of these genes are associated with predicted Fur binding sites, implicating them as direct targets of Fur-mediated repression. These data were validated by β-galactosidase and EMSA assays for two operons encoding putative transporters. The role of Fur as a positive regulator is also evident, given that 27 genes were downregulated both by mutation of *fur* and under low-iron condition. As expected, this group includes many genes involved in energy metabolism, mostly iron-using enzymes. Surprisingly, included in this group are also TonB-dependent receptors genes and the genes *fixK*, *fixT* and *ftrB* encoding an oxygen signaling network required for growth during hypoxia. Bioinformatics analyses suggest that positive regulation by Fur is mainly indirect. In addition to the Fur modulon, iron limitation altered expression of 113 more genes, including induction of genes involved in Fe-S cluster assembly, oxidative stress and heat shock response, as well as repression of genes implicated in amino acid metabolism, chemotaxis and motility.

**Conclusions:** Using a global transcriptional approach, we determined the *C. crescentus* iron stimulon. Many but not all of iron responsive genes were directly or indirectly controlled by Fur. The iron limitation stimulon overlaps with other regulatory systems, such as the RpoH and FixK regulons. Altogether, our results showed that adaptation of *C. crescentus* to iron limitation not only involves increasing the transcription of iron-acquisition systems and decreasing the production of iron-using proteins, but also includes novel genes and regulatory mechanisms.

**Keywords:** *Caulobacter crescentus*, Iron stimulon, Fur regulon, Transcriptome, Iron homeostasis, TonB-dependent receptor
Background
Iron is an essential micronutrient required for almost all organisms, functioning as a cofactor for proteins that are involved in a number of fundamental metabolic and enzymatic functions. Despite its high abundance, iron is a limiting nutrient in most biological systems due to its poor solubility under physiological conditions or because it is tightly sequestered by high-affinity proteins, such as transferrin and lactoferrin in eukaryotic hosts [1,2]. On the other hand, high iron levels can generate toxic hydroxyl radicals by the Fenton reaction [3]. Thus, organisms have evolved multiple strategies to maintain accurate control over intracellular iron levels.

In most bacteria, iron homeostasis is mediated by Fur (ferric uptake regulator), an iron-sensing repressor protein, that controls the expression of genes involved in iron uptake, storage and usage. Under iron sufficiency, Fe^{2+}-Fur (holo-Fur) binds at operator sites (Fur boxes) in the promoters of multiple iron-responsive genes, and represses their transcription [4]. In a few bacterial species, Fur seems to have a broader scope of regulation, acting also as a direct transcriptional activator [5-7] or as an apo-regulator (apo-Fur) [8,9]. However, the most common Fur-mediated activation mechanism occurs indirectly via small regulatory RNAs (sRNA), such as RyhB in Escherichia coli [10], PrrF1 and PrrF2 in Pseudomonas aeruginosa [11], NrrF in Neisseria meningitidis [12] and FsrA in Bacillus subtilis [13]. In all these cases, the sRNAs inhibit the production of non-essential iron-using proteins under iron limitation, allowing relocalization of the intracellular iron for essential proteins [14].

The Fur protein is the most widely found and best-studied iron-responsive regulator in bacteria from diverse taxonomic groups, such as subdivisions γ, β, δ and ε of proteobacteria and bacilli [4]. However, in α-proteobacteria iron regulation is still little studied and appears to be mediated by regulators different from Fur. Direct experimental data, available mostly to Rhizobiales, indicate that RirA and Irr are the master regulators of iron homeostasis while a Fur-like protein, named Mur, regulates only a manganese transporter [15,16]. It has been suggested, based on bioinformatics and phylogenetic analyses, that RirA and Irr emerged as the main iron regulators in the common ancestor of the Rhizobiales and Rhodobacteriales, whereas in more basal lineages of α-proteobacteria (Caulobacterales, Rhodospirillales and Sphingomonadales), Fur remained as the global iron regulator [17]. This in silico prediction was recently confirmed by experimental data for at least two α-proteobacteria, Caulobacter crescentus [6] and the magnetotactic bacterium Magnetospirillum gryphiswaldense [18,19].

We have previously demonstrated, using an in silico approach combined with experimental data, that Fur controls iron homeostasis in C. crescentus by regulating many iron-responsive genes, and protect this freshwater oligotrophic bacterium from oxidative stress [6]. However, the response of C. crescentus to iron limitation and a comprehensive investigation of its Fur regulon remain to be determined on a global scale. In this work, we performed DNA microarray analysis to determine the transcriptional response of C. crescentus to iron availability, using wild-type cells growing under iron-replete versus iron-limiting conditions. We also used transcriptional profiling, comparing wild-type versus fur-mutant strains, to find novel members of the C. crescentus Fur regulon.

Results and discussion
Effect of iron and Fur on the C. crescentus transcriptome
Whole genome transcriptional profiling using DNA microarrays were performed to identify iron-responsive and Fur-regulated genes in C. crescentus. Two sets of microarray experiments were conducted in duplicate using RNA samples prepared from two independent biological cultures. First, to define the C. crescentus iron limitation stimulon, we compared the transcriptome of wild-type cells treated with 100 μM FeSO₄ (iron sufficiency) with that of wild-type cells treated with 100 μM 2,2-dipyridyl for 2 hours (iron limitation), an experimental condition previously established to study gene expression of iron-responsive genes in C. crescentus [6]. Iron limitation altered expression of 182 genes of which 108 were upregulated and 74 downregulated (Figure 1). Second, to identify Fur-regulated genes, we compared the transcriptome of wild-type cells with that of a fur mutant both cultivated in iron sufficiency. The expression of 121 genes was found to be significantly changed by the fur mutation (58 upregulated genes and 63 downregulated genes) (Figure 1).

The up- and down-regulated genes, identified in these two microarray experiments, were compared to identify genes regulated by both iron limitation and fur mutation or genes affected by only one of these conditions (Figure 1). We found 42 genes upregulated both under iron limitation and in the fur mutant (Fe^{2+}-Fur repressed genes) and 27 genes that were found to be downregulated on both these conditions (Fe^{2+}-Fur activated genes), indicating that Fur has a major role on controlling expression of iron-responsive genes in C. crescentus. We also found many genes regulated exclusively in response to iron limitation, namely 66 upregulated genes and 47 downregulated genes, suggesting that the C. crescentus iron limitation stimulon is controlled by additional regulatory mechanisms.

Lastly, a group of genes showed differential expression in the fur mutant (16 up- and 36 downregulated genes) independent of iron availability (Figure 1; Additional file 1: Table S1). We were unable to determine whether
these transcriptional changes are secondary effects or are mediated directly by Fur in an iron-independent manner. Nevertheless, the most upregulated genes in the fur mutant are the genes involved in transport (CC0859-60-61) and catabolism (CC1296, CC1298, CC1299 and CC1302) of myo-inositol in C. crescentus, belonging to the IolR regulon [20]. As expected, the level of fur mRNA (CC0057) was severely reduced in the fur mutant (7.4 fold). Interestingly, the sodB gene (CC3557) encoding an iron/manganese superoxide dismutase was 2.2-fold downregulated in the fur mutant (Additional file 1: Table S1), although its iron-dependent regulation verified in other bacteria [21] was not observed in our microarrays.

The repertoire of iron-responsive and Fur-regulated genes in C. crescentus

The genes regulated by both iron limitation and Fur are those showed in Tables 1 and 2 (in Figure 2A and 2B, genes indicated in blue). In addition to those genes, we observed that expression of some genes assumed as significantly up or downregulated under only one of the tested conditions changed to levels very close to our cutoff criterion (twofold change) in the other condition (Tables 3, 4 and Additional file 1: Table S1 and in Figure 2, genes indicated in orange). Thus, it is probable that these genes are also responsive to both iron levels and Fur, especially if one considers that most of them are in putative operons with genes whose expression was significantly changed under both iron limitation and fur mutation. Therefore, these genes were discussed here as part of the repertory of iron-responsive and Fur-regulated genes.

The genes upregulated by both iron limitation and fur mutation (Fe²⁺-Fur repressed genes) were grouped into functional categories and according to their transcriptional organization in the chromosome (Table 1; Figure 2A). Many of these genes are organized in large clusters that contain at least one gene predicted to be involved in transport, implicating them in iron-acquisition associated functions (Figure 2A). These include four gene clusters containing TonB-dependent receptors, which are outer membrane proteins probably involved in Fe³⁺-siderophore acquisition (CC0028-27-26, CC0139, CC2194-95-96-97 and CC2928-27-26), the operon encoding the ferrous iron transporter FeoAB (CC0711-12) as well as two gene clusters encoding predicted ABC transporters (CC3692-93-94-95-96 and CC0683-84) and two gene clusters encoding hypothetical proteins that are putative transporters (CC2193-92-91 and CC3059-60-61-62-63) (these last two operons are discussed below). Although none of these putative transporters have been characterized yet, their high derepression by both iron limitation and fur mutation (Table 1) indicates that they could play a major role in the adaptation of C. crescentus to low-iron conditions. Unexpectedly, it has been shown, using hyper-saturated transposon mutagenesis, that feoAB is an essential operon in C. crescentus even for growth on rich media (iron sufficiency) [22], highlighting the vital role of iron acquisition in this bacterium.

In addition to these putative iron acquisition systems, a riboflavin biosynthesis operon (CC0885-86-87-88-89) as well as the bfd gene (CC3263) encoding a ferredoxin associated with bacterioferritin were upregulated by both iron limitation and fur mutation (Table 1; Figure 2A). It has been reported for Helicobacter pylori
| Gene CB15 | Gene NA1000 | Predicted function | Fold change<sup>b</sup> |
|-----------|-------------|--------------------|-----------------|
| **Transport** | | | |
| CC_0026  | CCNA_00026  | PAS-family sensor histidine kinase (heme) | 4.70 | 5.95 |
| CC_0027  | CCNA_00027  | PKHD-type hydroxylase (FeII) | 15.65 | 26.39 |
| CC_0028<sup>c</sup> | CCNA_00028  | TonB-dependent receptor | 28.27 | 55.08 |
| CC_0029  | CCNA_00029  | Lysine exporter protein | 2.18 | 2.03 |
| CC_0139  | CCNA_00138  | TonB-dependent receptor | 20.21 | 33.27 |
| CC_0683  | CCNA_00719  | Type I secretion adaptor protein hlyD | 2.42 | 2.81 |
| CC_0684  | CCNA_00720  | Type I protein secretion ATP-binding protein | 2.25 | 2.80 |
| CC_0711  | CCNA_00748  | Ferrous iron transport protein A | 9.10 | 9.04 |
| CC_0712  | CCNA_00749  | Ferrous iron transport protein B | 6.13 | 5.96 |
| CC_2191  | CCNA_02272  | Hypothetical protein | 4.49 | 7.10 |
| CC_2192  | CCNA_02273  | Glutathione peroxidase (DUF3297) | 6.27 | 9.09 |
| CC_2193  | CCNA_02274/75 | EF hand protein/hypothetical protein (DUF4198) | 64.82 | 167.73 |
| CC_2194  | CCNA_02277  | Hemin receptor (TonB-dependent receptor) | 17.90 | 25.29 |
| CC_2195  | CCNA_02278  | Putative membrane-associated alkaline phosphatase | 4.49 | 7.27 |
| CC_2196  | CCNA_02279  | Disulfide bond formation protein B | 2.51 | 3.17 |
| CC_2197  | CCNA_02280  | Ubiquinone biosynthesis protein COQ7 (Iron) | 2.52 | 3.23 |
| CC_2927  | CCNA_03022  | Transporter | 27.09 | 34.54 |
| CC_2928  | CCNA_03023  | TonB-dependent receptor | 15.36 | 22.72 |
| CC_3059  | CCNA_03155  | Transporter | 23.57 | 22.29 |
| CC_3060  | CCNA_03156  | Putative periplasmic protein (DUF2271) | 24.53 | 32.44 |
| CC_3061  | CCNA_03157  | Putative membrane spanning protein (DUF4198) | 44.13 | 51.11 |
| CC_3062  | CCNA_03158  | Iron-sulfur cluster assembly/repair protein ApbE | 17.44 | 24.66 |
| CC_3063  | CCNA_03159  | Sulfite reductase (NADPH) flavoprotein (Heme) | 12.25 | 16.47 |
| CC_3693  | CCNA_03807  | Organic solvent resistance transport system Ttg2D protein | 6.48 | 2.50 |
| CC_3694  | CCNA_03808  | Organic solvent resistance transport system Ttg2C protein | 5.62 | 2.19 |
| **Riboflavin biosynthesis** | | | |
| CC_0885  | CCNA_00929  | Diaminohydroxyporphobilinopyrimidine deaminase | 10.83 | 4.99 |
| CC_0886  | CCNA_00930  | Riboflavin synthase alpha chain | 8.01 | 3.57 |
| CC_0887  | CCNA_00931  | 3,4-dihydroxy-2-butanoate-4-phosphate synthase | 12.18 | 3.70 |
| CC_0888  | CCNA_00932  | 6,7-dimethyl-8-ribityllumazine synthase | 13.72 | 4.33 |
| CC_0889  | CCNA_00933  | Putative peptidase | 5.75 | 3.08 |
| **Miscellaneous** | | | |
| CC_0220  | CCNA_00220  | Thiol-disulfide isomerase and thioredoxin | 3.80 | 3.31 |
| CC_0884  | CCNA_00928  | Transcriptional regulator, GntR family | 5.16 | 2.45 |
| CC_1968  | CCNA_02046  | Nitrogen regulatory protein P-II GlnB | 2.87 | 3.45 |
| CC_1969  | CCNA_02047  | Glutamine synthetase GlnA | 2.32 | 2.33 |
| CC_3263  | CCNA_03372  | Bacterioferritin-associated ferredoxin (Fe-S cluster) | 56.96 | 40.04 |
| **Hypothetical** | | | |
| CC_0135  | CCNA_00154  | Hypothetical protein DUF2061 (predicted membrane) | 13.07 | 5.99 |
| CC_0681  | unannotated | Hypothetical protein | 7.97 | 3.77 |
| CC_0682  | unannotated | Hypothetical protein | 10.03 | 3.88 |
| CC_0719  | CCNA_00756  | Hypothetical protein | 9.81 | 2.89 |
### Table 1 Genes upregulated under iron-limiting condition and in the fur mutant (Continued)

| Gene    | Gene NA | Predicted function                      | WT DP/WT Fe | Δfur Fe/WT Fe |
|---------|---------|----------------------------------------|-------------|---------------|
| CC_2367 | CCNA_02452 | Hypothetical protein                    | 23.64       | 26.74         |
| CC_2904 | CCNA_02998 | Hypothetical protein                    | 9.64        | 13.35         |
| CC_3452 | CCNA_03566 | Hypothetical protein                    | 2.52        | 2.51          |

a The terms in parenthesis are Pfam domains found in hypothetical proteins or metals predicted to bind the proteins. Metal cofactors were found by searching the ExPASy and Brenda databases.

b Values are fold changes in the expression levels comparing wild type cells exposed to iron-limiting versus iron-replete conditions (WT DP/WT Fe) or comparing fur mutant strain versus wild type strain both exposed to iron-replete condition (Δfur Fe/WT Fe). The values were obtained as the average of the four last probes for each gene.

c According to previously proposed in earlier work [6], the most probable initiation codon of CC0028 is at position +234 relative to the initiation codon annotated in the genome. Thus, the last four probes designed for CC0028 are not useful to measure its expression. The values showed for this gene correspond to the average of four initial probes of the CC0027 gene, which hybridize within the final portion of CC0028.

### Table 2 Genes downregulated under iron-limiting conditions and in the fur mutant

| Gene CB15 | Gene NA1000 | Predicted function                      | Fold changea |
|-----------|-------------|----------------------------------------|--------------|
| Transport |             |                                        | WT DP/WT Fe  | Δfur Fe/WT Fe |
| CC_0925   | CCNA_00974  | OAR protein precursor (OmpA-like protein) | −8.22        | −2.74         |
| CC_0991   | CCNA_01042  | TonB-dependent receptor                 | −2.47        | −2.27         |
| CC_1099   | CCNA_01155  | TonB-dependent outer membrane receptor  | −2.22        | −2.06         |
| CC_2485   | CCNA_02570  | Transporter (Major Facilitator Superfamily) | −2.49        | −3.24         |
| CC_2486   | CCNA_02571  | Transporter (Major Facilitator Superfamily) | −2.16        | −2.24         |
| CC_2804   | CCNA_02895  | TonB-dependent receptor                 | −2.41        | −2.22         |
| CC_3161   | CCNA_03263  | TonB-dependent receptor                 | −2.89        | −2.68         |
| CC_3335   | reannotated | Hypothetical protein                    | −4.54        | −5.88         |
| CC_3336   | CCNA_03444  | TonB-dependent receptor                 | −2.51        | −2.38         |

Energy Metabolism

| Gene    | Gene NA | Predicted function                      | Fold changea |
|---------|---------|----------------------------------------|--------------|
| CC_0277 | CCNA_00279 | NAD(P)H dehydrogenase (quinone)        | −3.11        | −4.74         |
| CC_1401 | CCNA_01467 | Cytochrome cbb3 oxidase subunit I ccoN | −2.76        | −6.07         |
| CC_1951 | CCNA_02028 | NTF2 enzyme family protein             | −2.11        | −2.23         |
| CC_1952 | CCNA_02029 | NADH-quinone oxidoreductase chain D    | −2.18        | −2.23         |
| CC_1954 | CCNA_02031 | NADH-quinone oxidoreductase chain C    | −2.09        | −2.16         |
| CC_2115 | CCNA_02200 | Cytochrome c-family protein            | −3.02        | −4.61         |
| CC_2494 | CCNA_02579 | Cytochrome P450 (Heme)                 | −3.31        | −4.43         |
| CC_3526 | CCNA_03641 | Succinate dehydrogenase iron-sulfur protein (Fe-S cluster) | −2.35        | −3.12         |
| CC_3527 | CCNA_03642 | Succinate dehydrogenase flavoprotein subunit | −2.84        | −2.95         |
| CC_3528 | CCNA_03643 | Succinate dehydrogenase membrane anchor subunit | −3.11        | −3.29         |
| CC_3529 | CCNA_03644 | Succinate dehydrogenase cytochrome B-556 subunit | −2.87        | −2.89         |

Miscellaneous

| Gene    | Gene NA | Predicted function                      | Fold changea |
|---------|---------|----------------------------------------|--------------|
| CC_1363 | CCNA_01425 | H+ translocating pyrophosphatase        | −3.35        | −2.78         |
| CC_2479 | CCNA_02564 | acyl-CoA dehydrogenase, short-chain specific | −2.38        | −2.29         |
| CC_2518 | CCNA_02603 | Phosphatidylethanolamine decarboxylase (DUF1254) | −3.05        | −4.79         |
| CC_3085 | CCNA_03181 | Alcohol dehydrogenase (Zinc or iron)   | −4.05        | −2.28         |

Regulators

| Gene    | Gene NA | Predicted function                      | Fold changea |
|---------|---------|----------------------------------------|--------------|
| CC_0752 | CCNA_00789 | Hypoxia transcriptional regulator FixK | −3.02        | −4.08         |
| CC_0753 | CCNA_00790 | Hypoxia negative feedback regulator FixT | −2.63        | −3.65         |
| CC_1410 | CCNA_01476 | CRP-family transcription regulator FtrB | −6.56        | −13.47        |

a Values are fold changes in the expression levels as described in Table 1. Negative values denote downregulation.
Figure 2 Genomic organization of the *C. crescentus* Fur regulon. The chromosomal clusters of the iron-responsive and Fur-regulated genes are organized in functional categories grouped in separate panels. Genes are also grouped as upregulated (A) or downregulated (B) under both iron limitation and *fur* mutation. The arrows indicate each open reading frame and their orientation on the chromosome. Differentially expressed genes are indicated in blue. Genes that have been experimentally shown to be directly regulated by Fur [6] are indicated in red. Selected genes that were either iron or Fur regulated (Tables 3, 4 and Additional file 1: Table S1) and showed expression change very close to our cutoff criterion on the other condition are shown in orange. Vertical blocks indicate the location of the Fur binding sites detected by the MEME search described in Figure 1, where sites predicted in silico are white and experimentally validated by EMSA are black [6].
and Campylobacter jejuni that the production of riboflavin is also regulated by iron and Fur and secreted riboflavin has a role in Fe\textsuperscript{3+} reduction and hence in iron acquisition [23,24]. Genes involved in oxidative stress response (CC0220), RNA processing (CC0835), transcriptional regulation (CC0884) and ammonia assimilation (CC1968-69) were also Fe\textsuperscript{2+}-Fur repressed. A tight connection between iron homeostasis and nitrogen metabolism has been reported for the nitrogen-fixing cyanobacterium Anabaena sp. [25].

Finally, seven genes encoding hypothetical proteins were also upregulated by both iron limitation and fur mutation, of which two genes are of particular interest (CC0681 and CC0682). A previous report, based on tiled microarray analysis, suggested the existence of two candidate small regulatory RNAs (sRNAs) located in the intergenic regions between CC0680-CC0681 and C00681-CC0682, but attempts to validate these sRNAs by Northern blot allowed the detection of only a large transcript comprising all this region [26]. Considering that the putative operon CC0682-sRNA1-CC0681-sRNA2 was found to be Fe\textsuperscript{2+}-Fur repressed in our microarray analyses (Table 1, Figure 2A) we are tempted to speculate that it could be processed under iron limitation, generating two sRNAs and two mRNAs translated to small proteins. These components could mediate the iron sparing response in C. crescentus, similarly to what was observed in Bacillus subtilis in which a sRNA (FsrA) and three small basic proteins (FbpA, FbpB e FbpC) act in conjunction to repress the expression of iron-rich proteins [13].

Additionally to these Fe\textsuperscript{2+}-Fur repressed genes, our microarray analyses allowed us to identify the genes positively regulated by Fe\textsuperscript{2+}-Fur, in other words, the genes that were downregulated by both iron limitation and fur mutation (Table 2; Figure 2B). As expected, many of these genes encode iron-containing enzymes. These included succinate dehydrogenase (sdh operon, CC3529-28-27-26-25), NADH ubiquinone oxidoreductase (nuo operon, CC1956-55-54-53-52-51-50), cytochrome (CC0762, CC1401 and CC2115), cytochrome P450 enzyme (CC2494), glutamate synthase (CC3607), a hypothetical protein predicted as catalase and a hypothetical protein with a ferritin-like domain (CC0556-57). This mechanism of repressing iron-rich enzymes to prioritize iron usage when this metal is scarce, sometimes referred as iron sparing response, has been described in many bacteria, such as E. coli [10,21,27], P. aeruginosa [11] and B. subtilis [13,28].

Unexpectedly, a large number of genes encoding proteins involved in transport were also downregulated by both iron limitation and fur mutation (Table 2; Figure 2B). Among these, there are transporters belonging to the major facilitator superfamily (MFS) (CC1628, CC2485-86), porins (CC0925 and CC1409) and many TonB-dependent receptors. At least six of these genes (CC3336, CC3161, CC3461, CC0991, CC2804 and CC2485) are also highly induced by carbon limitation [29] and are positively regulated by CfrA, a sRNA that regulates adaptation to carbon starvation in C. crescentus [30]. Although the reason for these genes to be repressed by iron limitation and induced by carbon starvation is still not clear, it is reasonable to suppose that these TonB-dependent receptors are required for uptake of carbohydrates instead of Fe\textsuperscript{3+}-siderophore complexes, since it has recently been shown that novel substrates, such as nickel and different carbohydrates, are transported via TonB-dependent receptors [31].

Importantly, three genes (fixK, fixT and ftrB) encoding regulatory proteins that specify an oxygen signaling network required for C. crescentus growth under hypoxia [32] were found to be downregulated by both iron limitation and fur mutation (Table 2; Figure 2B). The C. crescentus Fix signaling system consists of the sensor histidine kinase FixL (a heme-binding oxygen sensor), its cognate response regulator FixJ, the transcriptional regulator FixK, and the kinase inhibitor FixT (the core FixLJ–FixK–FixT), besides the downstream regulators FtrA and FtrB [32]. Consistent with downregulation of fixK, many hypoxia-dependent FixK-activated genes containing a FixK binding site [32], were also downregulated by both iron limitation and fur mutation, including CC1409 (ompW), CC1410 (ftrB), CC0762 (cydA), CC1401 (ecoN), CC0753 (fixT), CC2115 and CC0277 (Table 2; Figure 2B). Therefore, the FixK-dependent hypoxia stress response seems to be positively regulated by Fe\textsuperscript{2+}-Fur under iron sufficiency and repressed in iron limitation condition, similarly to what was described for the anaerobic regulator Fnr in E. coli [21] and Salmonella enterica serovar Typhimurium [33]. The regulatory link between oxygen and iron availability could be mediated by the histidine kinase FixL, that senses oxygen through its heme-containing amino-terminal PAS domain [32].

To further discriminate whether regulation by Fur was direct or indirect, we conducted in silico searches in the upstream region of all up- and down-regulated genes identified in the microarray experiments (Figure 1). MEME-based analyses, including all genes together or each group of genes separately, identified a motif very similar to the Fur binding site previously detected in C. crescentus [6]. These Fur binding sites were detected only for genes regulated by both iron and Fur (Figure 1). As indicated in Figure 2, sixteen Fur binding sites were identified in the group of the genes upregulated by both iron limitation and fur mutation, indicating that most of these genes (37 out of 47 genes) are direct target for Fur-mediated repression. In contrast, only three Fur binding sites were detected in the group of the genes downregulated by both iron limitation and fur mutation,
suggesting that Fur indirectly mediates positive regulation of many genes, in addition to the direct positive regulation previously demonstrated [6].

Fur-independent regulation of \textit{C. crescentus} iron-responsive genes

In addition to the Fur modulin iron limitation also affected the \textit{C. crescentus} transcriptome in a Fur-independent manner, given that 66 genes were upregulated (Table 3) and 47 genes were downregulated (Table 4) during growth in iron-limitation condition that were not affected by the \textit{fur} mutation (Figure 1).

Among the genes strongly upregulated exclusively in response to iron limitation there is a large gene cluster (CC1866-65-64-63-62-61-60-59-58-57), which encodes the transcriptional repressor IscR (CC1866) and enzymes of the Suf system of Fe-S cluster biogenesis (Table 3). \textit{E. coli} possesses two operons implicated in Fe-S cluster assembly, \textit{iscSLA-iscBA-fdx}, encoding the housekeeping Fe-S cluster biogenesis pathway and \textit{sufABCDSE}, which synthesize Fe-S clusters under iron limitation or oxidative stress conditions [34,35], whereas \textit{C. crescentus} appears to have only one operon that contains a combination of \textit{isc} (CC1866-65, \textit{iscRS}) and \textit{suf} (CC1864-62-61-60, \textit{sufBCDS}) genes. In \textit{E. coli} both \textit{isc} and \textit{suf} operons are induced by iron limitation and oxidative stress, but while the \textit{isc} genes are regulated by IscR, the \textit{suf} genes are under control of OxyR and Fur [21,34-36]. In \textit{C. crescentus} upregulation of this large operon by iron limitation is Fur-independent and we postulate that it could be mediated by IscR via an IscR binding site previously predicted upstream of the CC1866 gene [17]. Because IscR senses damage to the Fe-S clusters of the cell, it is possible that iron limitation is generating some kind of stress in \textit{C. crescentus} which is able to damage Fe-S clusters.

In agreement with this assumption, many of the genes upregulated exclusively by iron limitation are related to various stress responses (Table 3) and were found to be induced when \textit{C. crescentus} was submitted to heavy metal stress [37]. Among the genes induced by both iron limitation and heavy metal stress (mainly cadmium stress), there are those related to oxidative stress defense (CC0141, CC0994, CC3161), detoxification efflux pumps (CC3195, CC3197), DNA repair (CC2590) and nucleotide biosynthesis (CC0260, CC3492) (Table 3). Interestingly, 12 heat shock genes, encoding chaperones, proteases and small heat shock proteins, were also upregulated by iron limitation, as well as some genes encoding peptidases containing metals as cofactors (Table 3), what is consistent with previous observations in \textit{Shewanella oneidensis} [38]. Induction of these genes might be directly mediated by the heat shock sigma factor RpoH (\textit{\(\sigma^{32}\)}) for the reason that the own \textit{rpoH} gene (CC3098) is upregulated in iron limitation (Table 3). Moreover, a predicted \(\sigma^{32}\)-binding motif (m_6 motif), which has been identified upstream of cadmium-induced genes [39], was found here upstream of nearly half (15 sites upstream of 30 genes/operons) of the 63 genes upregulated in iron limitation (Table 3), indicating induction of the RpoH regulon by iron limitation. The \textit{C. crescentus} \textit{rpoH} gene is transcribed from two promoters, a \(\sigma^{70}\)-dependent P1 promoter and a heat shock autoregulated \(\sigma^{32}\)-dependent P2 promoter [40]. It remains to be determined how these different signals (cadmium stress and iron limitation) could increase transcription of \textit{rpoH} in \textit{C. crescentus}, activating its regulon.

When the genes downregulated exclusively in iron limitation are grouped into functional categories, the most prominent groups of genes are involved in amino acid metabolism, chemotaxis and motility, and energy metabolism (Table 4). Among the enzymes of amino acid biosynthesis pathways repressed by iron limitation there are many involved in methionine biosynthesis, such as methionine synthases (CC0482, CC2137, CC2138), adenosylmethionine synthetase (CC0050), S-adenosyl-L-homocysteine hydrolase (CC0257) and methylenetetrahydrofolate reductase (CC2140), which is required to produce 5-methyltetrahydrofolate as methyl-group donor for methionine synthesis. Pathways of protein catabolism were also repressed by iron limitation as revealed by downregulation of many genes encoding peptidases (CC0167, CC0984, CC1048, CC2480 and CC3246) (Table 4). Furthermore, some genes for flagella assembly (CC0901-02, CC1456) and chemotaxis (CC0430-31-32-33, CC1399 and CC2847) were downregulated in iron limitation. Repression of motility and chemotaxis genes by iron limitation has been described in \textit{Sinorhizobium meliloti} [41] and \textit{Acinetobacter baumannii} [42]. Finally, some known Fe\textsuperscript{3+}-Fur activated genes [6,13] were downregulated in iron limitation, but not in the \textit{fur} mutant in this work. Of these, there are genes encoding the Fe-S clusters-containing enzymes aconitate hydratase (CC3667), NADH ubiquinone oxidoreductase (\textit{nuo} genes CC1946, CC1944-43-42), glutamate synthase (CC3606) and dihydroxy-acid dehydratase (CC3044) (Table 4). In some cases, at least part of the operons (\textit{nuo} and CC3607) was downregulated by both iron limitation and \textit{fur} mutation (Figure 2B). A possible explanation is that the Fe\textsuperscript{3+}-Fur activated genes showed modest differential expression (approximately 2 fold) (Table 2), thus small experimental fluctuations could exclude some genes based on our cutoff criteria for differential expression in the microarray analyses.

Comparing our microarray data with other large-scale transcriptomic studies performed under iron-limiting condition in bacteria from diverse taxonomic groups [21,28,38,41,42], we observed that, in spite of the multiplicity of regulatory mechanisms, the core of iron-regulated genes is extremely conserved, including mainly
### Table 3 Genes upregulated exclusively in response to iron limitation

| Gene CB15 | Gene NA1000 | Predicted function | Fold change$^a$ |
|-----------|--------------|-------------------|----------------|
| **Amino acid metabolism** | | | |
| CC_0013 | CCNA_00013 | Protein-PII uridylyltransferase GlnD | 2.62 |
| CC_0272 | CCNA_00273 | Peptide deformylase (FeII) | 2.81 |
| CC_0977$^b$ | CCNA_01028 | Cytosol aminopeptidase (Zinc or Manganese) | 4.62 |
| CC_1612 | CCNA_01684 | Phenylalanine-4-hydroxylase (Iron) | 2.33 |
| CC_2481$^b$ | CCNA_02566 | Membrane alanine aminopeptidase (Zinc) | 2.90 |
| CC_2532 | CCNA_02615 | Homogentisate 1,2-dioxygenase (Iron) | 2.38 |
| CC_2533 | CCNA_02616 | 4-hydroxyphenylpyruvate dioxygenase (Iron) | 2.49 |
| CC_3686 | CCNA_03800 | Diaminopimelate epimerase | 2.25 |
| **Iron-sulfur cluster assembly/repair** | | | |
| CC_0061 | CCNA_00059 | Oxygen-insensitive NADH nitroreductase | 3.31 |
| CC_0062 | CCNA_00060 | Mitochondrial-type Fe-S cluster assembly protein NFU | 4.60 |
| CC_0132$^b$ | CCNA_00131 | Rrt2 family protein | 3.30 |
| CC_1857 | CCNA_01933 | Hypothetical protein | 5.66 |
| CC_1858 | CCNA_01934 | HesB protein family | 5.66 |
| CC_1859 | CCNA_01935 | FeS assembly SUF system protein | 5.50 |
| CC_1860 | CCNA_01936 | Cysteine desulfurase/Selenocysteine lyase | 7.58 |
| CC_1861 | CCNA_01937 | SudD protein | 5.90 |
| CC_1862 | CCNA_01938 | ATP-dependent transporter sufC | 7.89 |
| CC_1863 | CCNA_01939 | ADP-ribosylglycohydrolase | 8.30 |
| CC_1864 | CCNA_01940 | ABC transporter-associated protein sufB | 7.81 |
| CC_1865 | CCNA_01941 | Cysteine desulphhydrase/Selenocysteine lyase | 7.09 |
| CC_1866$^b$ | CCNA_01942 | Rrt2 family transcriptional regulator | 7.98 |
| **Oxidative stress** | | | |
| CC_0141 | CCNA_00140 | Glutathione synthetase | 2.55 |
| CC_0993 | CCNA_01045 | Conserved hypothetical cytosolic protein (DUF419) | 2.84 |
| CC_0994$^b$ | CCNA_01046 | Peptide methionine sulfoxide reductase msrA | 3.07 |
| CC_1315 | CCNA_01375 | Lactoylglutathione lyase | 2.97 |
| CC_1316$^b$ | CCNA_01376 | Glutathione S-transferase | 3.93 |
| **Heat shock response** | | | |
| CC_0685 | CCNA_00721 | Chaperonin GroEL | 2.33 |
| CC_0686$^b$ | CCNA_00722 | Co-chaperonin GroES | 2.27 |
| CC_0878 | CCNA_00922 | ClpB protein | 2.71 |
| CC_2258 | CCNA_02341 | Small heat shock protein | 5.50 |
| CC_2467 | CCNA_02552 | ATP-dependent Clp protease adaptor protein ClpS | 2.42 |
| CC_2468 | CCNA_02553 | ATP-dependent clp protease ATP-binding subunit ClpA | 2.63 |
| CC_2509 | CCNA_02594 | Endopeptidase htpX | 8.09 |
| CC_2510$^b$ | CCNA_02595 | Hypothetical protein | 8.94 |
| CC_3098$^b$ | CCNA_03195 | RNA polymerase sigma factor RpoH | 5.16 |
| CC_3592$^b$ | CCNA_03706 | Small heat shock protein | 3.42 |
| CC_3727$^b$ | CCNA_03843 | ATP-dependent endopeptidase hsl proteolytic subunit hslV | 3.48 |
| CC_3728 | CCNA_03844 | ATP-dependent endopeptidase hsl ATP-binding subunit hslU | 2.49 |
those related to transport, use and storage of this metal. Some responses seems to be confined to few bacteria, such as upregulation of the heat shock response, also described in \textit{S. oneidensis}\cite{38} and downregulation of chemotaxis and motility, observed in \textit{S. meliloti}\cite{41} and \textit{A. baumannii}\cite{42}.

However, our study expands the range of genes involved in iron homeostasis when we consider physiological processes unique to the \textit{C. crescentus} lifestyle, such as adaptation to growing in oligotrophic environments and under different oxygen tensions. In fact, many TonB-dependent receptors, predicted to be required for sugar transport, and the hypoxia FixK regulon were surprisingly downregulated by both iron limitation and \textit{fur} mutation.

### Verification of iron- and Fur-dependent expression of the CC2193 and CC3059 operons

Nearly all of the genes previously identified as members of the \textit{C. crescentus} Fur regulon\cite{6} were found to be differentially expressed by microarray analyses (Figure 2, red arrows), validating the experimental procedure. To further confirm our microarray data, we selected genes located in two clusters that encode putative transporters for validation by $\beta$-galactosidase activity assays and EMSA. The first cluster (CC2193-92-91) encodes a hypothetical protein containing an EF hand motif (CC2193), a putative glutathione peroxidase (CC2192) and a hypothetical protein (CC2191). The CC2193 gene appears to have
Table 4 Genes downregulated exclusively in response to iron limitation

| Gene CB15 | Gene NA1000 | Predicted function | Fold change |
|-----------|-------------|--------------------|-------------|
| **Amino acid metabolism** | | | |
| CC_0049 | CCNA_00047 | tRNA m7-G46 methyltransferase | −3.12 |
| CC_0050 | CCNA_00048 | S-adenosylmethionine synthetase | −3.05 |
| CC_0167 | CCNA_00166 | Hypothetical protein (transglutaminase-like cysteine proteinase) | −2.22 |
| CC_0257 | CCNA_00257 | Adenosylhomocysteinate | −3.12 |
| CC_0482 | CCNA_00515 | Cobalamin-independent methionine synthase (Zinc) | −2.49 |
| CC_0984 | CCNA_01035 | Gamma-glutamyltranspeptidase | −2.68 |
| CC_1048 | CCNA_01100 | Acylamino-acid-releasing enzyme | −2.73 |
| CC_2137 | CCNA_02221 | Methionine synthase I meth (Zinc) | −2.52 |
| CC_2138 | CCNA_02222 | 5-methyltetrahydrofolate | −2.72 |
| CC_2139 | CCNA_02223 | Beta-lactamase, type II (Zinc) | −2.83 |
| CC_2140 | CCNA_02224 | Methylene tetrahydrofolate reductase | −2.39 |
| CC_2840 | CCNA_02933 | Aminopeptidase | −2.14 |
| CC_3044 | CCNA_03139 | Dihydroxy-acid dehydratase (Fe-S cluster) | −3.35 |
| CC_3606 | CCNA_03721 | Glutamate synthase (NADPH) small chain | −2.30 |
| CC_3607b | CCNA_03722 | Glutamate synthase (NADPH) large chain (Fe-S cluster) | −2.51 |
| **Chemotaxis and motility** | | | |
| CC_0430 | CCNA_00439 | Methyl-accepting chemotaxis protein | −2.97 |
| CC_0432 | CCNA_00440 | CheX protein | −2.50 |
| CC_0433 | CCNA_00441 | Chemotaxis receiver domain protein cheYI | −2.31 |
| CC_0901 | CCNA_00946 | Basal-body rod modification protein FlgD | −2.38 |
| CC_0902 | CCNA_00947 | Flagellar hook protein FlgE | −2.23 |
| CC_1399 | CCNA_01465 | Methyl-accepting chemotaxis protein | −2.22 |
| CC_1456 | CCNA_01523 | Acetyltransferase flmA | −2.63 |
| CC_2846 | CCNA_02939 | Conserved hypothetical protein | −5.24 |
| CC_2847 | CCNA_02940 | Methyl-accepting chemotaxis protein | −3.44 |
| **Energy Metabolism** | | | |
| CC_1942 | CCNA_02020 | NADH-quinone oxidoreductase chain I (Fe-S cluster) | −2.12 |
| CC_1943 | Unannotated | Hypothetical protein | −2.20 |
| CC_1944 | CCNA_02021 | Hypothetical protein | −2.26 |
| CC_1946 | CCNA_02023 | NADH-quinone oxidoreductase chain G (Fe-S cluster) | −2.05 |
| CC_1953b | CCNA_02030 | Hypothetical protein | −2.19 |
| CC_3525b | CCNA_03640 | Ferredoxin reductase subunit (Fe-S cluster) | −2.30 |
| CC_3659 | CCNA_03774 | Citrate lyase beta chain/citryl-CoA lyase subunit | −2.00 |
| CC_3667 | CCNA_03781 | Aconitate hydratase (Fe-S cluster) | −2.30 |
| **Miscellaneous** | | | |
| CC_0566 | CCNA_00601 | MoxR-like ATPase | −2.04 |
| CC_1409b | CCNA_01475 | OmpW family outer membrane protein | −3.21 |
| CC_1754b | CCNA_01830 | TonB-dependent receptor | −2.07 |
| CC_2389 | CCNA_02472 | Cobalt-zinc-cadmium resistance protein czcB | −2.42 |
| CC_3081 | CCNA_03177 | Methylmalonyl-CoA mutase MeaA-like protein | −2.55 |
been incorrectly annotated in the CB15 strain given that in the chromosome of the *C. crescentus* NA1000 strain, recently sequenced [43], two open reading frames were annotated in this region, CCNA02274 (encoding a shorter EF hand protein) and CCNA02275, encoding a hypothetical protein with a domain of unknown function (DUF4198). The second cluster (CC3059-60-61) contains three genes encoding a putative transporter (CC3059-60-61), and two genes involved in iron-related functions (sulfite reductase iron-flavoprotein and Fe-S cluster repair protein) (Figure 2; Table 1). Interestingly, the genes of these two clusters most highly upregulated in iron limitation and *fur* mutant (CC2193-corresponding to CCNA02275 in NA1000, and CC30561) (Table 1) encode two paralogous proteins belonging to the widespread Pfam family DUF4198. Although the proteins of this family are widely distributed in various groups of bacteria (750 sequences in 486 species, Pfam database February 2013), nothing is known about their function or regulation.

The promoter regions of CC2193 and CC3059 were cloned in a lacZ reporter plasmid and the constructions were introduced into the wild type and *fur* mutant strains. Beta-galactosidase activity assays indicated that the expression of these two genes was induced under iron limitation and derepressed in the *fur* mutant strain since their expression changes were very close to our cutoff criterion.

### Conclusions

Using DNA microarray analyses, we have defined the global transcriptional response of *Caulobacter crescentus* to iron availability, providing an overview of the physiological strategies that this oligotrophic α-proteobacterium employs for survival in iron limiting conditions (Figure 4). Our data reveal that the iron stimulon in *C. crescentus* is larger than the Fur regulon previously identified [6], involving a more complex regulatory network. Among the responses mediated by Fur it is worth pointing out the upregulation of genes involved in iron acquisition systems and biosynthesis of riboflavin in iron limiting condition, as well as the downregulation of genes encoding many iron-using enzymes involved in energy metabolism (Figure 4). Fur binding site prediction suggests that Fur acts mainly as a direct transcriptional repressor, whereas positive regulation could be mediated either directly by Fur in a few cases or indirectly for most genes. In many cases this indirect effect was provoked by downregulation of the hypoxia regulator FixK, causing decreased expression of FixK-activated genes in iron limitation condition (Figure 4). Other genes could be indirectly activated by Fur via an unidentified iron-responsive sRNA. While the Fe$^{2+}$-Fur mediated repression of some genes encoding TonB-dependent receptors confirmed our previous data [6], the Fe$^{2+}$-Fur mediated activation of many other TonB-dependent receptors putatively associated with sugar transport was unexpected. Fur-independent regulation of *C. crescentus* iron-responsive genes was also observed, indicating an overlap with other regulatory pathways (Figure 4). It is

### Table 4 Genes downregulated exclusively in response to iron limitation (Continued)

| Gene | Description                                      | Fold Change |
|------|--------------------------------------------------|-------------|
| NC_00527 | CCNA_03227 TonB-dependent receptor                | −2.41       |
| NC_00134 | CCNA_03524 Di-tripeptide transporter (Major Facilitator Superfamily) | −2.48       |
| NC_00361 | CCNA_03574 TonB-dependent receptor                | −2.70       |

Hypothetical

| Gene | Description                                      | Fold Change |
|------|--------------------------------------------------|-------------|
| NC_0060 | CCNA_00636 Hypothetical protein                   | −2.16       |
| NC_0106 | CCNA_01121 Conserved hypothetical protein         | −2.40       |
| NC_0110 | CCNA_01158 Hypothetical protein                   | −2.47       |
| NC_0274 | NCNA_02831 Conserved hypothetical protein (DUF2272) | −3.14       |
| NC_0341 | CCNA_03532 Hypothetical protein (Acetyltransferase (GNAT) family) | −2.18       |

* Values are fold changes in the expression levels as described in Table 3. Negative values denote downregulation.

b These genes are probably also downregulated in the *fur* mutant since their expression changes were very close to our cutoff criterion.
worth mentioning that iron limitation caused upregulation of the heat shock sigma factor RpoH with consequent activation of its regulon, and upregulation of the IscR regulon, whose genes are involved in Fe-S cluster biogenesis. Since most of these iron-responsive genes identified in this work have not been experimentally investigated in C. crescentus, they are good targets for future studies.

Methods

Bacterial strains and growth conditions

Caulobacter crescentus, also known as Caulobacter vibrioides [44], strains NA1000 (wild-type) [45] and SP0057 (fur mutant) [6] were grown aerobically at 30°C in peptone-yeast extract (PYE) medium [46]. Iron-replete and iron-limiting conditions were achieved by supplementing PYE medium with 100 μM FeSO₄ (Fe) or 100 μM 2,2-dipyridyl (DP) for two hours. The β-galactosidase activity generated by these lacZ fusions was determined. The experiments were performed in duplicate from three independent biological cultures. Fur binds directly to the promoter of the CC3059 operon. EMSAs were performed using the purified His-Fur protein and a probe containing the promoter region of CC3059. The 3²P-labeled probe was incubated with increasing concentrations of protein (0, 50, 200, 500 and 1000 nM) (left). A competition assay using 250 nM Fur and the labeled CC3059 probe was performed, where binding of Fur was challenged with a 30-fold excess of unlabeled DNA fragments of the same region (SE) or the 16S RNA coding region (SI) as competitors (right). Below is shown the promoter region of the CC3059 operon, indicating the previously identified transcriptional start site (+1) and conserved −35 and −10 sequences of Caulobacter σ⁷⁰ promoters (TTGAC-16 bp-G/CCTANA) [39]. The initiation codon (GTG) is underlined. The Fur binding site predicted in silico is shaded.

Microarray analysis

For the DNA microarray experiments, overnight C. crescentus cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 in 35 ml of PYE medium. Cells were grown up to midlog phase (OD₆₀₀ ~ 0.5) and the cultures were divided and treated with either 100 μM FeSO₄ (iron sufficiency) or 100 μM DP (iron limitation). The incubation was continued for two hours prior to RNA isolation as previously described [6]. Total RNA was extracted using Trizol Reagent (Invitrogen), according to the manufacturer’s instructions. RNA samples were treated with RNase-free DNase I (Fermentas) to digest residual chromosomal DNA and then precipitated using sodium acetate/ethanol prior to spectrophotometric quantification and visualization on formaldehyde-agarose gels. RNA samples were isolated from two independent bacterial cultures for each strain or condition analyzed as biological replicates. Amino allyl modified cDNA was generated by reverse transcription from 20 μg of total RNA and labeled with either Cy3 or Cy5 mono-reactive fluorescent dyes using the FairPlay III Microarray Labeling System (Stratagene). Labeled cDNA samples were hybridized to a custom-designed DNA oligo microarray (Agilent) (each gene is covered by 9–11
probes located −300 to +200 relative to the translational start site) using a protocol previously described [47,48]. The arrays were scanned for the Cy3 and Cy5 fluorescent signals with an Agilent High Resolution Microarray Scanner. Data extraction and normalization was performed with the Feature Extraction Software 9.0 (Agilent). A gene was considered as upregulated or downregulated if it showed 2-fold change relative to the control considering at least three out of four last probes (that are downstream of the translational start site) in both biological replicates. The values for the relative expression of each gene were obtained as the average of the four last probes. The microarray data have been deposited in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo) under accession number GSE45653.

Fur binding site detection

Bioinformatics analyses were performed using the Multiple Em for Motif Elicitation (MEME) tool [49] to identify motifs within the promoter regions of iron regulated genes. Putative gene regulatory regions (−200 to +50 bp relative to the start codon) were searched using the following parameters: motifs size from 6 to 50 bp; zero or one motif per sequence; search given strand only; palindromic and nonpalindromic models were tested. Sequence logos were generated using WebLogo [50].

lacZ fusions and β-Galactosidase assays

DNA fragments covering the promoter regions of CC2193 (193 bp) and CC3059 (183 bp) were PCR-amplified using primer pairs CC2193-fw (5'-TGGATCCCGGCGAAGTTT

Figure 4 Schematic representation of the main changes in gene expression and cell processes under iron-limiting conditions. Upregulated genes and pathways are shown in red, downregulated are shown in green. Large arrows indicate activation and blunt-head lines indicate repression by the respective transcription regulator (Fur is represented as iron-bound). Thin arrows indicate enzyme reactions. Traced arrow indicates activation of the enzyme GlnB by GlnD via uridylylation. L-hCys: L-homocysteine, SAM: S-adenosylmethionine, SAhC: S-adenosylhomocysteine.
CAGGCGCGAC-3')/CC2193-rv (5'-TAAGCTTACGGAT
CATTGGAACAAAACC-3') and CC3059-fw (5'-TGGATC
CAGTTGCGGCAGCGATTCAGG-3')/CC3059-rv (5'-TA
AGCTTGCGGCAGCGATTTCAGG-3'), respectively.
These PCR products were cloned into pGEM-T Easy,
AGCTTGCGGCGGATTTCACAGG-3'), respective-
CAGTTGACGGCGCAATAGGCC-3')/CC3059-rv (5'-TA
CATTGGACAAACCC-3') and CC3059-fw (5'-TGGATC
CAGGCGCGAC-3')/CC2193-rv (5'-TAAGCTTACGGAT

Electrophoretic mobility shift assay (EMSA)
A probe corresponding the promoter region of CC3059
(5'-CCCGCTGAATGATGAAGGTC-3') and 16SA-rv
(5'-GCTGCTGGCACGAAGTTAGC-3'). For EMSA, pu-
For competition assay, a 101-bp 16S rRNA intragenic
DNA

Additional file

Additional file 1: Table S1. Differentially expressed genes in the fur

Competing interest
The authors declare that they have no competing interest.

Authors’ contributions
JFSN and MVM planned the experiments; JFSN performed the experimental

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