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3 **Marionette: *E. coli* containing 12 highly-optimized small molecule sensors**

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29   **Cellular processes are carried out by many interacting genes and their study and optimization requires**

30   **multiple levers by which they can be independently controlled. The most common method is via a**

31   **genetically-encoded sensor that responds to a small molecule (an "inducible system"). However, these**

32   **sensors are often suboptimal, exhibiting high background expression and low dynamic range. Further,**

33   **using multiple sensors in one cell is limited by cross-talk and the taxing of cellular resources. Here, we**

34   **have developed a directed evolution strategy to simultaneously select for less background, high**

35   **dynamic range, increased sensitivity, and low crosstalk. Libraries of the regulatory protein and output**

36   **promoter are built based on random and rationally-guided mutations. This is applied to generate a set**

37   **of 12 high-performance sensors, which exhibit >100-fold induction with low background and cross-**

38   **reactivity. These are combined to build a single "sensor array" and inserted into the genomes of *E. coli***

39   **MG1655 (wild-type), DH10B (cloning), and BL21 (protein expression). These "Marionette" strains allow**

40   **for the independent control of gene expression using 2,4-diacetylphophloroglucinol (DAPG), cuminic**

41   **acid (Cuma), 3-oxohexanoyl-homoserine lactone (OC6), vanillic acid (Van), isopropyl β-D-1-**

42   **thiogalactopyranoside (IPTG), anhydrotetracycline (aTc), L-arabinose (Ara), choline chloride (Cho),**

43   **naringenin (Nar), 3,4-dihydroxybenzoic acid (DHBA), sodium salicylate (Sal), and 3-**

44   **hydroxytetradecanoyl-homoserine lactone (OHC14).**

45   Advances in biology are often tied to new methods that use external stimuli to control the levels

46   of gene expression[1-3]. Pioneered in the early 1980s, so-called inducible systems were developed that allow

47   genes to be turned on by adding a small molecule inducer to the growth media[4]. These consist of a protein

48   transcription factor (*e.g.*, LacI) whose binding to a DNA operator in a promoter is controlled by the inducer

49   (*e.g.*, IPTG). Initially co-opted from natural regulatory networks, over the years many versions were

50   designed to improve performance. In the 1990s, additional systems were developed that responded to

51   other inducers, notably arabinose and aTc, which became common tools in the field. In 1997, Lutz and

52   Bujard published a seminal paper that combined three (IPTG, arabinose, aTc) that could be easily

53   interchanged on a two-plasmid system[5]. Its organizational simplicity, compatibility, and quantified

54   response functions were revolutionary. Beyond providing a new tool to biologists to control multiple

55   genes with independent "strings," it facilitated researchers with quantitative backgrounds to enter

56   biology[6-7]. Armed with the new ability to control two genes with precision, physicists and engineers built

57   the first synthetic genetic circuits, performed single molecule experiments inside cells, deconstructed the

58   origins of noise in gene expression, determined how enzyme balancing impacts metabolic flux, elucidated

59   rules underlying the assembly of molecular machines, and built synthetic symbiotic microbial

60   communities, just to highlight a few[8-19].

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61   Sensor performance is quantified by its response function; in other words, how the concentration

62   of inducer changes the activity of the output promoter (Figure 1a). Often, this promoter retains a residual

63   activity in the absence of inducer ("leakiness"). This hampers the ability to explore low expression levels

64   or keep a gene in the off state, particularly needed for toxic proteins[20-21]. Another important parameter is

65   the dynamic range, defined as the ratio of promoter activity in the on and off states. When this is large,

66   both high and low expression can be explored as well as many intermediate states. The sensitivity is the

67   concentration of inducer that turns a sensor on (defined as 50% activation). A lower sensitivity reduces

68   the amount of a chemical that must be added to the media. Further, when multiple sensors are combined

69   into one cell, they can interfere with each other's response functions (Figure 1b). Some small molecules

70   bind non-cognate regulators and this can lead to off-target activation (cross reactivity) or competitive

71   inhibition with the cognate small molecule (antagonism)[22-27]. Finally, each sensor requires cellular

72   resources (*e.g.*, ribosomes) to function and the activation of one sensor can influence another indirectly

73   due to resource competition[28]. Each sensor also requires ~1-2 kb of DNA and this becomes increasingly

74   difficult to carry on plasmids. These challenges limit the number of sensors that can be put in a single cell

75   and the maximum reported to date is four[29].

76   Sensors can be improved using biophysical models, rational engineering and directed evolution[5,

77   23, 26, 30-36]. Improving performance requires that screens or selections be performed in the presence and

78   absence of inducer[37]. Such dual selections have been accomplished by sorting cells based low and high

79   fluorescence, utilizing proteins that can be both toxic and selective (*e.g.,* TetA or HSV-TK), or by deploying

80   separate positive and negative selections[26, 35, 37-44]. This has been applied to improving the response

81   functions and eliminating cross-reactivity between pairs of regulators[23, 26, 35, 45].

82   Here, we have developed a selection methodology that allows us to intervene at multiple steps in

83   order to simultaneously select for improved response functions and decreased crosstalk (Figure 1c). First,

84   we built 12 sensors that respond to different small molecules and made directed changes in order to

85   improve function. These sensors were then subjected to multiple rounds of negative-positive selection.

86   Negative selection is facilitated by a promiscuous A294G mutant of the phenylalanine aminoacyl tRNA-

87   synthetase (PheS)[46]. In the absence of inducer, leaky transcription of PheS leads to the charging of

88   phenylalanyl-tRNA with the non-canonical amino acid 4-Chloro-DL-phenylalanine (Cl-Phe). Adding more

89   Cl-Phe increases the stringency of the selection by making PheS transcription more toxic[43, 47]. Positive

90   selection is facilitated by a thermostable DNA polymerase (DNAP), either KOD DNAP from the archaea

91   *Thermococcus kodakarensis*[48] (for stringent replication) or the engineered PK6 DNAP[49] (to introduce

92   mutations). In the presence of inducer, the level of DNAP can be quantified by PCR amplification of the

93   sensor library after the cells are emulsified with primers[50-51]. Multiple properties of the response function

94   can be simultaneously improved during one cycle of negative-positive selection and stringency altered by

95   changing the concentrations of Cl-Phe and inducer. Further, cross reactions can be selected against by

96   adding the inducer or regulator from a problematic system. The dual selection is applied over multiple

97   rounds to create a highly-optimized set of sensors. From these, we identify 12 that can be used together

98   in a single strain, and these are combined and integrated into the genomes of *E. coli* MG1655, DH10B, and

99   BL21 in order to create the Marionette family of strains. Genome integration increases stability and

100   reduces the cellular resources required to maintain regulator expression. It also simplifies the use of the

101   inducible systems, where only the output promoters need to be incorporated into a design (*e.g.*, for the

102   expression of multiple proteins from a plasmid).

103   An initial set of 17 putative sensors were designed. Each sensor consists of a weak constitutive

104   promoter (P_{LacI}, Supplementary Table 1) driving the expression of the regulatory gene and an output

105   promoter that is acted on by the regulator. The regulatory genes were either codon optimized and

106   synthesized or cloned (Methods and Supplementary Table 4).   The output promoters were either

107   obtained from the literature or, in the case of P_{Van}, rationally designed by inserting cognate operator

108   sequences into unregulated promoters (Supplementary Table 1). The activity of the output promoter was

109   measured through the expression of yellow fluorescent protein (YFP) using flow cytometry and reported

110   in relative promoter units (RPUs) (Supplementary Figure 1 and Methods). Each complete sensor was

111   cloned into the same sensor plasmid architecture (Supplementary Figure 2). Some sensors require

112   additional genes, which are encoded as an operon with the regulatory protein. For the Ara-inducible

113   system, the transporter *araE* was included in order to produce a graded response[52]. For the Ery-inducible

114   system, the ribosome methylase *ery^R* was included to confer resistance to Ery[53].

115   Rational mutations were made to improve some sensors prior to performing the direction

116   evolution experiments. Multiple versions of each sensor, each with different a promoter and RBS used to

117   drive the expression of the regulator, were tested (Supplementary Figure 3). The version with the largest

118   dynamic range was chosen for further optimization. Then, a number of potential improvements gleaned

119   from the literature or rationally designed were evaluated, the results of which are shown in

120   Supplementary Figure 4. After this step, we reduced the set of sensors to 14, removing copper, glucaric

121   acid and paraquat inducible systems because their responses were too small for subsequent optimization.

122   The response functions of the initial sensors are shown in Figure 2 (grey curves). Each function was

123   obtained by fitting the experimental data to the equation

124      $$y = y_{min} + (y_{max} - y_{min})\frac{x^n}{K^n + x^n} \quad , \tag{1}$$

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125   where y is the promoter activity in RPU, x is the concentration of the small molecule, y_{min} is the leakiness,

126   y_{max}/y_{min} is the dynamic range, K is the sensitivity, and n is the cooperativity. The raw data points, including

127   error bars, used for this fit are shown in Supplementary Figure 5. While they all show some response, the

128   high leakiness, low dynamic range and low sensitivity are apparent for many. Of the 14, the aTc- and OC6-

129   sensors produced a good enough response to not require additional optimization (Table 1).

130   The remaining 12 were then subjected to directed evolution using the dual selection (Figure 1c,

131   Supplementary Figure 6). For each sensor, a library was constructed and cloned upstream of the operon

132   containing PheS and DNAP (Figure 1a) on a dual selection plasmid. The initial library contained a mixture

133   of random and rational mutations. For a subset of sensors, the output promoter was mutagenized:

134   selected bases in the -10 box and -35 box were randomized.   To control regulator expression, critical

135   bases in the ribosome binding site (RBS) were randomized. For LacI and AraC, we partially mutagenized

136   key amino acid residues based on prior work[23, 33-34]. Specifically, the genes were PCR amplified with

137   primers of limited degeneracy (*e.g.,* VNA, WKK, or NDC) thus allowing LacI Q18, F161, W220, Q291, and

138   L296 and AraC L133, E165, E169, and C280 to sample a subset of possible amino acids (Methods). The

139   specific mutations made to the initial library for each sensor are shown in Supplementary Appendix 2.

140   Multiple rounds of the dual selection were performed with the initial library. Between 4 and 23

141   rounds were performed, depending on how many issues needed to be corrected for each sensor.

142   Different interventions were performed during each round to bias solutions to address problems

143   identified for each sensor. The conditions for each round are presented in detail in Supplementary

144   Appendix 2. Typically, the stringency of the negative selection was increased at a particular round by

145   increasing the concentration of Cl-Phe from 2 mM to 4 mM. This biases against leakiness in the absence

146   of inducer. During positive selection, inducer was added to the surviving cells, leading to the expression

147   of DNAP. During early rounds, the maximum amount of inducer was added. For some sensors, we sought

148   to increase the sensitivity of the response by reducing the amount of inducer after each round; for

149   example, BetI was induced with 1 mM Cho in the final round of selection, down from 5 mM Cho in the

150   first round. After induction, the cells were encapsulated, lysed, and PCR amplified using the DNAP

151   expressed by the sensor (Methods). In early rounds, additional random mutations throughout the sensor

152   were introduced by using the PK6 DNAP during the positive selection (yielding an average of 1-2 mutations

153   per kilobase). In later rounds, the stringent KOD DNAP was used to reduce the diversity in the population.

154   After the amplification step of the positive selection, the constructs were recloned into the selection

155   plasmid. Recloning allows the selection plasmid to be reset each round, thus preventing the accumulation

156   of cheaters, and offers the opportunity to change the DNAP as needed. In some cases, in an effort to

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157   combine multiple beneficial mutations into a single variant, gene shuffling[54] was used between rounds

158   (Methods).

159   During the selection rounds, additional interventions were included in order to reduce crosstalk

160   between systems. For example, crosstalk between the IPTG- and Ara- sensors is well known, where IPTG

161   reduces the output of the AraC/P_{BAD} system[23] (Supplementary Figure 7). To identify mutants that reduce

162   this, 1000 μM IPTG was included during all rounds of selection for the Ara sensor. IPTG was also included

163   in the negative selection to prevent the evolution of an IPTG-induced AraC mutant. Crosstalk between

164   Van and DHBA was also observed and eliminated through the negative selection (Figure 2b,

165   Supplementary Figure 8).   Salycilate was also found to antagonize the Cuma sensor (Figure 2c,

166   Supplementary Figure 7) and cross react with the DAPG sensor (Figure 2b, Supplementary Figure 8) and

167   was thus added to both the positive and negative selections.

168   After all of the rounds of selection are complete, the library was assembled into a YFP screening

169   plasmid (Supplementary Figure 6). Several clones were picked and assayed for output expression in the

170   presence and absence of inducer (Supplementary Appendix 2). In addition, when selecting against

171   crosstalk, the clones were screened for induction by these molecules. The mutants showing highest

172   improvement (leakiness, sensitivity, dynamic range, and orthogonality) were identified and sequenced

173   (Supplementary Appendix 2). Based on the mutations observed, one or more consensus sequences were

174   constructed and re-screened to identify the best variant. The final sequences of the evolved sensors,

175   including the mutations identified are provided in Supplementary Table 4. Mutations were identified

176   throughout the sensors, including the promoter/RBS controlling regulator expression, synonymous and

177   non-synonymous mutations throughout the regulator genes, and mutations/substitutions in the output

178   promoters (Figure 2a). On average, about eight cumulative mutations were made to each sensor as a

179   result of the rounds of dual selection.

180   The improvements in the response functions are shown in Figure 2a (blue curves). The fit

181   parameters to Equation 1 are provide in Table 1 (raw data are provided in Supplementary Figure 5). There

182   is marked improvement in many of the response functions, sometimes showing orders of magnitude

183   changes in the leakiness, dynamic range, and sensitivity. The Ery- and Acu- sensors showed slight

184   improvements in their response functions, but were not chosen to be part of the final set because of their

185   low dynamic range.

186   CymR, NahR, VanR, PhlF, and PcaU each responds to a substituted benzene. Therefore, we

187   examined the activity of this set of sensors against all five inducers. The optimized sensors showed

188   significant reduction in the crosstalk while maintaining high activity with their cognate inducer (Figure 2b,

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189   Supplementary Figure 8). Improvements in the antagonism between CymR and Sal were also tested

190   (Figure 2c, Supplementary Figure 7). In the presence of 100 μM Sal, the ability for Cuma to induce its

191   sensor drops by 1200-fold. The sensor obtained by rounds of positive selection in the presence of Cuma

192   reduces this by two orders of magnitude. There is also slight antagonism of AraC by IPTG, which also

193   improved as a result of the selection (Supplementary Figure 7). Collectively, these improvements allow

194   for all of these sensors to be used simultaneously in a single cell.

195   The best 12 sensors were then combined to form a "sensor array" that was inserted into the

196   genome of *E. coli* MG1655 to create "Marionette-Wild" (Figure 3 and Supplementary Table 6). Genomic

197   insertion has several benefits: it stabilizes the cluster and simplifies the use of multiple systems without

198   building large plasmids containing the regulators. The array consists of the 12 regulatory genes and *araE*

199   transporter organized into several operons (Figure 3a and Supplementary Figure 9). The genes were

200   organized into three operons controlled by three medium-strength constitutive promoters. Each gene is

201   encoded by its own ribosome binding site (RBS), which was rationally designed using the RBS Calculator[55]

202   in order to achieve equivalent expression as when encoded on the plasmid (Methods and Supplementary

203   Figure 10). Strong terminators were included before and after the sensor array in order to insulate the

204   array from context effects. Phage transduction was used to move the sensor array to create two

205   additional cell lines: the recA-deficient *E. coli* DH10B strain for cloning "Marionette-Clo" and the protease-

206   deficient *E. coli* BL21 for protein expression "Marionette-Pro" (Methods).

207   The responses of the 12 genomically-encoded sensors in Marionette-Wild are shown in Figure 3b,

208   the parameters derived from the fits to Equation 1 are shown in Table 1, and the raw data points are

209   provided in Supplementary Figure 11 and Supplementary Appendix 1. Each response was measured by

210   transforming the strain with a p15a (Supplementary Figure 2) plasmid containing the output promoter

211   fused to YFP. Each response function shows at least 100-fold induction with similar levels of on- and off-

212   states. The technical information for the use of each sensor is organized as a series of datasheets in the

213   Supplementary Information. The response of each sensor was also measured in Marionette-Clo and

214   Marionette-Pro (Supplementary Appendix 1). The performances of the sensors closely match that of

215   Marionette-Wild with several exceptions; notably, the responses to IPTG (P_{Tac}) and choline (P_{BetI}) are

216   leakier.

217   All of the sensors follow similar induction dynamics, with induction after 15 minutes and full

218   induction by 2 hours. Interestingly, those sensors based on activators were slower to turn on, as

219   compared to those based on repressors (Supplementary Figure 12). There is little cross reactivity from

220   the non-cognate inducers (Figure 3c and Supplementary Figure 13). The response functions for three

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221   different promoters were also measured in the presence of the maximum levels of all 11 other inducers,

222   and there was little change in dynamic range (Supplementary Figure 14). The sensor responses were

223   measured during exponential growth. To evaluate performance in stationary phase, cells were grown

224   overnight (~20 hours) and response functions were measured (Methods). The responses closely match

225   those measured during exponential growth, with several exceptions (Supplementary Figure 12). Induction

226   on plates where the inducers are added to LB-agar resulted in similar responses as those observed in early

227   stationary phase (Supplementary Figure 15).

228   We tested whether carrying the sensor array impacts the growth rate of the three Marionette

229   strains (Supplementary Figure 16). The Marionette-Wild strain grows with a doubling time of 27.8 ± 1.3

230   min as compared 25.3 ± 1.3 for wild-type *E. coli* MG1655 (Methods). The other two Marionette strains

231   grow slightly faster, albeit within error (Supplementary Figure 16). Growth of Marionette-Wild was also

232   evaluated in the presence of all 12 inducers at maximum levels and only a modest effect was observed

233   (Supplementary Figure 16).

234   Carrying the sensor array requires the continuous expression of 12 regulatory proteins and a

235   transporter. This could lead to a draw on cellular resources that confers a selective advantage to

236   eliminating the array. While genomic insertion improves evolutionary stability[21, 56-57], it could still be

237   disrupted over time, particularly for the *recA*-positive[58] Marionette-Wild. To address this, we performed

238   three independent experiments to assess the evolutionary stability of Marionette-Wild.   First, we

239   determined whether Marionette could reliably control a plasmid based promoter, even after extended

240   passage. The 12 reporter strains were passaged for 14 days in liquid culture without inducer, diluting the

241   cells 10^6-fold each day (10^{84}-fold dilution total). On each day, a subset of cells from each line were grown

242   and assayed with and without inducer (Figure 3d and Supplementary Figure 17). Second, the Marionette-

243   Wild strain was passaged for nine days, streaking cultures on agar plates and inoculating single colonies

244   into liquid culture each day. On the tenth day, the culture was transformed with each of the 12 reporter

245   plasmids and assayed with and without the cognate inducer for each reporter. Third, we determined if

246   serial transfer would lead to the emergences of subpopulations[59]. We passaged the Marionette-Wild

247   strain for nine days in liquid culture, diluting the cells 10^6-fold each day (10^{54}-fold dilution total). On the

248   tenth day, the culture was transformed with each of the 12 reporter plasmids and assayed with and

249   without the cognate inducer for each reporter. For all three evolution experiments, the sensors perform

250   indistinguishably after growth and passaging (Supplementary Figure 17). There is no decline in the fold-

251   induction over time and there was no emergence of "broken" subpopulations by flow cytometry

252   (Supplementary Figure 18).

253   This work represents a dramatic expansion in our ability to study and control genes in cells. The

254   Marionette strains enable the modular control of up to 12 genes, simply by placing each one under the

255   control of a small (50 to 289 base pair) inducible promoter. This means that a single construct can be built

256   and then the expression levels perturbed in many ways through the combination of different small

257   molecules. This could be to determine the role of proteins in a natural system, picking apart the

258   stoichiometric requirements for a molecular machine[60-61]. This can also be part of rapid optimization of

259   metabolic pathways, where the ideal stoichiometry can be identified without the need to build megabase-

260   scale libraries[62-63]. Through the use of CRISPRi[64], sRNA[65], or other tools, endogenous genes can be inducibly

261   down-regulated as well as up-regulated, thus enabling exquisite control of natural processes and

262   metabolic flux. Further, dynamic systems can be probed by controlling the timing of the induction of each

263   component in order to determine the role of ordered gene expression[15, 66]. The ability to control gene

264   expression has been a major limitation in genetic engineering; now, pulling the strings on Marionette

265   enables unprecedented genetic control.

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267   **Acknowledgements**
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273   **Accession**

274   The following plasmids and strains can be acquired from Addgene: pAJM.711 (P_{PhlF}-YFP) 108512;
275   pAJM.712 (P_{CymRC}-YFP) 108513; pAJM.713 (P_{LuxB}-YFP) 108514; pAJM.714 (P_{VanCC}-YFP) 108515; pAJM.715
276   (P_{Tac}-YFP) 108516; pAJM.717 (P_{Tet*}-YFP) 108517; pAJM.716 (P_{BAD}-YFP) 108518; pAJM.718 (P_{BetI}-YFP)
277   108519; pAJM.719 (P_{Ttg}-YFP) 108520; pAJM.1459 (P_{3B5C}-YFP) 108521; pAJM.721 (P_{SalTTC}-YFP) 108522;
278   pAJM.944 (P_{Cin}-YFP) 108523; pAJM.847 (PhlF^{AM} + P_{PhlF}-YFP) 108524; pAJM.657 (CymR^{AM} + P_{CymRC}-YFP)
279   108525; pAJM.474 (LuxR + P_{LuxB}-YFP) 108526; pAJM.773 (VanR^{AM} + P_{VanCC}-YFP) 108527; pAJM.336 (LacI^{AM}
280   + P_{Tac}-YFP) 108528; pAJM.011 (TetR + P_{Tet*}-YFP) 108529; pAJM.677 (AraC^{AM} + AraE + P_{BAD}-YFP) 108530;
281   pAJM.683 (BetI^{AM} + P_{BetI}-YFP) 108531; pAJM.661 (TtgR^{AM} + P_{Ttg}-YFP) 108532; pAJM.690 (PcaU^{AM} + P_{3B5B}-
282   YFP) 108533; pAJM.771 (NahR^{AM} + P_{SalTTC}-YFP) 108534; pAJM.1642 (CinR^{AM} + P_{Cin}-YFP) 108535; pAJM.884
283   (AcuR^{AM} + P_{Acu}-YFP) 108536; pAJM.969 (MphR^{AM} + EryR + P_{Mph}-YFP) 108537; sAJM.1504 (Marionette-Clo)
284   108251; sAJM.1505 (Marionette-Pro) 108253; sAJM.1506 (Marionette-Wild) 108254.
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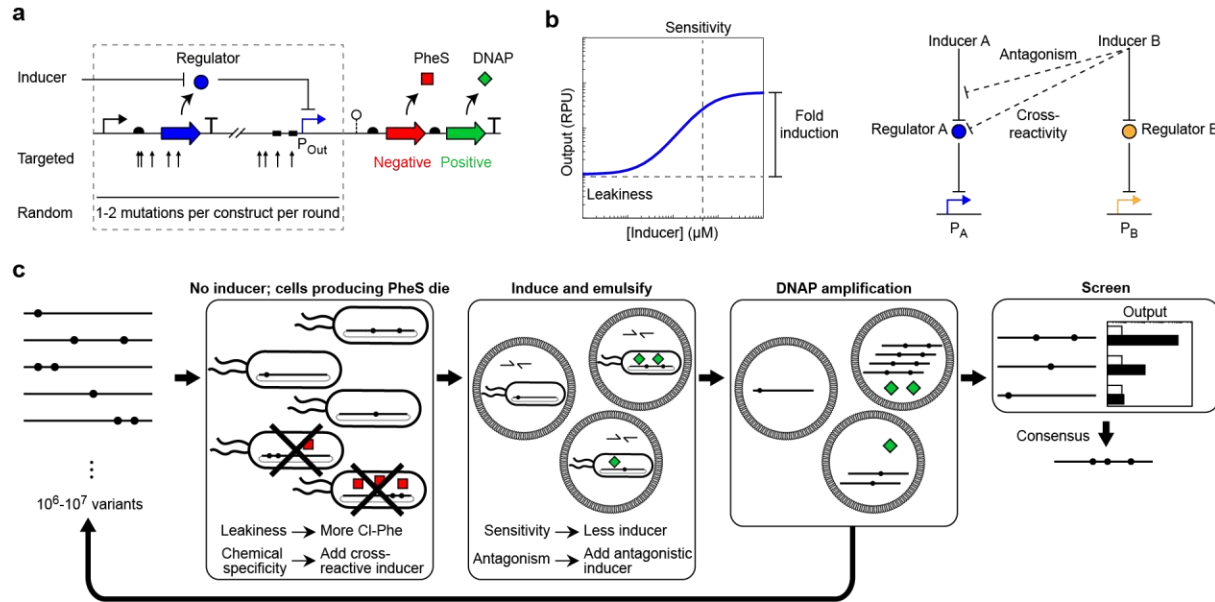
Figure 1: A dual selection for sensor optimization. a) A regulator (blue circle) is expressed from a weak constitutive promoter and controls expression from the output promoter (P_{Out}) while itself being controlled by an externally applied inducer molecule. Transcription from P_{Out} determines the expression level of an aminoacyl-tRNA synthetase (PheS; red square) and a DNA polymerase (KOD or PK6; green diamond). Initial libraries may contain targeted degeneracy in the regulator RBS, regulator CDS, or P_{Out} (arrows), and random mutations are added throughout the entire library during each round of selection. **b)** A response function captures the activity of P_{Out} at various levels of inducer. Regulator A affects promoter A, and is itself affected by inducer A. Inducer B may affect Regulator A (chemical cross-reactivty) or interfere with inducer A's action (antagonism). **c)** The dual selection schema is shown (see text for details). Dots denote mutations; half arrows represent PCR primers.

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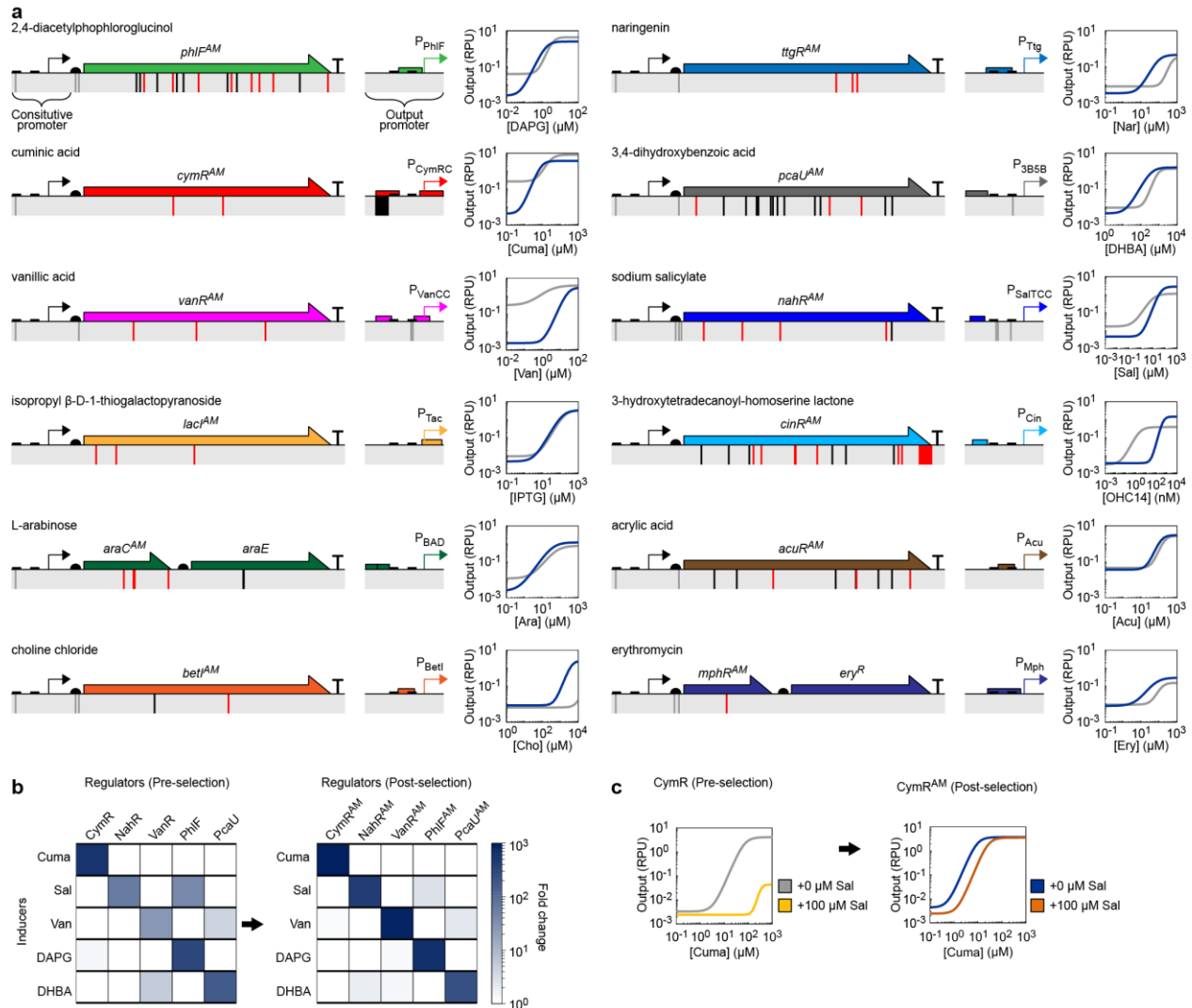
Figure 2: Improved sensor performance. a) Each genetically encoded sensor is shown, with coding (red) and non-coding (grey/black) mutations noted. Mutations in grey were also applied to the parental sensor. The corresponding response functions comparing the evolved (blue) and parental (grey) sensors. The fit of Equation 1 to the mean of three replicates performed on different days is shown (see Supplementary Figure 5 for data). The response function parameters for evolved sensors are provided in Table 1. **b)** Chemical cross-reactivity heat-map of reference (left) and evolved (right) sensors. Inducer concentrations were: 100 µM Cuma, 100 µM Sal, 100 µM Van, 10 µM DAPG, and 1 mM DHBA. The mean of three replicates performed on different days is shown (see Supplementary Figure 8 for data). **c)** Response function with Sal (light/dark orange) and without Sal (grey/blue) for parental CymR (left) and evolved CymR^{AM} (right). The fit of Equation 1 to the mean of three replicates from different days is shown (see Supplementary Figure 7 for data). Sequences of promoters and regulators are provided in Supplementary Table 1 and 4.

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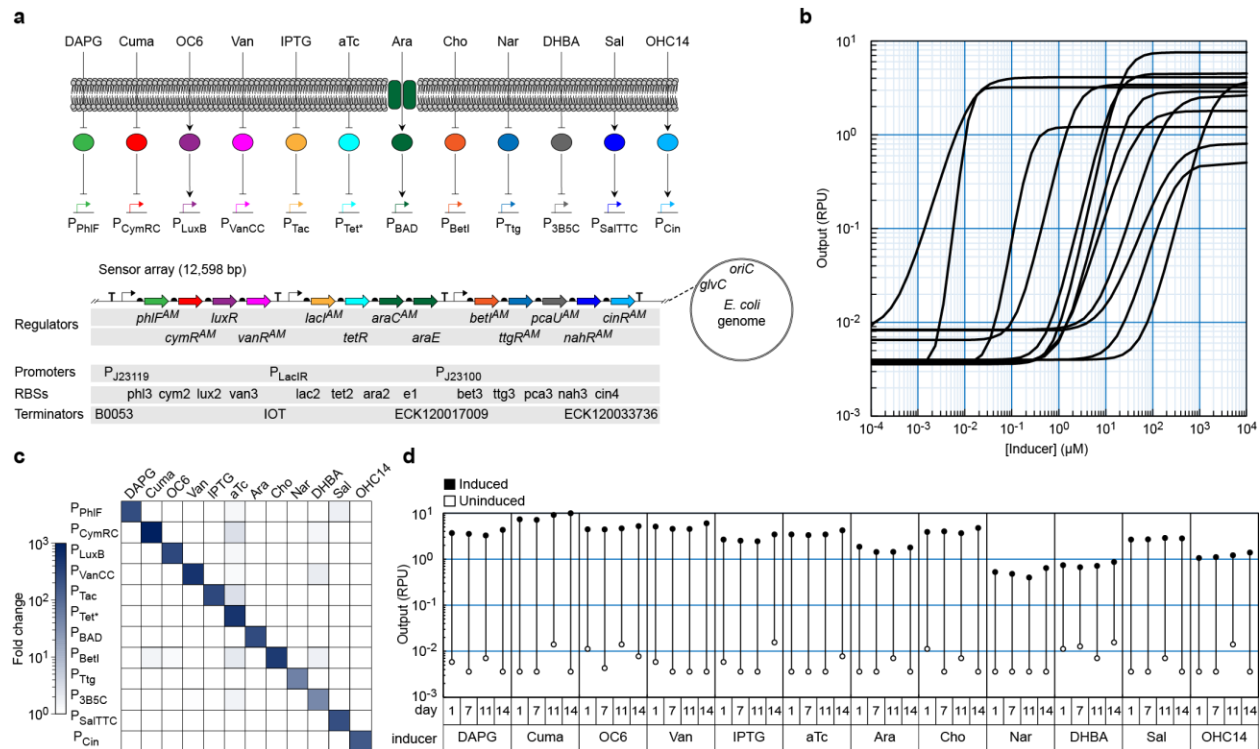
Figure 3: Marionette-Wild performance. a) The molecular and genetic schematic of the Marionette cluster and its location in the *E. coli* genome. The cluster was inserted in the direction of leading strand replication, between 3,860,010 and 3,861,627 in *E. coli* MG1655 (NCBI accession number NC_000913), between 3,957,594 and 3,959,211 in *E. coli* DH10B (NC_010473), and between 3,720,027 and 3,721,644 in *E. coli* BL21 (CP010816). **b)** Overlaid response functions for each of the 12 output promoters with their cognate inducers. The fit of Equation 1 to the mean of three replicates performed on different days is shown (see Supplementary Figure 11 for data). The response function parameters are provided in Table 1. **c)** Chemical cross-reactivity heat-map of the 12 output promoters with each inducer. Inducer concentrations were: 25 μM DAPG, 500 μM Cuma, 10 μM OC6, 100 μM Van, 1 mM IPTG, 200 nM aTc, 4 mM Ara, 10 mM Cho, 1 mM Nar, 2.5 mM DHBA, 250 μM Sal, and 10 μM OHC14. The mean of three replicates performed on different days is shown (see Supplementary Figure 13 for data). **d)** Output of uninduced (open circles) and induced (closed circles) cultures on day 1, 7, 11, and 14 days of passaging. Inducer concentrations were: 25 μM DAPG, 500 μM Cuma, 10 μM OC6, 100 μM Van, 1 mM IPTG, 200 nM aTc, 4 mM Ara, 10 mM Cho, 1 mM Nar, 2.5 mM DHBA, 250 μM Sal, and 10 μM OHC14. A single evolutionary trajectory is shown. Passaging details and data from other days are provided in Supplementary Figure 17.

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334 **Table 1. Sensor response function parameters[a]**

Inducer[d]		Max inducer (µM)[b]	Genome-based regulator (Marionette-Wild[c])				Plasmid-based regulator (*E. coli* DH10B)			
			y_{max} (RPU)	y_{min} (RPUx10³)	K (µM)	n	y_{max} (RPU)	y_{min} (RPUx10³)	K (µM)	n
DAPG	2,4-Diacetylphophloroglucinol	25	3.4	6.5	2.1	2.3	2.5	2.5	1.7	2.1
Cuma	Cuminic acid	100	7.6	3.6	22	2.3	3.7	4.3	8.9	2.4
OC6	3OC6-AHL	10	4.1	8.6	0.012	1.7	1.3	2.4	0.12	1.8
Van	Vanillic acid	100	4.5	4.0	14	2.1	3.0	2.4	26	2.3
IPTG	Isopropyl-beta-D-thiogalactoside	1000	2.6	8.3	190	1.7	3.3	4.8	140	1.8
aTc	Anhydrotetracycline HCl	0.2	3.2	3.6	0.012	4.4	2.4	4.9	0.013	3.8
Ara	L-Arabinose	4000	1.8	3.6	43	1.7	1.2	2.4	37	1.5
Cho	Choline chloride	10000	3.7	4.0	1900	2.0	2.6	8.5	4100	2.7
Nar	Naringenin	1000	0.5	4.0	280	2.3	0.5	3.4	95	1.9
DHBA	3,4-Dihydroxybenzoic acid	1000	0.8	8.0	240	1.5	1.6	4.5	370	1.8
Sal	Sodium salicylate	100	2.9	3.8	29	2.1	2.8	4.7	43	1.8
OHC14	3OHC14:1-AHL	10	1.2	3.6	0.25	3.0	1.5	3.0	0.43	2.3
Acu	Acrylic acid	1000					3.1	37.0	130	2.5
Ery	Erythromycin	125					0.3	8.0	65	1.5

335   a.   Response data from at least days were averaged and fit to Equation 1 (Methods). Full response functions are provided in Supplementary Appendix 1.

336   b.   Growth defects are observed above this concentration.

337   c.   Based on *E. coli* MG1655. Data for Marionette-Clo (DH10B) and Marionette-Pro (BL21) are provided in Supplementary Appendix 1.

338   d.   DAPG-Santa Cruz sc-206518; Cuma-Sigma 268402; OC6-Sigma K3007; Van-Sigma 94770; IPTG-Gold I2481C25; aTc-Sigma 37919; Ara-Sigma A3256; Cho-
339   Sigma C7017; Nar-Sigma N5893; DHBA-Sigma 37580; Sal-Sigma S3007; OHC14-Sigma 51481; Acu-Sigma 147230; Ery-Sigma E5389.

340

341

13

Methods

Strains, plasmids, and media. *Escherichia coli* DH10B (New England Biolabs, Ipswich, MA – USA) was used for all routine cloning and directed evolution. Plasmid-based regulator systems were characterized in *E. coli* DH10B. Marionette-Wild, -Clo, and -Pro were derived from *E. coli* MG1655[67], *E. coli* DH10B, and *E. coli* BL21 (New England Biolabs, Ipswich, MA – USA) cells respectively. *E. coli* JTK164H was used to clone RK6 suicide vectors[68]. All other plasmids contain p15A origins of replication and kanamycin resistance (Supplementary Figures 2 and 6). LB-Miller media (BD, Franklin Lakes, NJ - USA) was used for directed evolution, stability assays, and cytometry assays unless specifically noted. 2xYT liquid media (BD, Franklin Lakes, NJ - USA) and LB + 1.5% agar (BD, Franklin Lakes, NJ – USA) plates were used for routine cloning and strain maintenance. M9 media (1x M9 Salts [Millipore Sigma, St. Louis, MO - USA], 2 mM $MgSO_4$, 100 μM $CaCl_2$, and 0.2% Casamino acids) supplemented with either 0.4% glucose or 0.4% glycerol was used for cytometry assays where noted.

Chemical transformation. For routine transformations, strains were made competent for chemical transformation. Overnight cultures (250 μl for *E. coli* DH10B derived cells, 100 μl for *E. coli* MG1655 and BL21 derived cells) were subcultured into 100 ml SOB media (BD, Franklin Lakes, NJ – USA) and grown at 37 °C, 250 rpm for 3 hours. Cultures were centrifuged (4500g, 4 °C, 10 minutes) and pellets were resuspended in 15 ml TFBI buffer[51] (30 mM KOAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, and 15% v/v glycerol, pH 5.0). After 1 hour on ice, cells were centrifuged (4500g, 4 °C, 10 minutes) and pellets were resuspended in 2 ml TFBII buffer (10 mM NaMOPS pH 7.0, 75 mM $CaCl_2$, 10 mM RbCl, and 15% v/v glycerol). Competent cells were stored at -80 °C until use.

Response function measurements (mid-log phase). All measurements shown were taken by cytometry of cells in mid-log growth except when noted. Glycerol stocks of strains containing the plasmids of interest were streaked on LB + 1.5% Agar plates and grown overnight at 37 °C. Single colonies were inoculated into 1 ml LB + antibiotics in 2-ml 96-deepwell plates (USA Scientific, Orlando, FL - USA) sealed with an AeraSeal film (Excel Scientific, Victorville, CA - USA) and grown at 37 °C, 900 rpm overnight in a Multitron Pro shaker incubator (INFORS HT, Bottmingen, Switzerland). The overnight growths were diluted 1:200 into 1 ml LB + antibiotics in 2-ml 96-deepwell plates + AeraSeal film and grown at 37 °C, 900 rpm. After 2 hours the growths were diluted (*E. coli* DH10B/Marionette-Clo 1:500; *E. coli* BL21/Marionette-Pro 1:2,000; *E. coli* MG1655/Marionette-Wild 1:5,000) into prewarmed LB + antibiotics + inducer where necessary in

373   2-ml 96-deepwell plates + AeraSeal film and grown at 37 °C, 900 rpm for 5 hours. After growth, 20 μl of

374   culture sample was diluted into 180 μl PBS + 200 μg/ml kanamycin to inhibit translation.

375

376   **Response function measurements (stationary phase).** Measurements were taken from cells in stationary

377   phase to generate data shown in Supplementary Appendix 1 and Supplementary Figures 12 and 13.

378   Glycerol stocks of strains containing the plasmids of interest were streaked on LB + 1.5% Agar plates and

379   grown overnight at 37 °C. Single colonies were inoculated into 1 ml LB + antibiotics in 2-ml 96-deepwell

380   plates (USA Scientific, Orlando, FL - USA) sealed with an AeraSeal film (Excel Scientific, Victorville, CA -

381   USA) and grown at 37 °C, 900 rpm overnight in a Multitron Pro shaker incubator (INFORS HT, Bottmingen,

382   Switzerland). The overnight growths were diluted 1:200 into 1 ml LB + antibiotics in 2-ml 96-deepwell

383   plates + AeraSeal film and grown at 37 °C, 900 rpm. After 2 hours the growths were diluted (*E. coli*

384   DH10B/Marionette-Clo 1:500; *E. coli* BL21/Marionette-Pro 1:2,000; *E. coli* MG1655/Marionette-Wild

385   1:5,000) into prewarmed LB + antibiotics + inducer where necessary in 2-ml 96-deepwell plates + AeraSeal

386   film and grown at 37 °C, 900 rpm for 20 hours. After growth, 2 μl of culture sample was diluted into 198

387   μl PBS + 200 μg/ml kanamycin to inhibit translation.

388

389   **Time course (mid-log phase)**. For Supplemental Figure 12: mid-log induction time course, glycerol stocks

390   of strains containing the plasmids of interest were streaked on LB + 1.5% Agar plates and grown overnight

391   at 37 °C. Single colonies were inoculated into 1 ml LB + antibiotics in 2-ml 96-deepwell plates (USA

392   Scientific, Orlando, FL - USA) sealed with an AeraSeal film (Excel Scientific, Victorville, CA - USA) and grown

393   at 37 °C, 900 rpm overnight in a Multitron Pro shaker incubator (INFORS HT, Bottmingen, Switzerland).

394   The overnight growths were diluted 1:200 into 1 ml LB + antibiotics in 2-ml 96-deepwell plates + AeraSeal

395   film and grown at 37 °C, 900 rpm. After 2 hours the growths were diluted 1:500 into prewarmed LB +

396   antibiotics. After 0, 1, 2, 3, 3.5, 4, 4.25, 4.5, or 4.75 hours, cultures were further diluted 1:10 into

397   prewarmed LB + antibiotics + inducer where necessary in 2-ml 96-deepwell plates + AeraSeal film and

398   grown at 37 °C, 900 rpm for 5, 4, 3, 2, 1.5, 1, 0.75, 0.5, or 0.25 hours (5 hours total after the initial growth).

399   After growth, 20 μl of culture sample was diluted into 180 μl PBS + 200 μg/ml kanamycin to inhibit

400   translation.

401

402   **Time course (mid-log phase to stationary phase)**.   For Supplemental Figure 12: mid-log to stationary

403   induction time course, glycerol stocks of strains containing the plasmids of interest were streaked on LB

404   + 1.5% Agar plates and grown overnight at 37 °C. Single colonies were inoculated into 1 ml LB + antibiotics

405   in 2-ml 96-deepwell plates (USA Scientific, Orlando, FL - USA) sealed with an AeraSeal film (Excel Scientific,

406   Victorville, CA - USA) and grown at 37 °C, 900 rpm overnight in a Multitron Pro shaker incubator (INFORS

407   HT, Bottmingen, Switzerland). The overnight growths were diluted 1:200 into 1 ml LB + antibiotics in 2-ml

408   96-deepwell plates + AeraSeal film and grown at 37 °C, 900 rpm. After 2 hours the growths were diluted

409   1:5000 into prewarmed LB + antibiotics + inducer where necessary in 2-ml 96-deepwell plates + AeraSeal

410   film and grown at 37 °C, 900 rpm for 5, 6, 7, 8, 9, 10, or 20 hours. After growth, 2 to 20 μl of culture

411   sample was diluted into 180-198 μl PBS + 200 μg/ml kanamycin to inhibit translation.

412

413   **Cytometry analysis.** Fluorescence characterization with cytometry was performed on a BD LSR Fortessa

414   flow cytometer with HTS attachment (BD, Franklin Lakes, NJ - USA). Cells diluted in PBS + kanamycin were

415   run at a rate of 0.5 μl/s. The events were gated by forward scatter height (mid-log: 1,000-10,000;

416   stationary: 500-5000) and side scatter area (mid-log: 1,000-10,000; stationary: 500-5,000) to reduce false

417   events. After gating, thousands of events were used for analysis. For each sample, the median YFP

418   fluorescence was calculated. All output values are reported in terms of relative promoter units (RPU). For

419   a given promoter measurement, the strain (*E. coli* DH10B, Marionette-Wild, *etc*) is transformed with the

420   plasmid.   The strain is then assayed alongside a strain containing the RPU standard plasmid

421   (Supplementary Figure 1, Supplementary Table 8) as well as an autofluorescence control. The median

422   autofluorescence value is subtracted from the all other median fluorescence values, including that of the

423   RPU standard. The experimental sample value is then divided by the RPU standard value.

424

425   **Library generation.** Portions of the initial libraries were created by PCR using degenerate oligonucleotides

426   (Integrated DNA Technologies Coralville, IA - USA). These fragments were joined into a degenerate, full-

427   length sensor module by overlap PCR. Sensor modules were assembled into selection vectors by Golden

428   Gate assembly[70]. Linear insert and plasmid selection vector were mixed at 1:1 molar ratio totaling ~ 1 μg

429   DNA along with 5 μl 10x T4 ligase Buffer, 1 μl T4 DNA ligase (2,000,000 U/ml), 2 μl BbsI (10,000 U/ml) (all

430   from New England Biolabs, Ipswich, MA - USA) in 50 μl total. Reactions were cycled 45 times between 2

431   minutes at 37 °C and 5 minutes at 16 °C, and then incubated for 30 minutes at 50 °C, 30 minutes at 37 °C,

432   and 10 minutes at 80 °C in a DNA Engine cycler (Bio-Rad, Hercules, CA – USA). An additional 1 μl BbsI was

433   then added, and the assembly was incubated for 1 hour at 37 °C. Assemblies were then purified using

434   Zymo Spin I columns (Zymo Research, Irvine, CA - USA). Host cells were transformed with library plasmid

435   by electroporation. Supplementary Appendix 2 contains a depiction of all degeneracy found in the "Initial

436   library" for each selection.

437

438   **Negative selection.** LB media + 8 mM Cl-Phe (4-Chloro-DL-phenylalanine [Millipore Sigma, St. Louis, MO

439   - USA]) was mixed, autoclaved, and stored at room temperature. Cl-Phe has a tendency to adhere to

440   glassware, and care was taken to avoid disturbing the water + LB powder + Cl-Phe powder mixture prior

441   to autoclaving. LB media + 8 mM Cl-Phe was mixed with plain LB media to achieve the desired

442   concentration of Cl-Phe (See Interventions:[Cl-Phe] for each selection in Supplementary Appendix 2).

443   Following transformation and outgrowth, cultures were diluted into 7 ml LB + Cl-Phe supplemented with

444   antibiotics as well as any cross-reactive inducers (See Interventions:[Negative ligand] for each selection in

445   Supplementary Appendix 2). Cultures were grown at 37 °C for 12-16 hours.

446

447   **Positive selection.** Following negative selection, cultures were diluted 1:100 into 2 ml LB + antibiotics in

448   culture tubes and grown at 37 °C, 250 rpm for 2 hours. The cultures were then diluted 1:100 into 2 ml

449   prewarmed LB + antibiotics + inducer (See Interventions:[Positive ligand] for each selection in

450   Supplementary Appendix 2) and grown at 37 °C, 250 rpm for 4 hours. Following induction, 1 ml of culture

451   was centrifuged at (5,000g, 25 °C, 10 minutes). Supernatant was removed and the cell pellet was

452   resuspended in 50 µl 1x CPR buffer (50 mM Tris-HCl pH 8.8, 10 mM KCl, 2 mM MgSO$_4$, 10 mM (NH$_4$)$_2$SO$_4$).

453   5 µl resuspension was added to 95 µl of 1 x CPR buffer plus 0.4 µM CPR primers and 200 µM dNTPs. This

454   aqueous phase added to a 2 ml centrifuge tube containing 438 µl Tegosoft DEC (Evonik, Essen, Germany),

455   42 µl AbilWE09 (Evonik, Essen, Germany) and 120 µl mineral oil (Millipore Sigma, St. Louis, MO - USA) and

456   the rubber stopped from a 1 ml syringe. The mixture was vortexed at maximum setting for 2 minutes.

457   The emulsion was split evenly five 0.2 ml PCR tubes, and thermal cycled (95 °C for 5 minutes; 20 cycles of

458   [95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 2 minutes/kb]; and 72 °C for 5 min). Emulsions were

459   then centrifuged (10,000g, 25 °C, 10 minutes) and the upper (oil) phase was removed. Then, 100 µl H$_2$O

460   and 500 µl chloroform were added and the mixture was pipetted to disrupt the pellet. The resuspension

461   was then transfered to a 1.5 ml heavy-gel phase-lock tube (5 Prime, San Francisco, CA - USA) and

462   centrifuged (16,000g, 25 °C, 2 minutes). The upper (aqueous) phase was collected, and DNA was purified

463   using Zymo Spin I columns. The library was amplified in a recovery PCR using Accuprime *Pfx* and nested

464   recovery primers, gel purified, and assembled as described above. A depiction of the CPR primer and

465   recovery primer amplification are provided in Supplementary Figure 6.

466

467   **Library shuffling.** Between some rounds of selection (See Interventions:Notes for each selection in

468   Supplementary Appendix 2), libraries were shuffled upon themselves such that fragments of each mutant

17

469 mutually serve as both primer and template for a primer-less PCR amplification[54]. 1 μg linear, library DNA

470 (from the recovery PCR) was added to a mild DNAse reaction (500 mM Tris pH 7.4, 100 mM MnCl2, 0.5 U

471 DNAse [New England Biolabs, Ipswich, MA - USA]) and lightly digested for 3 minutes at 15 °C. Fragmented

472 DNA was purified using Zymo Spin I columns and reassembled in a primer-less PCR in 1 x KAPA HiFi Master

473 Mix (KAPA Biosystems, Wilmington, MA – USA) by thermal cycling (95 ˚C for 2 minutes; 35 cycles of [95 ˚C

474 for 30 seconds, 65 ˚C for 90 seconds, 62 ˚C for 90 seconds, 59 ˚C for 90 seconds, 56 ˚C for 90 seconds, 53

475 ˚C for 90 seconds, 50 ˚C for 90 seconds, 47 ˚C for 90 seconds, 44 ˚C for 90 seconds, 41 ˚C for 90 seconds,

476 68 ˚C for 90 seconds]; and 72 ˚C for 4 minutes). The reassembly was purified using Zymo Spin I columns,

477 reamplified using Accuprime *Pfx* and CPR primers, gel purified, and assembled as described above.

478

479 **On/off screen.** At the end of each selection, libraries were assembled into the YFP screening plasmid

480 (Supplementary Figure 6). Host cells were transformed and plated on LB-agar. Between 35 and 92

481 individual clones were picked and assayed by cytometry as described. Cells were grown with no inducer,

482 in the presence of cognate inducer, and when necessary in the presence of relevant non-cognate inducers.

483 Measurements were made of cells in mid-log phase by cytometry as described. The most promising

484 clones, as judged by dynamic range and orthogonality, were mini-prepped and the sensor region was

485 sequenced.

486

487 **Genomic integration.** In preparation of recombineering, cells were transformed with a plasmid

488 containing Ara-inducible λ Red recombination machinery with a temperature sensitive origin of

489 replication[71]. 50 μl of overnight culture was subcultured in 50 ml LB medium and grown at 30 °C, 250 rpm

490 for 2 hours. 2 mM Ara was added, and the culture continued to grow at 30 °C, 250 rpm for 3 hours. The

491 culture was then centrifuged (4500*g*, 4 °C, 10 minutes) and washed with ice cold 10% glycerol four times,

492 with the fourth resuspension in 200 μl 10% glycerol. Recombineering-ready cells were stored at -80 °C

493 until use. For the first insertion, six genes (*phlF^AM*, *cymR^AM*, *luxR*, *vanR^AM*, *lacI^AM*, and *tetR*) were Golden

494 Gate assembled using BsaI into an RK6 suicide vector (which needs Pir protein in order to propagate)[68].

495 Pir-expressing *E. coli* JTK164H cells were transformed, and plasmids were purified, verified, and linearized

496 with BpiI leaving homology to the *glvC* pseudogene. Recombineering-ready *E. coli* MG1655 cells were

497 electroporated and transformed with gel-purified, linearized inserts. After an outgrowth of one hour at

498 37 °C, transformations were plated on LB-agar plates + antibiotic (5 μg/ml chloramphenicol). Colonies

499 were picked and grown at 37˚C in LB + antibiotic, and the presence of the insert was verified by colony

500 PCR. For the second insertion, this strain was made recombineering-ready and the process was repeated

501 with the next set of genes (*araC*[AM], *araE*, *betI*[AM], and *ttgR*[AM]) with the insert containing homology to *tetR*

502 and *glvC* and conferring resistance to 20 µg/ml spectinomycin. For the third insertion, this strain was

503 made recombineering-ready and the process was repeated with the final set of genes (*pcaU*[AM], *nahR*[AM],

504 and *cinR*[AM]) with the insert containing homology to *ttgR*[AM] and *glvC* and conferring resistance to 5 µg/ml

505 chloramphenicol. See Supplementary Figure 9 for schematic details.

506

507 **Phage transduction to transfer the Marionette cluster.** 50 µl of overnight culture of Marionette-Wild

508 was subcultured in 5 ml LB medium + 0.2% glucose and 5 mM $CaCl_2$ and grown at 37 °C, 250 rpm for 30

509 minutes. 100 µl p1 phage lysate (10^9 pfu/ml) was added and the culture was grown at 37 °C, 250 rpm

510 until lysis (3 hours)[72]. 50 µl chloroform was added and the culture continued at 37 °C, 250 rpm for 5

511 minutes. Culture was centrifuged (9200*g*, 25 °C, 10 minutes). Supernatant was filtered (0.45 µM) and

512 stored at 4 °C until use. 1.5 ml of overnight culture of *E. coli* MG1655, BL21, or DH10B (the DH10B cells

513 contained a temperature-sensitive plasmid transiently expressing RecA) was centrifuged (10,000*g*, 25 °C,

514 5 minutes). The cell pellet was resuspended in 750 µl P1 buffer (5 mM $MgSO_4$ and 10 mM $CalCl_2$) and up

515 to 100 µl Marionette-P1 phage lysate was added. After 30 minutes at 25 °C, 1 ml LB + 200 µl 1 M sodium

516 citrate was added and the culture was grown at 37 °C, 250 rpm for 30 minutes. The culture was

517 centrifuged (10,000*g*, 25 °C, 2 minutes) and the pellets were resuspended in 100 µl LB and plated on LB-

518 agar plates + 5 µg/ml chloramphenicol and 5 mM sodium citrate. Colonies were picked and grown at 37

519 °C in LB + 5 µg/ml chloramphenicol and 5 mM sodium citrate, and the presence of the insert was verified

520 by colony PCR. The entire Marionette cluster was verified by sequencing PCR amplicons of the cluster

521 from the genome of Marionette-Wild.

522

523 **Sensor induction on plates**. Cells were transformed with reporter plasmids and, following outgrowth at

524 37°C for one hour, plated on LB-agar (LB-Miller powder + 1.5 % agar [BD, Franklin Lakes, NJ – USA]) with

525 and without the appropriate inducer. After an overnight incubation at 37°C for 16 hours, plates were

526 incubated at 4°C for one hour and imaged using a ChemiDocMP Imaging system (Bio-Rad, Hercules, CA -

527 USA) and Image Lab 4.0 software (Bio-Rad, Hercules, CA - USA) employing blue epi illumination, a 530/28

528 filter and a 0.1 second exposure. Raw images were rotated and cropped using XnView (XnSoft, Reims,

529 France). Individual colonies were scraped from the plate, resuspended in 200 µl PBS + 200 µg/ml

530 kanamycin, and assayed by cytometry as described in Cytometry analysis.

531

532 **Plate reader assays to measure growth rates**. Glycerol stocks of strains of interest were streaked on LB +

533 1.5% Agar plates and grown overnight at 37 °C. Single colonies were inoculated into 1 ml LB in 2-ml 96-

534 deepwell plates (USA Scientific, Orlando, FL - USA) sealed with an AeraSeal film (Excel Scientific, Victorville,

535 CA - USA) and grown at 37 °C, 900 rpm overnight in a Multitron Pro shaker incubator (INFORS HT,

536 Bottmingen, Switzerland). The overnight growths were diluted 1:200 into 1 ml LB in 2-ml 96-deepwell

537 plates + AeraSeal film and grown at 37 °C, 900 rpm. After 2 hours the growths were diluted (*E. coli*

538 DH10B/Marionette-Clo 1:500; *E. coli* BL21/Marionette-Pro 1:2,000; *E. coli* MG1655/Marionette-Wild

539 1:5,000) into prewarmed LB + inducer where necessary in 2-ml 96-deepwell plates. 100 µl of this culture

540 was immediately transferred to a 300-µl 96-well black walled optical bottom plates (Thermo Scientific

541 Nunc, Waltham, MA – USA) sealed with a BreathEasy film (Sigma-Aldrich, St. Louis, MO – USA), and grown

542 in a Synergy H1 plate reader (BioTek, Winooski, VT – USA) at 37 °C, 1000 rpm. OD_{600} was measured every

543 20 minutes over 12 hours of growth. OD_{600} readings were also taken from wells containing media with no

544 cells, and for each time point, readings from such wells were subtracted from the appropriate sample

545 measurements to remove background. OD_{600} values were converted to equivalent 1 cm path length

546 measurements using a standard curve.

547

548 **Calculation of growth rates.** The doubling time for each 1 hour sliding window was calculated assuming

549 exponential growth during that period. Doubling time is calculated as elapsed time (60 minutes) divided

550 by the number of doublings that occurred in that time ($\log_2[\text{final } OD_{600}/\text{initial } OD_{600}]$). The fastest doubling

551 time (excepting the first three hours) was reported as the doubling time.

552

553 **Evolutionary stability (passaging with reporters).** Marionette-Wild was transformed with each of the 12

554 reporter plasmids and plated on LB-agar. Single colonies were inoculated into 1 ml LB + antibiotics in 2-

555 ml 96-deepwell plates (USA Scientific, Orlando, FL - USA) sealed with an AeraSeal film (Excel Scientific,

556 Victorville, CA - USA) and grown at 37 °C, 900 rpm overnight in a Multitron Pro shaker incubator (INFORS

557 HT, Bottmingen, Switzerland). The overnight growths were diluted 1:200 into 1 ml LB + antibiotics in 2-ml

558 96-deepwell plates + AeraSeal film and grown at 37 °C, 900 rpm. After 2 hours, the growths were diluted

559 1:5000 into prewarmed LB + antibiotics + inducer where necessary in 2-ml 96-deepwell plates + AeraSeal

560 film and grown at 37 °C, 900 rpm for 5 hours. After growth, 20 µl of culture sample was diluted into 180

561 µl PBS + 200 µg/ml kanamycin to inhibit translation and assayed by cytometry as described in Cytometry

562 analysis. Uninduced cultures were allowed to grow at 37 °C overnight (17 hours) and the process

563 (beginning with the 1:200 dilution of the overnight culture) was repeated each day for 14 days.

564

565   **Evolutionary stability (passaging on plates).** A single colony of Marionette-Wild was inoculated into 1 ml

566   LB in 2-ml 96-deepwell plates (USA Scientific, Orlando, FL - USA) sealed with an AeraSeal film (Excel

567   Scientific, Victorville, CA - USA) and grown at 37 °C, 900 rpm for 12 hours in a Multitron Pro shaker

568   incubator (INFORS HT, Bottmingen, Switzerland). The overnight growth was streaked onto LB-agar plates

569   and grown at 37 °C for 12 hours. This process was repeated 9 times. The final culture was made

570   chemically competent as described in Chemical transformation, transformed with relevant reporter

571   plasmids, and assayed with cytometry as described in Cytometry analysis.

572

573   **Evolutionary stability (passaging in liquid culture).** A single colony of Marionette-Wild was inoculated

574   into 1 ml LB in 2-ml 96-deepwell plates (USA Scientific, Orlando, FL - USA) sealed with an AeraSeal film

575   (Excel Scientific, Victorville, CA - USA) and grown at 37 °C, 900 rpm for 12 hours in a Multitron Pro shaker

576   incubator (INFORS HT, Bottmingen, Switzerland). The overnight growths were diluted 1:200 into 1 ml LB

577   in 2-ml 96-deepwell plates + AeraSeal film and grown at 37 °C, 900 rpm. After 2 hours, the growths were

578   diluted 1:5000 into prewarmed LB in 2-ml 96-deepwell plates + AeraSeal film and grown at 37 °C, 900 rpm

579   for 22 hours. This process was repeated 9 times. The final culture was made chemically competent as

580   described in Chemical transformation, transformed with relevant reporter plasmids, and assayed with

581   cytometry as described in Cytometry analysis.

582

583   **Computational methods (fitting to the response function).** To parameterize the response function, error

584   minimization was performed using the Solver function in Excel software (Microsoft, Redmond, WA - USA).

585   Equation 1 was entered with y_{min} , y_{max} , K, and n as tunable parameters, x (inducer concentration) as the

586   independent variable and y as the output. For each x, the error between the measured RPU value and

587   the output of the function was determined. The total error was determined by summing the normalized

588   square of the error ($[y$-measured RPU value$]^2 /y$) for each x. The Solver function minimized the total error

589   by tuning y_{min} , y_{max} , K, and n.

590

591   **Computational methods (partial randomization of amino acids).** Initial libraries for the selection of LacI

592   and AraC utilized partial randomization of amino acids. Previous literature informed decisions regarding

593   the desirability of including each possible residue in the library. The CASTER 2.0 tool[73] was used to design

594   degenerate oligodeoxynucleotides that can sample the desired amino acids while limiting undesired

595   amino acids and stop codons.

596

597   **Computational methods (RBS design).** The initial RBS for each regulator in the Marionette cluster was

598   designed using the RBS Calculator v1.1[55] using the *E. coli* DH10B 16S rRNA setting. The "Pre-Sequence"

599   included the last 100 bp of the upstream CDS (where appropriate) as well as any scars used in Golden

600   Gate assembly. The "Target Translation Initiation Rates" were chosen based on the RBS strength of the

601   plasmid-based sensor (found using the "Reverse Engineer RBSs" tool) and compensating for the reduction

602   in copy number and increase in promoter strength associated with the move to the genomic system. After

603   initial assembly and testing, a decision was made based on the appearance of the response function as to

604   whether the RBS was too low, too high, or good. Low RBSs were rationally mutated to more closely

605   resemble the consensus Shine-Dalgarno sequence (TAAGGAGGT) while high RBSs were rationally mutated

606   to less closely resemble the consensus Shine-Dalgarno sequence. All rationally designed RBS variants

607   were checked using the "Reverse Engineer RBSs" to guard against making dramatic, unanticipated

608   changes to the RBS strength. Rational mutations are noted in Supplementary Table 2. Translation

609   Initiation Rate (in arbitrary units) for each RBS variant is provided in Supplementary Figure 10.

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610

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