A Multimodal Strategy Used By A Large c-di-GMP Network

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1 **Abstract**

2   The *Pseudomonas fluorescens* genome encodes for 50+ proteins involved in c-di-GMP

3 signaling. Here, we demonstrate that when tested across 188 nutrients, these enzymes and

4 effectors appear capable of impacting biofilm formation. Transcriptional analysis of network

5 members across ~50 nutrient conditions indicates that altered gene expression can explain a

6 subset, but not all, of biofilm-formation responses to the nutrients. Additional organization of

7 the network is likely achieved through physical interaction, as determined via probing ~2000

8 interactions by bacterial two-hybrid assays. Our analysis revealed a multimodal regulatory

9 strategy, using combinations of ligand-mediated signals, protein-protein interaction and/or

10 transcriptional regulation to further fine-tune c-di-GMP-mediated responses. These results create

11 a profile of a large c-di-GMP network that is used to make important cellular decisions, opening

12 the door to future model building and the ability to engineer this complex circuitry in other

13 bacteria.

14

15 **Abstract Importance**

16   Cyclic diguanylate (c-di-GMP) is a key signalling molecule regulating bacterial biofilm

17 formation, and many microbes have up to dozens of proteins that make, break or bind this

18 dinucleotide. Thus, a major open question in the field is how signalling specificity is conferred

19 in this context with a soluble signalling molecule. Here, we take a systems approach, using

20 mutational analysis, transcriptional studies and bacterial two-hybrid analysis to interrogate this

21 network. We find that the network typically combines two or more modes of regulation (i.e.,

22 transcriptional control with protein-protein interaction) to generate an observed output.

23

Introduction

Cyclic diguanylate (c-di-GMP) is a second messenger used across a wide range of bacterial species to control important life style decisions. c-di-GMP is produced by enzymes called diguanylate cyclases (DGCs) with the canonical GGDEF domain, and is in turn degraded by phosphodiesterases (PDEs) with the canonical EAL domain. This second messenger governs cellular processes by binding to and activating effector proteins that govern such behaviors as biofilm formation, motility, virulence activation, macromolecular synthesis, cell division, and other processes (1-4).

Many bacteria have large c-di-GMP signaling networks, with dozens of DGCs, PDEs, and possible effectors. Several models have been developed to help explain how large c-di-GMP signaling networks may effectively communicate with specific effector proteins at the right time or location in the cell. One model relies on global levels of c-di-GMP signaling to different effectors based on their binding affinity. Pultz *et al.* demonstrated that two *Salmonella Typhimurium* effector proteins with PilZ domains bound c-di-GMP with over a 40-fold difference in affinity (5). This finding was generalized to *Pseudomonas aeruginosa* where over a 140-fold difference was found among its eight PilZ domain-containing proteins (5, 6). Consistent with this "affinity model", modulating global pools of c-di-GMP has been shown to alter expression and activity of c-di-GMP-metabolizing enzymes, as well as outputs of the network such as exopolysaccharide production and control of flagellar synthesis (7-11). A second model put forth makes use of DGCs, PDEs, and effectors that physically interact with one another allowing for local signaling. Evidence for physical interaction among c-di-GMP enzymes has been found in *Yersinia pestis* (12), and examples of physical interaction being required for signaling include a DGC-PDE interaction in *Escherichia coli* and a DGC-effector interaction in

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47   *Pseudomonas fluorescens* (13, 14). Transcriptional control of the genes coding for c-di-GMP

48   related enzymes, including *rapA* in *P. fluorescens* and several DGCs and PDEs in *Vibrio*

49   *cholerae*, provides a third mechanism of signaling specificity (15-18). Finally, ligand binding

50   may mediate the activity state of various DGCs and PDEs as is the case in *E. coli* for the oxygen-

51   sensing DosC-DosP DGC-PDE pair, or the inactivating effect of zinc on DgcZ (19, 20). Both

52   transcriptional regulation and ligand-mediate activation could conceivable fit in either a global or

53   local signaling model. Thus, there does not appear to be any single mechanism used by bacteria

54   to assure a specific output from this complex network.

55   In order to understand how large c-di-GMP networks are organized and utilized, we

56   focused on *P. fluorescens* Pf01. The mechanism of biofilm formation in *P. fluorescens* is one of

57   the best understood of such signaling systems, from input to output (21, 22). The effector protein

58   LapD binds cytoplasmic c-di-GMP that in turns leads to an accumulation of the large adhesion

59   LapA on the cell surface via an inside-out signaling mechanism (23, 24). *P. fluorescens* contains

60   a large c-di-GMP network, with 21 GGDEF domain-containing proteins, 5 EAL-domain

61   containing proteins, and 17 dual-domain proteins containing both a GGDEF and EAL domain

62   (**Figure 1**). These dual-domain proteins may function as either DGCs, PDEs, both, or as

63   effectors that can bind c-di-GMP (23, 25-27). *P. fluorescens* also contains 6 proteins with PilZ

64   domains, a domain previously characterized as a c-di-GMP binding effector (28, 29). In a

65   previous study, a mutant library of *P. fluorescens* was assayed for a majority of these genes'

66   impact on biofilm formation on a single medium containing glycerol and tryptone. Four DGCs

67   and five putative PDEs appeared to impact biofilm formation under this minimal medium

68   condition, leaving most enzymes without an apparent function (30). This conundrum of "extra

69   enzymes" is not unique to *P. fluorescens*. Similar results have been obtained in *V. cholerae*, *P.*

70   *aeruginosa* and *E. coli* (31-33). This observation leads to two important questions. First, can

71   these other enzymes provide fine control over biofilm formation under non-laboratory

72   conditions? And second, in what ways can such a large network based around a diffusible

73   molecule be organized to provide order to the signaling process?

74   Here, we attempt resolve these questions using *P. fluorescens* Pf01 as a model system for

75   a large c-di-GMP signaling network. We find that when tested under a broad spectrum of

76   conditions, a large majority of the c-di-GMP-related enzymes and effectors of *P. fluorescens* can

77   impact biofilm formation. Further, we find that transcriptional regulation, non-transcriptional

78   responses to putative ligands and protein-protein interaction are common features among

79   network members. Furthermore, these modes of regulation can be combined, for example, with

80   some pairs of proteins interacting while also responding to common extracellular cues. These

81   findings provide a roadmap for approaching c-di-GMP signaling in bacteria of clinical and

82   environmental relevance, with particular emphasis on understanding how multimodal regulatory

83   strategies modulate biofilm formation in the context of a complex network.

84

85

86   **Results**

87   **A majority of proteins with predicted roles as c-di-GMP metabolizing enzymes or effectors**

88   **participate in biofilm formation.** A previous study from our group constructed a mutant library

89   of a majority of DGCs and PDEs in *P. fluorescens* and tested these mutants for biofilm formation

90   on our standard laboratory minimal medium with glycerol and tryptone (30). Four DGCs –

91   GcbA, GcbB, GcbC, and WspR – were found to positively contribute to biofilm formation under

92   these conditions. Mutations in the genes coding for five dual domain proteins – Pfl01_0192,

93   Pfl01_1887, Pfl01_2709, Pfl01_4086, and Pfl01_4876 – were found to increase biofilm

94   formation under these conditions, indicating that they likely act as PDEs. Given the large array

95   of remaining GGDEF and EAL domain-containing proteins that demonstrated no phenotype

96   under this single growth condition, we hypothesized that some number of these c-di-GMP

97   metabolizing enzymes and/or effectors may also contribute to biofilm formation under

98   conditions not previously tested. Further, in *P. fluorescens* most c-di-GMP related enzymes are

99   predicted to be fused to a variety of ligand-binding domains, including CACHE, CHASE, and

100   GAF domains (**Figure 1**). It therefore seemed possible that the c-di-GMP metabolizing enzymes

101   could be activated by growing *P. fluorescens* under different conditions. To this end, we tested

102   the WT and the 50 strains each carrying a mutation in an individual c-di-GMP-related gene

103   (**Table S1**) using Biolog plates (Biolog, Inc) to test 188 different nutrients (**Table S2**) for their

104   impact on biofilm formation. While we refer to the compounds in each of the wells of the Biolog

105   plates as "nutrients" because they are all organic compounds, these compounds could

106   conceivably serve as carbon sources, input signals, both or neither. The term "nutrients" is used

107   here as a convenient shorthand to generally describe the chemical compounds in the Biolog

108   plates.

6

109   The minimal medium typically used to grow *P. fluorescens* biofilms for our studies,

110   referred to as K10-T1 (K10), is a high phosphate medium containing tryptone and glycerol as

111   carbon and energy sources (30). For the purposes of testing biofilm formation in the Biolog

112   plates, we used a similar medium lacking the tryptone with its diverse array of carbon sources.

113   Because the nutrient sources tested here may not be metabolized by *P. fluorescens*, yet may still

114   impact the activity of the c-di-GMP network, glycerol was retained in this medium to permit

115   growth. We refer to this glycerol-containing medium as "base minimal medium" (BMM, see

116   Materials and Methods for details).

117   In-frame deletions of genes encoding the predicted DGCs, PDEs, and effectors as well as

118   the six genes predicted to encode PilZ domain-containing proteins and a FleQ homologue were

119   constructed. The remainder of tested strains originate from a previously reported (30) single-

120   crossover mutant library (**Table S1**). Each strain was grown overnight in LB with aeration,

121   normalized to OD_{600} of ~0.716, and added to the BMM with each carbon source found in the

122   Biolog plates before allowing biofilm formation to commence. The nutrients that were toxic to *P.*

123   *fluorescens*, as indicated by lack of growth of the wild-type strain (not shown), were eliminated

124   from the analysis. Of the remaining nutrients, it was clear that different nutrients have

125   differential effects on biofilm formation (**Figure S1**).

126   Of the 50 mutants tested, 44 mutants demonstrated a significant difference ($p > 0.001$)

127   from wild type when comparing their median ability to promote biofilm formation across all

128   nutrients tested (**Figure 2A**). Among strains lacking the GGDEF-containing proteins, 7 showed a

129   significant reduction in biofilm formation as might canonically be expected for this class of

130   mutants. Surprisingly, 11 of these mutants with mutations in DGC-encoding genes showed a

131   significant increase in biofilm formation, suggesting that some putative DGCs may be able to

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132   impact biofilm formation contrary to the manner canonically associated with their enzymatic

133   activity. Another 3 mutants in DGC-encoding genes showed no significant impact. Results from

134   strains lacking EAL-containing proteins were more straightforward, with 4 such mutants

135   showing elevated levels of biofilm formation, and a single strain that did not significantly differ

136   from wild type.

137   Analysis of strains lacking the dual-domain proteins revealed a range of results. 10 of the

138   mutant strains demonstrated elevated levels of biofilm formation, three showed a decrease, and

139   four had no significant impact (**Figure 2A**). While prediction of catalytic activity from sequence

140   alone can be unreliable, it is noteworthy that the bulk of mutants lacking dual-domain proteins

141   show elevated biofilm formation (**Figure 2A**). Given that *P. fluorescens* harbors 21 GGDEF

142   containing-proteins while having only five EAL domain-containing proteins, it is perhaps not

143   surprising that most of the dual domain proteins participate in c-di-GMP turnover.

144   Of the putative effectors, the PilZ domain-containing mutants of Pfl01_0958,

145   Pfl01_4008, and Pfl01_4884 showed significant increases across all carbon sources, mutants of

146   Pfl01_3860 and Pfl01_4257 showed no significant variation, and Pfl01_4150 mutant showed a

147   modest but significant decrease across all carbon sources compared to the wild type. When their

148   encoding genes were mutated, two of the effectors, the FleQ homolog Pfl01_1532 and LapD,

149   both showed significant loss of biofilm formation across a majority of the conditions tested,

150   demonstrating their general relevance to promoting biofilm formation.

151   Interestingly, 11 mutants that showed no aggregate change in biofilm formation across all

152   conditions. These include the GGDEF domain-containing Pfl01_0692, Pfl01_2176, Pfl01_2295,

153   Pfl01_4451 and Pfl01_5168; the EAL domain-containing Pfl01_2920; the dual domain-

154   containing Pfl01_1887, Pfl01_2709, Pfl01_5518, and Pfl01_5643; and the PilZ domain-

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155   containing Pfl01_4884. However, while there may be no global effect detected, there are

156   individual nutrients that appear to impact biofilm formation for these mutants. For example, the

157   Pfl01_1887 mutant showed no significant difference from wild-type in this overall comparison,

158   despite being previously identified as a critical contributor to biofilm formation in this organism

159   in the minimal K10 medium where its mutation results in elevated biofilm formation (30).

160   Taken together, our data suggest that while most genes encoding components of the c-di-GMP

161   network when mutated show a biofilm phenotype under at least some small set of nutrients, other

162   genes appear to play a much broader role in biofilm formation across many environments.

163

164   **Biolog data reveals heterogeneity in mutant-by-mutant response to nutrients**. To assess

165   trends across nutrient conditions and potential patterns across genes, all values for each genotype

166   were expressed as a ratio relative to that genotype's median biofilm formation across all

167   conditions. This treatment of the data converts each reading to a measure of how the biofilm in

168   that condition differed from a baseline biofilm formation for that strain. Each of these ratios was

169   then compared to that of WT, and any mutants that show a response to a particular condition as

170   evidenced by a difference in their ratio to baseline by a statistically significant amount ($p < 0.05$,

171   See Materials and Methods for details) are highlighted in **Figure S2** (red: higher ratio than WT;

172   blue: lower ratio than WT; black: not significantly different from WT). We therefore are able to

173   view data points from mutants which differ from how the wild-type behaved in the same carbon

174   source, while downplaying the global effect a mutation might have across all carbon sources

175   from driving the outcome of the analysis (see **Figure 2A**). Squares that are black therefore do not

176   necessarily represent nutrients conditions with no phenotype for that mutant, but represent

177   instances where the mutant did not respond significantly differently from WT to a given

178   condition. This analysis thus provided us an opportunity to examine how mutants behaved in

179   specific nutrients irrespective of their general phenotype.

180   An immediately apparent finding is the heterogeneity of phenotype that a given mutant

181   can have in particular carbon sources. No class of mutations in GGDEF-, EAL-, Dual- or PilZ-

182   encoding genes was exempt from producing higher and lower biofilm than expected compared to

183   wild type under particular nutrient conditions (**Figure S2**). Further, no class of nutrients tended

184   to generally promote significantly different biofilm levels. The lack of association between

185   particular kinds of nutrients and the phenotypes of particular classes of mutants may indicate that

186   different enzymes in the network respond to different input conditions.

187

188   **Differential impact of nutrients as a function of the genes in the c-di-GMP network**. To

189   display the relative impact of the different nutrients tested here on biofilm formation by strains

190   carrying mutations in the network, we plotted the percentage of conditions with a biofilm

191   phenotype (decreased or increased) as a function of each mutant (**Figure 2B**). These data show

192   that some mutants display a biofilm phenotype in a large number of the conditions tested. For

193   example, the *lapD* mutant shows significantly reduced biofilm formation in >20% of the

194   conditions tested, confirming its broad importance in biofilm formation. In contrast, other

195   mutants showed changes in biofilm formation in <5% of the conditions tested, with the strain

196   carrying a mutation in Pfl01_5643 showing a biofilm phenotype in only 1 of the 188 nutrients

197   tested. Interestingly, the mutants with increased biofilm formation are not uniformly disrupting

198   genes predicted to code for PDEs, and thus predicted to result in increased c-di-GMP and

199   enhanced biofilm formation. Similarly, the classes of proteins represented on the right side of

200   the figure also include DGCs (**Figure 2B**). These data speak to the differential impact of

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201   mutating different components of the network across the possible environments this microbe

202   may encounter.

203

204   **c-di-GMP-related genes are transcriptionally regulated in response to nutrients.** Given that

205   different DGCs, PDEs and effectors appeared to play a role in biofilm formation depending on

206   the conditions tested, we hypothesized that transcriptional regulation may be partly responsible

207   for determining which enzymes are present to contribute to c-di-GMP signaling. While very few

208   transcriptional studies of DGCs/PDEs/effectors have previously been conducted, there is some

209   precedence for transcriptional regulation. For example, transcription of the *rapA* gene, which

210   codes for a dual domain protein of *P. fluorescens* shown to have PDE activity, has been

211   previously demonstrated to be up-regulated when cells are grown in a low phosphate medium

212   (15).

213   Our goal was to capture possible roles for transcriptional regulation that may be

214   responsible for phenotypes observed in the Biolog plates. To this end, we ranked carbon sources

215   based on their ability to promote biofilm across all tested strains, and selected 14 "low" and

216   "medium" biofilm-promoting nutrients, 13 "high" biofilm-promoting carbon sources, and 18

217   "very high" biofilm-promoting carbon sources (**Table S3, Figure S1**). To assess transcription of

218   all the genes encoding DGCs, PDE, dual domain and PilZ proteins, cells were grown on the

219   BMM with the selected nutrients at a concentration of 2 mM unless, otherwise indicated (see

220   **Table S3**), for six hours before RNA was extracted. Expression analysis was conducted using the

221   Nanostring nCounter system, which directly measures transcript abundance without an

222   amplification step, and raw data were expressed as reads per 1000 counts (see Materials and

223   Methods, **Table S4 & S5**). This normalization strategy has been successfully employed

224   previously in *P. aeruginosa* (34), and we have adopted it here for the purposes of comparing

225   transcripts across many conditions.

226   To assess the impact of nutrients on expression of the genes in the c-di-GMP network, we

227   plotted the ratio of the transcript reads produced by each genes from each of the 46 nutrients

228   tested over the reads obtained in the BMM (e.g., ratio = reads on nutrient X/reads on base

229   medium). **Figure 3A** shows the individual data points with significant changes in expression as

230   determined indicated by the blue dots (see Materials and Method as for statistical analysis,

231   **Figure S3** for the data summarized as a heatmap, and **Figure S4A** for the figure with a legend

232   showing the nutrients). **Figure 3A** and **Figure S4A** shows that for most genes and for most

233   nutrients, the change in gene expression is <2-fold. However, there are several instances where

234   specific nutrients increase or decrease gene expression from 2-8-fold. We found that the gene

235   showing the highest fold-change encoded RapA, a verified PDE. The expression of *rapA* was

236   previously shown to be strongly up-regulated under low phosphate conditions (15), thus serving

237   to validate our transcriptional analysis. These data indicate that the degree of transcriptional

238   response to any particular nutrient, when such a response is detected, in quite modest (~2-fold).

239

240   **A few nutrients drive the largest changes in expression of genes in the network**. In a large

241   network of many related c-di-GMP proteins, one may consider two general methods of gene

242   regulation. The first is to have one or a small number of c-di-GMP-related genes dramatically

243   up- or down-regulated to a given condition, such as the response of *rapA* in low phosphate. An

244   alternative strategy would be to finely regulate a larger number of genes in the network at the

245   same time to produce a desired c-di-GMP output. To test this idea, we examined which nutrients

246   cause the greatest fold-increase and decrease in each gene compared to the BMM. If groups of

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247   genes are not regulated together under the same conditions, we would expect that the nutrients

248   causing the largest changes compared to the base medium to be different for each gene.

249   Conversely, if genes are regulated together, we would expect the same carbon sources to be

250   responsible for the largest fold changes in several genes across the network.

251   We found that six nutrients – m-tartaric acid, glycogen, D-ribose, D-galactose, L-proline,

252   and the low phosphate condition – are responsible for the highest recorded expression of 26

253   genes in the network (**Table S6** – bolded nutrients). Intriguingly, this same group of nutrients is

254   responsible for the lowest relative expression of an overlapping group of 29 genes. Fold-change

255   compared to the BMM among these genes varied greatly, from near baseline to up to five-fold.

256   Furthermore, m-tartaric acid and glycogen were found to be among the highest and lowest

257   biofilm-promoting nutrients we tested (**Table S3**). Notably, these two nutrients were responsible

258   for a majority of the peak high and low transcriptional values, impacting 18 and 10 genes,

259   respectively (**Table S6**). Overall, 12% of the tested nutrients appear to create the largest

260   transcriptional changes in half of the genes in the network, suggesting that at least for some

261   nutrients that do impact transcription, they may do so across many genes simultaneously.

262

263   **Growth on a surface minimally impacts expression of genes in the c-di-GMP network**.

264   Previous studies in pseudomonads showed that growth on a surface increases c-di-GMP levels

265   compared to growth as planktonic cells (35-37). Thus, we compared the expression of all genes

266   in the c-di-GMP network for a select set of nutrients in liquid medium versus the same medium

267   solidified with 1.5% agar. Specifically, we investigated gene expression on K10T-1, K10T-1

268   with low phosphate, BMM, as well as the BMM supplemented with citrate, pyruvate, and L-

269   methionine (**Table S7**). The data were plotted as a ratio of the high c-di-GMP to the low c-di-

13

270   GMP condition on a per-nutrient basis and analyzed by a Wilcoxon signed rank test with a

271   multiple comparisons correction (**Figure S4B**). Only three genes were found to have a

272   significantly different expression on a surface compared to a liquid (**Figure S4B**). The *gcbA*

273   gene, which has previously been described as being important in early surface attachment (38)

274   was significantly more highly expressed when cells were grown in liquid, while the genes

275   Pfl01_0050 and Pfl01_1336 were significantly more highly expressed when cells were grown on

276   a solid. The *gcbA* and Pfl01_ 1336 genes were both differentially regulated by ~2-4-fold, while

277   Pfl01_0050 exhibited less than a 2-fold change. Based on the modest dynamic range we

278   observed for most genes in the c-di-GMP network when exposed to different chemical

279   compounds (**Figure 3A**), it may possible that these differences in expression could be sufficient

280   to impact the role of these genes in the response of cells to a surface.

281

282   **Modulation of gene expression in the network by c-di-GMP**. In *P. aeruginosa*, increased

283   levels of c-di-GMP positively regulate the expression of exopolysaccharide production and

284   down-regulate expression of flagellar genes (8, 10, 39). Furthermore, previous reports have

285   shown c-di-GMP can regulate transcriptional levels of genes through riboswitches, although

286   there have been no reports of such structures in *P. fluorescens* (7, 40). Thus, we assessed the

287   impact of modulating c-di-GMP levels on expression of genes in the network by selecting two

288   strains of *P. fluorescens* that produce low and high levels of c-di-GMP, respectively. The

289   previously reported Δ4DGC mutant lacks the GcbA, GcbB, GcbC, and WspR DGCs, fails to

290   make a biofilm in K10 medium, and produces 2-fold lower c-di-GMP than the wild-type strain

291   (41). Conversely, the GcbC R366E mutant contains a point mutation in the auto-inhibitory site of

14

292   this DGC, producing over 10-fold more c-di-GMP than the Δ4DGC strain (41). Three biological

293   replicates of each strain were grown on BMM and analyzed via Nanostring (**Table S8**).

294   Most genes showed changes of <2-fold (**Table S8**), indicating that this large c-di-GMP

295   network does not broadly rely on a core global level of intracellular c-di-GMP to regulate the

296   transcription of other members of the network, although there is a small set of genes whose

297   expression is significant impacted by c-di-GMP levels (**Table S8**, in bold). The gene Pfl01_4451

298   (the homolog of the *P. aeruginosa sadC* gene) was up-regulated 2.4-fold in the high c-di-GMP

299   mutant compared to wild-type. This result was significant (p<0.01, t-test with Benjamini-

300   Hochberg correction comparing the low and high c-di-GMP producing strains). The *sadC* gene is

301   known to be important in early surface responses. Additionally, Pfl01_0190, Pfl01_5255, and

302   Pfl01_5518 also show significant and greater than 2-fold differences in expression, between the

303   low and high c-di-GMP strains tested here, indicating that transcription of only a small portion of

304   the network is sensitive to large swings in intracellular c-di-GMP concentrations.

305

306   **Broad trends observed in the transcriptional network of *P. fluorescens* are also seen in *P.**

307   ***aeruginosa***. Our analysis of the expression of c-di-GMP-related genes (CDG genes) in *P.*

308   *fluorescens* showed that, with the exception of *rapA* in low phosphate, these genes do not show

309   large variations across the conditions tested. To address whether a similar pattern of expression

310   of CDG genes is found in another related organism, we analyzed the large number of microarray

311   experiments that have been conducted in *P. aeruginosa*. Using the tools developed by Tan *et al.*

312   (42) we assembled a compendium of all published microarrays in *P. aeruginosa*. This

313   compendium includes data for 5549 genes in 1185 experiments including 78 different growth

314   media.

15

315 First, to assess whether the broad characteristics of expression of CDG genes in *P.*

316 *fluorescens* Pf01 are also seen in *P. aeruginosa* we compared the overall expression of CDG

317 genes in both organisms. To do this analysis, we normalised the expression of genes between

318 experiments in the compendium by converting all expression data to values between 0 and 1

319 according to how they compare to the highest and lowest reported value for each experiment,

320 with the highest value becoming 1 and the lowest value becoming 0. We compared summary

321 statistics of these normalized data to our counts per 1000 normalized Nanostring data (see **Table**

322 **S5**). We calculated the median expression for CDG genes in both datasets, as well as the median

323 of non-CDG genes, which we defined as genes coding for proteins without a GGDEF, EAL, HD-

324 GYP, or PilZ domain. We also included the Pho-regulon in the *P. aeruginosa* PAO1

325 compendium for a comparison with a group of genes that are under robust transcriptional

326 regulation. The pattern of expression observed for *P. fluorescens* Pf01, wherein most genes

327 encoding cdG-metabolizing enzymes show relatively low levels of expression while PilZ-

328 domain-encoding genes are more highly expressed (**Figure S5A**), is also observed for *P.*

329 *aeruginosa* PAO1 (**Figure S5B**), although the difference in magnitude of expression of the PilZ-

330 encoding genes is lower in *P. aeruginosa* PAO1.

331 Given that the methods used to assess gene expression differ between *P. aeruginosa*

332 PAO1 (microarrays) and *P. fluorescens* (Nanostring), we sought to use a common metric to

333 compare variation in gene expression between the two microbes. We calculated the coefficient

334 of variance for each data set by dividing the standard deviation by the mean of the expression of

335 each gene across conditions tested. Variability in the expression of these genes across conditions

336 was similar between the two organisms as judges by this metric, with both organisms exhibiting

337 lower variability in CDG gene than the median of non-CDG genes (**Figure S5C,D**). Conversely,

16

338   the variability of genes in the Pho regulon was higher than that of most non-CDG genes (**Figure**

339   **S5D**). Together these data suggest transcriptional regulation across multiple growth conditions is

340   not likely a major factor controlling the contribution of genes involved in the c-di-GMP network

341   in either *P. fluorescens* or *P. aeruginosa*.

342   To investigate potential transcriptional co-regulation common to both organisms, we

343   applied a simple pairwise Spearman correlation analysis of all genes in the compendium and

344   Nanostring datasets. In *P. fluorescens* we found that several genes seem to correlate strongly and

345   significantly with one another (**Figure S5E**), but with fewer genes being significantly and

346   strongly correlated with one another compared to *P. aeruginosa* (**Figure S5F**). These correlation

347   analyses suggest that while the magnitude of transcriptional changes are not large, groups of

348   CDG genes may be under related transcriptional regulation in both organisms.

349

350   **Evidence for nutrient-mediated, non-transcriptional control of proteins in the network**. We

351   next explored how much of the response of the network to the nutrients tested can be explained

352   by transcriptional regulation. That is, how often does a given mutant's defect when grown on

353   "nutrient X" correlate with a change in the transcription of that gene when grown on "nutrient

354   X"? To address this question, we assessed what portion of the conditions tested in both Biolog

355   and Nanostring were associated with changes in either biofilm formation, gene expression, both

356   or these phenotypes, or neither (**Figure 3B**).

357   In most cases that was no link between a change in biofilm formation in a particular

358   mutant and a change in expression of a c-di-GMP-related gene in that condition (**Figure 3B**).

359   However, we did observe instances of biofilm phenotypes that are associated with differences in

360   gene expression (**Figure 3B**), suggesting that gene expression may explain the impact of these

17

361   conditions. Data from the nutrients with both biofilm impact and gene expression difference are

362   displayed in **Figure 3C**. Interestingly, m-tartaric acid impacted both the biofilm formation and

363   expression of 13 different genes. m-Tartaric acid also induces one of the highest overall biofilm

364   tested, suggesting that m-tartaric acid may be an important signal or nutrient for *P. fluorescens*

365   and exert at least some degree of its impact on biofilm formation via transcriptional control. As

366   noted above, in a majority of circumstances, when biofilm formation occurs it can be attributed

367   to the chemical compound added in the absence of any detectable transcriptional change (**Figure**

368   **3B**). Given the large number of putative sensory domains present in many of the c-di-GMP-

369   related proteins in *P. fluorescens* Pf0-1, we suggest that nutrients may be impacting the c-di-

370   GMP network by controlling enzyme activity.

371

372   **Physical interaction is common for DGC and dual-domain proteins.** While patterns of

373   biofilm formation and transcriptional regulation emerged from the differing nutrient conditions

374   tested, these findings did not appear sufficient to explain how this larger network might be

375   ordered to prevent cross talk among its many enzymes and effectors. Furthermore, physical

376   interaction as a mechanism of signaling specificity has precedence in a number of bacterial c-di-

377   GMP systems including *E. coli*, *P. aeruginosa*, and *Xanthomonas axonopodis*. (13, 43, 44). In *P.*

378   *fluorescens*, physical interaction between the DGC GcbC and the effector LapD has previously

379   been shown to be required for specificity of GcbC signaling in biofilm formation (14). However,

380   it is largely unknown how common physical interaction is among enzymes and effectors in the

381   network, whether a given protein may have a single or many interaction partners, or if a given

382   type of c-di-GMP protein is more or less likely to form physical interactions than others.

383   To address these questions, each c-di-GMP related gene in *P. fluorescens* was cloned into

384   both "bait" and "prey" vectors of a bacterial two-hybrid (B2H) system to assess the capacity of

385   each protein for physical interaction. Each member of every protein type – GGDEF, EAL, dual-

386   domain, and PilZ – was tested against all members of the other types. Additionally, all dual-

387   domain proteins were also tested against all other dual-domain proteins as it is possible that such

388   proteins may act as DGCs, PDEs, both or as effectors. The summary of this analysis, which

389   represented close to 2000 bacterial two-hybrid assays, is presented in **Figure 4, Figure S6** and

390   **Table S9**.

391   Several dual-domain proteins showed a relatively high capacity for interaction with other

392   network members (**Figure 4A**). In particular LapD, Pfl01_0192, Pfl01_1252, Pfl01_2525,

393   Pfl01_2709, Pfl01_4086, and Pfl01_4487 interacted with 7 to 18 partners each. A subset of 15

394   proteins interacted with LapD (**Figure 4B**) and four additional dual-domain proteins interacted

395   with greater than 10 partners (**Table S9 and Figure S6**). Conversely, RapA and Pfl01_1887 are

396   not a part of these larger hubs, interacting with only two and one partners, respectively (**Figure**

397   **4A and Table S9**).

398   DGCs showed a highly variable propensity to interact (**Table S9**). 14 DGCs appear to

399   participate in physical interaction, having between one and eight interaction partners.

400   Interestingly, the four DGCs previously found to be necessary for biofilm formation under

401   K10T-1 media conditions have different interaction profiles. GcbA and GcbB failed to interact

402   with other proteins in this assay, while by contrast GcbC interacts with several dual-domain

403   proteins (including LapD) and with an EAL domain-containing protein. WspR has a single

404   interaction partner - Pfl01_1092. The remaining seven DGCs do not engage in detectable

405   physical interaction under our assay conditions.

19

406   Finally, little evidence was found to indicate that EAL and PilZ domain-containing

407   proteins interact with other members of the network (**Figure 4A**). Among the five EAL domain-

408   containing proteins, only Pfl01_2920 was found to interact with two DGCs and LapD. Likewise,

409   a single PilZ domain-containing protein, Pfl01_3860, interacts with the GGDEF domain-

410   containing protein Pfl01_3550 and the dual-domain protein Pfl01_0192. The Pfl01_3860 PilZ

411   domain-containing protein is part of a small node made up of a GGDEF domain and two dual-

412   domain proteins (**Fig. 4A**). Together, these data indicate that a number of the proteins in the

413   network have the potential to interact, and thereby potentially form signaling complexes.

414

415   **Relationship between interacting proteins and shared biofilm phenotypes.** Having shown

416   that some, but not all impacts of different conditions on biofilm formation may be explained by

417   transcriptional regulation, we next tried to determine how much of a role physical interaction

418   might play in contributing to signaling specificity in the network. To perform this analysis, we

419   assessed the conditions wherein both members of an interacting pair of proteins also displayed a

420   significant biofilm phenotype when mutated. We scored these biofilm phenotypes based on

421   whether they were in the same direction (i.e. both enhanced biofilm or both reduced biofilm) and

422   calculated the proportion of the total biofilm phenotypes each member of the interacting pairs

423   shared (**Figure 5A**). This analysis shows that most interaction partners impact biofilm in only

424   one or two of the same conditions, however some pairs do demonstrate a large overlap. For

425   example, the interaction pairs such as *wspR*-Pfl01_0192 and Pfl01_0692-Pfl01_1887, which only

426   impact biofilm formation in a few conditions, overlap in most of those conditions. Furthermore,

427   there are several instances wherein interacting pairs, when mutated, show opposite biofilm

428   phenotypes in a particular nutrient condition. Such a finding is likely physiologically relevant

429   given that the biofilm formation is impacted under a common growth condition. It is also not

430   surprising that overlapping conditions between interaction partners are generally consistent in

431   being all in the same direction or all in the opposite directions, as mutants generally only

432   impacted biofilm formation in one direction. However, it is worth noting that interaction partners

433   seem equally likely to act on biofilm formation in the same direction as they do in opposite

434   directions. These data suggest that there may be numerous examples of both antagonistic and

435   cooperative interactions in this network, often involving the same proteins, and possibly

436   modulated via physical interaction.

437

438   **Intersection between B2H and transcriptional control.** We next examined possible overlap

439   between proteins that interacted with one another and were coordinately expressed. We scored

440   the number of conditions where genes of an interacting pair changed in expression under the

441   same condition. A number of interacting pairs showed coordinated transcriptional changes under

442   one to seven conditions (**Figure 5B, Table S10**). Interesting, some genes that only showed

443   significantly different expression from the BMM in one or a small number of conditions (see

444   **Table S5**) did so under the same nutrient additions as genes encoding for their interaction

445   partner. For example, for the Pfl01_0192-Pfl01_4451 interacting pair, Pfl01_0192 shares all

446   seven conditions that produce differential expression from the BMM with the GGDEF domain-

447   containing Pfl01_4451. Together, these results suggest that in some cases, a combination of

448   transcriptional control and physical interactions may be utilized to modulate signalling

449   specificity.

21

Discussion

450

451   In this study, we investigated the c-di-GMP network of *P. fluorescens*. We selected this

452   model organism because it has one of the best-understood c-di-GMP circuits, from input to

453   output, with the control of localization of the cell-surface biofilm adhesion LapA as a key output

454   (21, 22). LapA appears to contribute to or be required for biofilm formation under every

455   condition we have examined to date, including the base minimal medium used in this study, and

456   this same medium supplemented with the 188 nutrients investigated here. Our analysis revealed

457   one simple, central conclusion: no one mode of regulation outlined above (nutrient input,

458   transcriptional regulation, protein-protein interaction) adequately describes the function of the

459   network. Instead, these modes of regulation can function in combination to contribute to tuning

460   of the network. We propose that this multimodal strategy of controlling c-di-GMP-mediated

461   outputs ultimately provides fidelity and specificity to the network.

462   We found that a majority of c-di-GMP related genes in *P. fluorescens* impact biofilm

463   formation when mutated and tested under a large array of conditions, indicating that context is

464   key for understanding the function of these large signaling networks. Further, this context

465   appears to shape the organization of the network in several ways. We found that transcriptional

466   regulation in *P. fluorescens* rarely results in over 5-fold changes in transcription across all tested

467   conditions. It was also rare for any given nutrient and any single gene that a transcriptional

468   change to be solely responsible for an observed biofilm defect. Despite this observation, we did

469   find that a small number of nutrients were responsible for the largest (albeit modest) change in

470   expression for many genes. For example, we identified several groups of genes whose expression

471   changed in the same nutrient conditions (i.e., m-tartaric acid, D-ribose, glycogen), possibly

472   suggesting co-regulation. Given this finding, we speculate that some genes in the c-di-GMP

22

473   network may be regulated as "suites", and coordinated 2- to 5-fold changes to a single nutrient

474   across 8 to 12 genes may be an effective method of governing the network by controlling which

475   proteins are present under that given environmental condition. Why would these nutrients in

476   particular regulate suites of genes? Interestingly, m-tartaric acid is produced and metabolized by

477   fluorescent pseudomonads (45) and plays a critical role in solubilizing inorganic phosphate (46);

478   phosphate is a key regulator of biofilm formation by this microbe (47). The physiological

479   relevance of D-ribose and glycogen is less clear. Finally, given that many of the members of the

480   c-di-GMP network, including those encoded by these suites of genes, also have putative ligand

481   binding domains, it is quite possible that more that one mechanism regulates the function and/or

482   specificity of these network members.

483   Physical interaction was also found to play a role in the network. In comparison to our

484   transcriptional results, we found many discrete examples where two genes, when mutated and

485   shared an altered biofilm phenotype on the same nutrient source also physically interact with one

486   another. Of the 85 pairs of interacting proteins analyzed only 6 shared no biofilm phenotypes,

487   and more than a quarter (24) of the pairs shared a biofilm phenotype in 25 of more nutrient

488   conditions. The remaining pairs shared phenotypes between 1 and 25 nutrient conditions. We

489   interpret the relatively common frequency of interacting pairs sharing biofilm phenotypes to

490   indicate that physical interaction occurs among network members that can exert their influence

491   in similar environmental conditions. Such a combination of common nutrient inputs among

492   interacting proteins provides one of the strongest findings supporting the concept of multimodal

493   regulation. In the case of those interaction pairs which show little or no overlap in their mutant's

494   biofilm phenotype, it possible that we have not yet identified the correct conditions for which the

23

495   interaction pair is relevant. Alternatively, some of these enzymes and effectors may interact but

496   are alternatively activated in differing environments.

497   The potential for physical interaction, especially among the DGCs and the LapD receptor

498   is potentially informative regarding signaling specificity. We reported previously that GcbC and

499   LapD interact (14), and a recent report by Cooley, O'Donnell and Sondermann (48) proposed

500   that LapD forms a dimer-of-dimers "basket", with space for a DGC to nestle within the basket –

501   it is possible, for example, that all DGCs that interact with LapD have the potential to form this

502   signaling complex.   Furthermore, given that many DGCs have associated ligand-binding

503   domains and show relatively few examples of ligand-mediated transcriptional control, we

504   propose that interaction of DGCs with the LapD receptor, coupled with ligand-mediated control

505   of DGC activity, could be a general mechanism of signaling specificity. Finally, the propensity

506   of members of the network to interact indicates the possibility that "local" signaling is a common

507   aspect of the network, and any models describing how the network functions cannot ignore this

508   feature (49).

509   We also identified a number of putative DGCs that worked 'against type', whereby their

510   absence actually resulted in an increase in biofilm formation in a number of conditions at varying

511   frequencies. Previous work has shown that the DGC GcbA in *P. aeruginosa* is partly responsible

512   for modulating a PDE linked to dispersal of biofilm (50), providing some precedence that DGCs

513   do not necessarily have to promote biofilm formation. However, we note here that two DGCs

514   (i.e., Pfl01_2049 and Pfl01_2297) that interact with LapD demonstrated this analogous

515   phenotype under some conditions, leading to the possibility that some DGCs may exert their

516   impact by binding and blocking effector proteins. Additionally, strains carrying mutations in

517   some DGCs were found to either enhance or reduce biofilm formation depending on the nutrient

518   tested, suggesting the mechanism of their impact on biofilm formation of some of these enzymes

519   may be different from what was reported for the *P. aeruginosa* GcbA enzyme. It is not

520   inconceivable that physical interaction may play a role in this phenomenon as well, with DGCs

521   blocking effectors until the correct nutrient/ligand activates the DGC, whereupon catalytic

522   activity is initiated or structural rearrangements cause the enzyme to change its interaction

523   profile.

524   While we have provided insight into the how a large c-di-GMP network is organized and

525   regulated, we note that there are also several hurdles to interpreting the results presented here.

526   One alternative explanation for the high percentage of c-di-GMP related proteins impacting

527   biofilm is that they are in fact responsible for different cellular process that indirectly have an

528   impact on biofilm formation. It is also likely that the nutrients tested here do not represent the

529   complete collection of environmental cues that these enzymes and effectors respond to in terms

530   of catalytic activation and transcriptional regulation. Indeed, the largest transcriptional change

531   observed was for *rapA*, the PDE up-regulated in a low phosphate condition, suggesting that

532   inputs other than organic compounds can impact the network. Further, we note that several

533   transcripts measured in our Nanostring experiments showed very low expression under most

534   conditions. Still, we find it compelling that a small subset of nutrients produced both the highest

535   and lowest transcriptional changes of a small number of genes, indicating that genes may be

536   regulated as suites in addition to being regulated individually by highly specific input signals.

537   Finally, we note that the bacterial two-hybrid only presents evidence of which proteins interact

538   with each other (or not) in a heterologous host under one growth condition (i.e., LB). That is, it is

539   conceivable that the ligand bound state of a DGC or PDE may not only activate/deactivate

540   enzyme activity, but it may also cause structural changes making the protein competent or

541   incompetent for interaction. Additionally, there may be further interactions we were not able to

542   observe in this system, either because the protein is unstable/unable to function outside of its

543   native host, or alternatively because the interaction was not observable under our assay

544   conditions.

545   Taken together, our results show that this large c-di-GMP network is a dynamic system

546   capable of responding in specific ways to a variety of inputs with individual members able to

547   take on different functions under differing circumstances. Importantly, we identified numerous

548   instances where combined modes of regulation are observed (nutrients inputs/protein-protein

549   interactions, transcription control/protein-protein interactions, nutrient inputs/transcriptional

550   control). Such multimodal regulation would allow the integration of multiple inputs to fine-tune

551   the output(s) of c-di-GMP-regulated processes, likely enhancing specificity of response by this

552   network. Future studies will be required to examine the detailed mechanisms of ligand

553   recognition, physical interaction among member proteins, and transcriptional regulation of genes

554   in the network.

555

556   **Materials and Methods**

557

558   **Strains and Media.** *P. fluorescens* Pfl01, *E. coli* S17 and *E. coli* BTH101 are used throughout

559   this study (51, 52). *P. fluorescens* and *E. coli* BTH101 were grown at 30°C on 1.5% agar LB

560   plates unless otherwise indicated. *E. coli* S17 was grown at 37°C. Media used in this study

561   includes K10T-1 as reported previously (52), and base minimal medium (BMM). BMM is

562   composed of 50 mM Tris HCL (pH 7.4), 7.56 mM $(NH_4)_2SO_4$, 0.15% glycerol, 1 mM K_2HPO_4,

563   and 0.6 mM $MgSO_4$. *P. fluorescens* strains harboring the pMQ72 expression plasmid were

564   grown overnight with 10 µg/ml gentamicin. Expression was induced during experimentation

565   with 0.2% arabinose. In-frame deletions were constructed as previously reported (53). Ligation

566   cloning of plasmids was conducted using standard laboratory techniques. All strains and

567   plasmids used in this study are listed in **Table S1**.

568

569   **Biofilm Assay.** *P. fluorescens* strains were struck out on LB plates overnight. In the case of

570   mutants coming from a previously generated mutant library, plates were supplemented with 30

571   µg/ml tetracycline (30). Single colonies were picked the following day to grow overnight in

572   liquid LB medium, and supplemented with15 µg/ml tetracycline when appropriate (see **Table**

573   **S1**). Cells densities were measured and normalized to $OD_{600} = 0.716$. Cells were mixed 1.5:100

574   with the BMM. 125 µl of the inoculated BMM was pipetted up and down in the Biolog PM1 and

575   PM2A nutrient plates; 100 µl of this mixture was then transferred from the Biolog plates to a

576   standard 96-well polystyrene plate (Costar) to perform the biofilm assays. Plates were covered

577   and placed in a humidified micro-chamber at 30°C for six hours, at which time the liquid in the

27

578   wells was discarded and the wells were stained with 125 µl of 0.1% crystal violet (CV) at room

579   temperature for ~20 minutes, then rinsed 2-3 times with water. Wells were allowed to dry

580   overnight, and were de-stained the following day using 150 µl of a solution of water, methanol,

581   and acetic acid (45:45:10 ratio by volume) for 20 minutes at room temperature. 125 µl of the

582   solubilized CV solution was transferred to a flat-bottom 96 well plate and the OD was recorded

583   at 550 nm.

584

585   **Analysis of Biofilm Data.** For all analysis of experiments assaying biofilm formation using

586   nutrients from the Biolog plates, OD_{550} values were adjusted in the following ways: First, a

587   baseline staining was established for each condition by growing a strain lacking the genes *gcbA,*

588   *gcbB, gcbC, and wspR* (referred to as Δ4DGC) and subtracting each of these values from the

589   corresponding values in each condition from all other experiments. Second, the variability of WT

590   values for each conditions were assessed and conditions where WT exhibited a coefficient of

591   variance of greater than 0.35 were excluded from further analysis to reduce errors due to noisy

592   conditions. Third, one batch of data (Batch #11, **Table S2**) was higher than all others so that was

593   batch-normalized by multiplying all data from that day by the ratio between the WT median on

594   that day and the median WT value across all other days.

595   For all analyses except that for **Figure S2**, mutant values were compared to the mean WT

596   value for each condition and the difference between these values was expressed in terms of the

597   number of standard deviations of WT data in that condition. The portion of values in a normal

598   distribution that would be expected to be this many or more standard deviations from the mean

599   was expressed as a p value. All p values were adjusted using a Benjamini-Hochberg adjustment.

28

600   An example calculation of the P value is as follows: WT in carbon source X has mean =

601   0.3 and standard deviation = 0.1. Mutant A has a value of 0.496. Difference between WT mean

602   and value for mutant A = 0.496 - 0.3 = 0.196. The number of WT standard deviations this value

603   differs from WT mean = 0.196 / 0.1 = 1.96, and the portion of normally distributed population

604   one would expect to differ from the mean by 1.96 SDs or more = 0.05. Therefore the unadjusted

605   p value for Mutant A in condition X is 0.05.

606   For **Figure S2,** OD_{550} values for *P. fluorescens* Pf01 and each mutant in each condition

607   were expressed as a ratio compared to the median OD_{550} value of that mutant across all

608   conditions. This approach serves as a representation of the response to that condition relative to

609   an approximation of the baseline biofilm formation. A second ratio was then calculated by

610   comparing the ratio in each condition to the median of WT ratios in that condition. Using the

611   standard deviation of WT ratios for each condition as described above, we assigned a p value to

612   each value.

613

614   **Transcriptional Expression.** 11 replicates of 5 µl from overnight LB liquid cultures of the wild

615   type and mutant strains were spotted onto 1.5% agar plates containing either K10T-1 medium, or

616   the BMM alone or containing 2 mM of the desired nutrient sources. Exceptions were made for

617   nutrients that have previously been published as having biofilm phenotypes including arginine

618   (0.4%), citric acid (0.4%), and L-arabinose (0.2%), or where exact measurements were infeasible

619   (dextrin; soluble fraction – 0.2%). Cells were grown at $30^{o}C$ for six hours, scraped from the plate

620   and resuspended in TE buffer, and processed using the RNAeasy extraction kit (Qiagen). 75 ng

621   of each RNA sample was added to the Nanostring nCounter kit, and the protocol provided by the

622   manufacture was followed without modification (Nanostring).

623

624   **Transcriptional Analysis.** Raw reads from the Nanostring system were normalized as a fraction

625   on the total number of reads measured for targeted genes, expressed as reads per 1000 transcripts

626   as described previously (34). A K10T-1 sample and a base medium sample was processed on

627   each cartridge, yielding six biological replicates. The six replicates of the BMM were used to test

628   significance in the difference between BMM and BMM plus other compounds. This analysis was

629   conducted in the same way as for the biofilm data analysis described above. Briefly, each

630   reading in the BMM plus a compound was compared to the mean value in base medium alone.

631   The standard deviation of that gene in the base medium was then used to estimate the portion of

632   values one would expect to find to be as different from the mean as the value being tested or

633   more extreme. This percentage was taken to be the p value. All p values were adjusted using the

634   Benjamini-Hochberg method. Of the 62 genes analyzed, 48 showed less than 2-fold variation

635   from the lowest to highest count between six replicate experiments when grown on K10T-1

636   (**Table S11**), and another 12 showed variation between 2 and 3-fold. Two genes showed greater

637   than 3-fold change; these genes were Pfl01_1252 and *gcbC*; both of these genes were found to

638   have low counts across all experiments (**Table S4, S11**).

639

640   **Bacterial Two-Hybrid Studies and Analysis.** BTH101 cells were electroporated with 50 ng of

641   the puT18 and pKNT25 plasmids containing the construct to be tested. Cells were recovered for

642   1 hr in 1ml LB at 37°C. 50 μl was then spread on selective agar containing 50 μg/ml

643   carbenicillin, 50 μg/ml kanamycin, 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

30

644   (X-gal), and 0.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Plates were incubated at

645   30°C for 20 hours, and colonies were examined for a blue color. A leucine zipper protein served

646   as a positive control, and the empty plasmids acted as the negative control. Each gene of interest

647   was fused into both the plasmids, and a positive result in either orientation was recorded as an

648   interaction.

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666   **Author Contributions.** Project was conceptualized by GAO, KMD, and CSG. Data curation,

667   validation, and visualization was conducted by KMD and AJC. Formal Analysis was conducted

668   by KMD, AJC, JNT, and GD. Funding was acquired by GOA, CSG, and DAH. Investigation

669   was conducted by KMD, AJC, and TJG. Methodology was developed by KMD. Project was

670   administered, supervised, and resources provided by GAO. Software was contributed by AJC

671   and JNT. Writing the original manuscript was conducted by KMD, and review and editing was

672   conducted by KMD, AJC, GAO, and CSG.

673   **Competing Interests**. None.

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677

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679

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825   bacteria. Appl Environ Microbiol **72:**5027-5036.

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831   **Figure Legends**

832

833   **Figure 1. Domain-level depiction of c-di-GMP-related proteins in *P. fluorescens*.** All

834   proteins in *P. fluorescens* with a predicted EAL, GGDEF, PilZ, or other known c-di-GMP-

835   binding domain are depicted. Domains illustrated include those predicted by Pfam and SMART.

836   In cases where these two databases predicted different domain architecture over the same amino

837   acid span, the Pfam prediction was used and Pfam domain naming conventions are used in all

838   cases. If a gene/protein name has been assigned/reported, it is also shown here. All recurrent

839   domains are listed in the key, with the remainder of domains labeled on each protein. Models are

840   not drawn to scale, although large amino acid stretches without a domain depicted represent

841   space in the protein where no known domains are predicted. **A.** GGDEF domain-containing

842   proteins. **B.** Dual domain-containing proteins. **C.** EAL, PilZ, and FleQ domain-containing

843   proteins. The domain key is shown boxed.

844

845   **Figure 2. A majority of c-di-GMP-related genes show a defect in biofilm development when**

846   **mutated**. **A**. Data from biofilm formation assays conducted under all 188 tested nutrients for

847   each gene indicated are displayed as a boxplot. Each box is color-coded based on the domain

848   associated with the indicated gene (see legend on right side). Mutants were compared to the wild

849   type using a Wilcoxon signed rank test and p values were adjusted using a Bonferroni correction.

850   ***p < 0.001. **B**. Shown is a bar graph of each of the mutants versus the number of nutrient

851   conditions wherein the mutant shows a biofilm phenotype, defined as a value significantly

852   different than the mean WT value using the test described in the Materials and Methods. The

853   height of the bar above and below the origin indicates the number of conditions that each mutant

40

854   had a significantly enhanced and reduced biofilm, respectively. The bars are color coded to

855   match the domain of the protein encoded by the mutated gene with the legend shown on the right

856   side of the panel.

857

858   **Figure 3. Analysis of the expression of genes in the network**. **A**. Shown is the $\log_2$ expression

859   ratio for each gene in the network across the 50 nutrients tested. The ratio was calculated with

860   dividing the number of counts per 1000 normalized mRNA transcripts on BMM plus the

861   indicated nutrient by the number of mRNA transcripts in the BMM. A positive value indicates

862   an increase in expression in response to the nutrient, while a negative value indicates a decrease

863   in expression. All ratios were transformed to their $\log_2$ values then plotted. Each red dot

864   indicates a condition in which expression was not significantly different than the expression on

865   BMM alone, while blue dots indicate conditions in which expression was significantly different

866   from BMM (determined as described in the Materials and Methods). A version of this plot with

867   the individual dots assigned to nutrients is shown in **Figure S4A**. **B**. Shown is a plot relating the

868   number of nutrient conditions for which transcription of the indicated gene was changed AND a

869   biofilm defect in a strain carrying a mutation in that same gene was observed in that same

870   nutrient. Data are shown for all the genes in the network. **C.** Conditions where a gene with both a

871   significant change in transcription and the mutation of that gene had a significant impact on

872   biofilm formation are plotted and color coded by nutrient.

873

874   **Figure 4**. **Physical interaction map of c-di-GMP-related proteins in *P. fluorescens*. A**.

875   BTH101 *E. coli* cells were grown on LB medium supplemented with X-gal for 20 hours and

876   colonies were assessed for blue color. Protein pairs of each domain type were tested for physical

41

877   interaction in the bacterial two-hybrid system against each member of every other domain type,

878   with dual-domain proteins also being tested against each other. Each gene was tested on both the

879   pUT18 and pKNT25 vectors; a positive result was recorded if either the T18- or T25-fused

880   protein produced blue colonies with another member of the network. Proteins that failed to

881   interact are shown at bottom. FleQ was not tested in this analysis. The interaction map was

882   generated by Cytoscape v 3.5.1. **B**. LapD serves as a node of interaction among several proteins

883   shown here. The legend is shown on the bottom, right of the figure: GGDEF-containing proteins

884   are represented by green circles, EAL-containing proteins by orange diamonds, dual-domain

885   proteins by blue rectangles, and PilZ-containing proteins by purple triangles.

886

887   **Figure 5**. **Assessing the protein-protein interactions in the network**. **A**. Shown is a plot of

888   interacting pairs of proteins in the network versus the number of nutrient conditions wherein

889   each pair shared a biofilm phenotype. Orange coloration indicates the number of conditions

890   where both interactors affected biofilm formation in the same way, while blue color indicated the

891   number of conditions that interactors affected biofilm formation in opposite directions. **B.** The

892   stacked box plot shows the relationship between interacting pairs of proteins in the network

893   versus a change in expression of both genes in the interacting pair in the same nutrient.   Orange

894   coloration indicates the number of conditions where both interactors showed a change in

895   transcription in the same direction, while blue color indicated the number of conditions that

896   interactors showed a change in transcription in the opposite direction. Note the difference in the

897   scale bar between panels A and B.

898

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900   **Supplemental Figure Legends**

901

902   **Figure S1. Biofilm phenotypes vary with nutrients.** Shown is a histogram of the quantitified

903   biofilm for the WT as a function of the nutrient added to BMM. The histogram is arranged in

904   order from lowest biofilm formed to highest biofilm formed. The red bar indicated the biofilm

905   formed in BMM.

906

907   **Figure S2. Heat map of biofilm phenotypes for each mutant with each nutrient.** In this

908   heatmap, red indicates a higher ratio than WT and blue is lower ratio than WT. That is, cells that

909   are red indicate conditions where a mutant biofilm either increased more or decreased less than

910   WT in a condition relative to the median biofilm formed by that mutant. Black indicates values

911   not significantly different from WT.

912

913   **Figure S3. Heat map of gene expression for each gene in the network on 50 different**

914   **nutrients.** Expression of genes when grown on the indicated nutrient-supplemneted BBM in

915   liquid medium. Only values which are significantly different from expression on BMM are

916   colored (see Materials and Methods for details of statistical tests). Red cells indicate conditions

917   where genes were expressed at significantly higher than in BMM, while blue cells indicate

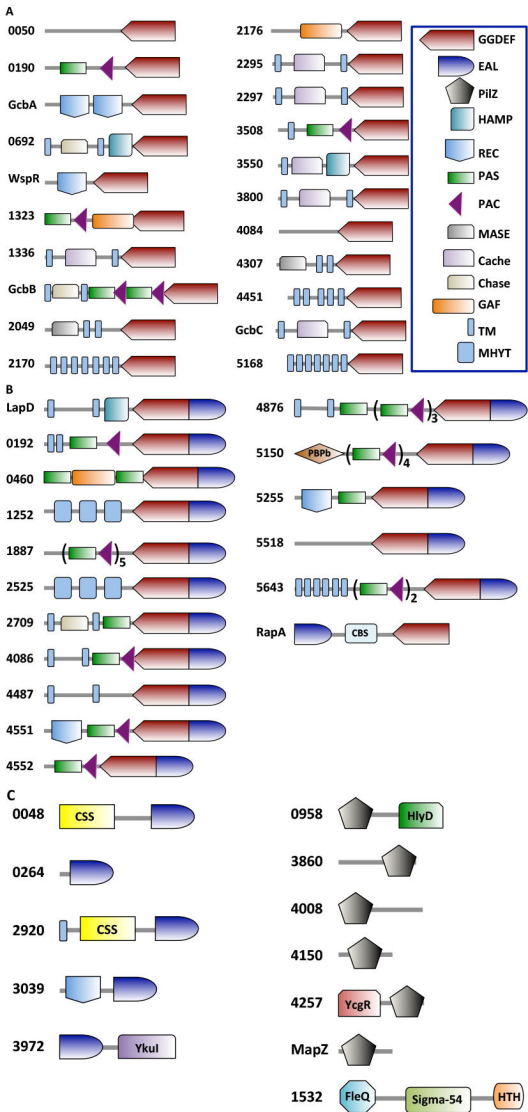918   conditions where genes were expressed at significantly lower levels compared to BMM.

919

920   **Figure S4. Summary of expression analysis. A.** Shown is the $\log_2$ expression ratio for each

921   gene in the network across the 50 nutrients tested. The ratio was calculated with dividing the

922   number of mRNA transcripts on BMM plus the indicated nutrient by the number of mRNA
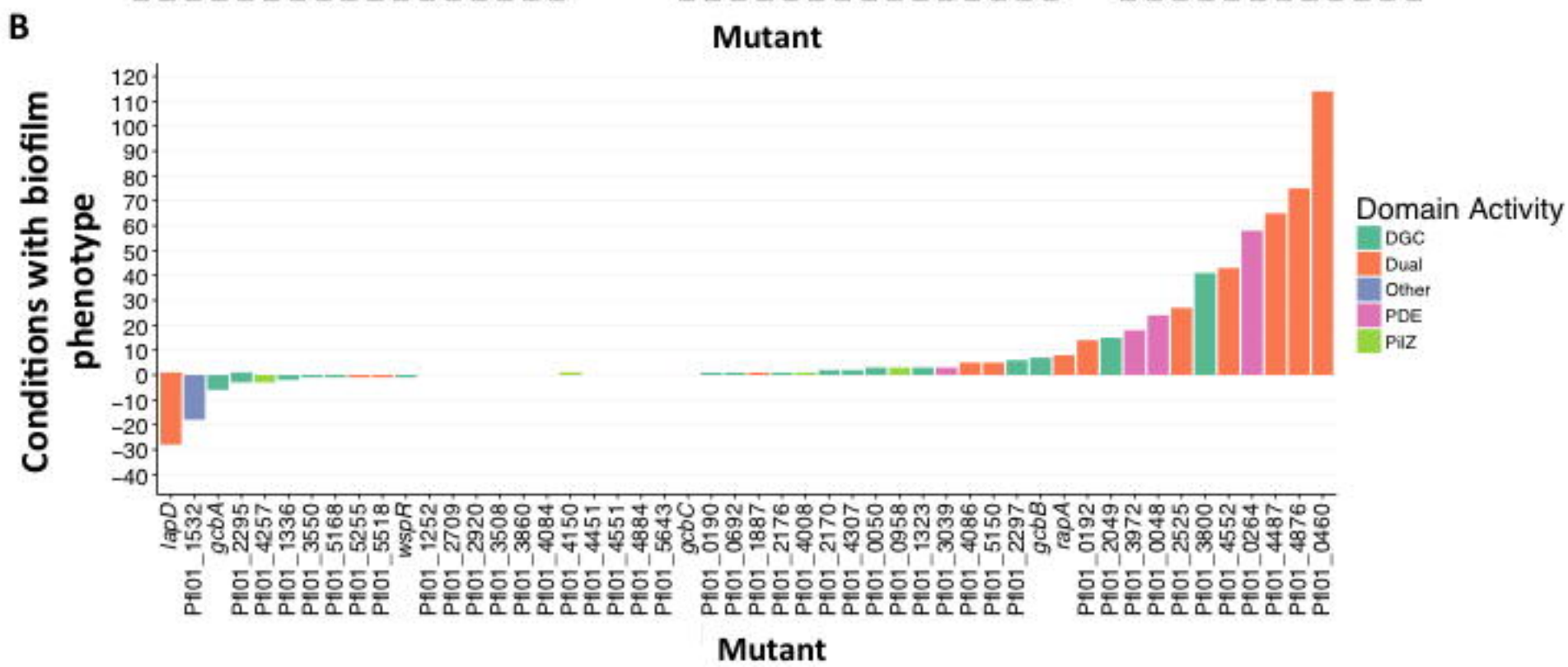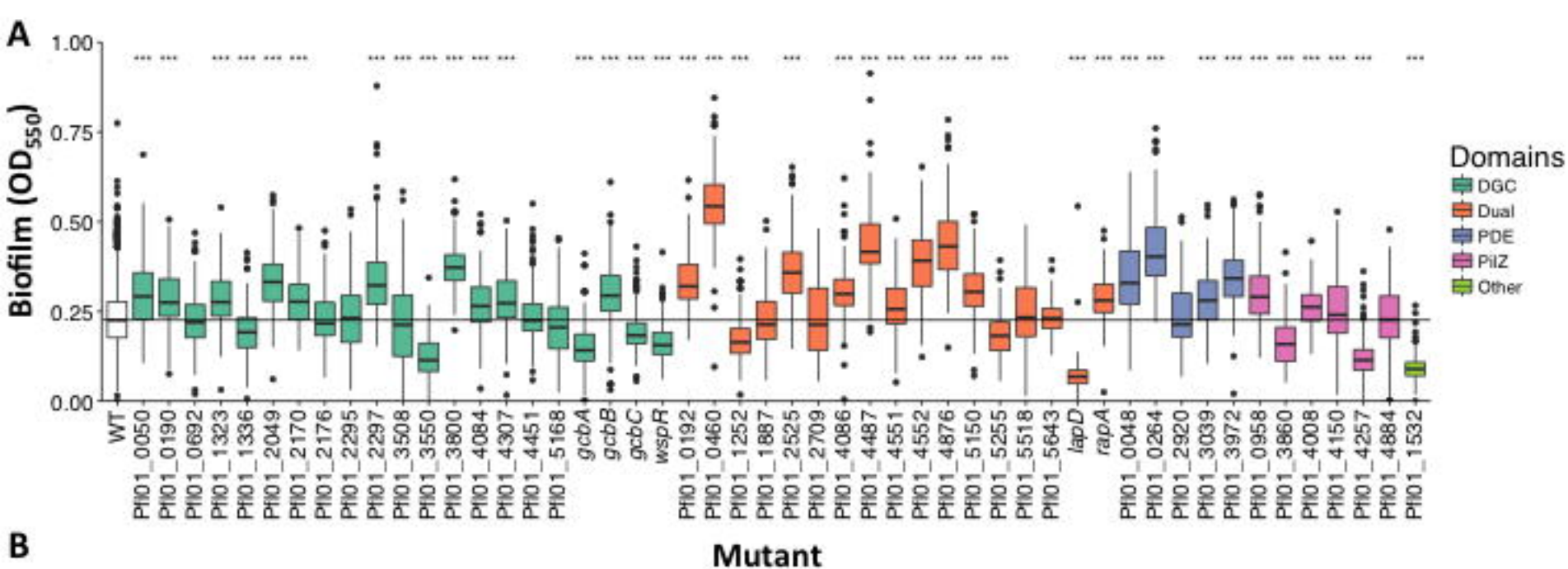
923   transcripts in the BMM. A positive value indicates an increase in expression in response to the

924   nutrient compared to BMM, while a negative value indicates a decrease in expression compared

925   to BMM. All ratios were transformed to their $\log_2$ values then plotted. Each colored dot

926   indicated the ratio of a different carbon source, with the key to the colors shown on the right. **B.**

927    $\log_2$ transformed ratio of expression of genes when cells were grown on a solid medium divided

928   by their expression in the same medium in broth. The carbon source added to the liquid or solid
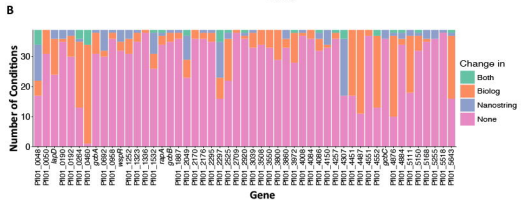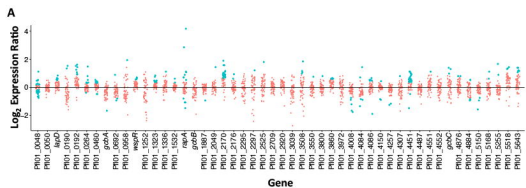
929   BMM is indicated. * $p<0.05$ Wilcoxon signed rank test.

930

931   **Figure S5. Comparing characteristics of c-di-GMP gene expression between organisms.** *P.*

932   *fluorescens* Pf01 Nanostring expression data were acquired as described in the Materials and

933   Methods. *P. aeruginosa* PAO1 expression data were retrieved from the NCBI database of

934   published microarray analyses using the tools developed by Tan *et al*. (42). The *P. aeruginosa*

935   PAO1 compendium data were normalized by assigning the highest value in each experiment the

936   value 1, and the lowest value the value 0. All other values in the experiment were scaled linearly

937   between those values using the following formula: for each value x: $z_i = (x_i - \min(x) / (\max(x) -$

938    $\min(x))$ where z_i is the normalized value of x_i. **A-D**. Genes were grouped according by predicted

939   proteins domains into the groups GGDEF, EAL, Dual, HD-GYP, and PilZ for c-di-GMP-related

940   genes. Genes not encoding one of those domains was termed "non-cdg". In addition, genes in the

941   Pho regulon (42) were included as an example of the characteristics of genes under

942   transcriptional regulation. **A-B.** The median normalized expression of each gene was calculated

943   and the distribution of those values is plotted as a boxplot with genes grouped according to the

944   characterization of the domains they encode. Panel **A** shows data from *P fluorescens* and panel **B**

945   from *P. aeruginosa* PAO1. **C-D.** The coefficient of variance of the normalized expression of

44

946   each gene was calculated by dividing the standard deviation by the mean, and the distribution of

947   those values is plotted as a boxplot with genes grouped according to the characterization of the

948   domain-containing proteins they encode. Panel **C** shows data from *P fluorescens* and panel **D**

949   from *P. aeruginosa* PAO1. **E-F.** Spearman correlation values for each pair of c-di-GMP-related

950   gene in each organism were calculated and plotted using the R package "corrplot". Significance

951   of these correlations was calculated using the "corrtest" function in this package and p values

952   were adjusted using a Bonferroni correction. Correlations with adjusted p values below 0.05

953   were not plotted. Panel **E** shows data from *P fluorescens* and panel **E** from *P. aeruginosa* PAO1.
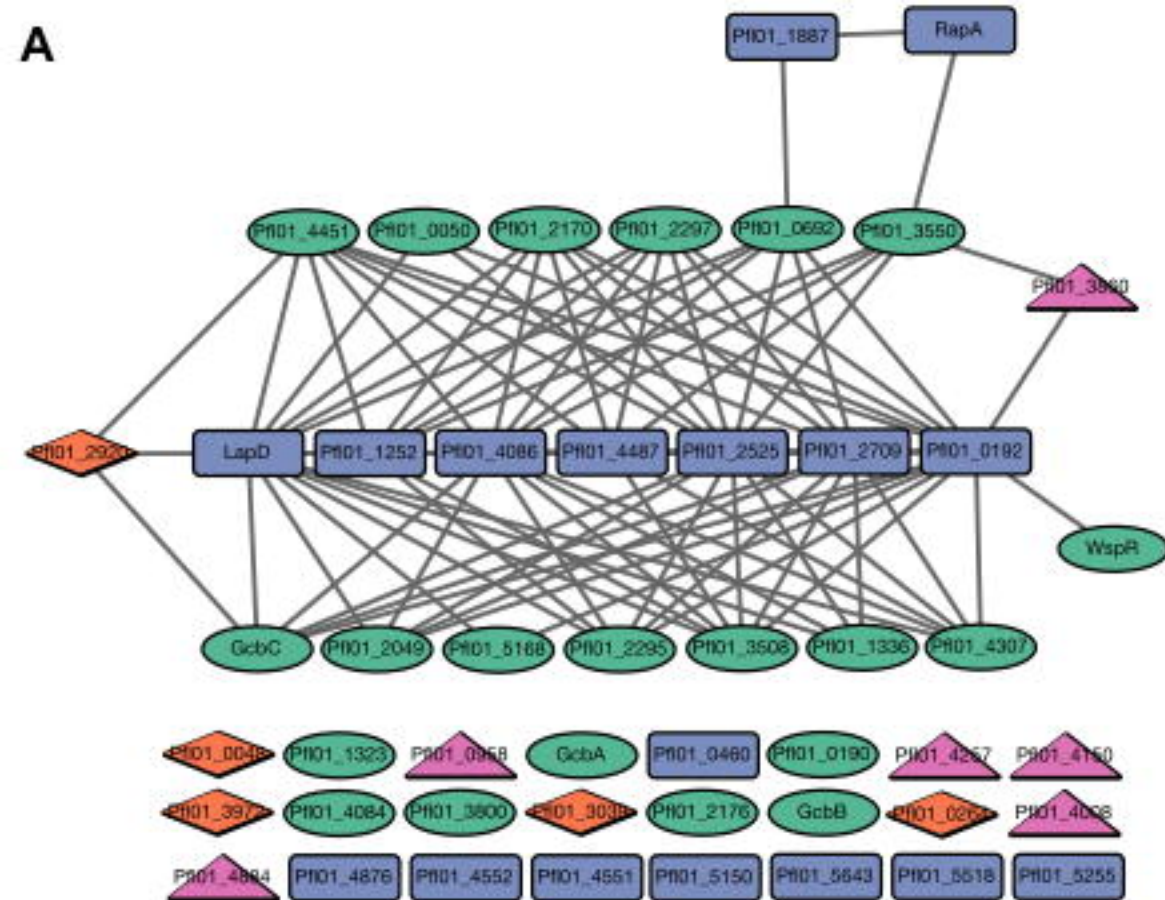
954

955   **Figure S6. Schematic of protein-protein interaction sub-networks. A.** Shown is the key for

956   each domain type shown in panels B-E. **B-E**. Small interaction networks showing proteins that

957   interact with a large number of other proteins. Such "hub" proteins are Pfl0_0192 (panel B),

958   Pfl0_2709 (panel C), Pfl0_2525 (panel D) and Pfl0_4068 (panel E). A summary of all such

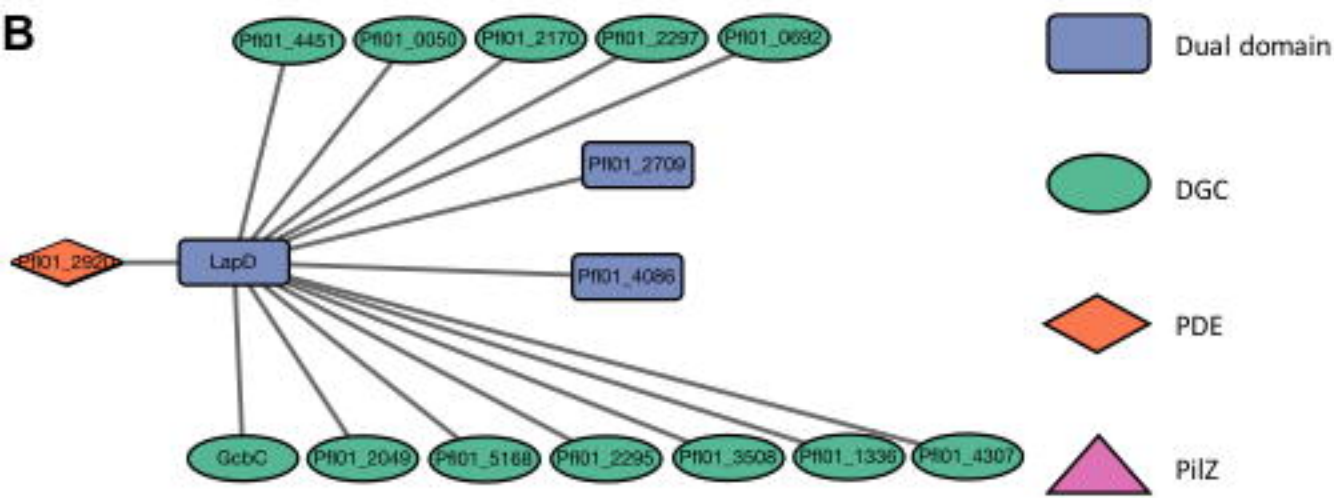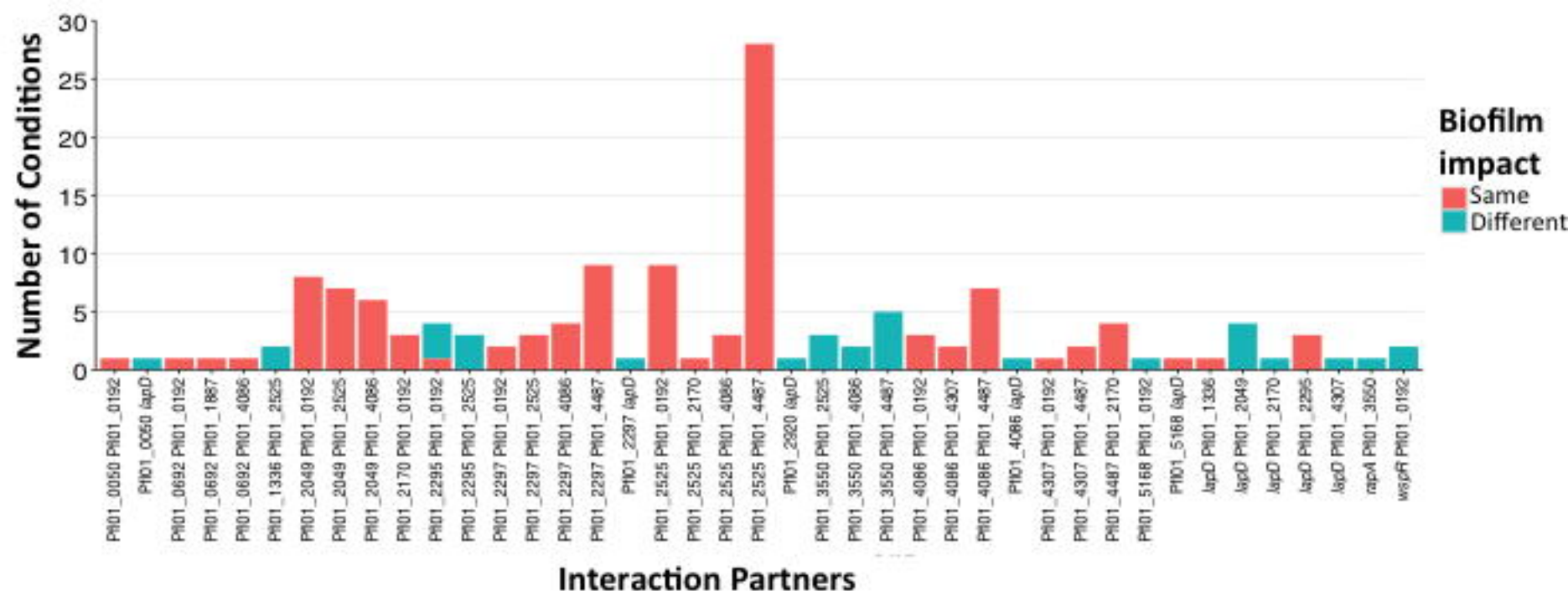959   interactions is presented in **Table S10** and **Figure 4**.

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0050
0190
GcbA
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WspR
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GcbB
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4307
4451
GcbC
5168

GGDEF
EAL
PilZ
HAMP
REC
PAS
PAC
MASE
Cache
Chase
GAF
TM
MHYT

B

LapD
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0460
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1887
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RapA

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MapZ
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