

1 **Genome-wide identification and characterization of Fur**
2 **binding sites in the cyanobacteria *Synechocystis* sp. PCC**
3 **6803 and PCC 6714**

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5 Matthias Riediger¹, Miguel A. Hernández-Prieto², Kuo Song¹, Wolfgang R. Hess^{1,*},
6 Matthias E. Futschik^{3,4,*}

7
8 ¹University of Freiburg, Faculty of Biology, Institute of Biology III, D-79104, Germany;

9 ²ARC Centre of Excellence for Translational Photosynthesis & School of Life and
10 Environmental Sciences, The University of Sydney, NSW 2006, Australia;

11 ³Sysbiolab, Centre of Marine Sciences (CCMAR), University of Algarve, 8005-139,
12 Faro, Portugal

13 ⁴MRC London Institute of Medical Sciences (LMS), Faculty of Medicine, Imperial
14 College London, London, W12 0NN, UK

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16 **Running title:** The Fur regulon in *Synechocystis* spp.

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18 *Corresponding authors:

19 Wolfgang R. Hess, University of Freiburg, Faculty of Biology, Institute of Biology III, D-
20 79104 Freiburg, Schänzlestr. 1, Germany; Tel. +49-761-2032796; FAX: +49-761-
21 2032745; Email: wolfgang.hess@biologie.uni-freiburg.de

22 and

23 Matthias E Futschik, MRC London Institute of Medical Sciences (LMS), Faculty of
24 Medicine, Imperial College London, London, W12 0NN, UK; Tel. +44-2083838024;
25 Email: m.futschik@imperial.ac.uk

27 **Abstract**

28 The Ferric uptake regulator (Fur) is crucial to both pathogenic and nonpathogenic
29 bacteria for the maintenance of iron homeostasis as well as the defense against
30 reactive oxygen species. Based on datasets from the genome-wide mapping of
31 transcriptional start sites and transcriptome data, we identified a high confidence
32 regulon controlled by Fur for the model cyanobacterium *Synechocystis* sp. PCC 6803
33 and its close relative, strain 6714, based on the conserved strong iron starvation
34 response and Fur binding site occurrence. This regulon comprises 33 protein-coding
35 genes and the sRNA *IsaR1* that are under the control of 16 or 14 individual promoters
36 in strains 6803 and 6714, respectively. The associated gene functions are mostly
37 restricted to transporters and enzymes involved in the uptake and storage of iron ions,
38 with few exceptions or unknown functional relevance. Within the *isiABC* operon, we
39 identified a previously neglected gene encoding a small cysteine-rich protein, which
40 we suggest calling, *IsiE*. The regulation of iron uptake, storage and utilization
41 ultimately results from the interplay between the Fur regulon, several other
42 transcription factors, the FtsH3 protease and the sRNA *IsaR1*.

43

44 **Keywords:** cyanobacteria, expression profiling, iron homeostasis, non-coding RNA,
45 transcriptional regulation

47 1. Introduction

48 Ferrous iron (Fe^{2+}) is an important and often essential enzymatic co-factor for many
49 enzymes. Therefore, most organisms have developed dedicated molecular acquisition
50 mechanisms for ferrous as well as ferric (Fe^{3+}) iron, which can be enzymatically
51 reduced to Fe^{2+} . At the same time, the uptake of iron needs to be tightly controlled
52 because of the tendency of free intracellular iron to participate in harmful Fenton
53 chemistry-dependent reactions, which can result in oxidative damage of cellular
54 components including DNA.¹ Due to this duality of iron as an essential prosthetic group
55 and as potential source of cellular damage, the amount of intracellular iron is typically
56 controlled by an intricate regulatory network.² The tight control of iron is of special
57 importance for photosynthetic organisms, which require large quantities of iron for the
58 photosynthetic apparatus.

59 Cyanobacteria are the only prokaryotes performing oxygenic photosynthesis. The
60 photosynthetic apparatus is particularly rich in iron-containing cofactors. Accordingly,
61 cyanobacteria have multiple mechanisms for iron uptake and sophisticated systems
62 for the regulation of iron metabolism. *Synechocystis* sp. PCC 6803 (from here:
63 *Synechocystis* 6803) was the first phototrophic and the third organism overall for which
64 a complete genome sequence was determined,³ takes up DNA spontaneously and
65 can be easily manipulated by homologous recombination and CRISPR-based tools.⁴⁻⁶
66 The genome of *Synechocystis* 6803 has been extensively curated by the research
67 community⁷ and dedicated databases have been developed providing information on
68 the regulation of gene expression at RNA level,⁸ the composition of protein-protein
69 and RNA-protein complexes,^{9,10} and the intracellular localization of proteins.¹¹ Thus,
70 *Synechocystis* 6803 has become one of the most popular cyanobacterial models.
71 Bacteria sense cytosolic iron (Fe^{2+}) in a concentration-dependent manner through its
72 interaction with the transcription factor Fur (Ferric uptake regulator). In its monomeric
73 form, Fur binds free iron, which promotes a dimerization of Fur and a higher binding
74 affinity for specific sites (so called Fur boxes) in the promotor region of genes blocking
75 their transcription.¹² The genome of *Synechocystis* 6803 encodes three proteins that
76 resemble the *E. coli* Fur protein. Of these proteins, only Fur encoded by *sll0567* seems
77 to be directly involved in iron sensing, while the paralogs PerR (*sll1738*) and Zur
78 (*sll1937*) have been related to oxidative stress¹³ and zinc sensing¹⁴, respectively.

79 Genome-level analyses showed that the expression of a large number of genes is
80 directly or indirectly affected by iron limitation^{15–19} but it has remained unknown which
81 of these genes are directly controlled by Fur.

82 Based on multiple transcriptomic data sets for iron-depleted cultures of *Synechocystis*
83 6803,^{15,17} we defined a set of genes which responded to changes in extracellular iron
84 concentration in a highly robust manner and identified the putative Fur-binding site
85 shared by all promoters of these genes. *Synechocystis* sp. PCC 6714 (from here:
86 *Synechocystis* 6714) is a strain closely related to *Synechocystis* 6803 indicated by a
87 16S rDNA identity of 99.4% and 2838 shared protein-coding genes,²⁰ making it ideal
88 for comparative analyses. Moreover, *Synechocystis* 6714 is also an established
89 laboratory strain used by the research community.^{21–23} Here, we used it to cross-
90 validate the relative localization of the identified motifs and the regulation of the
91 predicted Fur-controlled genes. The transcriptomic dataset for *Synechocystis* 6714
92 was generated from samples cultivated under the identical conditions as used for
93 strain 6803.²⁴ Based on the two datasets of experimentally mapped transcription start
94 sites (TSSs) and the validated Fur box, we identified the high confidence Fur regulon,
95 consisting of at least 33 protein-coding genes and one sRNA gene, whose Fur
96 regulation is conserved across the two strains, as well as ten or seven genes that are
97 strain-specifically regulated in strain 6803 or 6714, respectively.

98

99 2. Materials and methods

100 2.1. Motif finding

101 Three different components from the MEME Suite (v.5.1.1; available at [https://meme-](https://meme-suite.org/meme/)
102 [suite.org/meme/](https://meme-suite.org/meme/)) were used for the Fur box definition and identification of potential Fur
103 binding sites.²⁵ First, we used MEME to discover the Fur binding motifs based on
104 overrepresentation in the selected promotor regions for each strain individually,
105 applying default settings but allowing repetitions of motifs. As input sequences, we
106 selected the promoter regions of 11 high confidence Fur-regulated genes with
107 differential expression in *Synechocystis* 6803 and 6714, using a window of 200 nt up-
108 and downstream of the respective TSS. After obtaining two very similar palindromic
109 motifs for each strain, the analysis was repeated with the combined set of promoter

110 sequences of the two strains while restricting the search for palindromic sequences.
111 Next, we used TOMTOM,²⁶ to compare our motifs to experimentally verified Fur boxes
112 from cyanobacteria^{27–29} and motifs stored in the prokaryotic database CollecTF.³⁰
113 Finally, we used FIMO³¹ to identify binding sites on a genome-wide scale based on
114 the Fur motifs, matching the default p-value settings of $\leq 1.0E-04$. For a more
115 exhaustive comparison between the two strains, we also kept all predicted Fur boxes
116 with less stringent p-value settings of $\leq 1.0E-03$ to include specific cases, when a Fur
117 box fell under the default threshold in just one of the strains.

118 2.2. Comparative TSS pair determination in *Synechocystis* 6803 versus 6714

119 The assigned TSSs of all transcriptional units (TUs), their transcription profiles and
120 information about conserved non-coding TUs (nTUs) and antisense TUs (aTUs) as
121 well as information about orthologs between the two strains were taken from previous
122 analyses.^{17,20,24} However, we re-matched the TSSs to their associated TUs and also
123 allowed multiple TSSs to be assigned to the same TU. In particular, we included all
124 TSS ≤ 300 nt upstream of the assigned main TSS as well as all internal TSS (iTSS) \leq
125 20 nt downstream of the start codon as potential TSSs for each TU. If a gene did not
126 have an assigned TSS, all TSSs in a distance ≤ 300 nt upstream from the annotated
127 gene start were considered. In the case of multiple TSSs assigned to a gene, we
128 determined the correlation coefficients of the transcription profiles for each possible
129 TSS combination between the respective orthologs yielding a list of TSSs conserved
130 between the two strains. Subsequently, the correlation coefficients of the transcription
131 profiles of the orthologous TSSs to those of the respective coding regions was
132 determined. The TSS pairs which had the largest correlation coefficients between the
133 strains and those best matching the transcription profiles of the downstream coding
134 regions were selected for the comparison of conserved Fur box occurrence and iron
135 response between the strains.

136 2.3. Strain construction, cultivation, RNA and protein isolation and detection

137 *Synechocystis* 6803 strains overexpressing IsiE with or without a C-terminal triple
138 FLAG epitope tag under control of the *petE* promoter were constructed and cultivated,
139 as previously described for other small genes.³² The kanamycin resistance cassette
140 from plasmid pET28a was used to delete the *isiE* gene by homologous recombination.

141 To express IsiE-3xFLAG as a recombinant protein in *E. coli*, the vector backbone was
142 amplified from pET28a using primer pair OEflag-vec-F/R, while the coding sequence
143 of *isiE* was amplified from the genomic DNA of *Synechocystis* 6803 using primer pair
144 OEflag-IsiE-F/R. The two fragments were fused together using AQUA cloning, and the
145 resulting plasmid pOEIsiE-FLAG with ampicillin resistance was transferred into *E. coli*
146 BL21 (DE3) cells for the expression of IsiE-3xFLAG. The primers used to PCR-amplify
147 DNA fragments for the different cloning and mutagenesis steps are listed in
148 **Supplementary Table S1**. Samples for RNA and protein analysis were collected and
149 separated on denaturing formaldehyde-agarose gels or 15% glycine-SDS gels
150 respectively following published protocols.³² Nylon-membrane transferred RNA
151 samples were hybridized with ³²P-labelled single-stranded RNA probes that were
152 generated from amplified DNA fragments or else a directly labelled oligonucleotide, in
153 case of 5S rRNA (**Supplementary Table S1**). FLAG-tagged proteins were detected
154 by Western blot as described.³²

155 2.4. Purification of recombinant IsiE-3xFLAG and absorption spectra

156 *E. coli* BL21 (DE3) carrying the plasmid pOEIsiE-FLAG was cultured in LB medium at
157 37°C, 180 rpm to an OD600 of 0.8, and then induced with 0.5 mM IPTG for 4 hours.
158 Cells were collected by centrifugation at 5,000 rpm for 5 min, and washed with lysis
159 buffer (137 mM NaCl, 12 mM phosphate, 2.7 mM KCl, 1X cComplete™ protease
160 Inhibitor, pH 7.4,). Washed cells were spun again and suspended in 1/10 of the initial
161 culture volume in lysis buffer. Cells were physically disrupted by high pressure using
162 a Constant Systems Cell Disruptor. Cell lysate was centrifuged at 4°C, 13,000 rpm for
163 30 min to remove unbroken cell and membrane fractions. The supernatant was filtered
164 through a 0.22 µm pore size filter and used to purify IsiE-3xFLAG by specific binding
165 to ANTI-FLAG® M2 magnetic beads according to the manufacturer's instructions.
166 Absorption spectra were measured at room temperature using Specord® 250 Plus
167 (Analytik Jena) spectrophotometer. The purified IsiE-3xFLAG protein was dissolved in
168 PBS buffer (137 mM NaCl, 12 mM phosphate, 2.7 mM KCl, pH 7.4), while a solution
169 of 3X FLAG peptide using the same buffer was used as blank control.

170

171 3. Results

172 3.1. Selecting a high confidence set of Fur-regulated genes

173 The transcriptional acclimation of *Synechocystis* 6803 to iron starvation has been
174 analyzed in several studies using different technological platforms.^{15,16,18,19,29} The set
175 of genes detected as differentially expressed varied between the studies, likely due to
176 variations in the experimental protocols. While the small overlaps between these
177 studies were challenging for the consolidation of the results, they also provide the
178 opportunity for more robust inference of biological knowledge. Indeed, expression
179 patterns can be more biologically meaningful if they were observed across different
180 experiments and platforms.^{33,34} In this way, we aimed at exploiting the small overlap
181 between previous transcriptomics studies to more accurately define a Fur-binding
182 motif. We started with the results of a genome-wide mapping of TSSs and the definition
183 of 4,091 transcriptional units (TUs) representing mono- and multicistronic operons and
184 encompassing coding as well as non-coding genes under ten different growth
185 conditions, including iron starvation.¹⁷ To identify specifically upregulated TSSs, we
186 compared the number of reads obtained under iron limitation for every single TU with
187 those obtained under the nine different growth conditions. Next, genes associated with
188 the identified TSSs were inspected for iron depletion-dependent regulation across the
189 four microarray studies included in a meta-analysis of iron starvation¹⁵ and in
190 CyanoExpress³³. This crossover led to the identification of 13 TUs that displayed a
191 consistent behaviour upon iron depletion. We added the TU for the sRNA IsaR1, which
192 is an independently validated Fur-controlled gene.²⁹ The 14 TUs were filtered for their
193 presence, composition and the conservation of the iron depletion response in
194 *Synechocystis* 6714. This led to 11 TUs containing genes which were consistently
195 upregulated under iron limitation, and which clustered together in a meta-analysis of
196 115 environmental perturbations performed using CyanoEXpress (**Fig. S1A, B**).
197 These genes were also among those showing the highest induction under iron
198 depletion in the RNA-seq data compared to the other nine conditions. (**Fig. S1C**).

199 3.2. Computational identification of a Fur-binding motif

200 Using MEME, we detected in the promoter sequences of the 11 selected TUs
201 (**Supplementary Datasets 1 and 2**), a 21 nt long motif in the two strains (**Fig. 1A,**

202 **Table S2 and S3**). Both motifs tend to be palindromic with a single central nucleotide,
203 are AT rich, and exhibit only minor divergences in the underlying positional weight
204 matrices. The high motif similarity correlates with the strongly conserved Fur protein
205 sequence, differing in only three amino acids between the two strains (**Fig. S2**). Two
206 substitutions are conservative replacements, while on position 28, a threonine (with
207 uncharged side chain) in strain 6803 corresponds to a positively charged lysine in
208 strain 6714, which might lead to slight differences in the structure of the DNA binding
209 site. This may explain the minor differences between the Fur motifs of the two strains
210 (**Fig. 1A**). The amino acid at this position is frequently replaced, e.g., by a histidine in
211 *Anabaena* sp. PCC 7120 (**Fig. S2**). The MEME analysis using the combined promoter
212 set yielded a *Synechocystis* Fur-binding consensus motif of 23 nt with high statistical
213 confidence (**Fig. 1A**), which was used for all further analyses.

214 Our original choice of a search window 200 nt up- and downstream of the TSS,
215 respectively, was motivated by the location of a Fur-binding site, 163–186 nt
216 downstream of the TSS of *isiA*, whose relevance for de-repression under iron
217 starvation was demonstrated experimentally.^{27,29,35} The location of Fur binding sites
218 that far downstream of the TSS is consistent with Fur functioning as a transcriptional
219 roadblock, which in case of *isiA* leads to a prematurely terminated and clearly
220 detectable transcript.^{36,37} However, almost all Fur-regulated genes have a strong
221 tendency to harbor at least one Fur-binding site in a window restricted from 100 nt
222 upstream to 50 nt downstream of the respective TSS (**Fig. 1B**). Therefore, the
223 genome-wide motif search using FIMO was restricted to a 150 nt range to increase
224 specificity. When the *Synechocystis* consensus motif was compared with the
225 transcription factor binding sites in the CollecTF database,³⁰ 25 motifs were identified
226 with a p -value ≤ 0.01 , with the top 12 motifs corresponding to Fur box elements in
227 different species of bacteria (**Table S4**).

228 3.3. Correlation of iron response and Fur box conservation between 229 *Synechocystis* 6803 and 6714

230 The genome-wide search for potential Fur binding sites (p -value $\leq 1.0 \text{ E-}04$) identified
231 114 TSSs associated with such elements for *Synechocystis* 6803 (**Table S5**) and 120
232 TSSs for *Synechocystis* 6714 (**Table S6**), representing ~2% of all verified TSS. The
233 TSSs of both strains were paired according to their distance to the respective

234 orthologous genes, Fur box occurrence, and similarities in their activities under ten
235 different environmental conditions (**Fig. S3** and **Table S7**).

236 Under iron starvation, a total of 78 TSSs in strain 6803 and 53 TSSs in strain 6714
237 were differentially expressed (adj. p-value ≤ 0.05). Among the genes controlled by
238 these TSSs, there were 27 orthologous upregulated genes. Three orthologous protein-
239 coding genes (*slr0888*, *slr0889*, *slr1634*), the *isiA* antisense RNA *isrR*, and the sRNA
240 *pmgR1* showed conserved downregulation. Twenty of the conserved upregulated
241 genes also harbor Fur boxes in both strains (**Fig. 2A**). Of the conserved
242 downregulated genes, only *slr1634* has predicted potential Fur boxes in both strains
243 and might be activated by Fur (**Fig. 2B**). For one gene, *ank*, iron-dependent regulation
244 and a Fur-binding motif was detected only for the homolog in strain 6714 (**Fig. 2C**).
245 The gene pair *isiB/C* exhibited similar regulation in both strains, but a Fur box was only
246 detected in strain 6714 (**Fig. 2C**). Conversely, for the gene pair *exbD3/D4*, a Fur box
247 was only detected in strain 6803, given that the genes are transcribed by readthrough
248 from an upstream gene in strain 6714 (**Fig. 2D**). Finally, we identified a set of eight
249 iron-responsive genes with conserved differential expression but lacking a Fur box in
250 their promoters (**Fig. 2E**). Indeed, their expression is post-transcriptionally controlled
251 by the Fur-regulated sRNA IsaR1 in the case of *sufBCDS*,²⁹ inversely through co-
252 degradation with the Fur-regulated *isiA* mRNA in the case of *IsrR*,³⁶ or by currently
253 unknown alternative mechanisms.

254 3.4. Defining a high confidence set of genes regulated by Fur and their functions

255 The presence of a Fur box and the specific differential expression under iron starvation
256 resulting from the respective gene-specific promoter or as part of an operon is
257 summarized for both strains in **Fig. 3**. There are 34 high confidence Fur target genes,
258 including one sRNA gene, which exhibited a significant differential iron starvation
259 response and harbors Fur boxes in both strains. Another ten genes are specific or
260 specifically Fur-regulated only in strain 6803 and seven such genes in strain 6714
261 (**Fig. 3A**). Functional enrichment analysis identified 23 enriched GO terms, with an
262 adjusted p-value ≤ 0.05 , functionally assigned to 32 of these 51 genes (**Fig. 3B**).

263 To gain more detailed insights, we inspected the chromosomal regions of predicted
264 Fur regulated genes further and found several remarkable examples of conserved or
265 divergent Fur regulation.

266 3.5. Bidirectional promoters are a hallmark of Fur-mediated regulation

267 Visual inspection of predicted Fur box occurrences revealed that, besides the
268 respectively assigned TSS, additional iron-regulated TSSs were frequently located in
269 close proximity on the reverse strand, indicating bi-directional transcriptional
270 regulation. This feature is also present in giant iron-responsive gene clusters, ~32 kb
271 and 22 kb in size, which harbor the largest number of Fur-regulated genes (22 and
272 18), as well as 11 and 7 Fur boxes in the two studied strains, respectively (**Fig. 4**).

273 The genes located within this iron-responsive gene cluster encode most of the
274 components of the various ferric iron uptake systems. These are the FecB-FecC/D-
275 FecE system that consists of the periplasmic binding proteins FecB; the
276 transmembrane permease proteins, FecC and FecD; and the ATPase FecE that
277 allows the uptake of Fe-siderophores in conjunction with the FhuA and IutA TonB-
278 dependent (TBDT) receptor proteins.³⁸ IutA was suggested to be renamed to SchT for
279 schizokinen transporter.³⁹ These uptake systems function by coupling to the inner
280 membrane TonB–ExbB–ExbD protein complex⁴⁰ that energizes the transport by
281 building proton motive force. Indeed, one such complex is also encoded by genes of
282 this cluster (*tonB*, *slr1484*; *exbB1*, *sll1404*; *exbD1*, *sll1405*),⁴¹ while another set of
283 *exbB/exbD* genes is located in a different location (*exbB1*, *sll0477*; *exbD3*, *sll0478*;
284 *exbD4*, *sll0479*).⁴²

285 The central role of these proteins is reflected by the transcriptional organization of their
286 genes: *tonB* and *exbB1D1* are transcribed divergently from a bidirectional promoter
287 that is safeguarded by four clearly discernible Fur boxes in both strains. The distance
288 between the two TSSs are 223 nt in strain 6803 and 203 nt in strain 6714. The fact
289 that in TU36 (in strain 6803) and TU1050 (in strain 6714), short non-coding RNAs
290 possibly originate from this region makes the arrangement even more complex.

291 Other genes organized within the Fur-regulated operons of the gene cluster are
292 orthologs of the pyochelin siderophore transcriptional activator PchR,⁴³ the
293 methyltransferase Sll1407, and a few other uncharacterized periplasmic or

294 transmembrane proteins. Although the gene set differs between strains, with gene
295 *D082_09160* only occurring in strain 6714 (TU1055) and two larger insertions in strain
296 6803, the positions of Fur boxes and the iron starvation-dependent regulation are
297 strikingly well conserved (**Fig. 4**).

298 Another instance of bidirectional regulation was discovered for the Fur-regulated
299 genes *sll1878* and *slr1977*, encoding FutC⁴⁴ and a nucleoside phosphorylase-I family
300 (cl38914, e value = 1.23e-19) protein. The two major TSSs (TSS2 for both genes) are
301 just spaced 58 nt apart from each other; hence, this is an interesting instance of a
302 bidirectional promoter in the strictest sense (**Fig. S4**). TSS2 for *slr1977* leads to an
303 mRNA with its 5' end already within the coding region, rendering the annotated (and
304 conserved) start codon unlikely to start translation. It is tempting to speculate that
305 transcripts originating at TSS1 allow the translation of *slr1977* from the annotated start
306 codon, whereas TSS2 would lead to an N-terminally truncated, alternative protein. The
307 fact that the *futC-slr1977* arrangement is conserved in both strains supports its
308 possible functionality (**Fig. S4**).

309 3.6. Extension of the *isiABCD* operon and discovery of *isiE*

310 An intriguing example of conserved Fur regulation is the *isiABC* operon encoding the
311 iron stress-induced protein A (IsiA) and flavodoxin (IsiB)^{45,46} as well as a third
312 annotated gene, *sll0249*, encoding a protein with an alpha/beta hydrolase fold.
313 Previous work indicated that *sll0249* is co-transcribed with *isiAB* under iron-limited
314 conditions and was renamed as *isiC*.⁴⁷ Our data suggest that in both strains, three Fur
315 boxes control the transcription of this operon (**Fig. 5A**). One of these is located within
316 the 5'UTR, directly upstream of the conserved *isiA* GTG start codon, as previously
317 reported for strain 6803²⁷, and here identified also in strain 6714. Hence, a tight control
318 by Fur can be expected and indeed strong transcriptional upregulation was observed
319 under iron starvation starting at the *isiA* TSS.¹⁵ The mRNA coverage extended beyond
320 the *isiABC* genes and included the genes, *ssl0461* and *dfp*, encoding a DUF2555
321 domain-containing protein and the coenzyme A biosynthesis bifunctional protein
322 CoaBC. It has been suggested independently that these five genes belong to this
323 operon,⁴⁸ while *ssl0461* has recently been renamed to *isiD*.⁴⁹

324 While the general arrangement and transcriptional organization of this operon appears
325 to be common to both strains, a second TSS in strain 6803 at a position 17 nt
326 downstream of the *isiA* stop codon seems to separate *isiA* from *isiB*. This TSS is only
327 active during darkness; it does not appear to be active during iron starvation and the
328 *isiABCD-dfp* operon can be transcribed jointly (**Fig. 5A**). Directly downstream of the
329 *isiA* reading frame, a steep reduction is visible in the transcriptome coverage during
330 iron starvation in both strains, and a second descent at the end of the relatively long
331 (404 nt in strain 6803) intergenic spacer. We also noticed with *D082_02010*, a likely
332 short gene interspersed between the *isiA* and *isiB* homologs in strain 6714, but not in
333 strain 6803. *D082_02010* is annotated to encode a 45-amino acid protein, although
334 re-examination suggests a 59-amino acid cysteine-rich protein. Such short genes may
335 be artefacts of gene modelling; therefore, we considered the *isiA-isiB* intergenic spacer
336 in strain 6803 and found a 59-codon open reading frame (ORF) as well. Northern
337 analysis showed that the coding sequence is transcribed as part of the joint transcript
338 with *isiA* and *isiB* and that deletion of the coding sequence by a kanamycin resistance
339 cassette also affected the transcription of the downstream located *isiB* (**Fig. 5B**). We
340 therefore cloned this ORF under the control of an inducible promoter and engineered
341 a short sequence encoding a 3x FLAG tag at its C terminus. Upon conjugation into
342 *Synechocystis* 6803 and induction by adding Cu^{2+} , we observed the accumulation of
343 a short protein in three biological replicates (**Fig. 5C**). We conclude that the *isiA-isiB*
344 intergenic spacer contains a previously unknown protein-coding gene that we suggest
345 be named *isiE*. The IsiE amino acid sequence is extremely cysteine-rich (8/59 amino
346 acids in 6803 and 7/59 in 6714). Homologs can be identified in more than 100
347 cyanobacteria, both in syntenic and in different locations. Multiple sequence
348 alignments show that CXCC/CXXC is the most widely conserved motif among these
349 homologs (**Fig. 5D**). Similar motifs are known from metal-binding proteins and proteins
350 interacting with DNA or RNA including the Fur protein itself containing six cysteine
351 residues, two of which are arranged into a CXXC motif (**Fig. S2**). The corresponding
352 CXXC motif in Fur from *Anabaena* sp. PCC 7120 was shown to be functionally
353 critical.⁵⁰ Spectral analysis of the purified recombinant protein supported a possible
354 iron-binding function of IsiE, while structural modelling indicated a close spatial
355 arrangement of the cysteine residues protruding from the IsiE surface (**Fig. S5**).

356 Upstream of the *isiAEBCD-dfp* operon, a homolog of the ribosome biosynthesis
357 GTPase YlqF/RbgA (Slr0267) is encoded, exhibiting a bidirectional regulation via Fur
358 in strain 6714. Due to its low expression, there was no TSS defined for strain 6803;
359 thus, a possible bidirectional regulation via Fur cannot be excluded in both strains.

360 3.7. Iron-dependent regulation by gene insertion

361 Most of the differentially expressed genes are associated with the conservation of a
362 Fur motif. In the case of *ank* encoding an ankyrin-repeat-containing protein, we found
363 a strain-specific upregulation. Closer inspection suggested a gene insertion that
364 conferred Fur regulation to the *ank* gene exclusively in strain 6714 (**Fig. 6**). The
365 inserted gene, *D082_28000*, is transcribed from a bidirectional promoter with twin Fur
366 boxes that function in both directions. *D082_28000* encodes an iron-uptake porin
367 (cl41527, e-value = 0e+00). There is no closely related ortholog in strain 6803,
368 although it has six paralogous porin genes (Sll0772, Sll1271, Slr0042, Slr1908,
369 Slr1841)³⁸ and Sll1550 (this work).

370

371 4. Discussion

372 Accurate prediction of the Fur regulon is essential for our understanding of iron
373 homeostasis in cyanobacteria. Multiple transcriptomics experiments of iron depletion
374 in *Synechocystis* 6803 by different laboratories indicated a large number of genes,
375 which might be under the control of Fur. However, many of the observed expression
376 changes were likely not a direct consequence of Fur binding but might have been
377 secondary effects obscuring the primary response. To dissect primary (caused by
378 differential Fur binding) and secondary downstream effects in the existing
379 transcriptomic datasets, a faithful definition of the Fur-binding motif is key. Here, we
380 improved the accuracy for Fur box identification, by considering two closely related
381 *Synechocystis* strains and cross-validating the identified sites with their conserved
382 transcriptional response to iron starvation. The results of our analysis captured the
383 direct effects of the iron starvation response of *Synechocystis* mediated by Fur and
384 led to the construction of a high confidence Fur regulon (**Fig. 7**). Furthermore, the
385 detection of differences in the composition of the Fur regulon between the two closely
386 related strains 6803 and 6714 provides a valuable resource for identifying factors

387 important for strain divergence. While iron deficiency in cyanobacteria directly affects
388 Fe-S cluster biogenesis or general iron homeostasis and therefore a large number of
389 genes,^{18,51} the *Synechocystis* Fur regulon seems to be extended towards the
390 regulation of iron uptake. According to their functions, the genes belonging to the Fur
391 regulon can be assigned to four groups: (i) ferric iron transport, (ii) ferrous iron
392 transport systems, (iii) further iron stress-responsive genes and (iv) regulatory factors.

393 4.1. The ferric iron transport gene cluster

394 Our analysis indicates that Fur regulates all genes involved in ferric iron transport in
395 *Synechocystis* (**Fig. 4**). Interestingly, two large insertions in strain 6803 led to
396 additional FecB, FhuA and PchR paralogs, plus two more genes lacking direct
397 orthologs in strain 6714. The genomic organization of these insertions (*pchR-fhuA3-*
398 *fecB3* and *pcrR-fhuA1-fecB2-sll1203-sll1204*), consisting of a transcriptional regulator,
399 a TBDT receptor and a periplasmic siderophore-binding protein, suggests a conserved
400 role (**Fig. 7**). The multiple TBDT receptors and FecB paralogs might exhibit variations
401 in siderophore specificities and allow strain 6803 to be more flexible in utilizing different
402 types of siderophores for iron uptake.^{39,41} The role for PchR is not well-understood in
403 cyanobacteria, as well as the reason for its collective duplication in strain 6803
404 together with siderophore-uptake and -binding proteins. However, PchR in
405 *P. aeruginosa* acts as transcriptional activator of siderophore biosynthesis and export
406 genes, requiring siderophores as co-activator and being also repressed by Fur.⁴³
407 *Synechocystis* 6803 does not produce siderophores and no siderophore biosynthesis
408 genes have been detected.⁵² Therefore, it is tempting to speculate that the different
409 PchR paralogs in strain 6803 might respond to different siderophores as co-activators
410 for the regulation of genes involved in recycling and secretion of previously imported
411 siderophores. The Sll1407 methyltransferase might also contribute to the regulation of
412 processes involved in siderophore-mediated iron uptake. The co-transcription of *tonB*
413 with the genes encoding periplasmic protein Slr1485 and the ABC-transporter Slr1488,
414 as well as co-transcription of *fecB4* with the genes encoding the ABC-transporter
415 subunits Slr1494 and Slr1493, as well as of *fecB2* with *sll1204* and *sll1203* (strain 6803
416 only) suggest the existence of additional mechanisms for siderophore uptake and/or
417 secretion that are not described so far. Overall, the ferric iron transport system in strain
418 6803 seems to be more complex than in strain 6714, except for the occurrence of

419 *D082_09160* upstream of *fecE*. The functionality of multiple small ORFs (*D082_08980*,
420 *D082_08970* and *D082_09120*) in the ferric iron transport gene cluster of strain 6714
421 has yet to be validated.

422 4.2. Ferrous iron transport systems

423 Fur also regulates the ferrous iron transport systems, which are thought to be supplied
424 with Fe^{2+} diffusing through porins from the outer membrane (**Fig. 7**). While the Feo
425 system is exclusively restricted to Fe^{2+} uptake, there are controversies on the ion
426 affinities (Fe^{2+} or Fe^{3+}) of the Fut system.⁵³ In *Synechocystis*, the ferric iron uptake
427 (Fut) system possesses two periplasmatic iron-binding proteins, FutA1 and FutA2,
428 with distinct functions. While the former is largely responsible for the actual iron
429 transport, the latter may act as metallochaperone to establish an ion gradient to assist
430 in iron influx. It was also proposed that FutA2 is involved in iron reduction, possibly
431 interacting with the siderophore-dependent ferric iron transport system.⁵⁴ Notably, only
432 FutB, the integral membrane protein of the Fut system, seems to be not regulated via
433 Fur. The two Fur-regulated porins, *D082_28000* (strain 6714 only) and
434 *SII1550/D082_06170*, including the co-transcribed periplasmatic protein *SII1549/*
435 *D082_06180*, might be supplying the ferrous iron (Feo) transport systems with Fe^{2+} .
436 The Feo system surprisingly is restricted to strain 6803.

437 4.3. Comparison to other cyanobacteria and further iron starvation-responsive genes

438 Compared to the Fur regulons in more complex cyanobacteria, such as *Anabaena*,
439 the *Synechocystis* Fur regulon is more compact. In *Anabaena* sp. PCC 7120, FurA-
440 binding sites were identified upstream of 215 genes belonging to diverse functional
441 categories including iron homeostasis, photosynthesis and respiration, heterocyst
442 differentiation, oxidative stress defense and light-dependent signal transduction.⁵⁵ In
443 contrast, the Fur regulon in *Synechocystis*, as defined here, comprises 33 protein-
444 coding genes and the sRNA *IsaR1* that are under the control of 16 and 14 individual
445 promoters in strains 6803 and 6714, respectively. *Anabaena* spp. have a larger
446 number and bigger diversity of iron transporters and proteins involved in the
447 biosynthesis of siderophores. Therefore, it makes sense that the regulon controlled by
448 Fur is larger in these species. Still, our study revealed several novel Fur targets in
449 *Synechocystis* that are not directly involved in iron uptake, such as the bacterioferritin-

450 associated ferredoxin Ssl2250 (iron storage mechanisms), the nucleoside
451 phosphorylase Slr1977, the ankyrin-repeat-containing protein Slr1109/D082_27980
452 (potentially involved in heme trafficking to catalase,⁵⁶ Fur-regulated only in strain 6714),
453 the ribosome biogenesis GTPase YlqF/RbgA (only strain 6714), proteins encoded by
454 the *isiAEBCD-dfp* operon, and the polycopene isomerase CrhH in an operon with
455 *isaR1* (**Fig. 7**). Fur is well documented as a repressor of *isiA*.^{27,29,35,57} At the end of this
456 work, a search for “*isiA+Synechocystis*” on PubMed Central returned close to 300
457 publications showing that *isiA* is one of the best-studied genes in this organism.
458 Therefore, our discovery of the *isiE* gene in the intergenic spacer between *isiA* and
459 *isiB* demonstrates that the comparative analysis of regulatory mechanisms and
460 regulons can help to improve the fine mapping of even well-studied genomic regions,
461 such as the Fur regulated *isiAEBCD-dfp* operon. While IsiE could not be assigned to
462 any known protein family we noticed that the conserved CXCC motif (**Fig. 5D**) has
463 been characterized as a metal-binding element.⁵⁸ Indeed, spectroscopic analysis
464 yielded preliminary evidence that IsiE could be an iron-binding protein (**Fig. S5**).
465 Recently, the discoordination of operon expression during iron starvation was reported
466 for *isiA* and *isiB*, if ferredoxin 2 function was disturbed, and an unknown
467 posttranscriptional regulation was postulated.⁵⁹ IsiE might be involved in this process.
468 Alternatively, IsiE might play a role in iron recycling when photosystem I is structurally
469 re-organized at low iron or redox stress conditions and becomes functionally
470 associated with IsiA proteins.^{60,61} Photosystem I is essential for the survival of
471 cyanobacteria, requiring large quantities of iron-containing co-factors, thus a small
472 protein such as IsiE might transiently store released iron from damaged photosystem
473 I and release it when new photosystem subunits are being assembled. A similar role
474 is performed by the small high-light induced proteins (HLIPs) in the assembly of
475 chlorophylls to newly synthesized photosystems II.⁶²

476

477 4.4. Connection between Fur and other transcriptional regulators and regulatory 478 factors

479 Several Fur targets, namely those exhibiting strong induction during iron starvation,
480 such as the *tonB* system (**Fig. 4**) or *isiA* (**Fig. 5**), contain more than one predicted
481 binding site usually located upstream of the regulated genes, indicating an additive

16

482 effect of the binding of multiple Fur molecules. Additionally, Fur-regulated genes can
483 be controlled also by other transcriptional regulators. Again, this is exemplified by the
484 *isiA* promoter, which in addition to Fur is under control of the TetR-family
485 transcriptional regulator PfsR (SII1392)⁶³ and RpaB, integrating its expression with the
486 cellular redox status.³⁵ Most Fur boxes are located within a distance of less than 50 nt
487 upstream of the TSS. Surprisingly, we did not find a Fur box in the proximity of the *fur*
488 gene, which was inferred to be auto-regulated in *Anabaena* sp. PCC 7119.⁶⁴ This
489 indicates that the mechanism controlling the transcription of Fur potentially differs in
490 *Synechocystis*. A candidate is PfsR because *fur* transcription increased in a *pfsR*
491 deletion mutant during iron deprivation and recombinant PfsR was shown to bind to a
492 *fur* promoter fragment.⁶³ Homologs of PfsR are lacking in *Anabaena* strains PCC 7119
493 as well as PCC 7120 ($E < 10^{-6}$). Hence, the autoregulatory function of Fur present in
494 those species likely was replaced by the epistatic control through PfsR in
495 *Synechocystis* spp.

496 The interconnection between the regulons of Fur (this study) and those controlled by
497 other iron-related transcription factors, such as SufR,⁶⁵ and of secondary regulons,
498 such as those belonging to the Fur-controlled sRNA *IsaR1*,²⁹ *SII1408* (PcrR), *Slr1489*
499 and *SII1205* (both PchR), assure the physiological regulation of iron metabolism and
500 utilization (**Fig. 7**). In addition, paralogous transcription factors related to oxidative
501 stress responses and other metal stress responses, such as *PerR* (*slr1738*) and *Zur*
502 (*sII1937*),^{13,14} need to be taken into account. Finally, the Fur regulon in *Synechocystis*
503 6803 is interconnected via an epistatic control mechanism involving FtsH proteases to
504 photosynthesis and to other major transcriptional regulators. Fur becomes released
505 from DNA when Fe^{2+} is becomes scarce. The released Fur protein is then degraded
506 by the FtsH1/FtsH3 protease complex.⁵⁷ Interestingly, the manipulation of FtsH1/3
507 abundance leads to a drastic reduction in the transcriptional responses to different
508 types of nutrient starvation, mediated not only by Fur, but also by the Pho, NdhR, and
509 NtcA TFs.⁶⁶ Hence, the transcriptional regulation of iron, phosphorus, C and N
510 starvation responses appear to be interconnected at a higher level, with the activity of
511 the photosynthetic machinery through FtsH1/3 mediation illustrating the intriguing
512 complexity of regulatory systems in cyanobacteria.

513

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525

526 Conflict of interest

527 None declared.

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731 FtsH1/3 proteolytic complex suppresses the nutrient stress response in the
732 cyanobacterium *Synechocystis* PCC 6803. *Plant Cell*, **31**, 2912–28.
733

734 Figure Legends

735

736 **Figure 1. Definition and relative locations of putative Fur boxes in**
737 ***Synechocystis* 6803 and 6714. (A)** Sequence logos of putative Fur boxes in strains
738 6803 and 6714 determined by MEME motif prediction and resulting consensus for both
739 strains (bottom). The promoter sequences given in **Supplementary Datasets 1 and**
740 **2** were used as input for the motif prediction. The tabular output of the MEME motif
741 prediction used as basis for the individual sequence logos can be found in **Table S2**
742 and **S3**. The *Synechocystis* consensus Fur box was determined by performing a
743 combined analysis of the promoters of the two strains. **(B)** Graphical output of the
744 MEME motif prediction for 11 promoter regions conserved between *Synechocystis*
745 6803 (purple, left) and 6714 (orange, right). The Fur boxes of each strain are visualized
746 by rectangular boxes of their corresponding colors. Predicted elements just below the
747 threshold are indicated by less intense coloring. A dashed line boxes the region from
748 -100 to +50 nt, in which most Fur boxes are located. The TU numbers are given as
749 previously defined^{17,24}.

750

751 **Figure 2. Scatter plot showing the conservation of expression under iron**
752 **starvation between the two strains.** The plots display the transcription levels at low
753 iron, divided by the median of nine other conditions for strain 6803 (x axis) versus
754 strain 6714 (y axis), and indicate Fur box occurrence. **(A)** Genes with Fur boxes in
755 both strains (p-value $\leq 1.0E-04$). **(B)** Genes with potential Fur boxes in both strains (p-
756 value $\leq 1.0E-03$). **(C)** Genes with Fur boxes in strain 6803 (p-value $\leq 1.0E-04$). **(D)**
757 Genes with Fur boxes in strain 6714 (p-value $\leq 1.0E-04$). **(E)** Genes with iron-
758 dependent regulation but lacking Fur boxes. Data points of genes which were
759 differentially expressed during iron starvation (p-value ≤ 0.05) are colored: red =
760 differentially expressed in both strains; purple = differentially expressed only in 6803;
761 orange = differentially expressed only in 6714. Text labels of the *Synechocystis* 6803
762 orthologs are given for all differentially expressed genes with adjusted p-values ≤ 0.05
763 in at least one of the strains (except for panel E, adjusted p-value ≤ 0.05 in both strains
764 only).

765

766 **Figure 3. High-confidence Fur target genes and their functions. (A)** Genomic
767 organization of high-confidence Fur target genes, including information of ortholog
768 conservation, relevant TUs, Fur box occurrence and differential expression under iron
769 starvation for both strains. Conserved Fur regulated orthologs are highlighted in red,
770 strain 6803 specific are in purple, and strain 6714 specific in orange. Genes with grey
771 background are likely, but not verifiably Fur targets, since these are linked to instances
772 of gene duplication events in strain 6803 and do not have an assigned TSS (compare
773 with **Fig. 4**). Instances of read-through transcription are highlighted by the vertical
774 arrows. Key examples are further outlined in the genome plots in **Fig. 4–6 and S4. (B)**
775 Functional enrichment analysis of the Fur target genes using Gene Ontology (GO)
776 terms. Genes without functionally enriched GO terms are in grey.

777

778 **Figure 4. Fur regulation of the ferric iron transport gene cluster.** The expression
779 under iron starvation is shown for *Synechocystis* **(A)** 6803 and **(B)** 6714. Fur box
780 locations are indicated with triangles. High-confidence Fur-regulated TUs are
781 highlighted: red, if regulated in both strains; purple, if regulated only in strain 6803;
782 and orange, if regulated only in strain 6714. Two insertions of several genes in strain
783 6803 compared to strain 6714 are indicated and an instance of two recombined genes
784 boxed by a dashed line. This figure illustrates that bidirectional transcriptional
785 regulation is a common feature of Fur. The conservation of Fur regulation even after
786 complex recombination events is illustrated, as seen in the separation of *fecC/D* from
787 *fecE/B* in strain 6714.

788

789 **Figure 5. Extension of the Fur-regulated *isiA* operon. (A)** The expression under
790 iron starvation is shown for *Synechocystis* 6803 and 6714. Conserved genomic
791 regions between the strains are boxed light grey. Fur box locations are indicated by
792 red triangles. High-confidence Fur-regulated TUs are highlighted together with gene
793 names and read coverage under iron starvation. The TSS separating *isiA* and *isiB* in
794 strain 6803 and the TSS following *isiC* in both strains play no roles under iron
795 starvation conditions. Therefore, genes of TU1555 and TU1554 were added to the

796 conserved Fur regulon. The newly defined *isiE* genes (this study) are connected by
797 the bidirectional arrow. **(B)** Northern hybridization for the verification of *isiA*, *isiE* and
798 *isiB* transcript accumulation in the $\Delta isiE$ and wild type (WT) strains during a time course
799 of iron starvation. A size marker is given on the right, a 5S rRNA control hybridization
800 is shown underneath. **(C)** Western blot for the detection of FLAG-tagged IsiE in three
801 independent biological replicates C1–C3 but not in the wild type (WT). **(D)** IsiE
802 homologs can be predicted in several cyanobacteria (strain acronyms and Genbank
803 accession numbers are indicated). The multiple sequence alignment shows the
804 conservation of a region containing two cysteine-rich motifs.

805

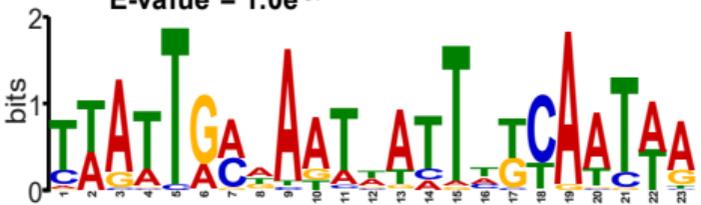
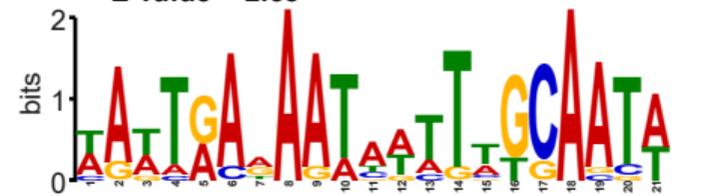
806 **Figure 6. Regulation by Fur via gene insertion in strain 6714.** The expression
807 under iron starvation is shown for *Synechocystis* **(A)** 6803 and **(B)** 6714. Fur box
808 locations are indicated by orange triangles. High-confidence Fur-regulated TUs are
809 highlighted in orange. The insertion of the two genes between *gltD* and *ank* triggers
810 Fur dependent de-repression of gene expression under iron starvation conditions
811 specifically in strain 6714.

812

813 **Figure 7. Extent of Fur-dependent regulation in *Synechocystis* strains 6803 and**
814 **6714 and functional association of the predicted target genes.** Proteins colored in
815 red: regulation is predicted for the orthologs in both strains, only the gene name from
816 *Synechocystis* 6803 is given. Proteins in purple or with orange background: Gene has
817 no ortholog in the other strain, or only the gene in the indicated strain is controlled by
818 Fur (purple, *Synechocystis* 6803; orange: *Synechocystis* 6714). The interaction
819 between the Fur regulon and the sRNA IsaR1-associated subregulon²⁹ is indicated.
820 The role of secondary regulons controlled by PcrR and PchR homologs in iron
821 homeostasis is currently unknown but might connect to later or additional stress
822 responses. OM, IM and TM; outer, inner and thylakoid membrane.

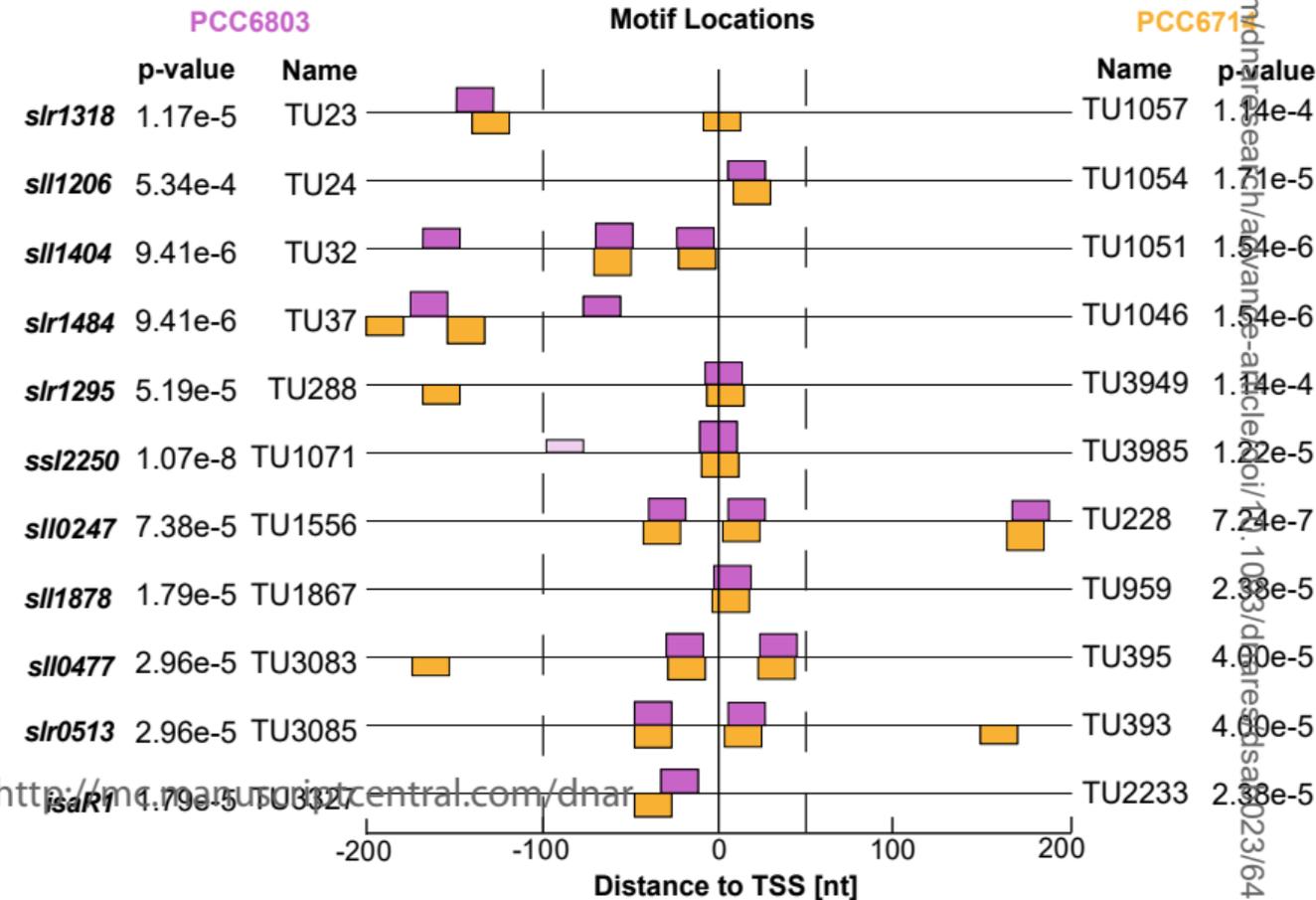
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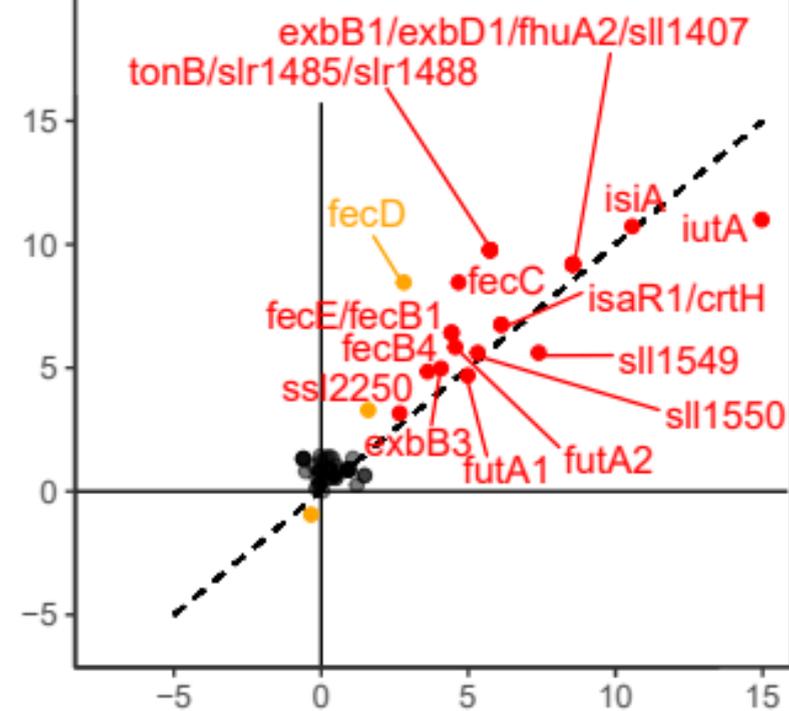
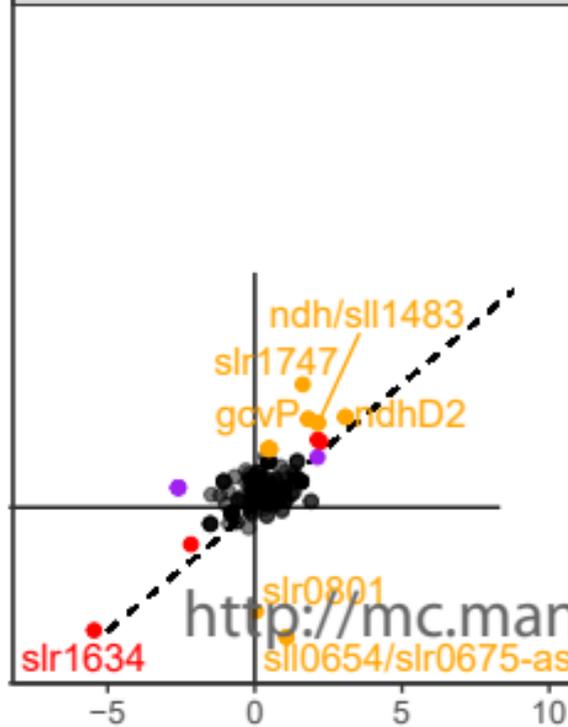
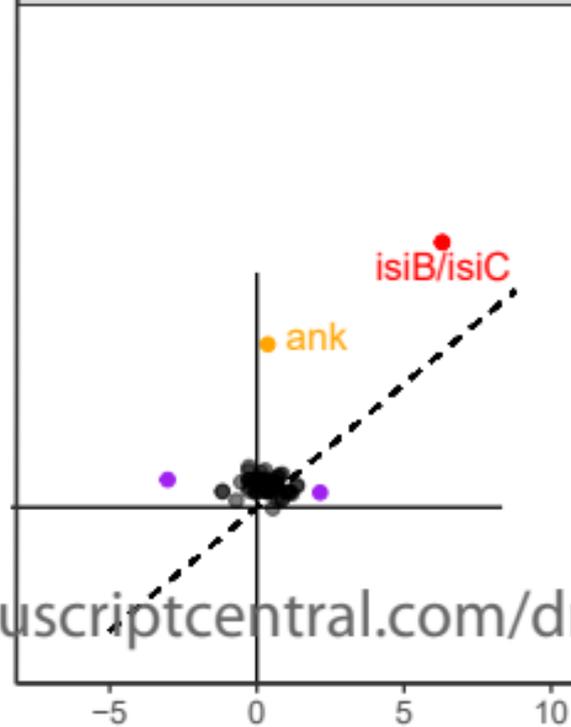
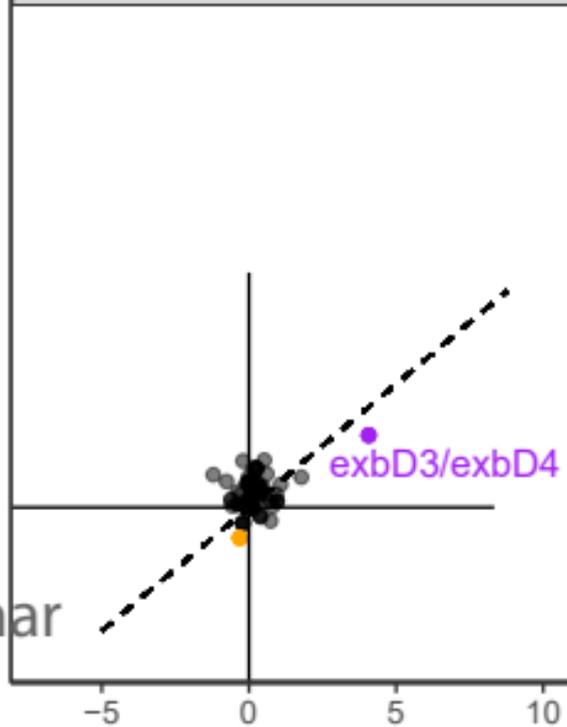
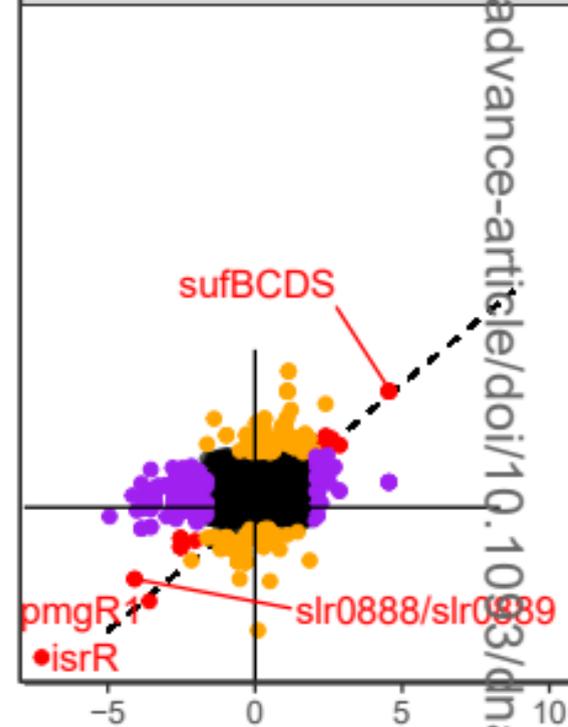
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TTATTGACnAATnATTnTCAATAA
 (Palindromic consensus sequence)

B Submission for DNA Research

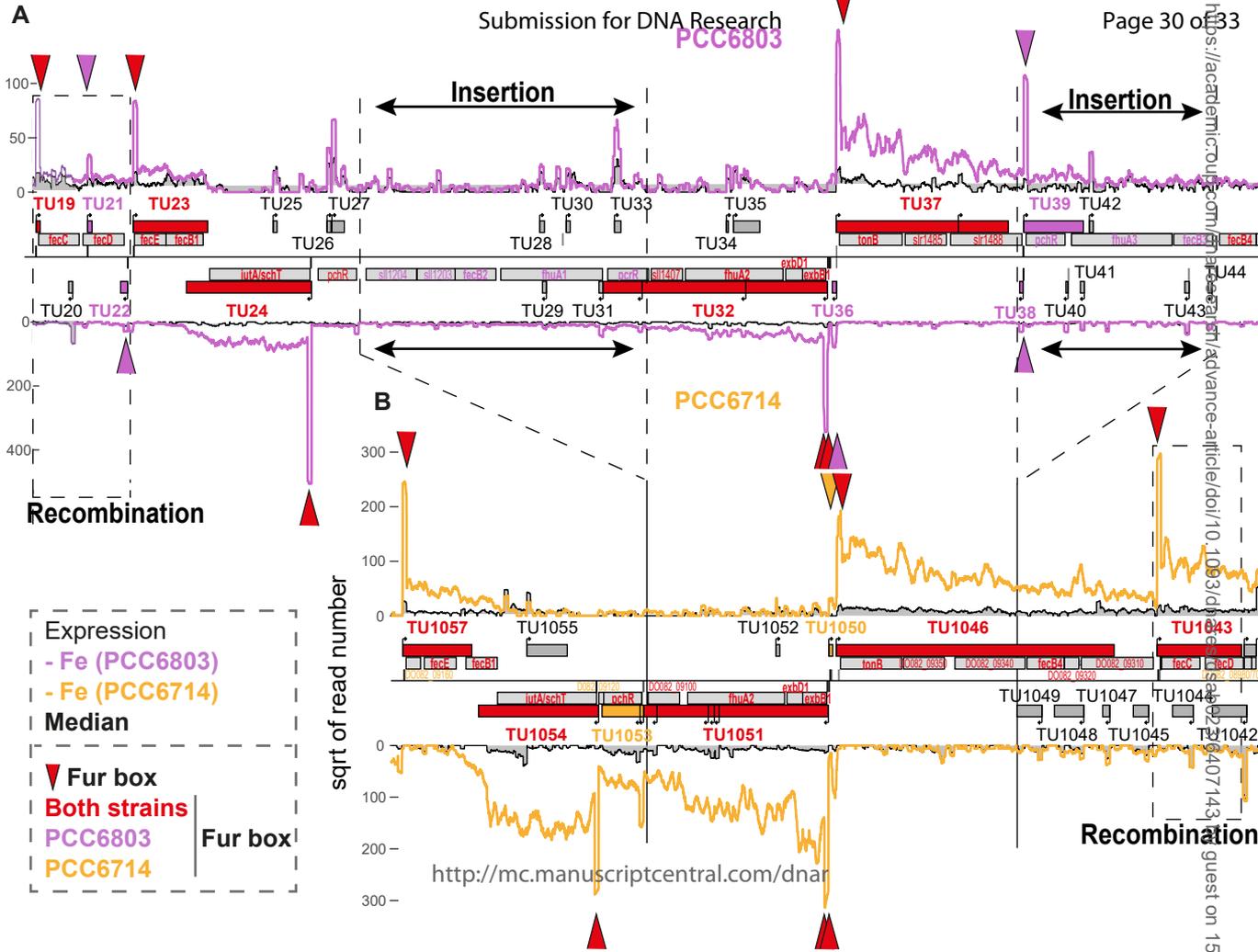


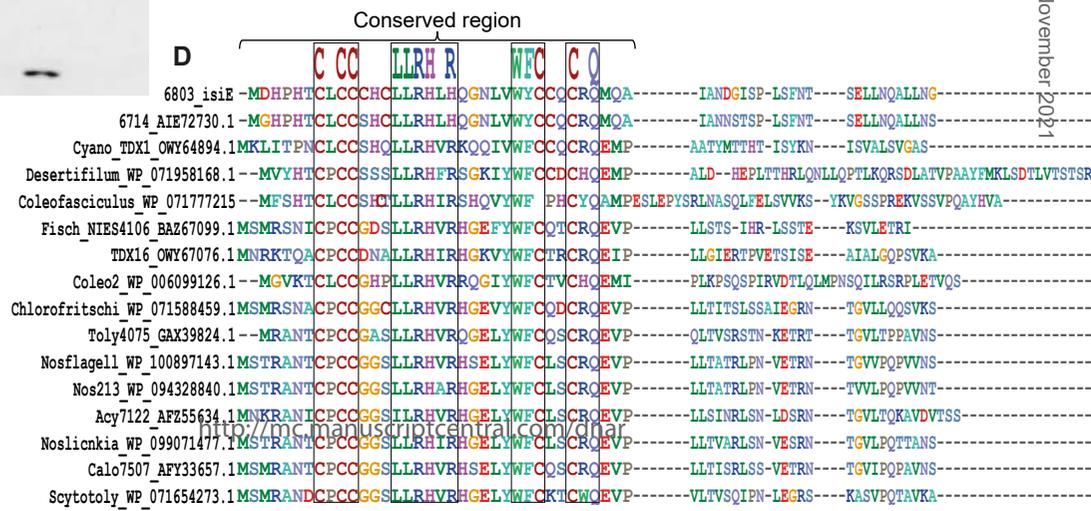
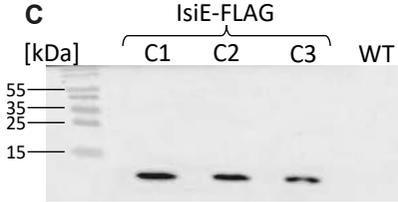
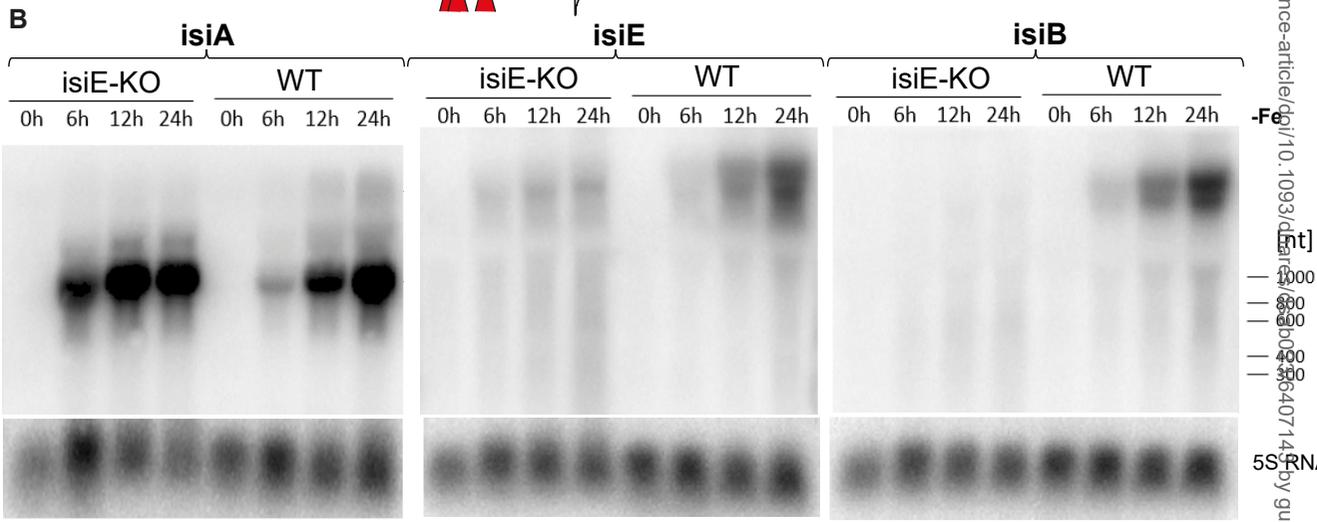
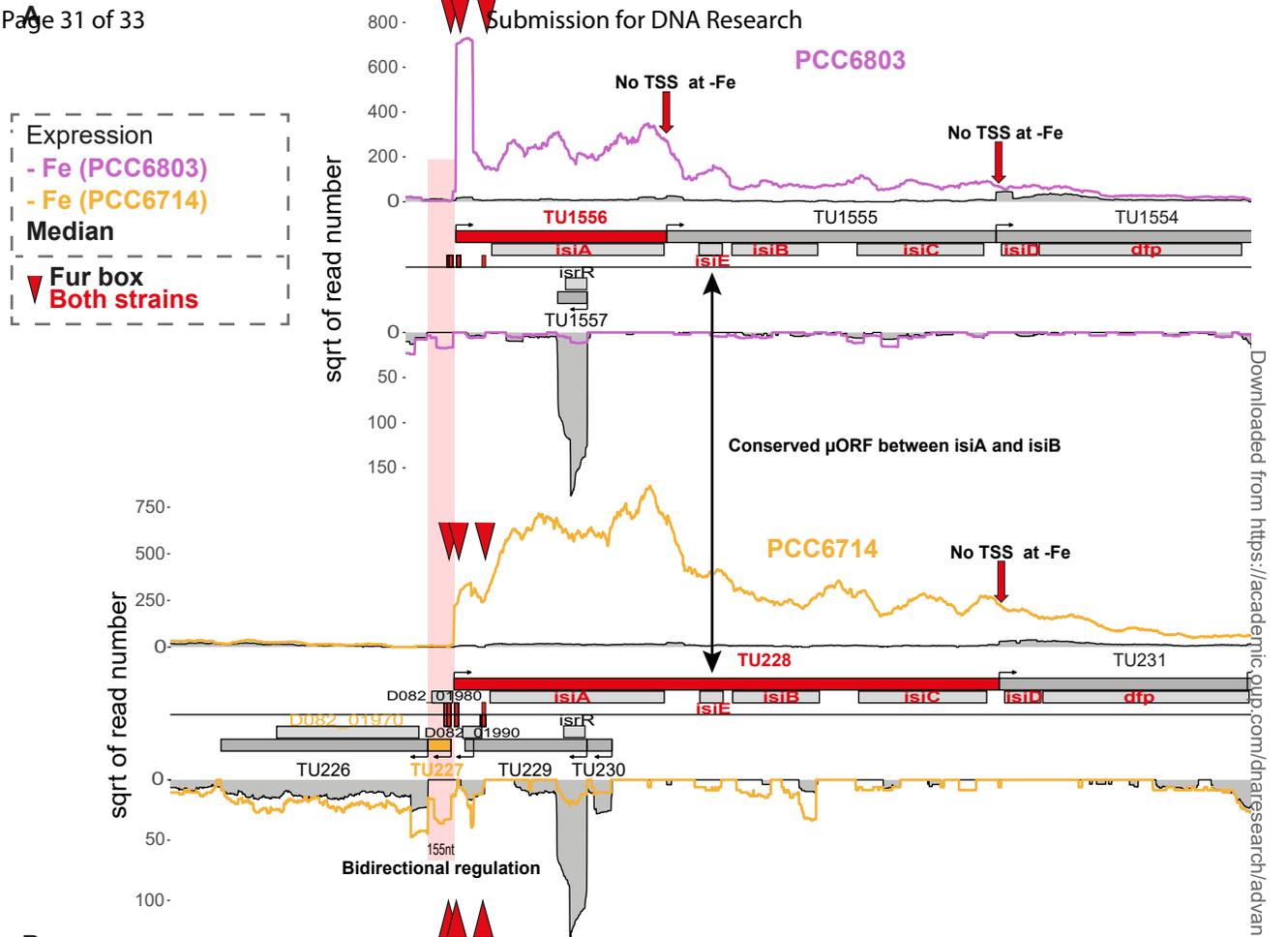
A Fur box in both strains**B** Potential Fur box in both strains**C** Fur box in PCC 6714 only**D** Fur box in PCC 6803 only**E** No Fur box

PCC6803 Relative Expression [Log2(-Fe / Median)]

<http://mc.manuscriptcentral.com/dnar>

PCC6803





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Expression

- Fe (PCC6803)

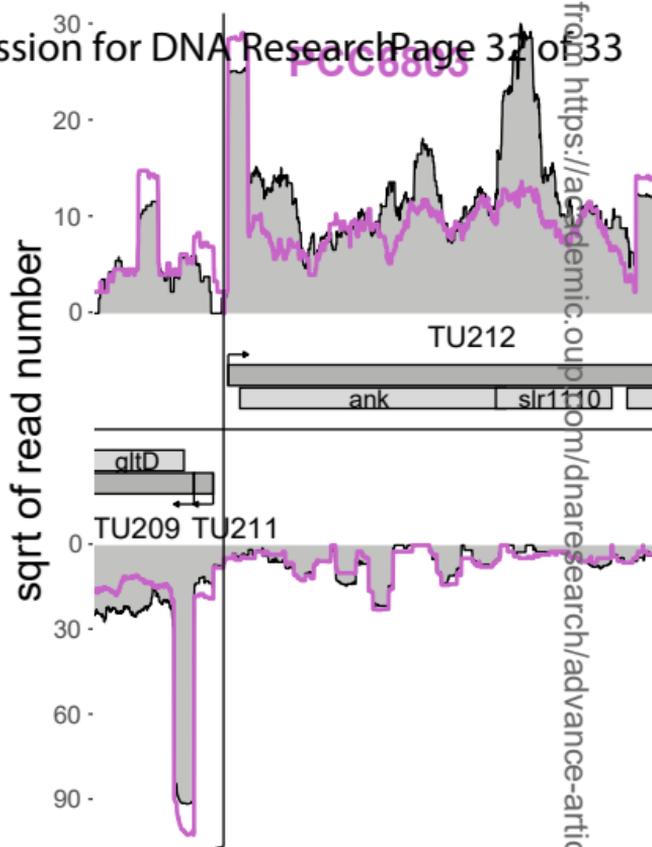
- Fe (PCC6714)

Median

▼ Fur box

PCC6714

sqrt of read number



B

sqrt of read number

