

1 **Genetic-metabolic coupling for targeted metabolic engineering**

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8 **SUMMARY**

9 **To produce chemicals, microbes typically employ potent biosynthetic enzymes that interact with**
10 **native metabolism to affect cell fitness as well as chemical production. However, production**
11 **optimization largely relies on data collected from wild type strains in the absence of metabolic**
12 **perturbations, thus limiting their relevance to specific process scenarios. Here, we address this**
13 **issue by coupling cell fitness to the production of thiamine diphosphate in *Escherichia coli* using a**
14 **synthetic RNA biosensor.**

15 **We apply this system to interrogate a library of transposon mutants to elucidate the native gene**
16 **network influencing both cell fitness and thiamine production. Specifically, we identify**
17 **uncharacterized effectors of the OxyR-SoxR stress response that limit thiamine biosynthesis via**
18 **alternative regulation of iron storage and Fe-S-cluster inclusion in enzymes.**

19 **Our study represents a new generalizable approach for the reliable high-throughput**
20 **identification of genetic targets of both known and unknown function that are directly relevant to**
21 **a specific biosynthetic process.**

22

23 **Keywords**

24 RNA biosensors; thiamine biosynthesis; transposon mutagenesis; stress response; cofactors.

25

26 **INTRODUCTION**

27 A common problem in the field of biotechnology is the unpredictability inherent in engineering
28 complex biological systems. The use of a whole-system approach can prevent some of the obstacles
29 encountered in bioengineering, including toxicity due to metabolite over-production (Fletcher et al.,
30 2016), metabolic bottlenecks (Lechner et al., 2016), and low product titers (Otero and Nielsen, 2013).
31 Systems metabolic engineering relies on the generation and analysis of large datasets to identify key
32 genetic components that contribute to high-yield production phenotypes. However, this methodology
33 often assumes an unperturbed system, and our capacity to elucidate all metabolic and regulatory
34 perturbations affecting the production of a given metabolite is limited.

35 The impact of engineering a cellular system to produce a primary metabolite can be substantial.
36 The biosynthetic pathways of primary metabolites often utilize specialized enzymes of high catalytic
37 rate and substrate affinity; these attributes support high flux through the pathway, even at low enzyme
38 concentrations (Nam et al., 2012). When these enzymes are over-expressed, the supply of energy,
39 cofactors, and carbon building blocks quickly become limiting and can lead to a strong metabolic
40 burden on the cell. Indeed, metabolic burden can lead to low cell density and reduced titers during the
41 production of recombinant vitamins, such as cobalamin (vitamin B₁₂) (Biedendieck et al., 2010) or
42 riboflavin (vitamin B₂) (Lin et al., 2014). Furthermore, central cell metabolism, which provides building
43 blocks for vitamin biosynthesis, is subject to complex regulation that can affect pathway optimization
44 (Lin et al., 2014).

45 The effect of producing a metabolite is strongly dependent on the individual metabolite and how it
46 affects cell metabolism and its regulation. Understanding the relevance of this context dependency at
47 the genome level remains a challenge in the field of biotechnology. New approaches enabling high-
48 throughput characterization of fitness and pathway yields are key to achieving this goal. Tagged
49 transposon mutagenesis (Oh et al., 2010) and synthetic RNA-based biosensors (Townshend et al., 2015)
50 are genetic tools that have been applied to study the condition-dependent contributions of genes to cell
51 growth and synthetic pathway output.

52 Here, we present a methodology for characterizing the metabolic perturbations that are a direct
53 result of the over-production of a primary metabolite in a bacterial cell. Genetic-metabolic coupling is
54 based on the concurrent expression of a synthetic metabolic pathway and an end-product biosensor in a
55 population of bar coded single-gene transposon insertion mutants. Mutant fitness data quantified by
56 deep sequencing are used to identify which genes provide growth advantage exclusively upon selection
57 for the end product. We use genetic-metabolic coupling to assess the influence of *Escherichia coli* cell
58 functions during biosynthesis of a derivative of vitamin B₁ – thiamine diphosphate (TPP).

59 We found that the OxyR-Fur-mediated regulation of Fe-S cluster formation strongly affects TPP
60 output. Ultimately, genetic-metabolic coupling allowed the identification of a small set of genes, several
61 of which of uncharacterized or predicted function, with strong population-wide fitness phenotypes from
62 over 2000 tested genes. When introducing specific knockouts in a clean genetic background, the
63 identified genes resulted in the predicted changes to the TPP titers with different production plasmids
64 and in different host strains. We finally demonstrate reverse tuning of several identified genetic targets
65 with multi-copy plasmids further supporting the broad application of genetic-metabolic coupling for
66 metabolic engineering.

67

68 **RESULTS**

69 **Coupling population genetics to thiamine metabolic engineering**

70 To create a TPP-overproducing strain for our analysis, we assembled the TPP biosynthetic genes
71 on a plasmid. The genes were arranged in two operons that encoded the two different cellular branches
72 required for the biosynthetic pathway. Four genes are involved in the synthesis of thiazole
73 monophosphate (THZ-P) from L-cysteine, L-tyrosine and deoxy-D-xylulose-5-phosphate (DXP):
74 *thiFSGH*, *thiC* for the synthesis of 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP-P) from 5-
75 aminoimidazole riboside (AIR), *thiE* for coupling THZ-P to HMP-PP, and either *thiD*, the kinase that
76 phosphorylates HMP-P to HMP-PP (plasmid pTHId), or *thiM* (plasmid pTHIm), a salvage enzyme that
77 phosphorylates THZ. In the latter case, the strain relies on the native chromosomal *thiD* gene for HMP-
78 P phosphorylation (details are provided in Sup. Fig. 1A-B). The synthesis of TPP relies on several
79 cellular biosynthetic pathways for substrates and in particular purine nucleotides biosynthesis for AIR,
80 cysteine biosynthesis, and S-adenosylmethionine (SAM) and NADPH as cofactors.

81 To quantitatively relay the intracellular TPP concentration at the single-cell level, we used a TPP
82 biosensor (plasmid pTPP_Bios) based on an engineered TPP riboswitch (Genee et al., 2016). This
83 riboswitch is used to activate the expression of two genes conferring resistance to the antibiotics
84 chloramphenicol (CAM) and spectinomycin (SpeR). This dual selection design allows for the reliable
85 selection of high TPP producers using both the pTHIm and pTHId plasmids with very low false-
86 positive rate (Genee et al., 2016). The extracellular titers of *de novo*-produced TPP in *E. coli*
87 MG1655:: Δ *tbpA* Δ *thiM* (BS134), which carries deletions of the thiamine salvage and import pathways,
88 were ~200 and ~800 μ g/l for pTHIm and pTHId, respectively (Fig. 1A). The overexpression of
89 thiamine biosynthetic genes reduced the growth rates by 10% and 35% for pTHIm and pTHId,
90 respectively.

91
92 To understand the cellular components impacting the *E. coli* TPP biosynthetic capacity, we used
93 whole-genome transposon (Tn) mutagenesis to construct libraries of *E. coli* MG1655 Tn5 mutant
94 strains harboring the thiamine selection system (pTPP_Bios) and the plasmid carrying both *de novo* and
95 salvage biosynthetic genes with a lower cell burden (pTHIm), or the plasmid without biosynthetic
96 pathway, constructing the respective libraries ITpp and IMck (Figure 1B). Insertions were evenly
97 distributed across the genome and through the lengths of open reading frames (Sup. Fig. 1C-D)
98 (RefSeq. NC_000913.3). To characterize the impact of transposon insertions within various *E. coli*
99 genes on the intracellular excess of TPP, we subjected the IMck and ITpp mutant libraries to a
100 competitive fitness assay for 16 hours in shake flasks, obtaining robust fitness estimates for 1857 genes
101 in the IMck library and 2054 genes in the ITpp library with high reproducibility across biological
102 replicates (Sup. Fig. 1E). The relative gene fitness in each condition was calculated from strain bar code

103 abundance (\log_2 fitness) using the relative abundance in the Tn libraries at time zero (Wetmore et al.,
104 2015).

105 **Iron and sulfur significantly impact TPP production**

106 We applied a two-step methodology for identifying genes and biological functions with significant
107 condition-specific fitness phenotype (top/bottom 5%, see also Sup. Methods), or a significantly
108 reduced/increased relative abundance of TPP-producing Tn mutants compared to time zero or mutants
109 carrying the backbone-only plasmid (IMck). First, extreme fitness values were identified by setting top
110 and bottom thresholds for each condition. These values were used to build a factorized matrix with +1
111 or -1 values corresponding to significantly increased or decreased fitness, respectively, which was later
112 used to establish gene-to-condition associations for functional enrichment (DAVID) (Huang et al.,
113 2008) (Fig. 2A).

114 We focused our analysis on two effects: the effect of having biosynthetic genes for TPP compared
115 to control backbone-only plasmid and the effect of selecting for intracellular TPP compared to no
116 selection. In both cases we expected that the deletion of a gene encoding factors important for the
117 biosynthesis of TPP, which was not provided in the cultivation media, or for replenishing cellular
118 resources would lead to relatively reduced fitness compared to the full population.

119 In the presence of the TPP heterologous pathway but without selective pressure (fTpp), we
120 identified 138 genes with significant fitness changes, of which 102 exhibited reduced and 36 exhibited
121 increased fitness (Sup. Fig. 2A and Sup. Table 1). Knockouts of genes affecting cofactor or substrate
122 supply to the TPP pathway showed fitness defects: *rtm* (ΔF : -1.0, ΔF : fitness difference to control) and
123 *fucA* (ΔF : -1.3), implicated in ribose metabolism, *bfr* (ΔF : -1.1) and *yaaA* (ΔF : -0.8) in iron
124 homeostasis, and *dxs*, involved in DXP biosynthesis. We also found enrichment for knockouts of genes
125 involved in protein folding (Sup. Table 1, $P < 0.05$, with Bonferroni correction for multi-hypotheses
126 testing). During selection for TPP without the TPP heterologous pathway (fMck^{CS}), several additional
127 genes involved in cellular iron homeostasis (*fes*, *ftnA* and *cueO*) and the Gene Ontology (GO) class
128 “water-soluble vitamin biosynthesis functions” (GO:0006767, $P < 0.05$, Sup. Fig. 2B and Sup. Table 1)
129 showed reduced fitness. Overall, this result indicates that TPP biosynthesis relies strongly on the cell
130 protein and vitamin synthesis apparatus, especially cellular iron availability. This result also highlights
131 that our synthetic TPP biosensor is sensitive to the concentration of endogenous TPP.

132

133 We next studied the effect of selecting for TPP in the presence of the heterologous TPP pathway
134 (fTpp^{CS}). We compiled a list of 45 genes with significant fitness changes, of which 27 were specific to
135 this condition (e.g., with a neutral phenotype in the absence of selection or biosynthetic genes; Fig. 2B
136 and Sup. Fig. 2C-D). This set of genes was functionally enriched for iron and glutathione ABC

137 transporters (Kyoto Encyclopedia of Genes and Genomes [KEGG] pathway, DAVID Bonferroni-
138 corrected $P < 0.01$), involvement in the sulfur-compound biosynthetic process (GO:0044272 $P \leq 0.05$),
139 and DNA restriction and modification (GO:0009307, $P < 0.01$). In addition, similar phenotypes were
140 shared by functionally related genes: reduced Fe-S cluster availability (*csdE*, *cysE*, *ccmC* mutations)
141 presented reduced fitness (Fig. 2B –bottom), while gene knockouts that were involved in sulfur
142 assimilation, major redox regulators OxyR and SoxR, and HSD DNA restriction enzymes resulted in a
143 positive fitness shift (Fig. 2B). Indeed, the pTHId pathway was more stable in a Δ *hsdMR* knockout
144 compared to wild type (Sup. Fig. 3), possibly suggesting direct cleavage by this endonuclease.

145 SAM turnover during TPP biosynthesis requires extensive NADPH reductive power (Chen et al.,
146 2015). NADP⁺ can increase cellular oxidation, impair Fe-S cluster formation, and cause accumulation
147 of hydrogen peroxide and reactive oxygen species (ROS) all tightly controlled by OxyR-SoxR regulons
148 (Imlay, 2015). We tested the effect on TPP titer from limiting Fe-S clusters and the activation of OxyR-
149 SoxR regulons. Sequestration of intracellular unbound iron by iron chelators o-phenanthroline and 2,2'-
150 dipyridyl (Chupka et al., 1988) led to a small but significant reduction in TPP titer already at low
151 concentrations (~25 μ M) whereas thiourea showed near-normal TPP levels (Fig. 2C, $P < 0.02$, red
152 bars). These effects were more significant at 5-fold higher compounds concentration, (Fig. 2C, cyan
153 bars), indicating that free iron and Fe-S clusters are limiting during TPP production and the removal of
154 ROS has an effect similar to the OxyR knockouts and regulon down-regulation.

155

156 **Elucidation of the Fe-S cluster biogenesis network**

157 We used a machine-learning algorithm to mine RefSeq gene descriptions (Tatusova et al., 2014)
158 and quantified for each condition the effects of knocking out genes with biological functions related to
159 iron, sulfur, cysteine, tyrosine and ribose, which are cofactors or substrates involved in TPP
160 biosynthesis (Chatterjee et al., 2008; Kriek et al., 2007). We also looked at cellular redox regulation,
161 which is believed to influence cellular vitamin metabolism (Dougherty and Downs, 2006). We
162 identified 108 genes in the *E. coli* genome related to these functions, among which 57 genes (~53%)
163 showed significant fitness shifts during selection for TPP production: 35 were associated with cofactor
164 supply, and 25 were associated with cellular redox regulation (fTpp^{CS} and fTPP, $P < 0.1$ compared to a
165 random set by random sampling with replacement or bootstrapping, Fig. 3A). The presence of a
166 significant number of genes involved in redox regulation suggests that oxidative stress can be relevant
167 during TPP production.

168

169 To extend our analysis beyond a specific enzyme or transporter, we quantified the roles of the
170 production phenotypes of parent GO classes (Keseler et al., 2009) associated with genes with extreme

171 fitness phenotypes. We found that mutants of ‘Cellular Iron Ion Homeostasis’ (GO: 006879) genes had
172 a significant low-fitness phenotype during selection for TPP ($P < 0.05$, Sup. Table 2); however,
173 mutations in genes involved in iron, siderophores and ferric-enterobactin transport (GO: 00015682,
174 0015684, 0015685, 015891) were associated with significantly higher fitness ($P < 0.1$, Fig. 3Bi and
175 Sup. Fig. 4A-B).

176 In bacteria, sulfur is mainly assimilated in the cell in a reduced form, as hydrogen sulfide from
177 sulfate, and is incorporated into the amino acids cysteine and methionine. We found that the average
178 fitness of mutants in genes involved in ‘Sulfate Assimilation’ (GO: 0000103) had significantly
179 increased ($P < 0.01$) during selection for TPP (Fig. 3Bii, Sup. Fig. 4C). We also found that impairment
180 of genes related to ‘Methionine Biosynthetic Process’ (GO:0009086) but not cysteine biosynthesis
181 ($\Delta cysE$: -1.5 ΔF) led to increased fitness ($P < 0.1$) (Sup. Fig. 4D-E). In *E. coli*, the sulfur found in Fe-S
182 clusters is derived from cytosolic L-cysteine mainly through the enzyme *IscS* (cysteine desulfurase) and
183 the conversion of L-cysteine to L-alanine. We could not obtain fitness data for members of the ISC
184 complex, likely because of its essentiality, but the deletion of the second sulfur-accepting complex – the
185 CSD sulfur transfer system – showed reduced fitness upon selection for TPP (ΔF , $\Delta csdA$: -0.9, $\Delta csdE$: -
186 3.4) (Fig. 3Bii). Surprisingly, we found that mutants in the SUF system for iron-sulfur cluster assembly
187 that is employed under iron starvation or oxidative stress conditions (Outten et al., 2004) exhibited
188 higher fitness (ΔF , $\Delta sufS$: +0.4, $\Delta sufA$: +0.7) (Fig. 3Bii).

189

190 The process of inclusion of Fe-S clusters in enzymes in *E. coli* is highly sensitive to the
191 environment and its redox state (Crack et al., 2014; Ding et al., 2005). Transposon insertions in *soxR*
192 and *oxyR* both showed higher fitness during selection for TPP (ΔF , respectively, +1.0 and +1.13). In
193 addition to the *suf* operon, we found that insertions in other components of this response pathway had
194 similar effects: the iron chelators *yaaA* and *dps* (ΔF : +1.4 and +0.6, respectively), *yhcN* (ΔF : +0.6),
195 which is induced during peroxide stress (Lee et al., 2010), and the chaperone Hsp33 (ΔF : $\Delta hslO$ +0.4).
196 During oxidative stress, both OxyR and SoxR upregulate the *fur* regulon that is regarded to play a key
197 role in maintaining the stability of cellular iron levels (Seo et al., 2014). Ferritin and enterobactin have
198 respectively key iron storage and iron uptake functions in *E. coli*. Bacterioferritin, whose expression is
199 controlled by Fur via the small regulatory RNA RyhB, is important for sequestering iron thus limiting
200 cellular oxidative damage caused by Fenton chemistry (Bou-Abdallah et al., 2002). We found that
201 while Tn insertions in iron-storage ferritin and enterochelin esterase, which releases iron from ferric
202 enterobactin, resulted in fitness defects (ΔF : $\Delta ftnAB$ ~ -2.0, Δfes -3.3), the lack of bacterioferritin had a
203 positive effect on fitness during selection for TPP (ΔF : Δbfr +1.0) (Fig. 3Biv).

204 We found that the control of OxyR-SoxR activation also had a significant effect. OxyR is activated
205 by oxidation, likely by oxidized glutathione, for which just a Cys¹⁹⁹ – Cys²⁰⁸ disulfide bond appears to
206 be sufficient (Georgiou, 2002). We found that the impairment of glutathione import to the cytosol and
207 *E. coli* disulfide bond system enzymes all result in negative fitness (ΔF , $\Delta gsiA$: -1.4, $\Delta dsbA$: -2.0,
208 $\Delta dsbC$: -1.7). In contrast, the lack of *soxR* inactivation by the RSX Membrane Reducing System (Koo
209 et al., 2003) showed positive growth phenotypes (ΔF , $\Delta rsxA$: +1, $\Delta rsxD$: +0.8, $\Delta rsxE$: +0.6) (Fig. 3Biii).

210 Overall, our data suggest that TPP overproduction likely causes oxidative stress as previously
211 reported (Kriek et al., 2007) and probably iron deprivation, resulting in an activation of the OxyR-SoxR
212 and possibly Fur regulons, which could be responsible for the distinct effects on iron uptake and
213 sequestration (Fig. 3Biv and i) due to negative feedback (Seo et al., 2014).

214

215 **Population dynamics effectively identify metabolic targets**

216 We set out to examine whether population-based screening could be used to reliably identify
217 metabolic targets by coupling genetic modification to metabolism. We measured the extracellular titers
218 of TPP produced *de novo* from the plasmid pTHId in 24 genomic deletions of *E. coli* MG1655 BS134
219 strains selected from the characterized Fe-S biogenesis network (Fig. 3B). For comparison with the
220 newly identified genetic targets we selected the following five loci previously shown to affect thiamine
221 biosynthesis: *mrp* (Boyd et al., 2008), *purF* (Frodyma et al., 2000), *iscAU* (Agar et al., 2000), *iscR* (Giel
222 et al., 2013) and *yggX* (Gralnick and Downs, 2003). We expected to see reductions in biosynthetic
223 output for Δmrp , $\Delta purF$ and $\Delta iscAUS$, and an increase for $\Delta iscR$, a regulator that inhibits transcription
224 from the *iscAUS* operon (Giel et al., 2013). We created 14 single-gene knockouts and 10 multi-gene
225 locus lesions (Sup. Table 3). For the latter, we expected to observe an additive effect of the genes on
226 TPP output because their products did not appear to be part of enzyme cascades (Sup. Table 4).

227 We observed strong correlation between the shift in fitness of a gene knockout in the multiplexed
228 competitive growth assays and the extracellular TPP titer of the isolated gene knockout (correlation:
229 0.89 excluding the five test loci). Iron supply and availability strongly contributed to TPP output.
230 Disruption of iron storage in ferritin and mobilization from enterobactin completely disrupted the TPP
231 titer ($\Delta ftnB$ and $\Delta fes-entF$, Fig. 4A). In contrast, knockouts of two genes induced by Fur-OxyR regulons
232 – *bfr* and *yaaA* – whose mutants showed fitness improvements in competitive assays (Fig. 3Bi), yielded
233 higher extracellular TPP titers (Δbfr : +0.3 ± 0.2, $\Delta yaaA$: +0.4 ± 0.1, fold difference from mean titer, see
234 also Sup. Fig. 5) compared to the average titer across all knockouts and wild type.

235

236 We then investigated pathways involved in S-incorporation into Fe-S in *E. coli*, including free
237 cytosolic cysteine. TPP output decreased as expected from previous reports (Agar et al., 2000; Boyd et

238 al., 2008; Giel et al., 2013) in the two test loci Δmrp and $\Delta iscAUS$ (respectively, -0.8 ± 0.04 and
239 undetected, fold difference). In contrast, knockout of the repressor *iscR* resulted in a higher TPP titer
240 ($+0.7 \pm 0.06$, Fig. 4B). We found that disrupting cysteine formation and the mobilization of its sulfur by
241 the cellular sulfur transfer system CSD significantly decreased the TPP titer compared to the mean
242 knockout effect ($\Delta cysE$: undetected, $\Delta csdA-ygdK$: -2.7 ± 0.09), which is in agreement with the reduced
243 fitness of mutants in its components (Fig. 3Bii). We previously found that mutants in the *E. coli* SUF
244 sulfur transfer system and in various proteins involved in sulfate assimilation and export exhibited
245 improved growth during TPP selection (Fig. 3Bii). The TPP output of single-locus knockouts agreed
246 with these observations: the average extracellular TPP titers were higher in these knockouts compared
247 to other knockouts and wild type ($\Delta sufAS$: $+0.4 \pm 0.2$, $\Delta cysN$: $+0.6 \pm 0.1$, $\Delta cysP$: $+0.5 \pm 0.04$, $\Delta eamA$:
248 $+0.7 \pm 0.2$, fold difference) (Fig. 4B).

249 The remaining tested loci encoded proteins involved in redox control of a number of cellular
250 functions, particularly iron homeostasis, which is tightly regulated by the SoxR-OxyR-Fur regulons
251 (Jang and Imlay, 2011; Seo et al., 2014). The OxyR regulon is activated by an environment that favors
252 disulfide bonds, in which glutathione plays a key role (Georgiou, 2002), and by nitric oxide via S-
253 nitrosylation (Seth et al., 2012). Deletion of the *narVYZ* locus, for which a mild fitness effect was
254 calculated (ΔF : $+0.4$), showed TPP titers close to the knockouts average ($\Delta narVYZ$: 0.10 ± 0.3) (Fig.
255 4C). In contrast, we found that knockouts of the three predicted activators of the OxyR regulon
256 exhibited significantly reduced TPP titers: $\Delta dsbA$, which is involved in disulfide bond formation
257 (Depuydt et al., 2007) (-1.8 ± 0.2), $\Delta yliAC$ (-1.7 ± 0.3) and $\Delta napCG$ (undetected). Mutants in these loci
258 showed reduced fitness during TPP production, whereas those in the SoxR-OxyR regulons had higher
259 fitness. These observations suggest a role for the SoxR-OxyR regulon in diminishing thiamine
260 biosynthesis. Indeed, knockouts of three downstream genes activated by OxyR – the peroxide-response
261 genes *yhcN* (Lee et al., 2010) and *yggX* (Gralnick and Downs, 2003) – had higher extracellular TPP
262 titers ($\Delta yggX$: $+0.6 \pm 0.1$, $\Delta argR-yhcN$: $+1.3 \pm 0.1$, fold difference) (Fig. 4C), in agreement with the
263 shifts in fitness of their pooled mutants (Fig. 3Biii and Sup. Table 3).

264

265 **Tuning of central Fe-S control**

266 We examined the role of specific regulators of the complex *E. coli* SoxR-OxyR-Fur regulons
267 during TPP production. We quantified the extracellular TPP titer obtained with $\Delta soxR$, $\Delta oxyR$ and Δfur
268 single-gene knockouts introduced in *E. coli* strain TOP10, which lacks DNA restriction systems, and
269 the production strain BS134. We found that compared to the background strains, deletion of SoxR did
270 not lead to increased TPP titers, but the deletion of both OxyR and Fur dramatically increased pathway
271 output in both TOP10 and BS134 (Figure 5A-B, respectively). This result pinpointed the OxyR-Fur

272 regulon, which directs iron homeostasis (Seo et al., 2014), as central cellular response to TPP
273 production.

274 In order to determine whether this response and Fe-S cluster formation could be tuned via the
275 identified genetic targets, we over-expressed the following genes as GFP-fusions (Kitagawa et al.,
276 2005): *iscS*, *dsbA*, *csdA*, *oxyR*, *yhcN*, *yncD* and *tonB*, in the same strain BS134 used for knockouts.
277 Compared to knockouts, TPP titers resulting from gene over-expression showed an opposite shift. In
278 particular, the titer increased during over-expression of *iscS* and *dsbA* while decreased during over-
279 expression of *oxyR*, *yhcN* and *yncD* (Figure 5C).

280

281 **DISCUSSION**

282 The study shows that by coupling the growth of genome-wide bar coded transposon mutants to a
283 biosynthetic product it is possible to identify product-specific metabolic and regulatory bottlenecks. We
284 show that disruptions in the assimilation of certain forms of iron and sulfur led unexpectedly to
285 improved fitness upon selection for TPP production (Fig. 3Bi and 3Bii), a result that was supported by
286 measuring the extracellular TPP titer from individual gene knockouts (Fig. 4). Further, over-expression
287 of *tonB*, involved in the uptake of iron-siderophore chelates, led to a significant decrease in TPP titer
288 (Fig. 5C). We included in our validation genes that are poorly characterized or of only predicted
289 function, which constitute the so-called Y-genes within the *E. coli* genome, and have strong fitness
290 phenotypes with regard to TPP production. Two genes in particular have predicted function in
291 siderophore uptake (*yncD*, a TonB-dependent receptor) and aryl-sulfate assimilation (*ydjJ*, a predicted
292 sulfatase). Both *yncD* and *ydjJ* knockouts exhibited TPP titers 20-25% higher than that of wild type
293 cells (Fig. 4), further confirming that certain assimilatory activities may play a role in inhibiting TPP
294 biosynthesis possibly through the regulator Fur.

295

296 Applied to TPP biosynthesis, genetic-metabolic coupling uncovered that the regulation of Fe-S
297 homeostasis mediated by OxyR-Fur regulons diminishes biosynthetic output by depleting or
298 sequestering cellular Fe-S clusters. In fact, Fe-S clusters are critical for the function of TPP pathway
299 enzymes ThiC and ThiH. The effects of changing global regulators are notoriously idiosyncratic.
300 Nevertheless, TPP-coupled fitness data of mutants in the regulons could be reproduced in different *E.*
301 *coli* strains and production plasmids (Fig. 5A-B). Although mutants were not screened for fitness
302 against wild type cells at the population level, 9 of 10 selected high-fitness mutants when introduced in
303 wild type exhibited an extracellular TPP titer 0.2- to 2-fold higher (with the exception of *narVYZ*),
304 while excluding *ydjJ* all other low-fitness mutants exhibited significantly lower titers (Fig. 4). We also

305 demonstrate that population-wide mutant fitness data could serve as basis for tuning a heterologous
306 pathway through over-expression of genetic targets from multi-copy plasmids (Fig. 5C).

307

308 The characterization of how important parts of the *E. coli* stress response and nutrient assimilation
309 affect a complex heterologous pathway and the identification of poorly characterized genes, which
310 today still represent ~30% of the *E. coli* genome, contributing to this response demonstrate the power of
311 genetic-metabolic coupling for the identification of target genes for the metabolic engineering of a
312 specific biosynthetic product.

313

314 **EXPERIMENTAL PROCEDURES**

315 **Strains, plasmids and cultivation media**

316 Plasmid DNA, PCR products and gel extractions were prepared with appropriate kits (Qiagen ©).
317 Synthetic oligonucleotides were purchased from Integrated DNA Technologies (IDT, Inc). In-frame
318 GFP fusions of selected genes for expression from multi-copy plasmid were obtained from the ASKA
319 collection (Kitagawa et al., 2005). Single-locus deletions were constructed by employing lambda Red
320 recombineering. The thiamine selection system (pTPP_Bios) was described in a previous study from
321 our laboratory (Genee et al., 2016). The TPP biosynthetic pathway was assembled from native *E. coli*
322 MG1655 genes via PCR amplification. A detailed description of the molecular tools is provided in Sup.
323 Info.

324 **Analytical quantification of TPP**

325 Extracellular TPP concentration was measured using a modified thiochrome-high-performance liquid
326 chromatography (HPLC) assay procedure. Briefly, fresh colonies carrying either pTHId or pTHIm were
327 inoculated into 1 mL MOPS rich media lacking thiamine (see above) and containing 30 µg/mL
328 chloramphenicol and 50 µg/mL spectinomycin in deep-well culture plates and then cultivated twice to
329 saturation in a 48-hour period (Sup. Info.).

330 **Construction of Tn-mutants, TnSeq and BarSeq protocols**

331 For construction of tagged transposon mutants of *E. coli* MG1655 and bioinformatic analysis of NGS
332 data we relied on reported methodology and tools (Wetmore et al., 2015). BarSeq reads are converted
333 into a table reporting the number of times each bar code is observed using a custom Perl script
334 (MultiCodes.pl). Given a table of bar codes, where they map in the genome, and their counts in each
335 sample, strain and gene fitness values are estimated with a custom R script (FEBA.R) (Sup. Info.). The
336 bioinformatic tools can be accessed at <https://bitbucket.org/berkeleylab/feba>.

337 **Calculation of gene fitness**

338 The gene fitness is the weighted average of Tn mutant strains fitness within the gene, that is strains with
339 more reads have less noisy fitness estimates and are weighted more highly. In more detail, we first
340 selected a subset of strains and genes that have adequate coverage in the time-zero samples (2 reads per
341 strain and 20 reads per gene are considered adequate). Only strains that lie within the central 10 to 90%
342 of a gene coding region are considered. Then, for each sample:

$$343 \text{ Strain fitness} = \text{Log}_2(n_{After} + \sqrt{\psi}) - \text{Log}_2(n_0 + \sqrt{\frac{1}{\psi}})$$

344 where ψ is a “pseudocount” to control for very noisy estimates when read counts are very low. (Further
345 details can be found in Sup. Info. and Wetmore et al., 2015).

346

347 **ABBREVIATIONS**

348 KO: knockout, Tn: transposon, aa: amino acid, DNA: deoxyribonucleic acid, RNA: ribonucleic acid,
349 RBS: ribosome binding site.

350

351 **SUPPLEMENTAL INFORMATION**

352 Refer to the web version on PubMed Central for supplementary materials.

353

354 **AUTHOR CONTRIBUTIONS**

355 Conceptualization, S.C. and M.O.A.S.; Methodology, S.C. and M.O.A.S.; Investigation, S.C. and F.G.T.;
356 Writing – Original Draft, S.C.; Writing – Review & Editing, S.C. and M.O.A.S.; Funding Acquisition,
357 M.O.A.S.

358

359 **Competing financial interests**

360 The authors have no competing financial interests to declare.

361

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367

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374

375 REFERENCES

- 376 Agar, J.N., Krebs, C., Frazzon, J., Huynh, B.H., Dean, D.R., Johnson, M.K., April, R. V, Re, V., Recci, M., and
377 May, V. (2000). IscU as a Scaffold for Iron - Sulfur Cluster Biosynthesis: Sequential Assembly of [2Fe-2S] and [
378 4Fe-4S] Clusters in IscU †. 7856–7862.
- 379 Biedendieck, R., Malten, M., Barg, H., Bunk, B., Martens, J.H., Deery, E., Leech, H., Warren, M.J., and Jahn, D.
380 (2010). Metabolic engineering of cobalamin (vitamin B12) production in *Bacillus megaterium*. *Microb. Biotechnol.*
381 *3*, 24–37.
- 382 Bou-Abdallah, F., Lewin, A.C., Le Brun, N.E., Moore, G.R., and Dennis Chasteen, N. (2002). Iron detoxification
383 properties of *Escherichia coli* bacterioferritin. Attenuation of oxyradical chemistry. *J. Biol. Chem.* *277*, 37064–
384 37069.
- 385 Boyd, J.M., Pierik, A.J., Netz, D.J.A., Lill, R., and Downs, D.M. (2008). Bacterial ApbC can bind and effectively
386 transfer iron-sulfur clusters. *Biochemistry* *47*, 8195–8202.
- 387 Chatterjee, A., Li, Y., Zhang, Y., Grove, T.L., Lee, M., Krebs, C., Booker, S.J., Begley, T.P., and Ealick, S.E.
388 (2008). Reconstitution of ThiC in thiamine pyrimidine biosynthesis expands the radical SAM superfamily. *Nat.*
389 *Chem. Biol.* *4*, 1–8.
- 390 Chen, Y., Xu, D., Fan, L., Zhang, X., and Tan, T. (2015). Manipulating multi-system of NADPH regulation in
391 *Escherichia coli* for enhanced S - adenosylmethionine production. *RSC Adv.* *5*, 41103–41111.
- 392 Chupka, W.A., Jivery, W.T., and Introduction, I. (1988). Toxic DNA Damage by Hydrogen Peroxide Through the
393 Fenton Reaction in Vivo and in Vitro. *240*.
- 394 Crack, J.C., Thomson, A.J., and Brun, N.E. Le (2014). Iron – Sulfur Clusters as Biological Sensors: The
395 Chemistry of Reactions with Molecular Oxygen and Nitric Oxide. *Acc. Chem. Res.*
- 396 Depuydt, M., Leonard, S.E., Vertommen, D., Denoncin, K., Morsomme, P., Wahni, K., Messens, J., Carroll, K.S.,
397 and Collet, J. (2007). A Periplasmic Reducing System Protects Single Cysteine Residues from Oxidation. *Science*
398 (80-). 8–11.
- 399 Ding, H., Harrison, K., and Lu, J. (2005). Thioredoxin Reductase System Mediates Iron Binding in IscA and Iron
400 Delivery for the Iron-Sulfur Cluster Assembly in IscU *. *J. Biol. Chem.* *280*, 30432–30437.
- 401 Dougherty, M.J., and Downs, D.M. (2006). A connection between iron – sulfur cluster metabolism and the
402 biosynthesis of 4-amino-5- hydroxymethyl-2-methylpyrimidine pyrophosphate in *Salmonella enterica*. *Microbiology*
403 *2345–2353*.
- 404 Fletcher, E., Krivoruchko, A., and Nielsen, J. (2016). Industrial Systems Biology and Its Impact on Synthetic
405 Biology of Yeast Cell Factories. *Biotechnol. Bioeng.* *113*, 1164–1170.
- 406 Frodyma, M., Rubio, A., and Downs, D.M. (2000). Reduced Flux through the Purine Biosynthetic Pathway Results
407 in an Increased Requirement for Coenzyme A in Thiamine Synthesis in *Salmonella enterica* Serovar Typhimurium.
408 *J. Bacteriol.* *182*, 236–240.
- 409 Genee, H.J., Bali, A.P., Petersen, S.D., Siedler, S., Bonde, M.T., Gronenberg, L.S., Kristensen, M., Harrison, S.J.,
410 and Sommer, M.O.A. (2016). Functional mining of transporters using synthetic selections. *Nat. Chem. Biol.* *12*,
411 1015–1022.
- 412 Georgiou, G. (2002). How to Flip the (Redox) Switch Minireview. *Cell* *111*, 607–610.
- 413 Giel, J.L., Nesbit, A.D., Mettert, E.L., Fleischhacker, A.S., Wanta, B.T., and Kiley, P.J. (2013). Regulation of iron-
414 sulfur cluster homeostasis through transcriptional control of the Isc pathway by [2Fe-2S]-IscR in *Escherichia coli*.
415 *Mol. Microbiol.* *87*, 478–492.
- 416 Gralnick, J.A., and Downs, D.M. (2003). The YggX Protein of *Salmonella enterica* Is Involved in Fe (II)
417 Trafficking and Minimizes the DNA Damage Caused by. *J. Biol. Chem.* *278*, 20708–20715.

- 418 Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2008). Systematic and integrative analysis of large gene lists
419 using DAVID bioinformatics resources. *Nat. Protoc.*
- 420 Imlay, J.A. (2015). Transcription Factors That Defend Bacteria Against Reactive Oxygen Species. *Annu. Rev.*
421 *Microbiol.* 69.
- 422 Jang, S., and Imlay, J.A. (2011). Hydrogen peroxide inactivates the Escherichia coli Isc iron-sulfur assembly system,
423 and OxyR induces the Suf system to compensate. *Mol. Microbiol.* 78, 1448–1467.
- 424 Keseler, I.M., Bonavides-Martínez, C., Collado-Vides, J., Gama-Castro, S., Gunsalus, R.P., Johnson, D.A.,
425 Krummenacker, M., Nolan, L.M., Paley, S., Paulsen, I.T., et al. (2009). EcoCyc: a comprehensive view of
426 Escherichia coli biology. *Nucleic Acids Res.* 37, D464–D470.
- 427 Kitagawa, M., Ara, T., Arifuzzaman, M., and Ioka-nakamichi, T. (2005). Complete set of ORF clones of Escherichia
428 coli ASKA library (A Complete Set of E. coli K-12 ORF Archive): Unique Resources for Biological Research.
429 *DNA Res.* 299, 291–299.
- 430 Koo, M., Lee, J., Rah, S., Yeo, W., Lee, J., Lee, K., Koh, Y., Kang, S., and Roe, J. (2003). A reducing system of the
431 superoxide sensor SoxR in Escherichia coli. *EMBO J.* 22.
- 432 Kriek, M., Martins, F., Leonardi, R., Fairhurst, S.A., Lowe, D.J., and Roach, P.L. (2007). Thiazole Synthase from
433 Escherichia coli AN INVESTIGATION OF THE SUBSTRATES AND PURIFIED PROTEINS REQUIRED FOR.
434 *J. Biol. Chem.* 282, 17413–17423.
- 435 Lechner, A., Brunk, E., and Keasling, J. (2016). The Need for Integrated Approaches in Metabolic Engineering.
436 *Cold Spring Harb Perspect Biol.*
- 437 Lee, J., Hiibel, S.R., Reardon, K.F., and Wood, T.K. (2010). Identification of stress-related proteins in Escherichia
438 coli using the pollutant cis -dichloroethylene. *J. Appl. Microbiol.* 108, 2088–2102.
- 439 Lin, Z., Xu, Z., Li, Y., Wang, Z., Chen, T., and Zhao, X. (2014). Metabolic engineering of Escherichia coli for the
440 production of riboflavin. *Microb. Cell Fact.* 13.
- 441 Nam, H., Lewis, N.E., Lerman, J.A., Lee, D., Chang, R.L., Kim, D., and Palsson, B.O. (2012). Network Context and
442 Selection in the Evolution to Enzyme Specificity. *Science* (80-.). 6185.
- 443 Oh, J., Fung, E., Price, M.N., Dehal, P.S., Davis, R.W., Giaever, G., Nislow, C., Arkin, A.P., and Deutschbauer, A.
444 (2010). A universal TagModule collection for parallel genetic analysis of microorganisms. *Nucleic Acids Res.* 38,
445 e146.
- 446 Otero, M., and Nielsen, J. (2013). Genome-scale modeling enables metabolic engineering of Saccharomyces
447 cerevisiae for succinic acid production. *J. Microbiol. Biotechnol.* 735–747.
- 448 Outten, F.W., Djaman, O., and Storz, G. (2004). A suf operon requirement for Fe – S cluster assembly during iron
449 starvation in Escherichia coli. *Mol. Microbiol.* 2004, 861–872.
- 450 Seo, S.W., Kim, D., Latif, H., O’Brien, E.J., Szubin, R., and Palsson, B.O. (2014). Deciphering Fur transcriptional
451 regulatory network highlights its complex role beyond iron metabolism in Escherichia coli. *Nat. Commun.* 5, 4910.
- 452 Seth, D., Hausladen, A., Wang, Y., and Stamler, J.S. (2012). Endogenous Protein S-Nitrosylation in E. coli:
453 Regulation by OxyR. *Science* (80-.).
- 454 Tatusova, T., Ciufu, S., Fedorov, B., O’Neill, K., and Tolstoy, I. (2014). RefSeq microbial genomes database: New
455 representation and annotation strategy. *Nucleic Acids Res.* 42, 553–559.
- 456 Townshend, B., Kennedy, A.B., Xiang, J.S., and Smolke, C.D. (2015). High-throughput cellular RNA device
457 engineering. *Nat. Methods* 12.
- 458 Wetmore, K.M., Price, M.N., Waters, R.J., Lamson, J.S., He, J., Hoover, C. a, Blow, M.J., Bristow, J., Butland, G.,
459 and Arkin, A.P. (2015). Rapid Quantification of Mutant Fitness in Diverse Bacteria by Sequencing Randomly Bar-
460 Coded Transposons. *MBio* 6, 1–15.
- 461

FIGURE LEGENDS

Figure 1. Genetic-metabolic coupling design.

A) Quantification of extracellular TPP titer with pTHId and pTHIm (cyan and red, respectively) compared to a control (no plasmid, grey) in *E. coli* MG1655 and BS134 strains (n = 4-6) **B)** Graphical scheme of the experimental design. A wild type *E. coli* strain carrying the dual riboswitch (pTPP_Bios) is subject to mutagenesis via a tagged Tn5 transposon. Following library characterization, the TPP production plasmid (pTHIm) or the backbone plasmid (pMck) is electroporated in the pool of mutants to obtain respectively the fTpp and fMck libraries. These libraries are finally used for fitness assays in absence or presence (^{CS}) of selection for TPP production.

Figure 2. Gene knockouts with significant fitness shift during TPP production.

A) Data analysis pipeline: significant fitness values are identified and used to build a phenotypic binary map. Functional enrichment in gene clusters is examined by DAVID and by mining RefSeq gene descriptions for functional annotations. **B)** Hierarchical clustering of set of genes with significant fitness shift exclusively during TPP production and selection (right-most column fTpp^{CS}, +/- or red/cyan indicate significantly increased/decreased fitness value) (see also Sup. Fig. 2C). **C)** Extracellular TPP concentration from *E. coli* either untreated (-) or grown in presence of low (red bars, phe=20 μM, dpd=30 μM, thiU=100 μM) or high (5x) (cyan bars) concentrations of o-phenanthroline (phe and phe⁺), 2,2'-dipyridyl (dpd and dpd⁺) or thiourea (thiU or thiU⁺) (n = 4-5) (p: two-tailed Student's t-Test).

Figure 3. Diverse effects of Fe-S clusters metabolism

A) Venn diagram of sets of genes with significant fitness for each cultivation condition (p: the P-value of Fe-S metabolism enrichment for each condition found via mining gene annotations, *: significant, bootstrap n = 10000; null hypothesis: enrichments can occur with the same probability in a random set of genes of equal size). **B)** A map of the Fe-S biogenesis and regulatory network. Four sub-networks are represented: iron supply (*i*), sulfur supply (*ii*), redox regulation of oxyR-soxR regulons (*iii*) and Fe-S metabolism (*iv*) (orange/cyan arrowheads: improved/reduced fitness, small-size: 1-2 St. Dev. from mean, big-size: > 2 St. Dev. from mean) (see also Sup. Methods and Table 2).

Figure 4. TPP quantification in a set of genetic targets.

Extracellular titers for individual locus deletions compared to the average titers across all strains (fold difference, for absolute values see Sup. Fig. 5). Strains are grouped in three different sub-networks involved in Fe-S biogenesis: iron supply (**A**), sulfur supply (**B**) and oxidative regulation (**C**). (*: Test KO, fitness error bars are reported for multi-gene loci) (Error bars: St. Dev. n = 3-5).

Figure 5. Reverse-engineering OxyR and Fe-S regulation

Extracellular titers obtained by engineering central Fe-S cluster formation and the cellular stress response. (**A-B**) TPP titer measured in knockouts of central oxidative stress response in *E. coli* TOP10 (**A**) and BS134 (**B**) strains bearing pTHIm and pTHId production plasmids (respectively cyan and orange, pink dashed line: background strain) (n = 6-8). (**C**). Extracellular TPP titer quantified in *E. coli* BS134 over-expressing several identified genetic targets in Fe-S cluster metabolism and oxidative response (cyan bars) compared to their deletion (knockouts, orange bars) (Pink dashed line: average production) (n = 6-8).

Figure 1

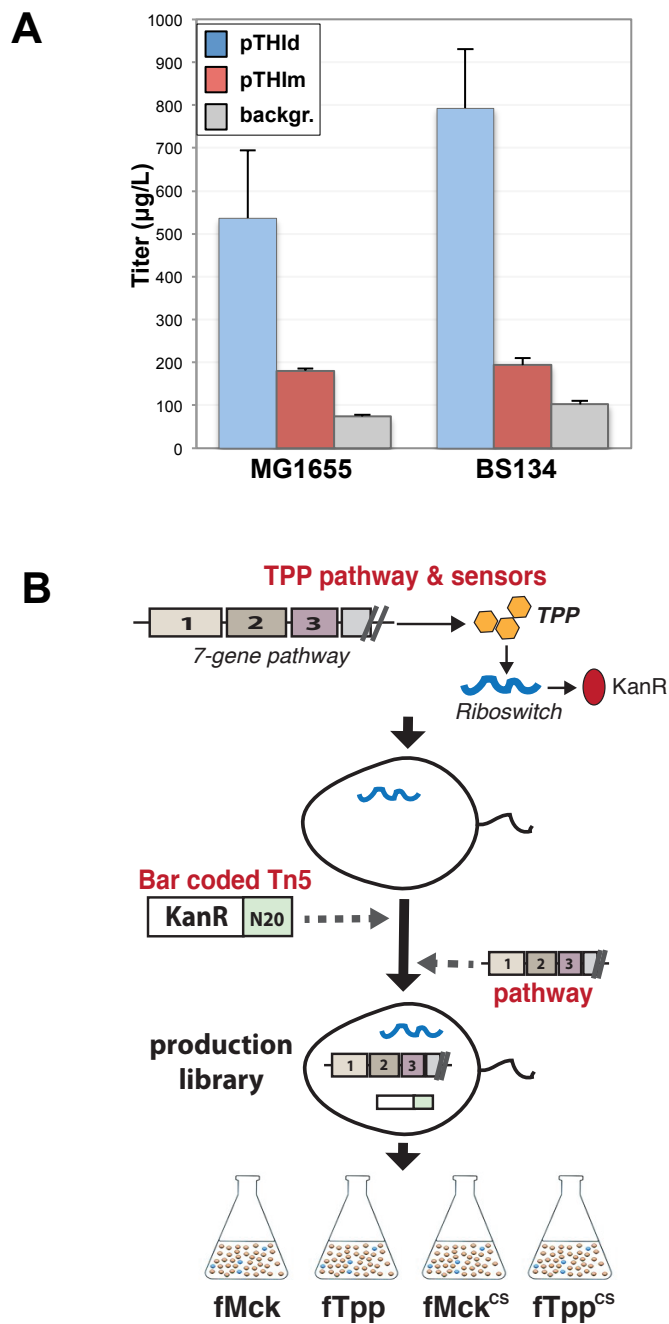


Figure 2

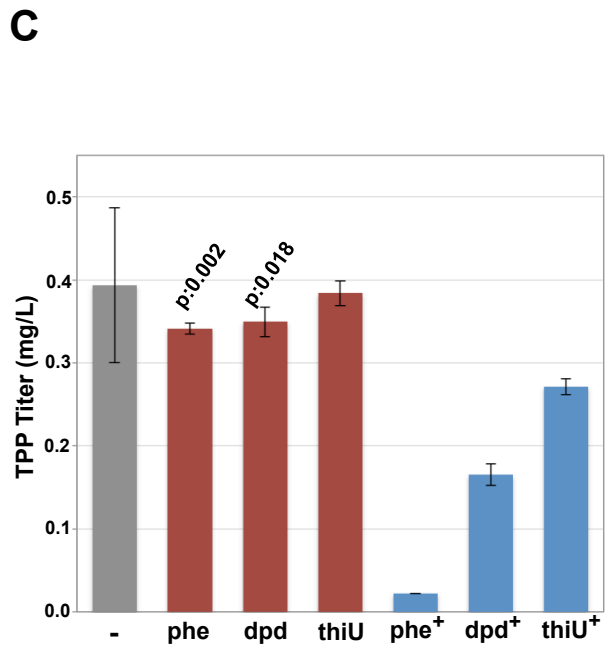
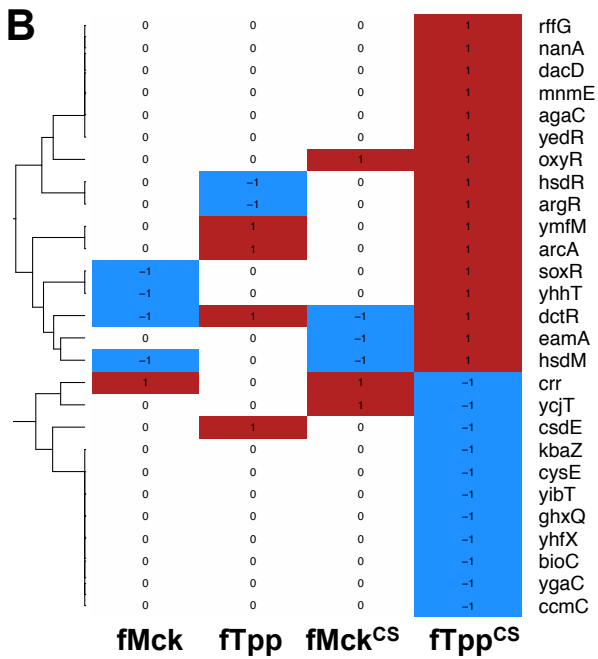
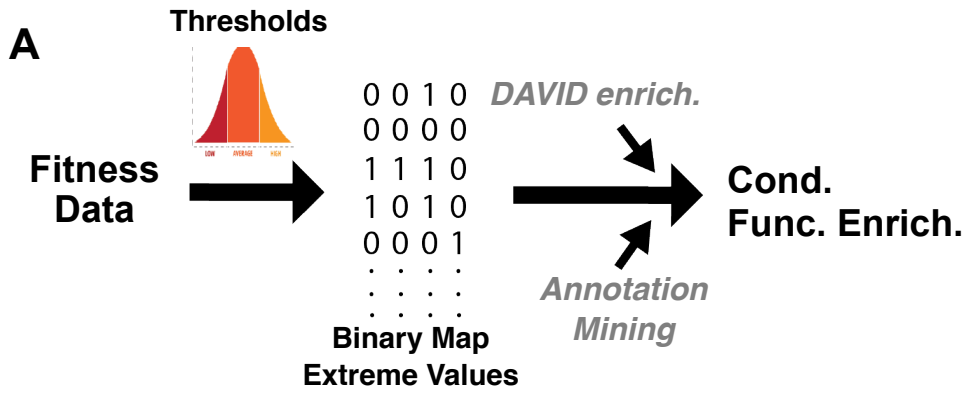


Figure 3

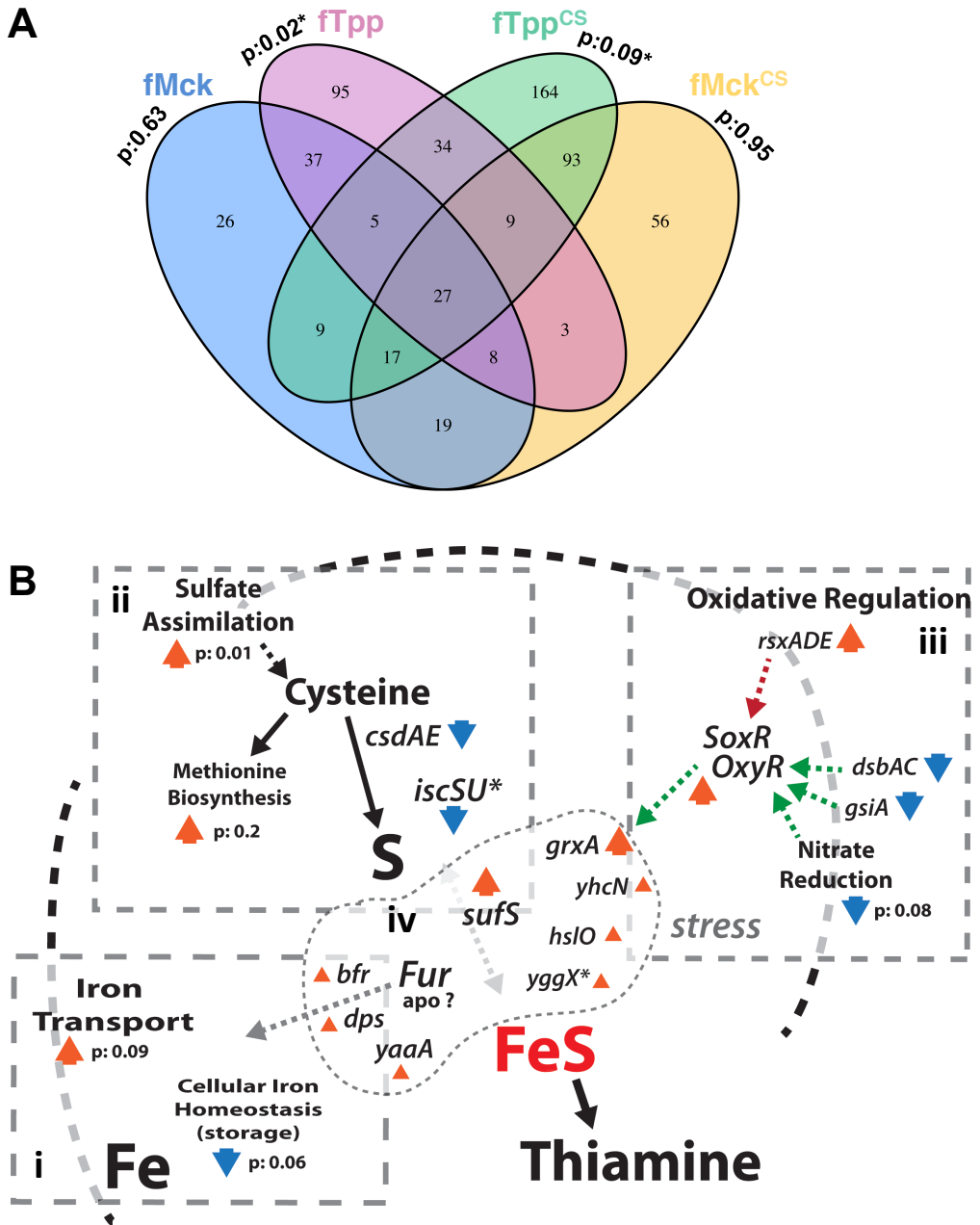


Figure 4

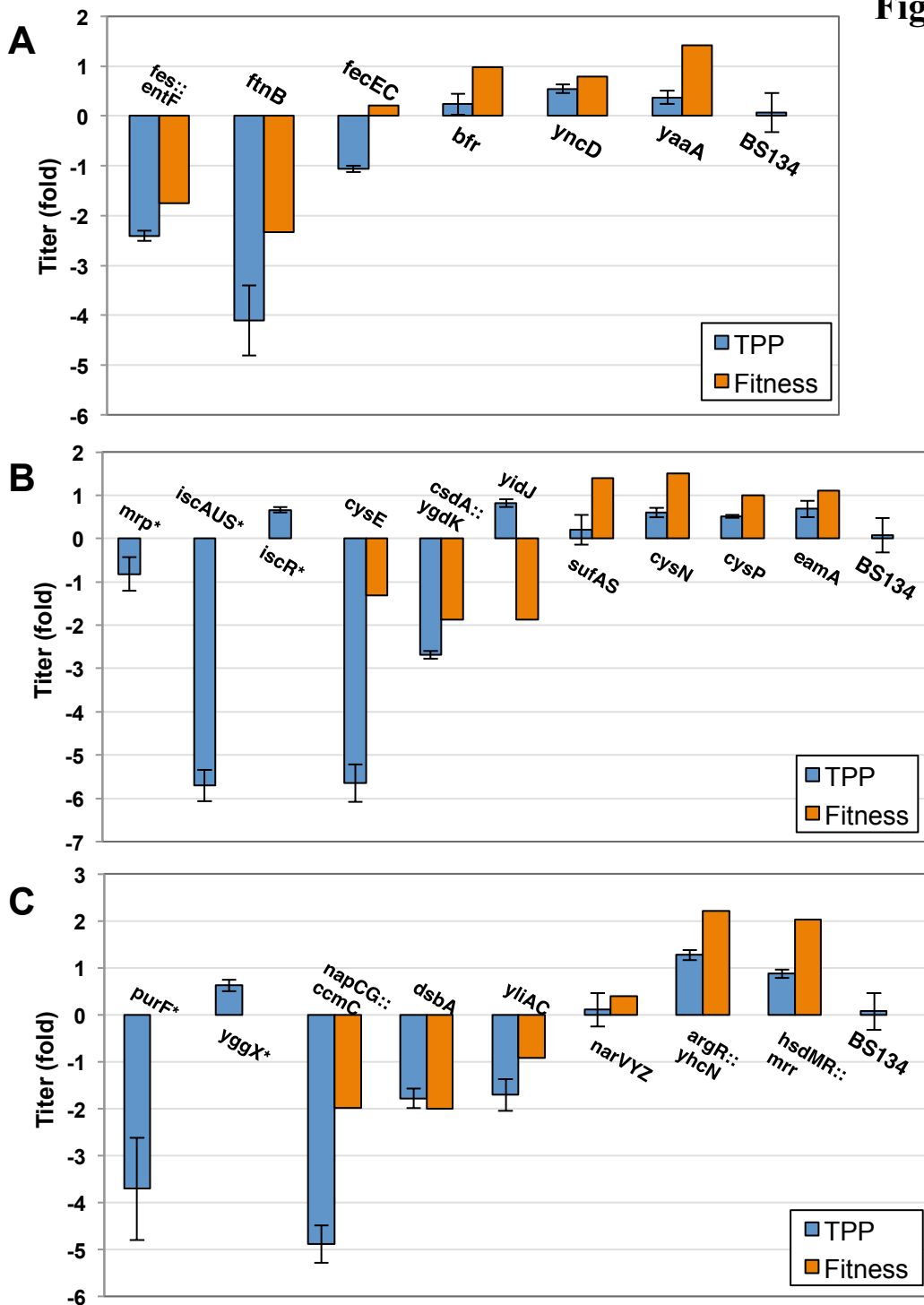


Figure 5

