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1 Robust characterization of two distinct glutarate sensing transcription factors of *Pseudomonas*
2 *putida* L-lysine metabolism

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32 **ABSTRACT**

33 A significant bottleneck in synthetic biology involves screening large genetically encoded
34 libraries for desirable phenotypes such as chemical production. However, transcription factor-
35 based biosensors can be leveraged to screen thousands of genetic designs for optimal chemical
36 production in engineered microbes. In this study we characterize two glutarate sensing
37 transcription factors (CsiR and GcdR) from *Pseudomonas putida*. The genomic contexts of *csiR*
38 homologs were analyzed and their DNA binding sites were bioinformatically predicted. Both
39 CsiR and GcdR were purified and shown to bind upstream of their coding sequencing *in vitro*.
40 CsiR was shown to dissociate from DNA *in vitro* when exogenous glutarate was added,
41 confirming that it acts as a genetic repressor. Both transcription factors and cognate promoters
42 were then cloned into broad host range vectors to create two glutarate biosensors. Their
43 respective sensing performance features were characterized, and more sensitive derivatives of the
44 GcdR biosensor were created by manipulating the expression of the transcription factor. Sensor
45 vectors were then reintroduced into *P. putida* and evaluated for their ability to respond to
46 glutarate and various lysine metabolites. Additionally, we developed a novel mathematical
47 approach to describe the usable range of detection for genetically encoded biosensors, which
48 may be broadly useful in future efforts to better characterize biosensor performance.

49

50 **KEYWORDS**

51 Biosensor, Transcription Factor, *Pseudomonas putida*, Glutarate, Monte Carlo Markov Chain

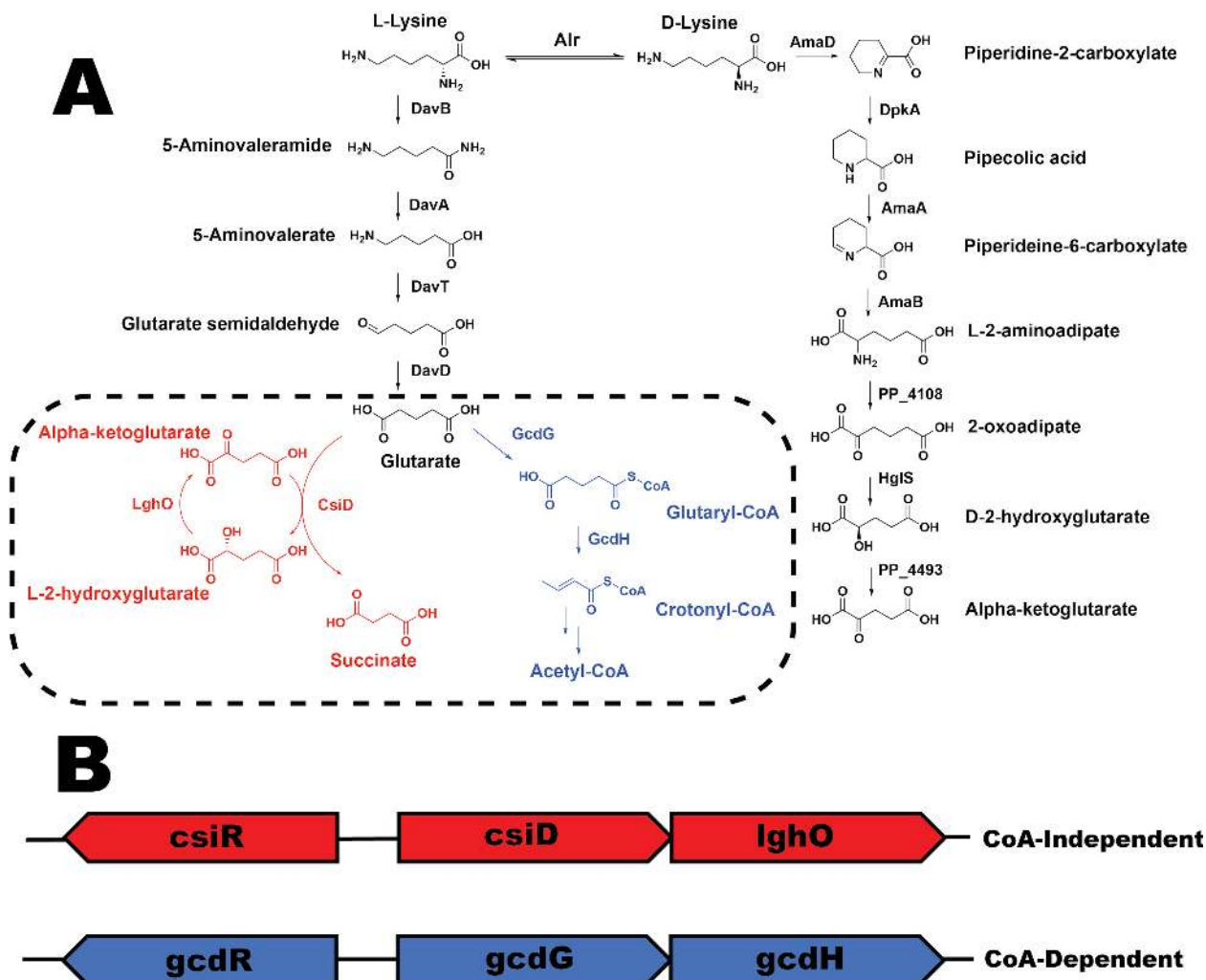
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53 **INTRODUCTION**

54 A rate limiting step in the design-build-test-learn cycle is often the test phase, wherein
55 hundreds or thousands of genetic designs need to be evaluated for their productivity^{1,2}. Though
56 recent advances in analytical chemistry have dramatically increased sample throughput³,
57 transcription factor-based biosensors still offer multiple advantages over traditional
58 chromatographic and mass-spectrometry based detection methods^{4,5}. One of the most attractive
59 benefits is the ability to rapidly screen constructs for the production of the target compounds via
60 either plate-based or flow-cytometry-based assays¹⁻³, which increases throughput by orders of
61 magnitude compared to mass-spectrometry based methods. Additionally, biosensors may offer
62 unmatched sensitivity towards specific ligands, with some sensors having picomolar affinity³.
63 The evolution of diverse microbial metabolism has provided researchers with the ability to sense
64 a wide array of ligands, ranging from complex natural products^{6,7} to small central metabolites
65^{1,8}.

66 Diacids, polyamines, and lactams are petrochemical derivatives used to produce various
67 polyester and nylon fibers^{9,10}. In an effort to make production of these chemicals sustainable,
68 many groups have developed engineered microbes to synthesize these precursors¹¹⁻¹⁴. The L-
69 lysine metabolism of *Pseudomonas putida* has been leveraged both in the native host and
70 heterologously to produce valerolactam¹⁵ the diacid glutarate^{16,17}. Recently, this utility has
71 inspired much work to uncover missing steps in the lysine catabolism of *P. putida*. These

72 missing steps included the discovery that glutarate is not only catabolized through the previously
 73 known coA-dependent route to acetyl-coA, but is also catabolized through a coA-independent
 74 route to succinate^{16,18} (Figure 1). Recent work has also demonstrated that both glutarate
 75 catabolic pathways are highly upregulated in the presence of glutarate¹⁸. The *Pseudomonas*
 76 *aeruginosa* homolog of the ketogenic pathway regulator (GcdR)¹⁹ and the *Escherichia coli*
 77 homolog of the glucogenic pathway regulator (CsiR) have both been characterized. Furthermore
 78 a rigorous investigation of the *P. putida* homologs has now been reported²⁰..



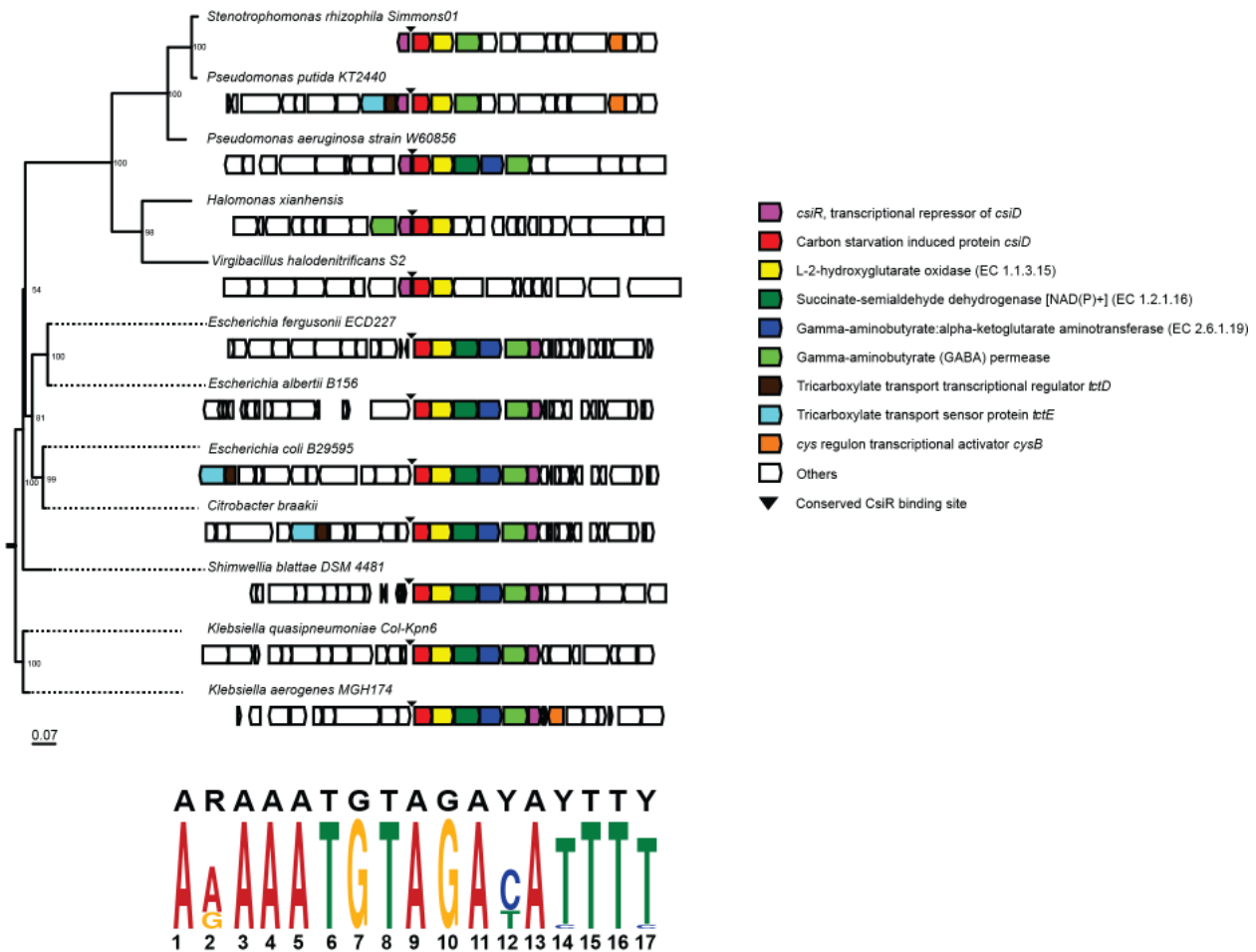
80 **Figure 1: A) The known lysine catabolism of *P. putida*. Dashed box shows the two known**
81 **pathways of glutarate catabolism in *P. putida*. Highlighted in red is the CoA-independent**
82 **route of glutarate catabolism, in blue the CoA-dependent route. Enzymes in the**
83 **metabolism are DavB - L-lysine monooxygenase, DavA - 5-aminopentanamidase, DavT - 5-**
84 **aminovalerate aminotransferase, DavD - glutaric semialdehyde dehydrogenase, CsiD -**
85 **glutarate hydroxylase, LghO - L-2-hydroxyglutarate oxidase, GcdG glutaryl-coA**
86 **transferase, GcdH - glutaryl-CoA dehydrogenase, Alr - alanine racemase, AmaD - D-lysine**
87 **oxidase, DpkA - Δ 1-piperidine-2-carboxylate reductase, AmaB - pipecolate oxidase, AmaA**
88 **- L-aminoadipate-semialdehyde dehydrogenase, PP_4108 - L-2-aminoadipate**
89 **aminotransferase, HglS - D-2-hydroxyglutarate synthase, PP_4493 - D-2-hydroxyglutarate**
90 **dehydrogenase. B) Operonic structure of the two routes of glutarate metabolism in *P.***
91 ***putida*.**

92 In this work we sought to also characterize the two putative local regulators of glutarate
93 catabolism in *P. putida*, *csiR* and *gcdR*. First, we compared the genomic context of *csiR*
94 homologs across bacteria to bioinformatically predict a conserved DNA binding site. We then
95 biochemically and genetically characterized both regulators. Secondly, we developed a novel
96 mathematical approach to rigorously determine the detection ranges for genetically encoded
97 biosensors that can be used to systematically compare biosensors. . Finally, we introduced RFP
98 transcriptional-fusions of the promoter for both catabolic pathways into *P. putida* and evaluated
99 their induction upon the addition of various lysine metabolites.

100 **RESULTS**

101 Genomic contexts of *csiR* and *gcdR* homologs and prediction of *P. putida* binding sites

102 Work in *Pseudomonas aeruginosa* has characterized the GcdR regulation of ketogenic
103 glutarate metabolism, and shown that the binding site is conserved across multiple bacterial
104 species¹⁹. While binding sites for CsiR in *E. coli* have been identified, it was yet to be
105 investigated whether there is a conserved binding site for homologs across bacterial species²¹. In
106 order to identify conserved binding sites of CsiR homologs, we compared the syntenic genomic
107 contexts of 12 selected genomes that contained neighboring *csiD* and *csiR* homologs. Genes
108 encoding *csiR* were found in two distinct genomic contexts, either transcribed divergently from
109 *csiD* as found in *P. putida*, or transcribed as the last gene in the *csiD* operon as in *E. coli* (Figure
110 2). The genomic regions upstream of the *csiD* homolog were extracted and Multiple EM for
111 Motif Elicitation (MEME) was used to identify a conserved CsiR binding motif²². A consensus
112 A(A/G)AAATCTAGA(C/T)ATTTT motif was identified upstream of each *csiD* homolog.
113 Previously, footprinting assays in *E. coli* BW25113 revealed two primary and two secondary
114 bindings sites with the sequences TTGTN₅TTTT and ATGTN₅TTTT respectively²¹. While this
115 manuscript was under review Zhang et al. demonstrated via DNase I footprinting that CsiR does
116 indeed binding at two locations upstream of *csiD*, including the conserved
117 A(A/G)AAATCTAGA(C/T)ATTTT motif²⁰. Our consensus motif agrees closely with the
118 secondary binding site identified, and highly suggests that the binding site of CsiR is conserved
119 across the bacteria where homologs are present.



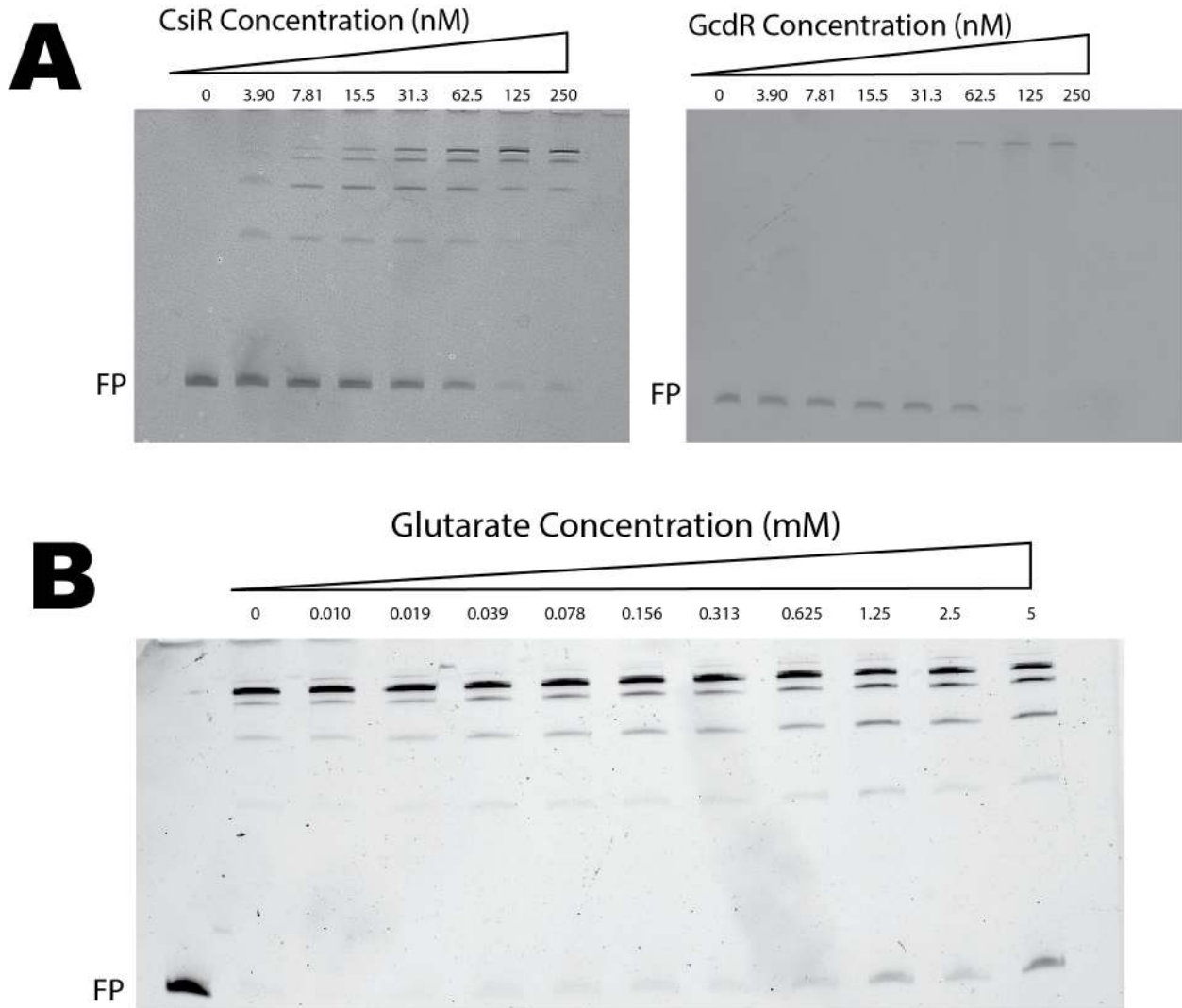
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121 **Figure 2: Genomic contexts of *csiR* homologs and predicted binding regions: Phylogeny of**
 122 **assorted gammaproteobacteria and the location of their *csiD* operons. MEME analysis of**
 123 **the intergenic regions upstream of the *csiD* operon resulted in the sequence motifs depicted**
 124 **below the tree.**

125 Biochemical characterization of CsiR and GcdR

126 To determine if the *P. putida* CsiR also acts as a regulator and to identify its putative
 127 binding sites, we biochemically characterized this protein using electrophoretic mobility shift
 128 assays (EMSAs). The CsiR protein was purified (Figure S1) and incubated with DNA probes
 129 consisting of the intergenic region between *csiR* and *csiD*. The assay showed multiple binding

130 sites in this intergenic region, as four distinct bands appeared. These results appear to confirm
131 previous work in *E. coli* in which four binding sites of the *E. coli* CsiR homolog were observed
132 ²¹. CsiR had a high affinity for the DNA probe, with a calculated K_d of approximately 30 nM
133 (Figure 3Aa), which is similar to the 10 nM CsiR/DNA K_d of the *E. coli* CsiR homolog, and
134 nearly identical to the results of Zhang et al. which showed complete probe shift at 6-fold molar
135 excess CsiR ^{20,21}. Purified GcdR (Figure S1) also bound to its cognate probe with a single
136 distinct shift and had an estimated K_d of approximately 62.5 nM (Figure 3A) ¹⁹. Again, these
137 results were consistent with Zhang et al., who showed near complete probe shifts at 8-fold molar
138 excess GcdR ²⁰. As CsiR is a GntR family transcriptional regulator, many of which act as
139 repressors ²³, we evaluated whether glutarate would decrease the DNA binding affinity of CsiR.
140 EMSA assays were repeated in the presence of increasing glutarate concentrations. Analysis by
141 gel electrophoresis revealed increasing quantities of free probe as glutarate concentrations
142 increased (Figure 3B). These results suggest that the *P. putida* CsiR is a glutarate-responsive
143 repressor of the *csiDlhgO* operon²¹. Zhang et al. also showed that in addition to dissociating from
144 the DNA probe at high concentrations of glutarate, CsiR was also responsive to 2-
145 hydroxyglutarate (2HG) ²⁰.



146

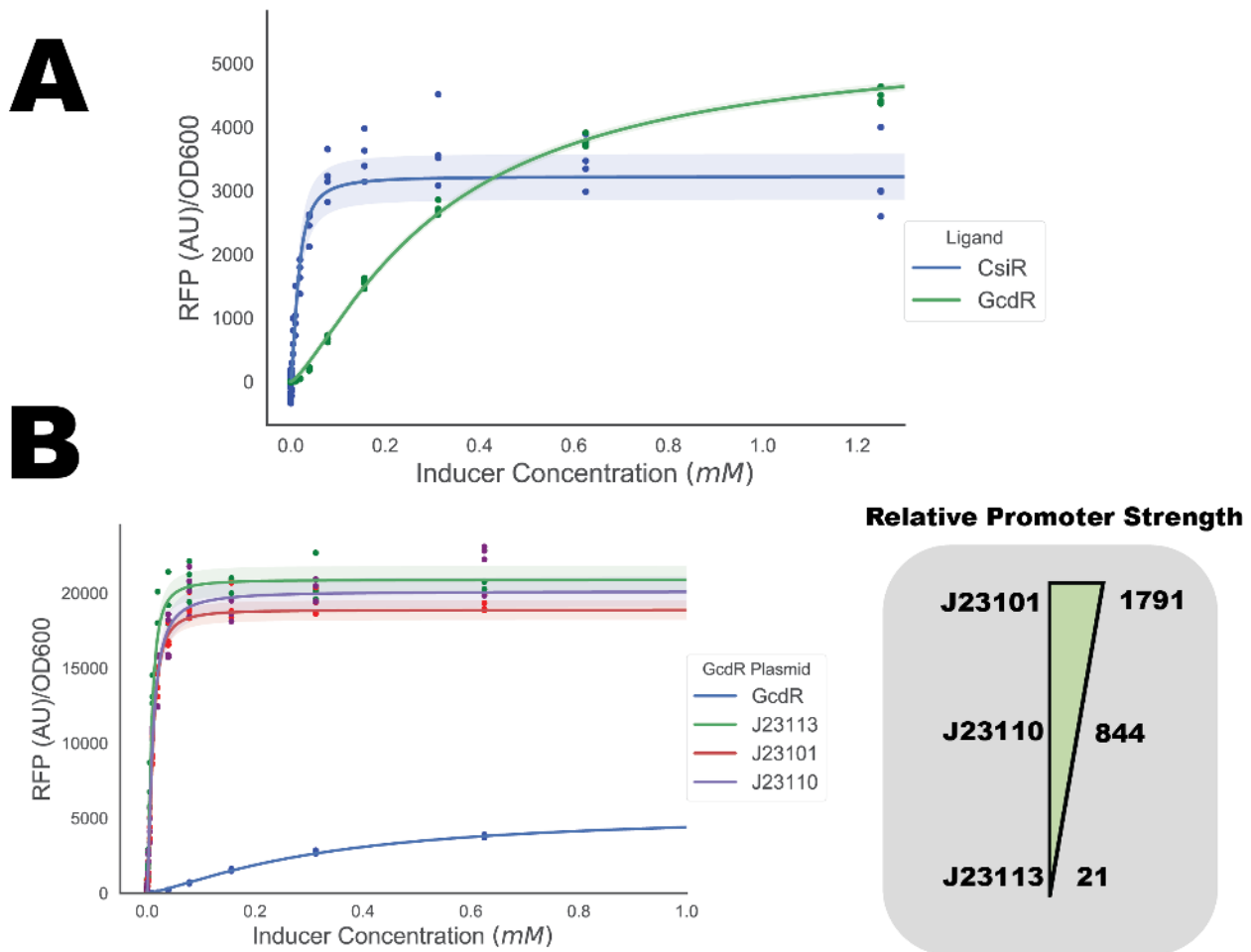
147 **Figure 3: Biochemical characterization of CsiR and GcdR. (A) EMSA of either purified**
148 **CsiR (left) or GcdR (right) incubated with 10 nM fluorescent probe of intergenic region**
149 **between *csiR* and *csiD*. Concentrations of CsiR protein range from 0 nM to 250 nM. FP**
150 **denotes free probe (B) EMSA showing 100 nM CsiR and 10 nM fluorescent probe**
151 **incubated with glutarate from concentrations of 0 mM to 5 mM. Far left lane shows free**
152 **probe with no CsiR. FP denotes free probe.**

153 Development of two glutarate biosensor vectors

154 In order to evaluate CsiR and GcdR as biosensors, we cloned both the regulator and
155 intergenic region of both the coA-independent and coA-dependent glutarate catabolism pathways
156 upstream of RFP on the broad host range vector pBADT. *E. coli* DH10B harboring either vector
157 were grown for 24 hours in EZ-Rich medium supplemented with concentrations of glutarate
158 ranging from 0.00015 mM to 2.5 mM, after which both OD600 and RFP fluorescence were
159 measured. At 2.5 mM glutarate, the GcdR and CsiR vectors demonstrated a maximal induction
160 over background of 55.5 and 1.5 times over uninduced cells, respectively; however, the CsiR
161 system showed considerable background signal (Table 1). Normalized RFP expression for each
162 sensor was fitted to the Hill equation to derive biosensor performance. The GcdR system was
163 found to have a Hill coefficient of 1.33, a K_d of 0.32 mM, and a maximum predicted normalized
164 RFP expression of 5403, while the CsiR system was shown to have a Hill coefficient of 1.61, a
165 K_d of 0.016 mM, and a maximum predicted normalized RFP expression of 3223 (Figure 4A). To
166 test for the ability of CsiR or GcdR to sense other diacids, *E. coli* harboring either vector were
167 grown in LB medium with 5 mM to 0.00015 mM of either succinate, adipate, or pimelate. While
168 pimelate and succinate were unable to induce either system, 2.5 mM adipate induced the CsiR
169 biosensor ~0.2x, and induced the GcdR system ~4x over background (Figure S2).

170 Given that GcdR showed a greater dynamic and substrate range with no response to 2-
171 HG, we tested whether the performance characteristics of these GcdR system could be altered.
172 The promoter region 50 bp upstream sequence of *gcdR* in the GcdR-sensing vector was replaced
173 by three different previously characterized constitutive promoters from the Anderson collection
174 (J23101, J23110, and J23113), representing a high (1791 RFP AU), medium (844 RFP AU), and
175 low (21 RFP AU) strengths²⁴. All of the engineered GcdR systems showed reduced limits of
176 detection and increased sensitivity to glutarate compared to natively regulated GcdR, with each

177 new vector showing a decreased K_d (J23113 : 0.008 mM, J23110 : 0.01 mM, J23101 : 0.01 mM)
178 (Figure 4B). All engineered GcdR vectors showed $\sim 4x$ the maximal expression compared to the
179 native system (Figure 4B), but also had nearly 30x greater basal RFP expression (Table 1).
180 While all three engineered GcdR vectors performed similarly, the limit of detection of J23110
181 was 5x less than the other two vectors, likely due to the lower basal expression of the vector
182 (Table 1).



183

184 **Figure 4: Development of glutarate responsive reporter vectors for *E. coli*. (A)**
185 **Fluorescence data fit to the Hill equation to derive biosensor performance characteristics**
186 **for native CsiR and GcdR systems. Points show individual experimental measurements.**

187 Shaded area represents (+/-) one standard deviation, n=4. (B) Fluorescence data fit to the
 188 Hill equation to derive biosensor performance characteristics for the engineered GcdR
 189 systems. Points show individual experimental measurements. Relative promoter strength is
 190 shown to the right. Shaded area represents (+/-) one standard deviation, n=4.

191 **Table 1: Biosensor performance parameters with standard deviations. Sensitivity: Hill**
 192 **coefficient of fitted data. Kd: Concentration of ligand to achieve half predicted maximal**
 193 **RFP expression. Max (Normalized RFP): Predicted maximal RFP expression after OD**
 194 **normalization and subtraction of uninduced expression. Dynamic range: Minimal and**
 195 **maximal experimental range of OD normalized RFP. Induction: Ratio of maximal**
 196 **experimental induction over basal expression. Inducer Range: Experimentally determined**
 197 **range of glutarate that can detected with biosensor.**

Report er	Sensitivi ty	Kd (mM)	Max (Normalized RFP)	Dynamic Range (RFP/OD600)	Induct ion	Inducer Range (mM)
CsiR-Native	1.61 (+/- 0.16)	0.016 (+/- 0.002)	3223 (+/- 72)	5704-8357	1.5	0.005-0.0195
GcdR-Native	1.33 (+/- 0.03)	0.32 (+/- 0.006)	5353 (+/- 38.5)	97-5403	55.5	0.01-2.5
GcdR-J23113	1.64 (+/- 0.09)	0.008 (+/- 0.0003)	20896 (+/- 184)	3272-24027	7.3	0.001-0.0195
GcdR-J23101	1.74 (+/- 0.07)	0.01 (+/- 0.0003)	18881 (+/- 131)	2428-20099	8.3	0.001-0.0195

GcdR- J23110	1.42 (+/- 0.09)	0.01 (+/- 0.0004)	20113 (+/- 214)	2113-20690	9.8	0.0002- 0.0195
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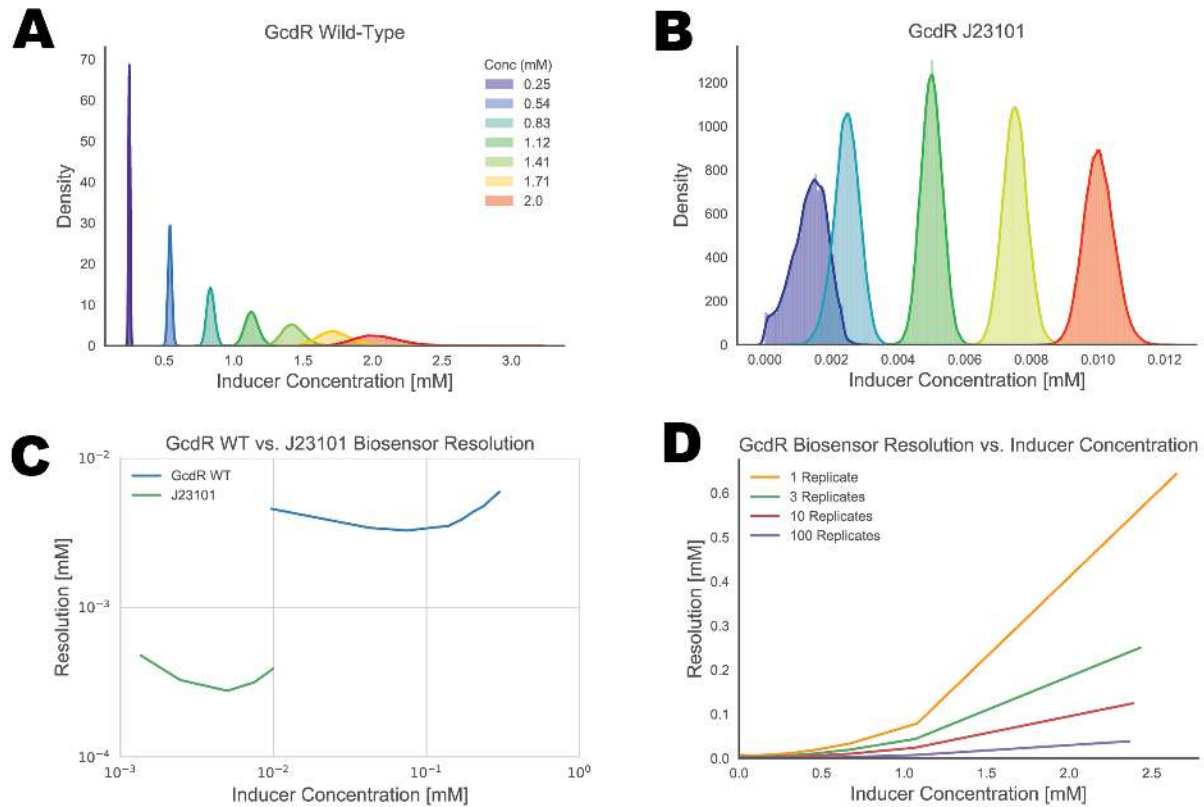
199 Developing metrics to quantify biosensor performance

200 In previously published work the mathematical basis for determining linear range or limit
201 of detection has often been obscure and non-systematic^{1,8,25}. Furthermore, a linear range may not
202 be able to adequately capture the performance characteristics of a biosensor over the range of
203 ligand concentrations where the sensor can still resolve differences. To address these
204 deficiencies, we sought to develop a mathematical method for evaluating the sensing
205 performances of genetically encoded transcription factors fitted to the Hill equation. Our
206 approach uses a probabilistic model to relate inducer concentration and corresponding
207 fluorescence measurements fit to the Hill equation assuming: (1) fluorescence measurements at a
208 particular concentration are normally distributed, (2) the variance of fluorescence measurements
209 is roughly constant over the range of measured values, and (3) the relationship between ligand
210 concentrations and fluorescence can be well modeled using the Hill function. This model allows
211 us to estimate the concentration of ligand compatible with our observed fluorescence data given
212 the variance of the data as determined via Markov Chain Monte Carlo (MCMC) sampling²⁶. A
213 detailed methodological description can be found in the supplemental Jupyter notebook, which
214 can be used to analyze other biosensor data.

215 By applying MCMC sampling to the model of our native GcdR biosensor, we can readily
216 produce the probability density functions (i.e. the probability that the ligand produces the
217 observed fluorescent response) of specific ligand concentrations (Figure 5A). At glutarate
218 concentrations of 0.25 mM, 0.68 mM, and 1.125 mM associated fluorescence values can be

219 resolved from one another (no overlap), however the biosensor is less able to resolve ligand
220 concentrations between 1.5 mM and 2 mM (Figure 5A). When we apply MCMC sampling to the
221 GcdR sensor being driven by the J23101 promoter, we observe that this system possesses the
222 resolution to distinguish between 0.004 mM and 0.011 mM, but is less able to distinguish
223 between higher concentrations (Figure 5B). A biosensor's resolution window, defined as the
224 width of the 95% prediction interval of inducer concentrations derived from a set of fluorescence
225 measurements, can then be expressed as a continuous function across a range of ligand
226 concentrations for a given biosensor (Figure 5C). Below concentrations of ~ 0.01 mM glutarate
227 the J23101 GcdR biosensor has greater resolution, while at higher concentrations the native
228 GcdR sensor system has greater resolution (Figure 5C). Another important aspect of our approach
229 is that it allows for the resolution window to be calculated as a function of the number of
230 replicates in a biosensor experiment. If either variance decreases or sample size increases, the
231 resolution of a biosensor also increases. By simulating sample sizes of 1 through 100 via MCMC
232 sampling, the theoretical resolution of the native GcdR dramatically increases (Figure 4D). This
233 "power" analysis may serve as a guide for experimental design when a certain biosensor
234 resolution is required for a given application. We believe this approach may be generally useful
235 to any dataset derived from fluorescent transcription factor based biosensors. To demonstrate this
236 we also applied our MCMC methodology to two well characterized BglBrick vectors, pBbBSk-
237 rfp and pBbBEk-rfp, which express RFP from arabinose-inducible vectors from SC101 or ColE1
238 origins respectively (Figure S3). Fluorescence data from each vector (Figure S3A), was fit to the
239 Hill question (Figure S3B), and demonstrated that both vectors had the highest resolution at an
240 arabinose concentration of $\sim 0.1\%$ w/v (Figure S3C).

241



242

243 **Figure 5: Development of analytics for biosensor performance. Probability density**
244 **functions of fluorescence values compatible with concentrations of glutarate for selected**
245 **ligand concentrations modelled to the Hill equation of the native GcdR system (A) or the**
246 **GcdR J23101 system (B). (C) Resolution of native GcdR or GcdR J23101 biosensor systems**
247 **over a select range of glutarate concentrations. (D) Theoretical resolution of the GcdR**
248 **native biosensor with differing number of replicates.**

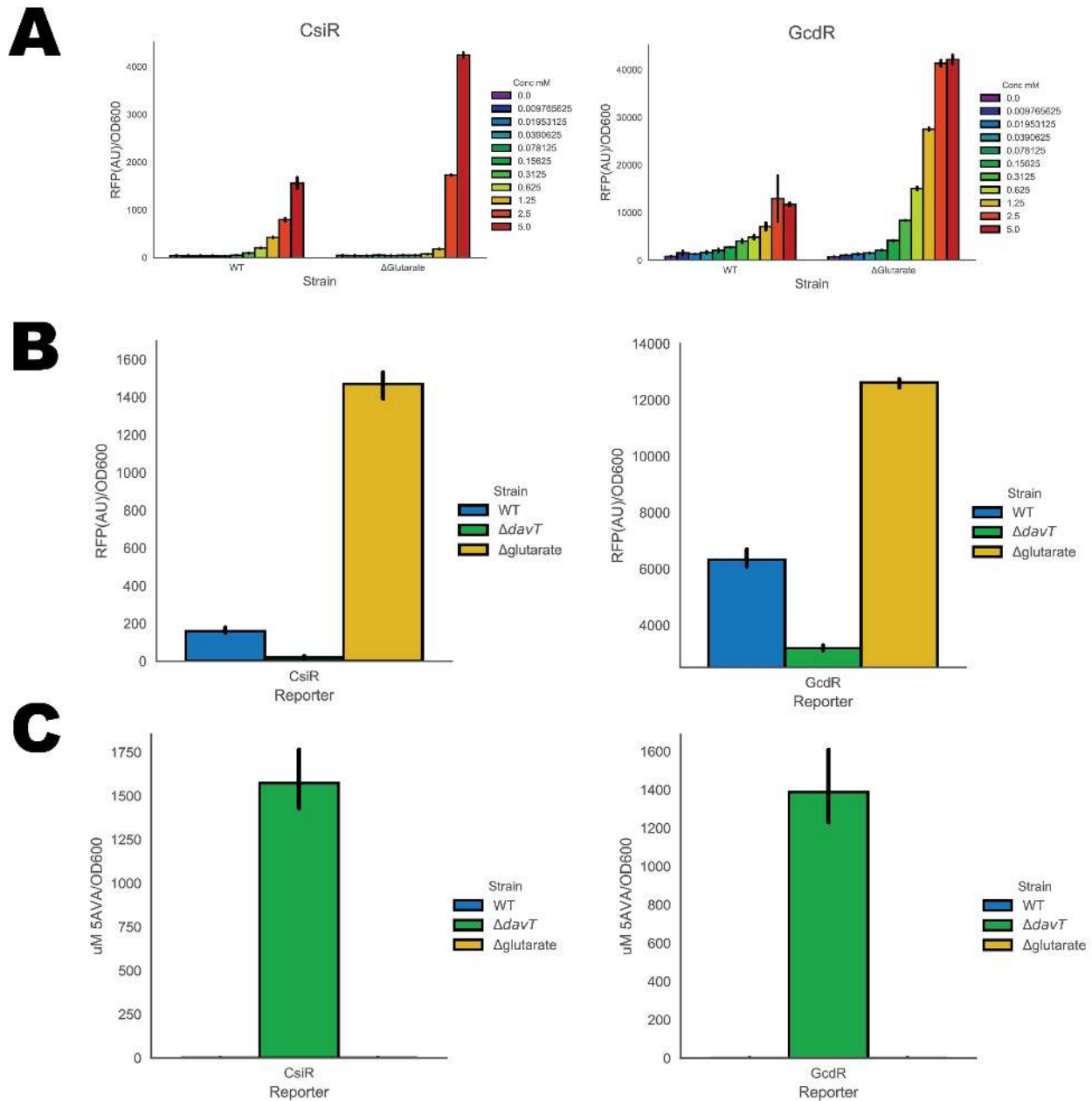
249 Responsiveness of glutarate biosensors to lysine metabolites in *P. putida*

250 To assess the ability of these vectors to function in *P. putida* both plasmids were
251 introduced into either wild type *P. putida* or a strain with both known pathways of glutarate
252 catabolism deleted (Δ *csiD* Δ *gcdH* - referred to as Δ glutarate). The resulting strains were grown

253 in MOPS minimal medium supplemented with 10 mM glucose and glutarate ranging from 5 mM
254 to 0.01 mM for 24 hours. Both vectors responded to increased concentrations of exogenously
255 applied glutarate, though the GcdR vector had ~10x greater fluorescence than the CsiR vector
256 (Figure 6A). The Δ glutarate strain showed increased levels of RFP induction using both the CsiR
257 and GcdR systems, suggesting both vectors are able to sense increased levels of glutarate (Figure
258 6A). To further examine the ability of these vectors to probe *P. putida* lysine metabolism, both
259 were also introduced into a Δ *davT* strain, which is unable to metabolize 5-aminovalerate to
260 glutarate semialdehyde (Figure 1A) and therefore precludes glutarate production. When wild
261 type, Δ *davT*, and Δ glutarate strains harboring either the CsiR or GcdR systems were grown on
262 minimal medium supplemented with 10 mM glucose and 10 mM 5-aminovalerate, both vectors
263 in the Δ *davT* strain showed decreased fluorescence compared to wild type, while vectors in the
264 Δ glutarate strains showed increased fluorescence (Figure 6B). Measurement of intracellular 5-
265 aminovalerate showed significant pools of the metabolite in the Δ *davT* strains (~1500
266 μ M/OD600) with no detectable 5-aminovalerate in the other genetic backgrounds (Figure 6C).
267 These results highly suggest that both GcdR and CsiR are insensitive to 5-aminovalerate, an
268 essential feature of these sensors if they are to be used in organisms that derive glutarate from a
269 5-aminovalerate precursor.

270 To evaluate the ability of both reporter vectors to monitor the catabolism of other lysine
271 metabolites, wild-type *P. putida* harboring either GcdR or CsiR were grown in minimal media
272 supplemented with either 10 mM glucose, L-lysine, D-lysine, 5-aminovalerate, or 2-
273 amino adipate for 48 hours with OD₆₀₀ and RFP fluorescence being measured continuously
274 (Figure S4A and Figure S4B). Neither vector was induced when the bacterium was grown on
275 glucose (Figure S4). The GcdR vector was strongly induced when grown on 5-aminovalerate,

276 and to a lesser extent 2-aminoadipate, D-lysine, and L-lysine (Figure S4A). Conversely, the
277 strain harboring the CsiR vector only displayed induction of RFP above background when grown
278 on 5-aminovalerate (Figure S4B). While 5-aminovalerate was able to induce RFP induction in
279 the strain harboring the CsiR vector, induction was extremely limited compared to induction of
280 RFP from the GcdR vector (Figure S4).



281

282 **Figure 6: Performance of CsiR and GcdR biosensors in *P. putida*. (A) RFP expression of**
283 **either wild type *P. putida* or *P. putida* with the ability to metabolize glutarate knocked out,**
284 **measured with either the CsiR or GcdR biosensor under different external glutarate**
285 **concentrations. Error bars represent 95% CI, n=4 (B) RFP expression of wild type, $\Delta davT$,**
286 **or $\Delta glutarate$ strains of *P. putida* harboring either the CsiR or GcdR biosensor when grown**
287 **on 10 mM glucose and 10 mM 5-aminovalerate (5AVA). Error bars represent 95% CI,**
288 **n=3. (C) Intracellular concentration of 5-aminovalerate of wild type, $\Delta davT$, or $\Delta glutarate$**
289 **strains of *P. putida* harboring either the CsiR or GcdR biosensor when grown on 10 mM**
290 **glucose and 10 mM 5-aminovalerate (5AVA). Error bars represent 95% CI, n=3.**

291 DISCUSSION

292 Recent advances in high-throughput functional genomics have allowed researchers to
293 rapidly identify novel metabolic pathways, and in turn infer the function of novel transcription
294 factors^{27,28}. Rigorous characterization of these regulators is a critical step to developing novel
295 parts for synthetic biology as well as useful tools for metabolic engineering. The discovery of
296 two distinct pathways for glutarate catabolism within *P. putida*, regulated by independent
297 transcription factors, presents an interesting opportunity to compare and contrast the relative
298 sensing properties of each system.

299 CsiR and GcdR homologs have now been characterized thoroughly in multiple bacteria,
300 and have demonstrated that CsiR acts as a repressor of *csiD* whereas GcdR acts as a positive
301 regulator of *gcdHG*¹⁹⁻²¹. Our bioinformatic analyses suggest that the binding site of CsiR is
302 highly conserved amongst bacteria that possess the regulator (Figure 2). While our CsiR
303 biosensor was shown to have a lower limit of detection of glutarate than the unengineered GcdR
304 biosensor (Table 1), Zhang et al. demonstrated that CsiR is also responsive to 2-HG²⁰. Given

305 that the GcdR biosensor can readily be engineered to have lower limits of detections, it is likely
306 the better choice to detect glutarate via a genetically encoded transcription factor.

307

308 Multiple works have demonstrated that engineering changes to the expression of a
309 transcription factor, the responsiveness of the transcriptional output can also be changed^{31,32}.
310 By engineering the promoter of the *gcdR* transcription factor, the sensitivity of all the vectors
311 were increased from ~1.3 to ~1.7, while the K_d was lowered from 0.32 mM to ~0.01 mM (Figure
312 4B). This sensitivity to glutarate may make this vector useful in prototyping novel routes to
313 biological production of the C5 diacid. However at high concentrations of adipate (>1.25 mM)
314 GcdR is also activated, which may confound results (Figure S2).

315 While our preliminary work with exogenously applied ligand is promising, further work
316 remains to be done to evaluate the ability of these sensors to detect flux of glutarate in living
317 cells. Multiple recent publications have shown that glutarate metabolism is widespread in
318 bacteria, and evaluating the ability of the CsiR and GcdR sensors to measure this flux will
319 require careful experimentation with C^{13} labelled substrates. Another possible confounding
320 factor in utilizing these vectors is the presence of CsiR or GcdR binding sites in the host
321 organism. The overexpression of either transcription factor may misregulate host metabolism.
322 This hypothesis is supported by the differences in growth observed between GcdR and CsiR
323 biosensor containing *P. putida* when grown on lysine metabolites (Figure S4).

324 The overarching goal of synthetic biology is to apply engineering principles to biological
325 systems so that outcomes of genetic manipulation can become more predictable and repeatable
326^{33,34}. While there has been a large body of work devoted to the characterization and application
327 of biosensors, there has been conspicuously few attempts to rigorously describe at which ligand

328 concentrations a biosensor is useful. Often the analysis merely states limit of detection, and a
329 ‘linear range’ with little in terms of a mathematical justification. Here we present an alternative
330 metric that allows for the calculation of ligand resolution across the entire range of detection for
331 a biosensor. By leveraging a MCMC approach to predicting ligand ranges compatible with
332 fluorescence values, researchers can more precisely describe a biosensors performance and
333 identify whether a given biosensing system is potentially useful for a given engineering task. The
334 MCMC approach also allows for the simulation of an increasing number of replicates, which
335 could inform the researcher of the replicates that may be required in an experimental design to
336 achieve a desired level of resolution. We hope that this initial work to better characterize
337 biosensor performance inspires other groups to develop even more sophisticated methods of
338 analysis.

339 In addition to their utility as biosensors for metabolic engineering, these sensors may be a
340 valuable tool in studying the carbon utilization in the native host *P. putida*. Work conducted here
341 demonstrates the ability of both CsiR and GcdR sensors to distinguish between glutarate
342 accumulating and mutants blocked in their ability to metabolize 5-aminovalerate to glutarate
343 (Figure 6). Lysine metabolism in *P. putida* is isomer specific, with each isomer being degraded
344 by a separate catabolic pathway¹⁸. While cross-feeding between the pathways has been proposed
345 previously³⁵, recent work by our group has proposed a molecular mechanism for metabolite
346 exchange between the D- and L- catabolic pathways¹⁸. The exchange relies on the 2-oxoacid
347 promiscuity of the non-heme Fe(II) oxidase CsiD, which normally catalyzes the hydroxylation of
348 glutarate using 2-ketoglutarate as a cosubstrate to yield 2-hydroxyglutarate and succinate. CsiD
349 can also use 2-oxoadipate, a D-lysine catabolic intermediate, as a 2-oxoacid cosubstrate to yield
350 2-hydroxyglutarate and glutarate as products. The glutarate from this reaction could then proceed

351 down the L-lysine catabolic pathway. When 2-aminoadipate was fed into *P. putida* harboring the
352 GcdR vector, fluorescence was observed in stationary phase. As 2-aminoadipate immediately
353 precedes 2-oxoadipate in the D-lysine catabolic pathway, these results support the hypothesis
354 that CsiD could act as a bridge between the two catabolic pathways. There has been substantial
355 interest in developing microbes to produce glutarate, with strains of *E. coli*, *P. putida*, and
356 *Corynebacterium glutamicum* all engineered to produce high titers^{16,36,37}. Further engineering of
357 the GcdR system may be able to extend the resolvable range to higher concentrations, furthering
358 its utility as a tool to achieve even higher titers of glutarate. Though glutarate is a valuable
359 commodity chemical, the C6 diacid adipate is used in much greater quantities primarily as a
360 monomer used to make nylons 33. This slight sensitivity of GcdR toward adipic acid is
361 especially interesting, as recent work has demonstrated the effectiveness of evolving
362 transcription factors to sense non-native ligands⁶. Such methods could be applied to GcdR in
363 order to expand its utility in sensing other industrially important diacids.

364 **METHODS**

365 Media, chemicals, and culture conditions

366 *E. coli* cultures were grown in Luria-Bertani (LB) Miller medium (BD Biosciences,
367 USA) at 37 °C while *P. putida* was grown at 30 °C. When indicated, *P. putida* and *E. coli* were
368 grown on modified MOPS minimal medium³⁹. Cultures were supplemented with kanamycin
369 (50 mg/L, Sigma Aldrich, USA), gentamicin (30 mg/L, Fisher Scientific, USA), or carbenicillin
370 (100 mg/L, Sigma Aldrich, USA), when indicated. All other compounds were purchased through
371 Sigma Aldrich (Sigma Aldrich, USA) .

372 Strains and plasmids

373 All bacterial strains and plasmids used in this work are listed in Table 3. All strains and
374 plasmids created in this work are available through the public instance of the JBEI registry.
375 (<https://public-registry.jbei.org/folders/390>). All plasmids were designed using DeviceEditor and
376 VectorEditor software, while all primers used for the construction of plasmids were designed
377 using j5 software⁴⁰⁻⁴². Plasmids were assembled via Gibson Assembly using standard protocols
378⁴³, or Golden Gate Assembly using standard protocols⁴⁴. Plasmids were routinely isolated using
379 the Qiaprep Spin Miniprep kit (Qiagen, USA), and all primers were purchased from Integrated
380 DNA Technologies (IDT, Coralville, IA). Construction of *P. putida* deletion mutants was
381 performed as described previously¹⁸.

382 Expression and purification of proteins

383 Proteins were purified as described previously⁴⁵. Briefly, 500 mL cultures of *E. coli*
384 BL21 (DE3) harboring expression plasmids were grown in LB medium at 37 °C to an OD of 0.6
385 then induced with 1mM isopropyl β-D-1-thiogalactopyranoside. Cells were allowed to express
386 for 18 hours at 30 °C before being harvested via centrifugation. Cell pellets were stored at -80 °C
387 until purification. Cell pellets were then resuspended in lysis buffer (50 mM sodium phosphate,
388 300 mM sodium chloride, 10 mM imidazole, 8% glycerol, pH 7.5) and sonicated to lyse cells.
389 Insolubles were pelleted via centrifugation (30 minutes at 40,000 x g). The supernatant was
390 applied to a fritted column containing Ni-NTA resin (Qiagen, USA) which had been pre-
391 equilibrated with several column volumes of lysis buffer. The resin was washed with lysis buffer
392 containing 50 mM imidazole, then the protein was eluted using a stepwise gradient of lysis
393 buffer containing increasing imidazole concentrations (100 mM, 200 mM, and 400 mM).
394 Fractions were collected and analyzed via SDS-PAGE. Purified proteins were concentrated using

395 Spin-X UF 20 (10 kDa MWCO) spin concentrators (Corning, Inc.). Concentrated protein was
396 stored at 4 °C until *in vitro* analysis.

397 Plate based growth and fluorescence assays in *P. putida*

398 Growth studies of bacterial strains were conducted via microplate reader kinetic assays.
399 Overnight cultures were inoculated into 10 mL of LB medium from single colonies, and grown
400 at 30 °C. These cultures were then washed twice with MOPS minimal medium without any
401 added carbon and diluted 1:100 into 500 uL of MOPS medium with 10 mM of a carbon source in
402 48-well plates (Falcon, 353072). Plates were sealed with a gas-permeable microplate adhesive
403 film (VWR, USA), and then optical density and fluorescence were monitored for 48 hours in a
404 Biotek Synergy 4 plate reader (BioTek, USA) at 30 °C with fast continuous shaking. Optical
405 density was measured at 600 nm, while fluorescence was measured using an excitation
406 wavelength of 485 nm and an emission wavelength of 620 nm with a manually set gain of 100.

407 Transcriptional fusion fluorescence assays

408 To measure RFP production in *E. coli*, fluorescence measurements were obtained from
409 single time points of cells grown in deep-well 96-well plates as described previously with minor
410 changes⁴⁶. Briefly, cells were grown in 500 µL of EZ-Rich medium supplemented with
411 kanamycin and a range of glutarate concentrations from 5 mM to 0 mM. Plates were sealed with
412 AeraSeal film (Excel Scientific, AC1201-02) and grown for 22 hours at 37 °C on a 200 rpm
413 shaker rack. After incubation, 100 µL from each well was aliquoted into a black, clear-bottom
414 96-well plate and fluorescence was measured with a Tecan Infinite F200 plate reader (Tecan,
415 USA). Optical density was measured at 600 nm, while fluorescence was measured using an
416 excitation wavelength of 535 nm and an emission wavelength of 620 nm with a manually set

417 gain of 60. Endpoint fluorescence assays in *P. putida* were carried out in LB media, and
418 measured by the same method.

419 Electrophoretic mobility shift assays

420 Electrophoretic mobility shift assays were performed as previously described⁷. 6-
421 Carboxyfluorescein labelled PCR products for CsiR probes were generated from the intergenic
422 region between PP_2908 and PP_2909 using primers csiRprobeFOR 5'-6-
423 FAM/AGTTCGATCTGCGTAAAG-3' and csiRprobeREV 5'-CCCGCTGAATGCTGAGTT-
424 3'), while probes for GcdR were generated from the intergenic region between PP_0157 and
425 PP_0158 with primers gcdRprobeFOR 5'-6-FAM/CGGGTCGATCCAGTTGAAA-3' and
426 gcdRprobeREV 5'-GCATGTACGTCAACCTCACT-3'. Primers were purchased from IDT
427 Technologies (IDT, Coralville, IA). PCR product was then purified with a QIAquick PCR
428 Purification Kit (Qiagen, USA), and the amount of DNA was quantified via a NanoDrop 2000C
429 (Thermo Fisher Scientific). Binding reactions were conducted with 10 ng of labelled probe,
430 which was added to 10 mM Tris-HCl (pH 8.0), 25 mM KCl, 2.5 mM MgCl₂, 1.0 mM DTT and
431 2 ug salmon sperm DNA in 20 uL reactions. CsiR was added to reactions in concentrations
432 ranging from 250 nM to ~4 nM, in addition to a control reaction without CsiR, and then allowed
433 to incubate at 22 °C for 20 minutes. Reactions were then loaded into 10% native polyacrylamide
434 gels buffered with 0.5x TBE. Afterwards, electrophoresis gels were imaged on an Alpha
435 Innotech MultiImage III (Alpha Innotech). Adobe Photoshop was used to average pixel intensity
436 over the entire band on EMSA gels in order to estimate the K_d.

437 Measurement of 5-aminovalerate

438 To measure intracellular concentrations of 5-aminovalerate, cells were quenched as
439 previously described⁴⁷. LC/MS analysis was performed on an Agilent 6120 single quadrupole

440 LC/MS equipped with a Waters Atlantis Hilic 5 μ M Silica column (4.6 x 150 mm). A linear
441 gradient of 100-30% 90:10 CH₃CN:H₂O with 10 mM ammonium formate and 0.1% formic acid
442 (v/v) over 20 min in 90:10 H₂O:CH₃CN with 10 mM ammonium formate and 0.1% formic acid
443 (v/v) at a flow rate of 1.0 mL/min was used. Extracted ion chromatograms were integrated and
444 peak area was used to construct a standard curve using an authentic 5-aminovalerate standard.
445 Concentrations of 5-aminovalerate within samples were interpolated from this curve.

446 Analysis of biosensor parameters

447 A model relating inducer concentrations and fluorescence measurements to characterize
448 the performance of a biosensor was generated under the following assumptions 1) the
449 relationship between analyte concentrations and fluorescence can be well modeled using the Hill
450 equation 2) fluorescence measurements at a particular concentration are normally distributed 3)
451 the variance of fluorescence measurements is roughly constant over the range of measured
452 values.

453 Under these assumptions we can phrase the following probabilistic model via equation 1
454 (Figure 7). Using the probabilistic model which captures our constraints on the problem the log
455 likelihood function is expressed as equation 2 (Figure 7). The log likelihood is used to express the
456 maximum likelihood estimation (MLE) problem as equation 3 (Figure 7), which when solved
457 results in the optimal parameters of the model given the characterization data. In order to
458 estimate the distribution of ligand concentrations that are compatible with experimental
459 fluorescence data, MCMC sampling was used to solve the MLE problem equation 4 (Figure 7).
460 We determined biosensor resolution by solving the above maximum likelihood estimation
461 problem iteratively over the range of observed fluorescences during the biosensor
462 characterization process. This can determine the relationship between an inducer concentration

463 estimate and the estimated standard deviation. The standard deviation of the estimate of inducer
464 concentration can be interpreted as the resolution window. In the case of this work, two standard
465 deviations is considered the resolution window of the sensor, as 95% of the compatible inducer
466 concentration estimates fall within the interval.

467 To determine minimal and maximal levels of ligand detection of a given biosensor, the
468 minimal detection limit was defined as the minimal concentration of inducer that resulted in a
469 OD600 normalized RFP value significantly (t-test pval <0.05) greater than that of uninduced
470 cultures, while the maximal detection limit was defined as the greatest concentration of inducer
471 that resulted in a OD600 normalized RFP value significantly (t-test pval <0.05) less than that of
472 cultures induced with the highest concentration of ligand experimentally tested (2.5 mM).

473 A comprehensive methodological description of calculating biosensor performance
474 parameters, as well as usable Jupyter notebooks can be found at
475 https://github.com/JBEI/biosensor_characterization_public.

Equation 1

$$P(F|C, \theta, \alpha) = \mathcal{N}(h_{\theta}(C), \sigma)$$

Equation 2

$$\ell(\theta, \sigma | \mathcal{D}) = \sum_{i=1}^N \log P(F = f_i | C = c_i, \sigma, \theta).$$

Equation 3

$$\hat{\theta}, \hat{\sigma} = \arg \max_{\theta, \sigma} \ell(\theta, \sigma | \mathcal{D}),$$

Equation 4

$$\hat{c} = \arg \max_c \sum_{i=1}^N \log P(F = f_i | C = c, \hat{\theta}, \hat{\sigma})$$

476

477 **Figure 7: Equations used in this study for MCMC analysis of biosensor data.**

478 Bioinformatic analysis

479 For the phylogenetic reconstructions, the best amino acid substitution model was selected
480 using ModelFinder as implemented on IQ-tree⁴⁸, phylogenetic trees were constructed using IQ-
481 tree, and nodes were supported with 10,000 bootstrap replicates. The final tree figures were
482 edited using FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>). Orthologous syntenic
483 regions of CsiR were identified with CORASON-BGC⁴⁹ and manually colored and annotated.
484 DNA-binding sites were predicted with MEME²².

485 **SUPPORTING INFORMATION**

486 The Supporting Information is available free of charge on the ACS Publications website.

487 S1.pdf contains supplementary figures.

488

489

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513 **Contributions**

514 Conceptualization, M.G.T.; Methodology, M.G.T., Z.C., J.M.B, P.C.M., N.H., W.S. Investigation,
515 M.G.T., Z.C., J.M.B, R.N.K, P.C.M, M.R.I., A.N.P.; Writing – Original Draft, M.G.T, M.R.I.;
516 Writing – Review and Editing, All authors.; Resources and supervision, J.D.K, P.S., H.M.

517 **Competing Interests**

518 J.D.K. has financial interests in Amyris, Lygos, Demetrix, Napigen, and Maple Bio.

519 **Table 2. Strains and Plasmids used in this work**

Strain	JBEI Part ID	Reference
<i>E. coli</i> DH10B		50
<i>E. coli</i> BL21(DE3)		Novagen
<i>E. coli</i> S17		ATCC 47055
<i>P. putida</i> KT2440		ATCC 47054
<i>P. putida</i> Δ <i>davT</i>	JPUB_013544	This work
<i>P. putida</i> Δ <i>gcdH</i> Δ <i>csiD</i>		18
Plasmids		
pET21b		Novagen
pBADT		51
pMQ30		52
pBADT- <i>gcdR</i> -P <i>gcdH</i> ::RFP	JPUB_010960	This work
pBADT- <i>csiR</i> -P <i>csiD</i> ::RFP	JPUB_010962	This work
pBADT- <i>gcdR</i> -J23101	JPUB_013546	This work
pBADT- <i>gcdR</i> -J23110	JPUB_013548	This work
pBADT- <i>gcdR</i> -J23113	JPUB_013550	This work
pMQ30 <i>davT</i>	JPUB_013544	This work
pET21b- <i>CsiR</i> _P <i>put</i>	JPUB_010964	This work

pET21b-GcdR_Pput	JPUB_010966	This work
pBbS8k-rfp		53
pBbE8k-rfp		53

520

521

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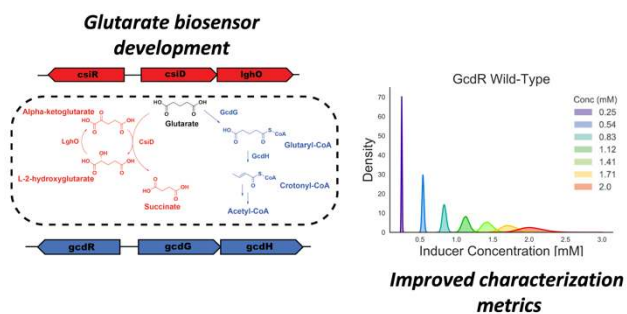
683 Robust characterization of two distinct glutarate sensing transcription factors of *Pseudomonas*
684 *putida* L-lysine metabolism

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