Regulation of Iron Metabolism by *Pyrococcus furiosus*

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Supplementary Material

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Table S1. ORFs whose expression is >2-fold up-regulated under iron-limited conditions and their potential operon arrangement.

ORF	Description	COM1c2 $\log_2 \pm$ SD	COM1c2 Fold change	ΔDTXR $\log_2 \pm$ SD	ΔDTXR Fold Change
PF0685	conserved hypothetical protein	1.2 ± 0.1	**2.2**	1.2 ± 0.4	**2.3**
PF0686	hypothetical protein	0.6 ± 0.2	1.5	0.9 ± 0.4	1.9
PF0687	Transcription factor B-2	1.0 ± 0.2	**2.0**	1.1 ± 0.4	**2.2**
PF0692	Prismane	1.1 ± 0.1	**2.1**	1.7 ± 0.3	**3.2**
PF0694	flavoprotein	0.4 ± 0.4	1.3	0.9 ± 0.8	1.8
PF0695	hypothetical protein	1.0 ± 0.2	**2.0**	1.8 ± 0.6	**3.5**
PF0696	carbohydrate-binding protein	0.5 ± 0.2	1.4	0.6 ± 0.9	1.5
[†]PF0723	Ftr1 iron permease	1.6 ± 0.2	**3.1**	0.8 ± 0.3	1.7
PF0728	hypothetical protein	1.1 ± 0.4	**2.1**	1.2 ± 0.4	**2.3**
PF0729	multi domain protein	0.3 ± 0.7	1.2	1.1 ± 0.5	**2.1**
Putative ferrous transporter					
[†]**PF0857**	ferrous iron transport protein B	1.0 ± 0.0	**2.0**	-0.1 ± 0.1	1.0
PF0858	conserved hypothetical	0.3 ± 0.3	1.2	-0.3 ± 0.3	1.2
Branched amino acids biosynthesis					
PF0934	hypothetical	1.8 ± 0.3	**3.6**	1.0 ± 0.5	1.9
PF0935	acetolactate synthase	2.2 ± 0.6	**4.6**	1.7 ± 0.4	**3.3**
PF0936	ketol-acid reductoisomerase	2.5 ± 0.6	**5.5**	1.8 ± 0.4	**3.4**
PF0937	2-isopropylmalate synthase	2.3 ± 0.5	**4.9**	1.7 ± 0.3	**3.3**
PF0938	3-isopropyl malate dehydratase II	2.2 ± 0.5	**4.7**	1.7 ± 0.4	**3.3**
PF0939	putative 3-isopropylmalate dehydratase	1.9 ± 0.5	**3.8**	1.4 ± 0.3	**2.7**
PF0940	probable isocitrate dehydrogenase	2.4 ± 0.5	**5.3**	1.5 ± 0.4	**2.8**
PF0941	alpha-isopropylmalate synthase	2.5 ± 0.5	**5.5**	1.7 ± 0.2	**3.3**
PF0942	dihydroxy-acid dehydratase	2.4 ± 0.5	**5.2**	1.5 ± 0.2	**2.8**
Putative phosphate transporter					
[†]**PF1020**	probable phosphate transport protein	1.6 ± 0.4	**3.1**	0.0 ± 0.5	1.0
[†]**PF1021**	conserved hypothetical protein	1.4 ± 0.3	**2.7**	0.1 ± 0.3	1.1

[†]PF1085	acetyl-CoA synthetase Q3 alpha	1.8 ± 0.2	**3.5**	0.6 ± 0.1	1.5
[†]PF1085.1	hypothetical	1.8 ± 0.4	**3.4**	0.1 ± 0.3	1.1
PF1404	proteasome, subunit beta	1.0 ± 0.4	2.0	0.8 ± 0.3	1.7
PF1405	polyadenylation specifity factor	1.4 ± 0.5	**2.7**	1.0 ± 0.3	**2.1**
PF1406	probable threonine synthase	1.0 ± 0.5	2.0	0.9 ± 0.3	1.9
PF1616	myo-inositol-1-phosphate synthetase	1.2 ± 0.2	**2.3**	0.8 ± 0.3	1.7
Histidine biosynthesis					
PF1657	histidyl-tRNA synthetase	1.1 ± 0.6	**2.2**	0.7 ± 0.3	1.6
PF1658	ATP phosphoribosyltransferase	0.9 ± 0.6	1.8	0.5 ± 0.3	1.4
PF1659	histidinol dehydrogenase	1.0 ± 0.5	2.0	0.7 ± 0.2	1.6
PF1660	imidazoleglycerolphosphatedehydratase	0.8 ± 0.6	1.8	0.7 ± 0.3	1.6
PF1661	glutamine amidotransferase	0.8 ± 0.5	1.7	0.5 ± 0.3	1.4
PF1662	HisA	1.1 ± 0.4	**2.1**	0.5 ± 0.3	1.4
PF1663	imidazoleglycerol-phosphate synthase	0.7 ± 0.4	1.7	0.4 ± 0.2	1.4
PF1664	phosphoribosyl-AMP cyclohydrolase	1.3 ± 0.4	**2.5**	0.6 ± 0.2	1.5
PF1665	histidinol-phosphate aminotransferase	1.0 ± 0.4	**2.0**	0.4 ± 0.2	1.3
PF1666	conserved hypothetical protein	1.0 ± 0.3	**2.0**	0.4 ± 0.2	1.3
PF1898	conserved hypothetical protein	1.2 ± 0.3	**2.3**	0.8 ± 0.3	1.7
[†]PF1951	Archaeal asparagine synthetase A	1.1 ± 0.2	**2.1**	-0.1 ± 0.2	1.1

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[a] The ORF description is derived from the annotation in NCBI and TIGR databases. Potential operons are indicated by bold entries within a group where the intergenic distances are less than 30 nt.

[b] Description derived from NCBI database.

[c] The intensity ratio (-Fe/+Fe) is expressed as a $\log_2$ value so that the standard deviation can be given. For comparison between ORFs, the apparent change in the expression level is also indicated. ORFs are listed that are more than twofold regulated or that are potentially part of an operon with twofold-regulated ORFs but which themselves are regulated by at least twofold.

[d] Calculated from the average $\log_2$ intensity ratio.

[†] Genes significantly differentially regulated in COM1c2 and ΔDTXR.

Table S2. ORFs whose expression is > 2-fold down-regulated under iron-limited conditions and their potential operon arrangement.

ORF[a]	Description[b]	COM1c2 $\log_2 \pm$ SD[c]	COM1c2 Fold Change[d]	ΔDTXR $\log_2 \pm$ SD[c]	ΔDTXR Fold Change[d]
Putative peptide transporter					
PF0191	peptide transporter	-0.8 ± 0.5	1.8	-0.6 ± 0.4	1.5
PF0192	oligopeptide transport	-1.3 ± 0.5	**2.5**	-1.1 ± 0.2	**2.2**
PF0193	putative ABC transport	-1.1 ± 0.6	**2.1**	-1.0 ± 0.2	**2.0**
PF0194	dipeptide ABC transporter	-1.2 ± 0.6	**2.2**	-1.1 ± 0.4	**2.1**
PF0195	conserved hypothetical protein	-0.7 ± 0.4	1.7	-0.8 ± 0.3	1.7
PF0196	glucose-6-phosphate isomerase	-0.4 ± 0.1	1.3	-0.3 ± 0.3	1.2
PF0341	conserved hypothetical protein	-1.0 ± 0.5	**2.0**	-0.7 ± 0.9	1.6
†PF0346	aldehyde ferredoxin oxidoreductase	-1.6 ± 0.4	**3.0**	-0.9 ± 0.3	1.8
Putative cobalt transporter					
PF0528	cobalt transport protein	-0.8 ± 0.3	1.7	0.2 ± 0.2	1.1
PF0529	conserved hypothetical protein	-0.5 ± 0.3	1.4	0.2 ± 0.2	1.1
PF0530	conserved hypothetical protein	-0.8 ± 0.3	1.7	0.0 ± 0.3	1.0
†**PF0531**	cobalamin biosynthesis protein	-1.0 ± 0.5	**2.0**	0.0 ± 0.2	1.0
PF0678	conserved hypothetical	-1.0 ± 0.4	**2.0**	-0.5 ± 0.2	1.4
PF1032	cys rich ORF	-1.2 ± 0.6	**2.3**	-0.7 ± 0.3	1.6
PF1033	peroxiredoxin	-2.1 ± 0.4	**4.3**	-2.0 ± 0.4	**4.0**
PF1242	molybdopterin oxidoreductase	-1.2 ± 0.1	**2.2**	-1.0 ± 0.3	**2.0**
PF1243	conserved hypothetical	-1.0 ± 0.3	2.0	-0.7 ± 0.4	1.7
PF1244	Hypothetical	-1.0 ± 0.2	1.9	-0.8 ± 0.4	1.8
PF1890	conserved hypothetical protein	-1.1 ± 0.1	**2.1**	-0.4 ± 0.3	1.4
Putative sugar transporter					
PF1936	putative sugar transport protein	-1.2 ± 0.5	**2.3**	-1.2 ± 0.5	**2.2**
PF1937	putative sugar transport protein	-1.3 ± 0.4	**2.4**	-1.1 ± 0.5	**2.2**

PF1938	maltodextrin binding protein	-1.2 ± 0.6	**2.3**	-1.1 ± 0.4	**2.1**

[a] The ORF description is derived either from the annotation in NCBI or TIGR databases. Potential operons are indicated by bold entries within a group where the intergenic distances are less than 30 nt.

[b] Description derived from NCBI database.

[c] The intensity ratio (-Fe/+Fe) is expressed as a $\log_2$ value so that the standard deviation can be given. For comparison between ORFs, the apparent change in the expression level is also indicated. ORFs are listed that are more than twofold regulated or that are potentially part of an operon with twofold-regulated ORFs but which themselves are not regulated by at least twofold.

[d] Calculated from the average $\log_2$ intensity ratio.

[†] Genes significantly differentially regulated in COM1c2 and ΔDTXR.

Table S3. *Pyrococcus furiosus* ORFs potentially involved in iron transport.

Locus[a]	**Name**	**Annotation**[b]	**TK homolog**[c]
Ferrous Transporters			
PF0723*	*ftr1*	Iron permease FTR1 family	-
PF0857*	*feoB*	ferrous iron transport protein b	TK0714/TK0957
PF0858	*feoA*	hypothetical protein	TK0715/TK0958
ABC-type Transporters			
PF0502		iron(III) dicitrate transport system permease	TK2208
PF0503		putative iron ABC transporter	TK2209
PF0909		ferric enterobactin transport ATP-binding protein	TK0708/TK2020
PF0910		iron (III) ABC transporter, permease protein	TK0707/TK2019
PF0911		iron (III) ABC transporter	TK0706/TK2018
PF1774		iron III ABC transporter, ATP-binding protein	TK0865/TK0706/ TK2018

[a] Adjacent genes in bold are predicted to be in the same operon.
[b] Annotation based on TIGR and NCBI databases.
[c] Homologs in *Thermococcus kodakarensis* found using BLAST.
*Genes significantly up-regulated in COM1c2 iron-limitation microarray.

Figure S1. Effect of iron on the growth of *P. furiosus*. Cells were grown under standard conditions on maltose except that the concentration of iron added to the medium was varied as indicated. Cultures were harvested at the end of the exponential growth phase and growth was determined by the amount of cellular protein. Typical growth curves are shown in Figure S2.

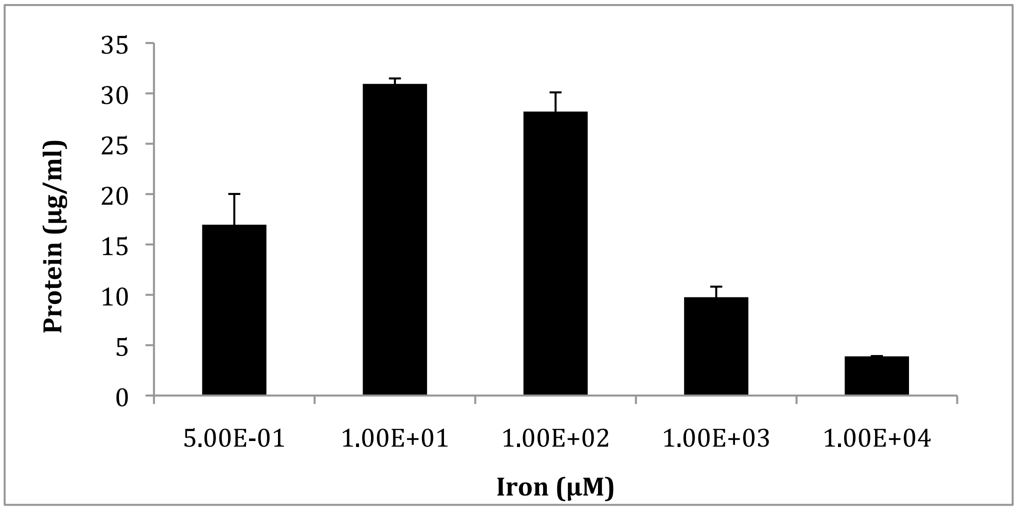

Figure S2. Characterization of the ΔFUR strain. (a) Growth of ΔFUR and COM1 in iron-sufficient and iron-limited conditions. Growth was monitored by assaying total cell protein at each time point. Results are shown for COM1 (circle), ΔFUR (triangle) in iron-sufficient (closed) and iron-limited (open) conditions. (b) The effect of iron on the transcription of genes annotated as iron transporters in ΔFUR was measured using quantitative PCR. Total RNA was prepared from ΔFUR and COM1 grown in iron-sufficient and iron-limited conditions. The constitutively expressed gene encoding the pyruvate ferredoxin oxidoreductase (POR) gamma subunit (PF0971) was used as internal control. Shown is the ratio of change in gene expression in response to iron limitation in COM1 (closed bar) and ΔFUR (open bar). The asterisk indicates qPCR confirmation of the deleted *fur* gene product in ΔFUR.

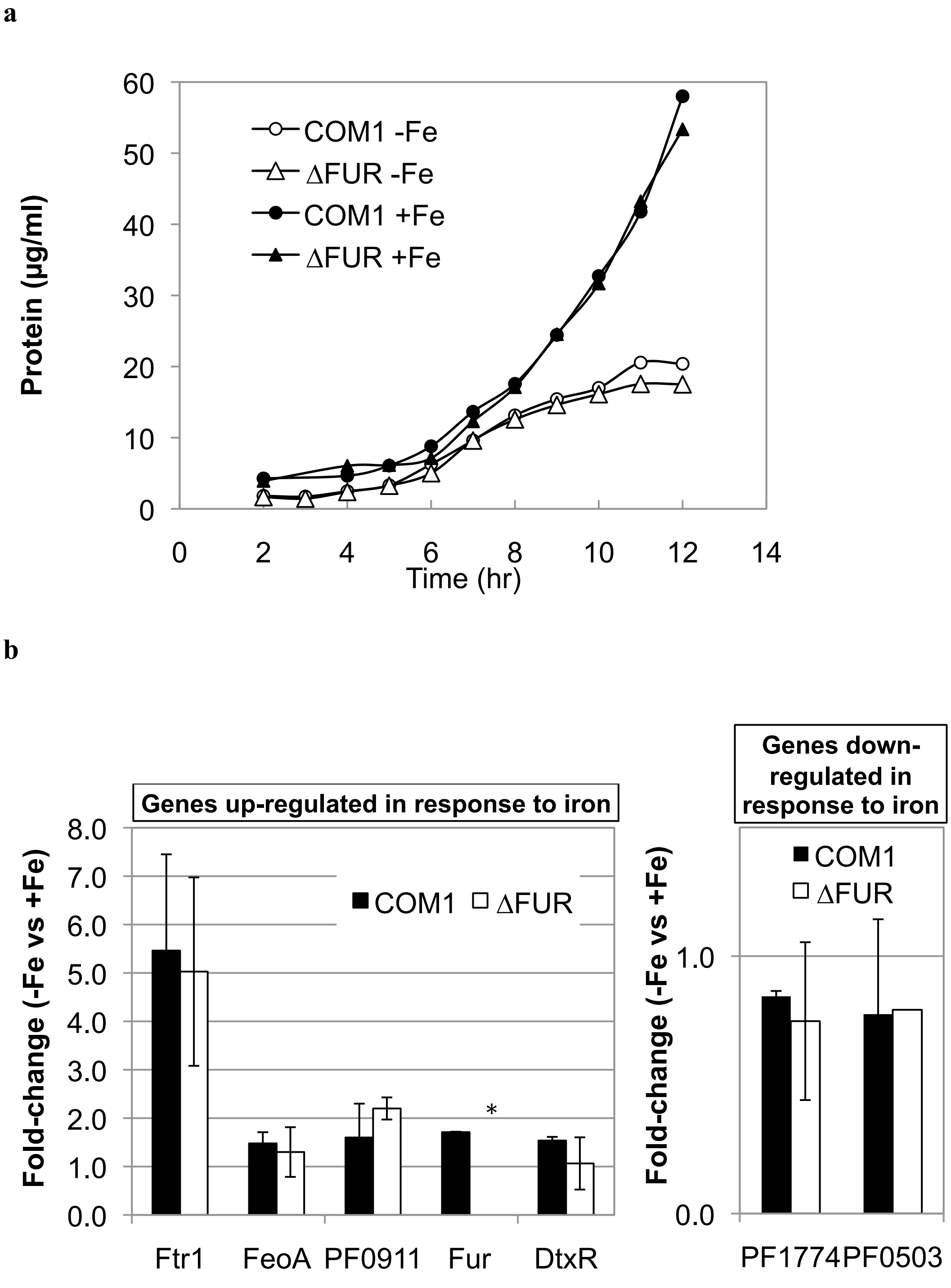
a
Protein (µg/ml)
0
10
20
30
40
50
60
COM1 -Fe
ΔFUR -Fe
COM1 +Fe
ΔFUR +Fe
0
2
4
6
8
10
12
14
Time (hr)
b
Genes up-regulated in response to iron
Fold-change (-Fe vs +Fe)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
COM1
ΔFUR
*
Ftr1
FeoA
PF0911
Fur
DtxR
Genes down-regulated in response to iron
Fold-change (-Fe vs +Fe)
0.0
1.0
COM1
ΔFUR
PF1774
PF0503

Figure S3. DtxR protein sequence comparisons. Protein sequence alignment of DtxRs (first ~60-bp in the N-terminus) in Thermococcaceae family species. Ton, *T. onnurineus*; Tk, *T. kadakarensis*; Tgam, *T. gammatolerans*; Termp, *T. barophilus*; Tsib, *T. sibiricus*; Ph, *P. horikoshii*; Pab, *P. abysii*; Pf, *P. furiosus*. Pf0851 indicates the *P. furiosus* DtxR sequence predicted by NCBI and TIGR; Pf0851+ indicates the corrected protein sequence with the extra 12 amino acids at the N-terminus. The first methionine (M) in Pf0851+ is encoded by TTG instead of ATG. Same amino acids in protein sequences are marked by asterisks, similar amino acids are marked by colons (strong similarity) and dots (family similarity). (b) Protein sequence alignment of the *B. subtilis* MntR and the *P. furiosus* DtxR with the corrected sequence. Metal-binding residues characterized in *B. subtilis* MntR (10) and the homologous residues in *P. furiosus* are displayed in bold. The 12-amino acid sequence in the N-terminus of *P. furiosus* DtxR is underlined. Sequence alignment generated using ClustalW2.

a

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Ton1956     MEISKREEEYLETIYILHKNKGIIRVKDIAKMMRVKPPSVVDALKKLNEKGLVEYEKYDR 60
Tk0107      MEVTKREEEYLETMYILHKNKGVIRVKDIAKALNVRPPSVVDALKKLAEKGLIEYEKYDR 60
Tgam0472    MQVSKREEEYLETMYLLYKSKGIIRVKDIAKRMNVKPPSVIDALKKLSSKGLVEYEKYDR 60
Termp01609  MEISKREEEYLEAMYLLYKRKGIIRIKDIAKKLRVRPPSVVDALKKLSEKGLVEYEKYDR 60
Tsib0121    MEISKREEEYLEVIYLLQKNKGVIRVKDISKRLGIRPPSVVDALKKLSEKGFVEYEKYDR 60
Ph1163      -MVSKREEEYLEVMYLLQKNKGVIRVKDIAKILKVKPPSVVDALKKLSKKGLVEYEKHDR 59
Pab0714     -MVSKREEEYLEVMYLLQKNKGVIRVKDIARVLKVKPPSVVDALKKLSKKGLIEYEKYDR 59
Pf0851      -------------MYILQKNKGVIRVKDIAKMMRVKPPTVVEALKKLRDKGFVKYEEHEH 47
Pf0851+     -MPSKREEEYLETMYILQKNKGVIRVKDIAKMMRVKPPTVVEALKKLRDKGFVKYEEHEH 59
               :******** :*:* * **:**:***:: : ::**:*::***** .**:::**::::
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b

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BsMntR   MTTPSMEDYIEQIYMLIEEKGYARVSDIAEALAVHPSSVTKMVQKLDKDEYLIYEKYRGLVLTSKGKKIGK
PfDtxR   MPSKREEEYLETMYILQKNKGVIRVKDIAKMMRVKPPTVVEALKKLRDKGFVKYEEHEHILLTEKGLEVAK
         *.:   *:*:* :*:* ::**  **.***: : *:*.:*.: ::** .. :: **::. ::**.** ::.*

BsMntR   RLVYRHELL-EQFLRIIGVDEEKIYNDVEGIEHHLSWNSIDRIGDLVQYFEEDD--ARKKDLKSIQKKTEH
PfDtxR   KTYSKHQLLTEFFINILGIPPEIAERDACQFEHYVSEVTVHRIREFISYIQQECPYALKQFLKKVREKDQA
         :   :*:** * *:.*:*:  *   .*.  :**::*  ::.** :::.*::::   * *: **.:::* :

BsMntR   HNQ
PfDtxR   VAK
           :
```

Figure S4. Electrophoretic Mobility Shift Assay results of recombinant t-DtxR and Fur with the putative promoter of *feoAB*. 100 nM promoter DNA (P_{ORF}, putative promoter region, which is the -200 to +50 bp sequence relative to the translation start site of the corresponding ORF) was incubated with truncated DtxR (t-DtxR) and Fur. Protein/Nucleic acid (P/N) ratios are listed above each lane.

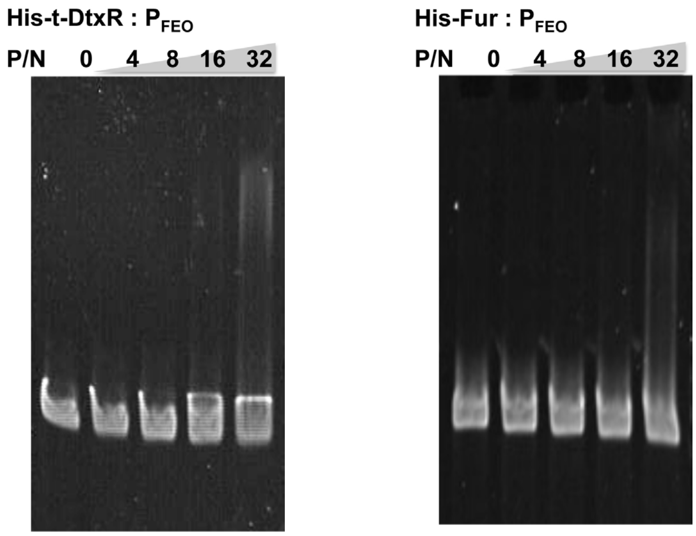


Figure S5. Electrophoretic Mobility Shift Assay results of recombinant DtxR and Fur with the putative promoters of *ftr1*, *feoAB* and *pf0849*. 100 nM promoter DNA (P_{ORF}, putative promoter region, which is the -200 to +50 bp sequence relative to the translation start site of the corresponding ORF) was incubated with full-length DtxR (lane 1-5) and with heparin added from 0.2 to 2000 ng/μL as a protein-binding competitor (lane 6-10). PF0849 promoter was used as a negative control. Protein/Nucleic acid (P/N) ratios are listed above each lane.

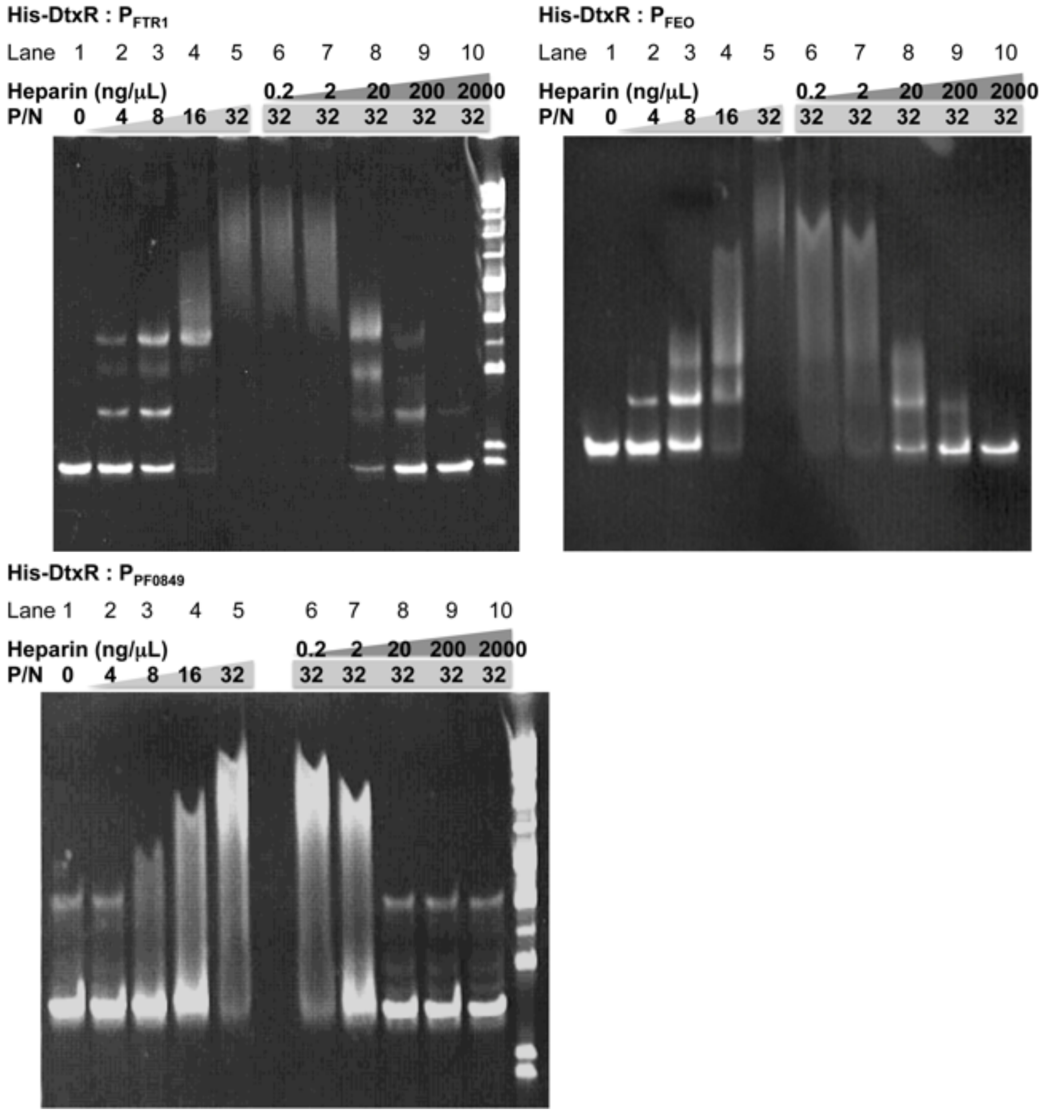


Figure S6. Transcription of annotated iron transporters in ΔFTR1 in response to iron. The effect of iron on the transcription of genes annotated as iron transporters in ΔFTR1 was measured using quantitative PCR. Total RNA was prepared from ΔFTR1 and COM1 grown in iron-sufficient and iron-limited conditions. The constitutively expressed gene encoding the pyruvate ferredoxin oxidoreductase (POR) gamma subunit (PF0971) was used as an internal control. Shown is the ratio of change in gene expression in response to iron limitation in COM1 (closed bar) and ΔFTR1 (open bar). The asterisk indicates qPCR confirmation of the deleted *ftr1* gene product in ΔFTR1.

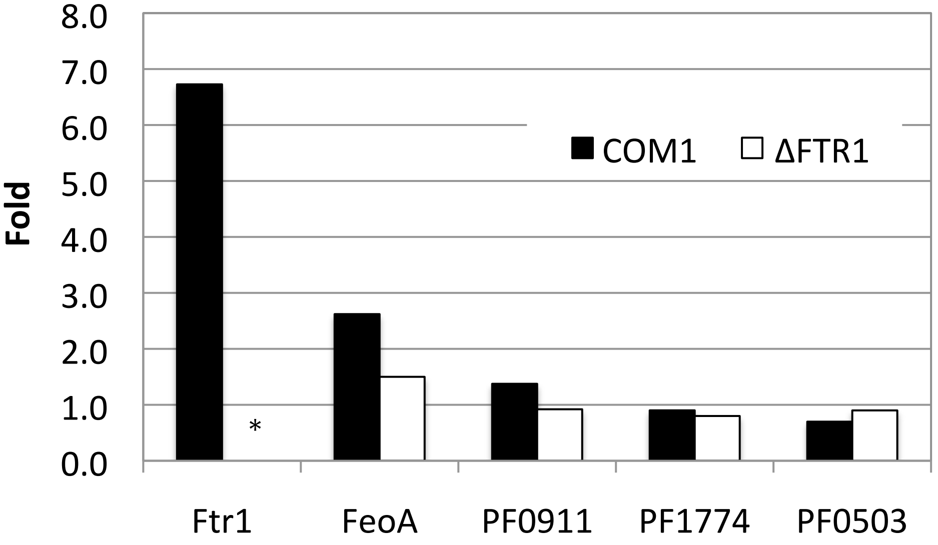
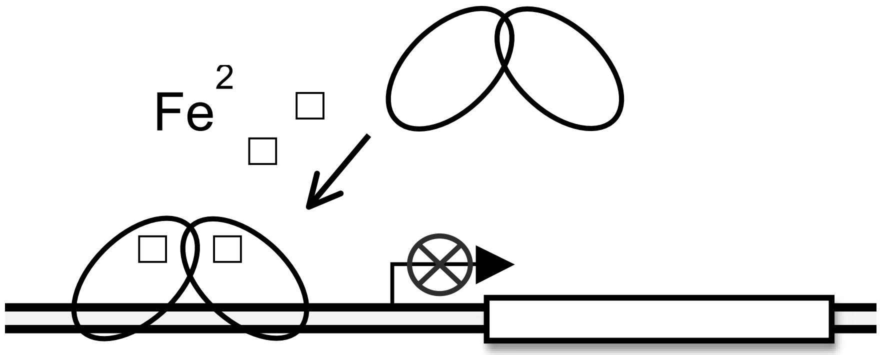
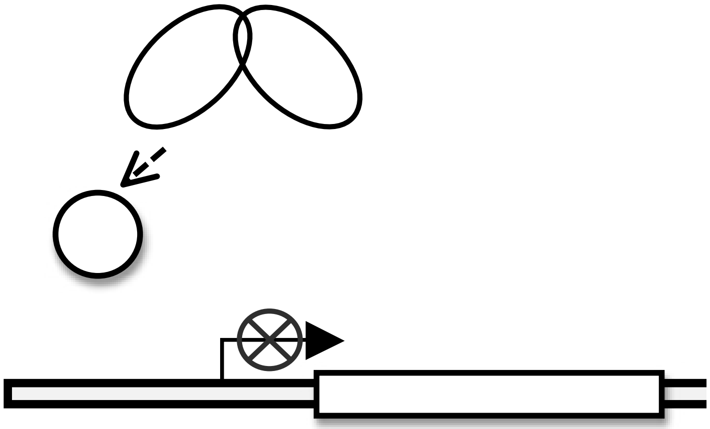


Figure S7. Schematic depiction of the role of DtxR in regulation of gene transcription in *P. furiosus*. DtxR represses the transcription of *ftr1* and *feoAB* in the presence of iron; it also represses the transcription of AOR and the putative cobalt transporter PF0528-PF0531 indirectly (dashed arrow) in the absence of iron.



Fe-bound Repression for Ftr1 and



Indirect Fe-free Repression for AOR and PF0528-PF0531