Conditional antagonism in co-cultures of *Pseudomonas aeruginosa* and *Candida albicans*: an intersection of ethanol and phosphate signaling distilled from dual-seq transcriptomics

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Running title: Conditional antagonism between *C. albicans* and *P. aeruginosa*

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Abstract

Pseudomonas aeruginosa and *Candida albicans* are opportunistic pathogens whose interactions involve the secreted products ethanol and phenazines. Here we describe the focal role of ethanol in mixed-species co-cultures by dual RNA-seq analyses. *P. aeruginosa* and *C. albicans* transcriptomes were assessed after growth in mono-culture or co-culture with either ethanol-producing *C. albicans* or a *C. albicans* mutant lacking the primary ethanol dehydrogenase, Adh1. Analyses using KEGG-pathways and the previously published eADAGE method revealed several *P. aeruginosa* responses to *C. albicans*-produced ethanol including the induction of a non-canonical low phosphate response mediated by PhoB. *C. albicans* wild-type, but not *C. albicans adh1*Δ/Δ, induces *P. aeruginosa* production of 5-methyl-phenazine-1-carboxylic acid (5-MPCA), which forms a red derivative within fungal cells. We first demonstrate that PhoB is required for this interaction and that PhoB hyperactivity, via deletion of *pstB,* leads to increased production of 5-MPCA even when phosphate concentrations are high, but only in the presence of ethanol. Second, we show that ethanol is only sufficient to promote 5-MPCA production at permissive phosphate concentrations. The intersection of ethanol and phosphate in co-culture is mirrored in *C. albicans*; the *adh1*Δ/Δ mutant had increased expression of genes regulated by Pho4, the *C. albicans* transcription factor that responds to low phosphate which we confirmed by showing the *adh1*Δ/Δ strain had elevated Pho4-dependent phosphatase activity. The dual-dependence on ethanol and phosphate concentrations for anti-fungal production highlights how environmental factors modulate microbial interactions and dictate antagonisms such as those between *P. aeruginosa* and *C. albicans*.

Author Summary

Pseudomonas aeruginosa and *Candida albicans* are opportunistic pathogens that are frequently isolated from co-infections. Using a Dual-Seq approach in combination with genetics approaches, we found that ethanol produced by *C. albicans* stimulates the PhoB regulon in *P. aeruginosa* asynchronously with activation of the Pho4 regulon in *C. albicans.* In doing so, we demonstrate that eADAGE-based analysis can improve the understanding of the *P. aeruginosa* response to ethanol-producing *C. albicans* as measured by transcriptomics: we identify a subset of PhoB-regulated genes as differentially expressed in response to ethanol. We validate our result by showing that PhoB is necessary for multiple roles in co-culture including the competition for phosphate and the production of 5-methyl-phenazine-1-carboxylic acid, and that the *P. aeruginosa* response to *C. albicans*-produced ethanol depends on phosphate availability. The conditional stimulation of virulence production in response to sub-inhibitory concentrations of ethanol only under phosphate limitation highlights the importance of considering nutrient concentrations in the analysis of co-culture interactions.

41 **Introduction**

42 *Pseudomonas aeruginosa* and *Candida albicans* are opportunistic pathogens that
43 are frequently isolated from co-infections [1-11]. These pathogens affect each other's
44 behaviors through competition for nutrients [12-17], physical contact [3, 4, 7, 13, 14],
45 diffusible signaling molecules [18-23] and antimicrobials [18, 21, 24-30]. Studies
46 highlighting the dynamic interactions between *P. aeruginosa* and *C. albicans* have
47 contributed to the growing understanding of how microbial interactions influence microbial
48 physiology and behavior as well as microbiological and pathological outcomes.

49 Like many fermentative organisms, *C. albicans* produces ethanol. Ethanol is a
50 biologically-active metabolite which, sub-inhibitory concentrations, modulates *P.*
51 *aeruginosa* behavior in multiple ways: it induces activity of the sigma factor AlgU through
52 ppGpp and DksA [31]; it promotes Pel matrix production through the Wsp system [29]; it
53 decreases flagellar-mediated motility through a pathway implicated in cell-surface
54 sensing [29, 32]; it affects pathways known to contribute to *P. aeruginosa* virulence [29,
55 33]; and it fuels fungal antagonism [29]. The broad effects of ethanol apply to many
56 contexts and the response of *P. aeruginosa* to *C. albicans*-produced ethanol can serve
57 as a model for how *P. aeruginosa* may respond to other fermentative fungi and bacteria.
58 We seek to further understand this response and identify common themes which may be
59 implicated in other microbial interactions.

60 To study the effects of ethanol in co-culture, we used *P. aeruginosa* anti-fungal
61 production as a readout of the ethanol response. Previous work has shown that ethanol
62 promotes the production and secretion of the phenazine 5-methyl-phenazine-carboxylic
63 acid (5-MPCA) by *P. aeruginosa* and that, in turn, *P. aeruginosa* phenazines cause an

64 increase in *C. albicans* fermentative metabolism and ethanol production [24-26]. *P.*
65 *aeruginosa* does not normally secrete 5-MPCA in axenic cultures but in co-culture it
66 secretes 5-MPCA through the MexGHI-OmpD efflux complex. Consequently, 5-MPCA
67 enters *C. albicans* cells wherein it reacts with the amine group of arginine, disrupting
68 protein function and forming a red pigment whose accumulation causes redox stress and
69 eventually death of *C. albicans* [24, 27, 34]. While it is known that ethanol production by
70 the fungus is necessary for *P. aeruginosa* 5-MPCA release, the mechanisms by which 5-
71 MPCA production is regulated have not yet been described. The mechanisms of
72 stimulation and ensuing consequences of *P. aeruginosa* 5-MPCA production and
73 accumulation of the red 5-MPCA-derivative within *C. albicans* cells is a scopic case study
74 for microbial interactions because it is an indicator of general antagonism.

75 Several studies have described the conditional production of antagonistic factors
76 in response to nutrient availability such as phosphate or iron limitation [35-52]. This is
77 often mediated transcriptionally, and such is the case for the low phosphate response
78 which is mediated by the PhoR-PhoB two-component system wherein inorganic
79 phosphate is sensed through the periplasmic domain of the phosphate transport complex,
80 PstS, which is dependent on the ATPases PstA and PstB. The failure to bind phosphate
81 in low phosphate environments causes the de-repression of the sensor kinase PhoR
82 which phosphorylates the response regulator PhoB and initiates PhoB DNA-binding to
83 the promoters of many genes.

84 This environmentally responsive regulation aids in the competition for essential
85 nutrients. For example, the *P. aeruginosa* low-phosphate response includes the secretion
86 of an arsenal of phosphatases, phospholipases and DNases that cleave phosphate from

87 diverse macromolecules [40]. However, the secretion of these enzymes renders
88 phosphate freely available to any nearby organism. Simultaneous production of
89 antagonistic factors could aid *P. aeruginosa* in the competition for phosphate amongst
90 other microbes. Indeed, in response to low phosphate and other complex stimuli, *P.*
91 *aeruginosa* produces antagonistic factors like phenazines and phospholipases which play
92 important roles in microbial interactions [39, 53, 54]. It has been reported that *P.*
93 *aeruginosa* tailors its low phosphate response to secondary stimuli: in *P. aeruginosa*
94 PhoB interacts with other regulators such as the transcription factor TctD [55] and the
95 sigma factor VreI [37] to orchestrate the expression of its target genes. However, the
96 mechanism by which PhoB exerts condition-specific control over its diverse regulon to
97 manage antagonistic factors in microbial interactions, is not yet fully understood.

98 Co-culture transcriptomics data from single species RNA-Seq or dual RNA-Seq
99 methods can be heterogeneous due to varying temporal and spatial relationships
100 between organisms. Variability can make such data difficult to analyze with traditional
101 statistical and pathway-based approaches. However, these data contain a wealth of
102 information about nutrient competition, synergy and antagonism in microbial interactions.
103 Therefore, it may be necessary to use techniques, such as recent machine learning based
104 methods, that allow for the detection of subtle and novel transcriptional signals in order
105 to render this important and complex data informative [53, 56-59].

106 Analysis of transcriptomic data from complex environments using curated
107 pathways can be challenging if the conditions are not well represented by the data used
108 for pathway definition. Furthermore, pathway definition relies on expert-contributed
109 annotations, yet ~38% (2,162 of 5,704) of genes for PAO1 reference strain

110 (pseudomonas.com) lack description. Recent methods are using unsupervised machine
111 learning to leverage large amounts of transcriptomic data and automatically identify sets
112 of genes with correlated expression across large compendia of samples, agnostic of gene
113 annotations and previously characterized pathways [56, 57, 59-62]. With over 2,000
114 transcriptional profiles of *P. aeruginosa* in the public sphere, such an approach has been
115 successfully implemented to make expression-based gene sets which can be used as
116 data-driven analytical tools that can bolster transcriptional analyses [53, 58, 63, 64]. In
117 particular, the data-driven tool eADAGE has identified transcriptional signals that contain
118 uncharacterized genes, manifest as small magnitude changes in expression, or are
119 condition-specific yet biologically informative.

120 Here we demonstrate that *P. aeruginosa* and *C. albicans* undergo transcriptional
121 changes in response to one another dependent on *C. albicans* ethanol production. Using
122 eADAGE analysis we identify a group of PhoB-regulated genes as differentially
123 expressed in response to ethanol and validate the result using genetic and biochemical
124 assays that show PhoB is necessary for accumulation of the red 5-MPCA-derivative in *C.*
125 *albicans* cells and that ethanol is sufficient to stimulate PhoB in mono-culture. We show
126 that in co-culture PhoB regulation of phosphate scavenging and 5-MPCA production are
127 independently necessary for *P. aeruginosa* fitness and antagonism against *C. albicans*
128 respectively. Further, by examining *C. albicans* gene expression profiles matched to
129 those of *P. aeruginosa* from the same co-cultures, we show that the *C. albicans* low
130 phosphate response is inversely correlated to that of *P. aeruginosa* linking the
131 coincidence of these stimuli in co-culture. By requiring dual-stimulation, In summary, we
132 show that *P. aeruginosa* only produces antifungal 5-MPCA when the death of neighboring

133 fungi would simultaneously remove a competitor and provide a source of the essential
134 nutrient phosphate: in both the presence of fermenting *C. albicans* and phosphate
135 limitation. We conclude that the enmity of *P. aeruginosa* – *C. albicans* interactions is
136 conditional upon ethanol and phosphate concentrations.
137

138 **Results**

139 **Ethanol is a defining factor in *P. aeruginosa* – *C. albicans* interactions stimulating**
140 **antagonism wrought by transcriptional changes in both organisms**

141 When grown on a lawn of *C. albicans*, *P. aeruginosa* produces the anti-fungal
142 phenazine 5-MPCA which is taken up by *C. albicans* and modified within the fungal cells
143 to form a red derivative [24, 27, 29] that can be seen first below and then as a halo
144 surrounding *P. aeruginosa* colonies (**Fig. 1A**). The 5-MPCA precursor phenazine-1-
145 carboxylic acid (PCA) can be synthesized via enzymes encoded in either of the two highly
146 similar operons, *phzA1B1C1D1E1F1G1* (*phz1*) and *phzA2B2C2D2E2F2G2* (*phz2*) with
147 different regulation; *phz1* contributes to phenazine production in liquid while *phz2* is
148 responsible for phenazine production in colony biofilms [65]. Analysis of phenazine
149 production in co-culture found that *phz1* was dispensable for the formation of red pigment,
150 while *phz2* was required (**Fig. 1A**). Consistent with previous results, 5-MPCA-derived red
151 pigment formation required *phzM* [27], *mexGHI-ompD* and *soxR* [24], and was over-
152 abundant in upon deletion of *phzS,* which catalyzes the conversion of 5-MPCA into
153 another phenazine, pyocyanin [66] (**Fig. S1 A**). As we have previously reported and
154 reproduced here, *P. aeruginosa* 5-MPCA production required *C. albicans* ethanol
155 production as the *C. albicans adh1*Δ/Δ, which lacks the major ethanol dehydrogenase,

156 did not elicit 5-MPCA-derived red pigment accumulation [29]. Chromosomal
157 complementation of a single copy of *ADH1* in *C. albicans* restored *P. aeruginosa* 5-MPCA
158 production (**Fig. 1A**).

159 To determine how *C. albicans* ethanol production influenced *P. aeruginosa* and
160 how 5-MPCA-derived red pigment accumulation influenced *C. albicans*, we took a dual
161 RNA-Seq approach in which we collected total RNA for simultaneous transcriptome-wide
162 analyses of both organisms from 16 h co-cultures of *P. aeruginosa* with *C. albicans* WT,
163 in which 5-MPCA-derivatives accumulated, and co-cultures of *P. aeruginosa* with *C.*
164 *albicans adh1*Δ/Δ, in which 5-MPCA products were not observed (see **Fig. 1B** for
165 experimental set up). Single-species *P. aeruginosa* and *C. albicans* colony biofilms grown
166 on YPD medium were also analyzed at the same time point. Principle component analysis
167 (PCA) of gene expression for each organism differentiated mono-culture and co-culture
168 with the first component PC1, which accounted for 40.1% and 44.7% of total variance for
169 *P. aeruginosa* and *C. albicans* respectively (**Fig. 1C,D**). The presence of *ADH1* in *C.*
170 *albicans* constituted a defining feature of co-culture in PC2 for both organisms, which
171 captured 18.8% and 28.2% of total variance for *P. aeruginosa* and *C. albicans*
172 respectively (**Fig. 1C,D**). Comparison of *P. aeruginosa* gene expression on either *C.*
173 *albicans* WT or *adh1*Δ/Δ to gene expression in mono-culture found 1,830 differentially
174 expressed genes (DEGs) with an absolute $\log_2$fold-change (logFC) > 1 and a corrected
175 p-value (FDR) < 0.05. Over half of the DEGs between *P. aeruginosa* in mono-culture and
176 on *C. albicans* WT were also DEGs between *P. aeruginosa* grown on *C. albicans* WT
177 compared to *adh1*Δ/Δ suggesting that a major portion of *P. aeruginosa* gene expression
178 in co-culture was influenced by *C. albicans* ethanol production (**Fig. 1E** and **Supp.**

179 **Dataset 1**). A similar trend was evident in *C. albicans* gene expression as approximately
180 half of the DEGs between mono- and co-culture with *P. aeruginosa* were also DEGs
181 between *C. albicans* WT and *C. albicans adh1*Δ/Δ from co-cultures (**Fig. 1F** and **Supp.**
182 **Dataset 2**). Here, expression patterns of DEGs in both *P. aeruginosa* and *C. albicans*
183 illustrated that ethanol played a defining role in *P. aeruginosa* – *C. albicans* interactions
184 from the perspectives of both organisms.
185

186 **How fungal ethanol shapes co-culture transcriptomes: the *C. albicans* perspective**

187 *C. albicans* Adh1 is responsible for reducing acetaldehyde to ethanol during
188 fermentation, and we thus expected metabolic shifts between *C. albicans* WT and
189 *adh1*Δ/Δ [29]. We identified DEGs between co-cultures of *C. albicans* WT and *adh1*Δ/Δ
190 with *P. aeruginosa* and conducted KEGG [67-69] pathway over-representation analysis.
191 The *C. albicans* KEGG pathway for fatty acid beta oxidation was over-represented in the
192 DEGs and the genes it contained (e.g. *FAA2-1*, *FAA2-3*) were more highly expressed in
193 *C. albicans* WT than in *C. albicans adh1*Δ/Δ (**Fig. 2, Supp. Dataset 3**). Since these
194 pathways were not over-represented in DEGs between *C. albicans* WT and *C. albicans*
195 *adh1*Δ/Δ in mono-culture, where there is no *P. aeruginosa*-produced 5-MPCA, these
196 results are consistent with a previous report of a phenazine and other mitochondrial
197 inhibitors increasing beta-oxidation in *C. albicans* WT as determined in metabolomics
198 studies [70].

199 Other KEGG pathways over-represented in DEGs in *C. albicans* from co-cultures
200 of *C. albicans* WT with *P. aeruginosa* compared to *C. albicans adh1*Δ/Δ with *P. aeruginosa*
201 were amino acid metabolism (e.g. *PUT2*, *GLT1*), sulfur metabolism (e.g. *MET15*) and

202 peroxisomal transport (e.g. *PEX1*, *PEX19*) (**Fig. 2, Supp. Dataset 3**). These pathways
203 converge on reactive oxygen species (ROS) mitigation (e.g. *GSH1*, *CAT1*) and, since
204 previous reports have shown phenazines causing redox stress to neighboring fungi [25,
205 26], the upregulation of these pathways could have been due to ethanol-induced *P.*
206 *aeruginosa* 5-MPCA production.

207 The KEGG pathway for glycolysis (e.g. *HXK2*, *PGI1*) was also over-represented
208 in DEGs between *C. albicans* WT in co-culture with *P. aeruginosa and C. albicans*
209 *adh1*Δ/Δ in co-culture with *P. aeruginosa,* but the genes within were more highly
210 expressed in *C. albicans adh1*Δ/Δ compared to *C. albicans* WT, perhaps as metabolic
211 compensation for the inability to ferment to ethanol (**Fig. 2, Supp. Dataset 3).** Similarly,
212 there was also over-representation of the KEGG pathway for iron scavenging (e.g. *FRP1*,
213 *FET99*) and these genes were again more highly expressed in *C. albicans adh1*Δ/Δ (**Fig.**
214 **2, Supp. Dataset 3).** Since the KEGG pathway for iron scavenging was not over-
215 represented in DEGs between *C. albicans* WT and *C. albicans adh1*Δ/Δ in mono-culture
216 (**Supp. Dataset 3)**, the increase in iron scavenging may have been due to a change in *P.*
217 *aeruginosa* behavior that affected iron availability.

218 In co-cultures of *P. aeruginosa* with ethanol-deficient *C. albicans adh1*Δ/Δ, which
219 did not promote 5-MPCA production*, C. albicans* also had higher expression of genes
220 involved in DNA damage repair (e.g. *RBT5, CSA1*) (**Fig. 2**). While not a result of *P.*
221 *aeruginosa* 5-MPCA, such damage may have been caused by another *P. aeruginosa*
222 antagonistic factor. However, the KEGG pathways for DNA replication and repair were
223 also over-represented in the DEGs between *C. albicans* and *adh1*Δ/Δ in mono-culture
224 (**Supp. Dataset 3),** so DNA damage may be a native consequence of the loss of Adh1 in

225 *C. albicans,* which is consistent with previous reports of an *adh1*Δ/Δ mutant having higher
226 intracellular concentrations of the DNA-damaging metabolic intermediate methylglyoxal
227 [71, 72].
228
229 **How fungal ethanol shapes co-culture transcriptomes: the *P. aeruginosa***
230 **perspective**
231 We identified *P. aeruginosa* DEGs when grown on *C. albicans* WT compared to
232 on *adh1*Δ/Δ. On *C. albicans* WT, *P. aeruginosa* upregulated genes involved in 5-MPCA
233 biosynthesis including *phzM* and genes within both the *phz1* and *phz2* operons, which is
234 consistent with differences in 5-MPCA formation between the two co-cultures (**Fig. 2**).
235 While the last four genes of the *phz* operons have fewer than three SNPs between each
236 gene pair and are thus not differentiated by alignment *phzA1, phzB1 and phzC1* have
237 substantial enough differences in sequence from *phzA2, phzB2 and phzC2* respectively
238 that the two operons can be distinguished, and we found that transcripts from both
239 operons were more highly abundant by at least 2-fold when *P. aeruginosa* was grown in
240 co-culture with *C. albicans* WT relative to with *adh1*Δ/Δ. While *phzS* and *phzH* are not
241 required for 5-MPCA biosynthesis [24, 29] they have been reported to have coordinated
242 expression with other phenazine genes [55, 73] and indeed we saw increases in their
243 expression on *C. albicans* WT compared to *C. albicans adh1*Δ/Δ as well (**Fig. 2**).
244 We again conducted KEGG pathway over-representation analysis and found over-
245 representation of three KEGG pathways in DEGs from *P. aeruginosa* grown in co-culture
246 with *C. albicans* WT compared to with *C. albicans adh1*Δ/Δ: phenazine biosynthesis,
247 quorum sensing (QS) and pyochelin biosynthesis (**Fig. 2, Supp. Dataset 3**). Over-

248 representation of the phenazine biosynthesis pathway was expected based on the
249 upregulation of the *phz* genes as described above. The identification of QS as an over-
250 represented pathway was also not surprising in light of the known regulation of phenazine
251 biosynthesis by QS in response to environmental cues [74, 75], including in *C. albicans*
252 co-culture [27]. *P. aeruginosa* QS involves three major transcriptional regulators: LasR
253 [76], PqsR [77] and RhlR [76]. RhlR and PqsR were necessary for phenazine production
254 (**Fig. S1B**). Although Δ*lasR* appeared to produce less 5-MPCA than *P. aeruginosa* WT
255 on *C. albicans* WT, consistent with previous data, it produced an abundance of the blue-
256 green phenazine pyocyanin, which is a 5-MPCA-derivative [18]. Upon examining the
257 expression of gene targets for these transcription factors (**Supp. Dataset 6**), we found
258 heterogenous expression patterns inconsistent with canonical, cell-density regulated QS
259 but reconcilable with activation of a subset of QS regulated genes that includes phenazine
260 biosynthesis genes (**Fig. S1C,D)**.

261 The third over-represented KEGG pathway was that for the biosynthesis of
262 pyochelin, a siderophore [51]. Expressly, genes involved in pyochelin biosynthesis, import
263 and regulation were lower in *P. aeruginosa* on *C. albicans* WT compared to on *C. albicans*
264 *adh1*Δ/Δ. Pyochelin and another siderophore, pyoverdine, are fluorescent, and we
265 supported the RNA-Seq data by showing increased *P. aeruginosa*-derived fluorescence
266 on *C. albicans adh1*Δ/Δ compared to on *C. albicans* WT (**Fig. 2,** inset). The over-
267 representation of low iron responsive genes in both *P. aeruginosa* and *C. albicans*
268 demonstrated that the organisms were experiencing simultaneous iron limitation. Taken
269 together these data are consistent with 1) *C. albicans* ethanol production stimulated *P.*
270 *aeruginosa* antagonistic 5-MPCA production which affected *C. albicans* metabolism and

271 ROS stress pathways and 2) increased glycolysis in *C. albicans adh1*Δ/Δ that
272 compensated for the inability to ferment coincided with a competition for iron.
273
274 **eADAGE analysis of *P. aeruginosa* transcriptome revealed additional pathways**
275 **differentially active in response to ethanol in co-culture with *C. albicans***
276 While the analysis of DEGs and the KEGG pathways over-represented therein
277 provided insight into two key *P. aeruginosa* – *C. albicans* interactions, only 19 of the 120
278 *P. aeruginosa* DEGs (|logFC > 2, FDR < 0.05) fell within the three statistically over-
279 represented KEGG pathways: QS (orange bar), phenazine biosynthesis (red bar) and
280 pyochelin biosynthesis (blue bar) (**Fig. 3A**). To look for additional processes that were
281 affected by *C. albicans* ethanol production, we further identified patterns in the RNA-Seq
282 data using eADAGE, a denoising autoencoder-based tool [53, 58, 64]. In eADAGE
283 analysis, the activities of previously-defined gene expression signatures are calculated
284 as a weighted sum of normalized gene expression values (TPM) where gene weights are
285 unique to each signature [64]. The eADAGE signatures were derived irrespective of
286 human curation, which allowed for the examination of gene sets with coherent expression
287 patterns but no annotation to date. The eADAGE-transformed *P. aeruginosa* – *C. albicans*
288 co-culture signature activity profiles had a higher clustering coefficient (CC) by condition
289 than was observed by normalized gene expression profiles; the CC for differentially active
290 eADAGE signatures (DASs) was 0.68 compared to 0.38 for gene expression data;
291 randomized data had CC values less than 0.14 (**Table S1**). The higher CC after eADAGE
292 signature transformation indicated that biological information was retained and signals
293 that differentiated sample types may be more clear at the pathway level. Using eADAGE,

294 we found 48 DASs in *P. aeruginosa* grown on *C. albicans* WT versus *P. aeruginosa* grown
295 on *C. albicans adh1*Δ/Δ (**Supp. Dataset 3**).

296 As predicted by the DEG analysis (**Fig. 3A**), there were multiple DASs in which
297 phenazine biosynthesis and pyochelin biosynthesis KEGG pathways were over-
298 represented (**Fig. 3B,** red and blue bars respectively). The detection of multiple
299 signatures enriched in these pathways was expected based on the presence of similar
300 signatures that are redundant in some contexts but discriminating in others [53]. There
301 were 32 DASs (**Fig. 3B,** black bar) with over-representations of 7 additional pathways (all
302 pathways over-represented in DASs shown in inset) and 16 DASs in which no pathway
303 was over-represented (**Fig. 3B,** grey bar), perhaps representing novel transcriptional
304 signals. As expected based on the larger differences in gene expression for phenazine
305 biosynthetic genes, multiple signatures that contained over-representations of phenazine
306 biosynthesis (annotated as phenazine or pyocyanin) (red) had increased activity in *P.*
307 *aeruginosa* grown on *C. albicans* WT compared to *P. aeruginosa* grown on *C. albicans*
308 *adh1*Δ/Δ, and together contained over-representations of pyochelin biosynthesis
309 (annotated as siderophore or iron transport) (blue) had decreased activity in *P.*
310 *aeruginosa* grown on *C. albicans* WT compared to *P. aeruginosa* grown on *C. albicans*
311 *adh1*Δ/Δ. Other pathways over-represented in DASs included amino acid metabolism,
312 styrene metabolism and zinc uptake (**Fig. 3B** inset, **Supp. Dataset S4**). Notably, one
313 differentially active signature, Node206neg (N206n), contained ethanol catabolism genes
314 (**Fig. 3C,** pink dot). The signature with the largest increase in activity, Node108neg
315 (N108n) (**Fig. 3C,** violet dot) was not enriched in any KEGG pathways. However, we had
316 previously identified Node108neg as significantly more active in low phosphate media

317 than phosphate replete media across the compendium of gene expression on which
318 eADAGE was trained [53]. Therefore, upon identifying Node108neg as the most activated
319 eADAGE signature in response to *C. albicans* ethanol production in co-culture, we further
320 investigated the genes within Node108neg and their connection to the *P. aeruginosa* low
321 phosphate response.
322

323 **eADAGE analysis suggests *P. aeruginosa* PhoB up-regulated genes in response to**
324 ***C. albicans* ethanol production**

325 In the most upregulated eADAGE signature, Node108neg, the set of PhoB-
326 regulated genes PhoB (i.e. the PhoB regulon) was significantly over-represented
327 (hypergeometric test: $p = 5.9 \times 10^{-9}$). The PhoB regulon has been extensively defined
328 through rigorous experimental methods including mutant transcriptomics, motif analysis
329 and chromatin immunoprecipitation assays [55]. Of the 32 genes in Node108neg, 11 were
330 also in the PhoB regulon (**Fig 3D**) and they all increased in expression in *P. aeruginosa*
331 grown on *C. albicans* WT compared to *adh1*Δ/Δ, but the Pho regulon defined in Bielecki
332 *et al*. was heterogeneously expressed overall (**Fig. 3E**). We examined DEGs in the
333 context of signatures more closely in order understand how changes in eADAGE
334 signature activities embodied the *P. aeruginosa* response to *C. albicans*-produced
335 ethanol and whether *P. aeruginosa* gene expression changes between growth on *C.*
336 *albicans* WT and *adh1*Δ/Δ signaled a low phosphate response.

337 We visualized relationships among DEGs in the eADAGE gene-gene network. The
338 full gene-gene network consists of the 5,549 *P. aeruginosa* genes used to create the
339 eADAGE model as vertices with similarities in transcriptional patterns as weighted edges

340 (shorter edges represent higher Pearson correlations between gene weights across all
341 signatures in the eADAGE model) [53, 58, 64]. Here we show strongly DEGs (logFC > 2,
342 p-value < 0.01) between *P. aeruginosa* grown on *C. albicans* WT and *adh1*Δ/Δ connected
343 by edges whose weight is drawn from the full gene-gene network (edge cutoff ± 0.5) (**Fig.**
344 **3F**). DEGs fell into cliques (**Table S2**) when visualized as a sub-network within the
345 eADAGE gene-gene network (**Fig. 3D**). The three largest cliques contained genes
346 relevant to the biological processes of phenazine biosynthesis (clique 1, 17 genes),
347 pyochelin biosynthesis (clique 2, 29 genes), and the low phosphate response (clique 3,
348 23 genes) (**Fig. 3F**). Other cliques contained genes involved in isoprenoid catabolism
349 (clique 4), magnesium flux across the membrane (clique 5), aconitate porins (clique 6),
350 pyrimidine metabolism (clique 7), pyocin biosynthesis (clique 8), spermidine biosynthesis
351 (clique 9), histidine metabolism (clique 10), the heat shock response (clique 11), and the
352 ROS stress response (clique 12). Most notably, many genes within clique 3, which were
353 related to the low phosphate response, were also in Node108neg.

354 Genes within clique 3 clustered into two groups by gene expression: four were
355 more highly expressed in the *P. aeruginosa* grown on *C. albicans adh1*Δ/Δ and 19 genes
356 were more highly expressed in *P. aeruginosa* grown on *C. albicans* WT, including those
357 in Node108neg (**Fig. 3G**). Notably, both groups of genes are regulated by PhoB, but are
358 in different operons. The group of *P. aeruginosa* genes that were more highly expressed
359 when grown on *C. albicans adh1*Δ/Δ fall within the neighboring operons *phoBR* and
360 *pstABC*. The group of *P. aeruginosa* genes that were more highly expressed when grown
361 on *C. albicans* WT belong to the consecutive operons that encode the Hxz type II
362 secretion system (PA14_55450, PA14_55460) and its substrate enzyme the

363 phosphatase *lapC* [78]. The latter group of genes also contained genes involved in TonB-
364 dependent transport (*exbB2*, *exbD2*) as well as the phosphatases *phoA*, and the
365 phospholipases *plcN*. All of these genes are canonically co-regulated by PhoB and
366 usually have positively correlated expression patterns but it appeared as if PhoB was
367 selectively promoting the expression of only a subset of its regulon. We demonstrated
368 that the non-canonical PhoB response identified by eADAGE was biologically meaningful
369 through genetic, biochemical and phenotypic experiments described below.
370

371 **The *P. aeruginosa* low phosphate response was activated in response to *C.***
372 ***albicans* ethanol production**

373 eADAGE analysis of the co-culture RNA-Seq data found higher levels of some
374 PhoB-regulated transcripts in *P. aeruginosa* co-cultured with *C. albicans* WT than in *P.*
375 *aeruginosa* co-cultured with *C. albicans adh1*Δ/Δ. We confirmed this result using
376 NanoString, a multiplex RNA analysis method, to measure the mRNA levels of
377 representative PhoB-regulated genes. The data shown are normalized to six house
378 keeping genes as described in the Methods section. Like in the RNA-Seq data, there was
379 a split in the expression of PhoB-regulated genes: phosphate transport-associated genes
380 *phoR*, *phoB*, *pstA* and *phoU* did not increase in expression (**Fig. 4A**, bottom section) but
381 those encoding phosphate scavenging enzymes did: glycosyl transferase PA14_53380,
382 glycerophosphoryl diester phosphodiesterase *glpQ*, TonB-dependent transport protein
383 *exbD2,* phosphonate transporter *phnD* and alkaline phosphatase *phoA*. As well as genes
384 for phenazine biosynthesis (**Fig. 4A**, top two sections). Additionally, ethanol catabolism

genes *exaA* and *exaB* showed PhoB-independent increases in expression (**Fig. 4A,** third section).

We assessed PhoB activity in co-culture using phosphate supplementation, the native suppressor of PhoB. The addition of 10 mM potassium phosphate to the medium underlying the co-cultures resulted in a decrease in the expression levels of PhoB regulated genes (**Fig. 4A,** top section), including phenazine genes (**Fig. 4A,** second section). Visualization of transcript levels by sample revealed some heterogeneity which we predicted was indicative of a dynamic response as both species grow. To additionally confirm the expression of these genes was PhoB-dependent we included Δ*phoB* in co-culture with *C. albicans* WT. The histogram to the right shows the mean signal of PhoB-dependence (*P. aeruginosa* WT on *C. albicans* WT / *P. aeruginosa* Δ*phoB* on *C. albicans* WT) and indicates that expression of expected PhoB targets but not ethanol catabolism genes was PhoB-dependent.

Complementing transcript abundance data, promoter activity in *P. aeruginosa* WT decreased in response to 10 mM phosphate in co-culture with *C. albicans* WT as measured by promoter fusion assay of the PhoB target *pdtA* [41], **Fig. 4B**). These data suggested that in co-culture, the bioavailability of phosphate modulated PhoB activity which may have affected co-culture interactions.

PhoB was necessary for 5-MPCA-derived red pigment accumulation in *P. aeruginosa* – *C. albicans* co-culture

Given the dependence on PhoB for the expression of phenazine biosynthesis genes, we determined if PhoB was necessary for the accumulation of 5-MPCA-derived

408 red pigment in co-culture with *C. albicans* WT. *P. aeruginosa* Δ*phoB* did not support
409 accumulation of the 5-MPCA-derivative as indicated by the lack of red pigment in co-
410 culture with ethanol-producing *C. albicans* WT, and this was restored by chromosomal
411 complementation with a wild-type copy of *phoB* in *P. aeruginosa,* provided *C. albicans*
412 had a functional *ADH1* gene (**Fig. 4C**). Phenazine production was also dependent on
413 PhoR, the known regulatory kinase of PhoB, and the Δ*phoR* phenotype was
414 complemented by a wild-type copy of *phoR* expressed on an extrachromosomal plasmid.
415 We further demonstrated that PhoB activity is required for 5-MPCA production as
416 phosphate supplementation led to a decrease in 5-MPCA-derived red pigment in *P.*
417 *aeruginosa* WT co-culture with *C. albicans* WT as seen across a gradient plate (**Fig. 4D**,
418 top). PhoB activity was sufficient to overcome suppression by phosphate supplementation
419 as a mutant lacking the phosphate transport ATPase *pstB* with constitutively active PhoB,
420 continued to produce 5-MPCA-derived red pigment despite the addition of phosphate
421 (**Fig. 4E**, second down). As expected, *P. aeruginosa* WT did not form any 5-MPCA-
422 derived pigment in co-culture with *C. albicans adh1*Δ/Δ at any phosphate concentration
423 tested (**Fig. 4E**, third down). *P. aeruginosa* Δ*pstB* grown on *adh1*Δ/Δ only slightly rescued
424 5-MPCA-derived red pigment formation (**Fig 4. D**, bottom). This suggested that, while
425 phosphate levels contributed to the control of PhoB activity, *C. albicans* Adh1 activity
426 provided an additional stimulus that created conditions conducive to PhoB-regulated *P.*
427 *aeruginosa* antifungal phenazine production.
428
429
430

431 **Ethanol was sufficient to activate PhoB at intermediate phosphate concentrations**

432 To determine if ethanol was sufficient to stimulate PhoB in *P. aeruginosa*, we
433 monitored PhoB-regulated alkaline phosphatase (AP) activity. AP activity can be
434 monitored by the conversion of 5-bromo-4-chloro-3-indolylphosphate (BCIP) into a
435 colorimetric substrate according to published methods [79-81]. On MOPS (3-
436 morpholinopropane-1-sulfonic acid) buffered minimal medium after growth for 16 h at
437 37°C, in *P. aeruginosa* Δ*phz* where all blue coloration can be attributable to BCIP
438 conversion and not phenazines, AP was detected up to approximately 0.55 mM
439 phosphate in the absence of ethanol (**Fig. 5A**). With the addition of 1% ethanol to the
440 medium, *P. aeruginosa* showed AP activity at higher phosphate concentrations, up to
441 approximately 0.73 mM phosphate (**Fig. 5A**). This induction was also seen in *P.*
442 *aeruginosa* WT but not Δ*phoB* at 0.7 mM phosphate on single concentrations plates, and
443 was restored upon complementation with a WT copy of *phoB* (**Fig. 5B**). Quantification of
444 AP activity under these conditions showed a 4-fold increase in the presence of 1% ethanol
445 for WT *P. aeruginosa*, only trace AP activity for *P. aeruginosa* Δ*phoB* and hyper activity
446 in Δ*pstB* (**Fig. 5C**).

447 To determine if PhoB was acting upon the same targets under ethanol
448 supplementation in mono-culture as shown for co-culture, we monitored the expression
449 of PhoB targets using the same Nanostring codeset described earlier. *P. aeruginosa* WT
450 and Δ*phoB* were grown in mono-culture on MOPS minimal medium with 0.7 mM
451 phosphate with and without 1% ethanol. The same subset of PhoB-regulated genes
452 indicated in co-culture expression analysis increased in *P. aeruginosa* WT upon ethanol
453 supplementation: *phoA*, an alkaline phosphatase; *phnD,* a phosphonate transporter;

454 *glpQ*, a glycerophosphoryl diester phosphodiesterase (**Fig. 5D,** top section). In mono-
455 culture, when ethanol was supplemented into the medium, we saw an increase in
456 phenazine biosynthesis genes however, unlike in co-culture, the magnitudes of their
457 logFC between *P. aeruginosa* WT and Δ*phoB* was not as high as those of other PhoB-
458 regulated genes suggesting their expression was also stimulated by PhoB-independent
459 factors (**Fig. 5D**, second panel). Interestingly, the set of PhoB-regulated genes whose
460 expression was heterogenous and did not trend upward in co-culture had expression that
461 trended downward in mono-culture upon ethanol supplementation (**Fig. 5D**, bottom
462 panel). Stimulation of PhoB by ethanol in mono-culture meant that ethanol could have
463 been one of the stimuli that activated PhoB in co-culture.
464

465 **Low phosphate and ethanol were additive stimulants for PhoB-mediated red**
466 **pigment formation**

467 We tested whether ethanol stimulation and canonical PhoB de-repression (Δ*pstB*)
468 had additive effects on 5-MPCA-derived red pigment formation in *P. aeruginosa* – *C.*
469 *albicans* co-culture. On non-ethanol producing *C. albicans adh1*Δ/Δ, the constitutive PhoB
470 activity in *P. aeruginosa* Δ*pstB* was not sufficient to stimulate red pigment as that on
471 ethanol-producing *C. albicans* WT (**Fig. 5E**). Strikingly, the addition of 1% ethanol to *C.*
472 *albicans* a*dh1*Δ/Δ lawns increased red pigment formation in *P. aeruginosa* Δ*pstB* but not
473 in *P. aeruginosa* WT or Δ*phoB* (**Fig. 5E**). This signified that PhoB activation both through
474 the canonical signaling pathway and by means of ethanol stimulation were necessary and
475 only combined were sufficient for 5-MPCA-derived red pigment formation in co-culture
476 (proposed model in **Fig. 5F**).

477 In light of the recent characterization of the *P. aeruginosa* response to exogenous
478 ethanol [31] that showed ppGpp, synthesized by RelA and SpoT, DksA, and AlgU
479 mounted a transcriptional response to ethanol, we assessed their role in ethanol-induced
480 PhoB activation in co-culture by monitoring red pigment accumulation and in mono-
481 culture via BCIP assay. We determined that these genes were not necessary to induce
482 PhoB activity in response to ethanol (**Table 1**). We also ruled out roles for known
483 mechanisms of alternative PhoB activation including contributions of the non-canonical
484 histidine kinase KinB in both co- and mono- culture [53] and the extra-cytoplasmic
485 function (ECF) sigma factor VreI in monoculture, but further investigation is required to
486 determine if VreI played a role in regulating 5-MPCA production in co-culture [37, 41]
487 (**Table 1**).

488 We tested the role of ethanol catabolism in PhoB activation in both mono- and co-
489 culture. Mutants in the ExaA- dependent pathway for ethanol catabolism through acetate
490 including *exaA* and *acsA* did not show increases in AP activity in response to 1% ethanol
491 (**Table 1**) indicating that ethanol catabolism was essential for PhoB activation in mono-
492 culture. We hypothesized that ethanol catabolism led to increased levels of acetyl
493 phosphate, a non-canonical phosphate donor of transcription factors including PhoB, but
494 neither acetyl phosphate biosynthesis mediated by AckA nor catabolism by Pta was
495 necessary for ethanol-induced PhoB activity (**Table 1**) [82-85]. Mutants defective in the
496 ExaA-dependent ethanol catabolic pathway showed only weak 5-MPCA production on *C.*
497 *albicans* lawns and the phenotype could be complemented (**Table 1**). Together these
498 data suggest that ethanol catabolism plays a role in PhoB activation, and that additional
499 pathways for ethanol catabolism may be present in co-culture conditions. Further

500 investigation is required to determine the mechanism for ethanol induction of PhoB
501 activity, but these results demonstrate ethanol stimulation and PhoB activation
502 participated in non-linear but intersecting pathways.
503

504 **The *C. albicans* low phosphate response was more active in *adh1*Δ/Δ than in WT**

505 Given the differences in *P. aeruginosa* PhoB activity in co-culture with *C. albicans*
506 WT compared *adh1*Δ/Δ, we sought to understand if *C. albicans* also experienced
507 differences in phosphate availability by examining its low phosphate response. In *C.*
508 *albicans*, low phosphate induces activity of the transcription factor Pho4 which regulates
509 genes involved in phosphate acquisition as well as the homeostasis of cations (e.g. iron),
510 tolerance of stresses including ROS and arsenic, and fitness in murine models [86-90].
511 Pho4 regulates 133 genes that were identified using transcriptomics as differentially
512 expressed both between *C. albicans* WT and *pho4*Δ/Δ in low phosphate and between *C.*
513 *albicans* WT in high and low phosphate [86] (see **Supp. Dataset 4** for gene list). We found
514 that Pho4-regulated genes were over-represented in DEGs between co-cultures of *C.*
515 *albicans* WT and *C. albicans adh1*Δ/Δ (logFC > 1, $p < 0.05$) (hypergeometric test $p =$
516 1.9×10^{-3}). Of the top ten most differentially expressed genes between *C. albicans* WT and
517 *pho4*Δ/Δ in low phosphate determined by Ikeh *et al.* [86], seven were also strongly
518 differentially expressed (logFC > 2) in co-cultures of *C. albicans* WT with *P. aeruginosa*
519 compared to *C. albicans adh1*Δ/Δ with *P. aeruginosa* including a secreted phospholipase
520 (*PHO100*) and two secreted phosphatases (*PHO112* and *PHO113*) (**Fig. 6A**, black bars).
521 Given the strong differential expression of these phosphatases, we assessed
522 phosphatase activity using BCIP supplementation as done for *P. aeruginosa*. Consistent

523 with the transcriptional data, higher phosphatase activity was observed in *C. albicans*
524 *adh1*Δ/Δ compared to *C. albicans* WT in the presence (**Fig. 6B**) of *P. aeruginosa*.
525 To determine if the activation of the *C. albicans* low phosphate response in
526 *adh1*Δ/Δ in co-culture was a consequence of competition for phosphate with *P.*
527 *aeruginosa*, we examined *C. albicans* gene expression and phosphatase activity in mono-
528 culture. The same Pho4-regulated genes that were DEGs in co-culture were also DEGs
529 between *C. albicans* WT and *C. albicans adh1*Δ/Δ even in the absence of *P. aeruginosa*
530 (**Fig. 6C**), and higher phosphatase activity was evident in *C. albicans adh1*Δ/Δ compared
531 to *C. albicans* WT or the complemented strain *adh1*Δ/*ADH1* in mono-culture by BCIP
532 assay (**Fig 6D**). As a control, we assayed *C. albicans pho4*Δ/Δ. The low amounts of
533 phosphatase activity seen for *C. albicans* WT and *adh1*Δ/*ADH1* was distinguishable from
534 the absence of phosphatase in *pho4*Δ/Δ indicating that that phosphatase activity was
535 Pho4-dependent (**Fig 6D**). The high Pho4 response in *C. albicans adh1*Δ/Δ, evident even
536 in mono-culture, suggested that Adh1 activity impacts *C. albicans* phosphate access,
537 requirements or Pho4 regulation. Since phosphatases produced by *C. albicans* as part of
538 its low phosphate response are secreted, we hypothesized that their production would
539 affect *P. aeruginosa* in co-culture, perhaps by providing access to phosphate freed from
540 macromolecules and this model is discussed further below.
541

542 **In co-culture with ethanol-producing *C. albicans*, *P. aeruginosa* PhoB-plays**
543 **independent roles in phosphate scavenging and phenazine-mediated antagonism**

544 PhoB was important for *P. aeruginosa* growth in co-culture as Δ*phoB* formed fewer
545 CFUs on *C. albicans* WT than *P. aeruginosa* WT (**Fig. 6E**). Notably, a comparable number

546 of *P. aeruginosa* CFUs were recovered from *P. aeruginosa* WT and Δ*phz* suggesting the
547 lack of phenazines was not a major reason for decreased CFU formation in Δ*phoB* but,
548 likely, phosphate acquisition defects explained decreased growth in co-culture (**Fig. 6E**).
549 The defect in CFU formation by Δ*phoB* compared to WT or Δ*phz* held true on *C. albicans*
550 with a complemented copy of *ADH1.* On the *C. albicans adh1*Δ/Δ mutant, however, *P.*
551 *aeruginosa* CFUs were similar for WT, Δ*phoB*, and Δ*phz* suggesting that in the absence
552 of *C. albicans* Adh1 activity, *P. aeruginosa* PhoB, and its roles in phosphate acquisition
553 or phenazine production, were not necessary for fitness. This finding is consistent with
554 the model that elevated phosphatase production by the *C. albicans adh1*Δ/Δ (**Fig. B and**
555 **D**) may eliminate the need for *P. aeruginosa* to produce phosphatases.
556 *C. albicans* CFUs were enumerated from the same co-cultures. Consistent with
557 previous reports on the antifungal properties of the phenazine 5-MPCA [25, 27], *C.*
558 *albicans* had lower CFUs upon co-culture with *P. aeruginosa* WT compared to when co-
559 cultured with Δ*phoB* or Δ*phz* (**Fig. 6F**). In *C. albicans adh1*Δ/Δ co-cultures that did not
560 support *P. aeruginosa* PhoB-dependent phenazine production (**Fig. 4**), *C. albicans* CFUs
561 were not different between co-cultures with *P. aeruginosa* WT, Δ*phoB* or Δ*phz*.
562
563 ***P. aeruginosa* and *C. albicans* asynchronously activate their low phosphate**
564 **responses dependent on *C. albicans* Adh1-mediated ethanol production**
565 While *P. aeruginosa* had higher activity of its low phosphate responsive regulator
566 PhoB in the presence of ethanol-producing *C. albicans* WT (**Fig. 3G** and **4A**), *C. albicans*
567 had higher activity of its low phosphate responsive transcription factor Pho4 in *C. albicans*
568 *adh1*Δ/Δ (**Fig. 6**). To explicitly examine the relationship between the *P. aeruginosa* and

569 *C. albicans* low phosphate responses in co-culture we performed a cross-species
570 correlation analysis on the co-culture gene expression data for a subset of *P. aeruginosa*
571 PhoB-regulated genes (clique 3) and a subset of *C. albicans* Pho4-regulated genes
572 (those also DEGs between *C. albicans* WT and *adh1*Δ/Δ shown in **Fig. 6A**). There was a
573 striking pattern of inverse correlations between *P. aeruginosa* PhoB-regulated genes and
574 *C. albicans* Pho4-regulated genes (**Fig. 7A**, upper triangle). In addition to being of high
575 magnitudes, many same-species correlations (white background) as well as cross-
576 species correlations (grey background) were statistically significant as indicated by circles
577 (**Fig. 7A**, lower triangle). Of the two groups of strongly correlated genes, the one
578 composed entirely of *P. aeruginosa* genes was more highly expressed in co-culture of *P.*
579 *aeruginosa* and *C. albicans* WT than co-cultures of *P. aeruginosa* and *C. albicans*
580 *adh1*Δ/Δ. Conversely, the group composed of mostly *C. albicans* genes and including the
581 four *P. aeruginosa* phosphate transport-associated genes was more highly expressed in
582 co-cultures of *P. aeruginosa* and *C. albicans adh1*Δ/Δ than in co-cultures of *P. aeruginosa*
583 and *C. albicans* WT (**Fig. 7A**, right-hand barplot).

584 The asynchronous and seemingly inverse activations of the *P. aeruginosa* and *C.*
585 *albicans* low phosphate responses in co-culture, in combination with the differences in
586 requirement for PhoB on *C. albicans* WT versus *adh1* (**Fig. 6E and 6F**), suggest that the
587 two microbes were not responding to common environmental stimuli but rather that they
588 influenced each other. The increased phosphatase activity in *C. albicans adh1*Δ/Δ even
589 in the absence of *P. aeruginosa* (**Fig. 6D**) suggested that activation of the *C. albicans* low
590 phosphate response may have inhibited activation of the *P. aeruginosa* low phosphate
591 response providing negative regulation that, in concert with the lack of ethanol production,

592 led to low PhoB activity in *P. aeruginosa* in co-culture with *C. albicans adh1*Δ/Δ and a
593 consequent lack of 5-MPCA-derived red pigment accumulation (**Fig. 7B)**.
594
595 **Discussion**
596 **Co-culture transcriptomics explored dynamic microbe-microbe interactions**
597 Whole genome transcriptional profiling has been used as a powerful tool to explore
598 microbe-microbe interactions. By considering the gene expression patterns of both *P.*
599 *aeruginosa* and *C. albicans* in co-culture, we found that the production of secreted
600 phosphatases, a common good, was anti-correlated between *P. aeruginosa* and *C.*
601 *albicans* depending on the ability of *C. albicans* to produce ethanol (**Fig 7A**). This
602 interesting observation suggests that the two microbes interact differently in high and low
603 phosphate environments and the dynamics of phosphate sensing, transport and
604 enzymatic release by one organism can influence the microbial behavior of the other in
605 complex ways that lead to the emergence of conditional antagonism.
606 Exploration of co-culture expression data sparked the investigation showing that
607 signals of biologically-available phosphate and sub-inhibitory concentrations of ethanol
608 were integrated into a PhoB-coordinated transcriptional response in *P. aeruginosa.*
609 Interestingly, analysis of *C. albicans* gene expression in co-culture suggested these
610 stimuli may be linked as well. Inorganic phosphate is well established as a negative
611 regulator of the low phosphate response via the phosphate transport complex in *P.*
612 *aeruginosa*, and here we show that ethanol, a common microbial fermentation product,
613 positively regulated the *P. aeruginosa* low phosphate response specifically promoting the
614 expression of secreted phosphatases, phospholipases, and antifungal phenazines. While

615 these enzymes are only a subset of the PhoB regulon, PhoB activity and their expression
616 were critical for P. aeruginosa in co-culture. In co-culture, this small armory of enzymes
617 could make phosphate available by actively sequestering it away from neighboring
618 microbes or by means of direct antagonism.
619

620 **eADAGE-based analysis identified novel transcriptional signals and increased**
621 **interpretability of *P. aeruginosa* co-culture transcriptomics**

622 The eADAGE model comprises 600 data-derived, weighted gene sets called
623 signatures that are defined by the features of a hidden layer in an ensemble denoising
624 autoencoder neural network (www.adage.greenelab.com). The signatures were learned
625 from an unlabeled compendium of *P. aeruginosa* gene expression data and thus were
626 defined solely on gene expression relationships rather than metadata. Fortuitously, many
627 eADAGE signatures are enriched in one or more KEGG pathways [64] but others are not
628 enriched in any. In this way, eADAGE provides an opportunity to expand the breadth of
629 gene expression analysis beyond previously characterized pathways.

630 Despite our incomplete understanding of condition-dependent PhoB activity, we
631 were able to identify a novel signal for a subset of PhoB-regulated genes through
632 transcriptional profiling of *P. aeruginosa* grown in co-culture with *C. albicans* using
633 eADAGE. Representation of the RNA-Seq data in reduced feature space yielded 48
634 differentially active signatures that distilled gene expression patterns amongst complex
635 and dynamic systems. By analyzing eADAGE node activity, we were able to identify
636 subtle signals for which genes did not individually meet the cutoffs for DEGs and whose
637 pathways were not detected through over-representation analysis. Subtle changes in

638 gene expression could still have dramatic phenotypic effects; for example, although the
639 ethanol catabolism genes were not included in any enriched pathway, they were present
640 in eADAGE differentially active signatures, and we identified ExaA-based ethanol
641 catabolism as a potential regulator of the PhoB-mediated ethanol response including 5-
642 MPCA production [91, 92]. Similarly, we observed enrichment of genes involved in
643 isoprenoid metabolism which may be indicative of *P. aeruginosa* response to *C. albicans*-
644 produced farnesol [18, 28, 93].

645 Of the 600 eADAGE signatures, only four contained over-representations of PhoB-
646 controlled genes and while Node108neg was the only signature that reached the
647 threshold for DASs, the activities of the other three trended upwards. The dynamics of
648 co-culture that result in the constant utilization and solubilizing of phosphate differ from
649 the steady state of phosphate limitation achievable in laboratory conditions. Therefore,
650 while the low phosphate responses described in functional databases and the
651 experimentally determined regulon did not show uniform increases in expression of the
652 relevant genes, the data-defined gene signatures of eADAGE suggested that a tightly
653 correlated sub-group of the Pho regulon is coordinately upregulated in response to
654 ethanol-producing *C. albicans*.

655 It is only through the gene expression patterns observed by machine learning
656 models trained on publicly available *P. aeruginosa* gene expression data that we were
657 able to identify co-culture induced, ethanol-dependent PhoB activity. Considering the
658 absence of samples from *P. aeruginosa* grown with *C. albicans* in the compendium of
659 publicly available data, the distillation of the transcriptional pattern in Node108neg and its
660 strong ethanol-induced activity in co-culture highlight the power of using unsupervised

661 machine learning methods, in conjunction with a compendium of versatile conditions, to
662 identify higher order gene expression patterns that are not evident in linear correlation
663 based analyses and have not yet been manually annotated or systematically described.
664

665 **A conditional antagonism: PhoB-regulated antifungal production was dependent**
666 **on *C. albicans* ethanol-production and phosphate limitation**

667 Here we have shown that *P. aeruginosa* production of the antifungal phenazine 5-
668 MPCA is dependent on PhoB, but canonical activation of PhoB by de-repression (Δ*pstB*)
669 is insufficient for 5-MPCA production. We found that *P. aeruginosa* 5-MPCA production
670 required ethanol, either produced by *C. albicans* or supplied exogenously, as an
671 additional stimulus. While the mechanism by which ethanol influences PhoB activity is
672 beyond the scope of this paper, PhoB has been implicated in cases of non-canonical
673 signaling including auto-phosphorylation, promiscuity with non-canonical histidine
674 kinases, activation in response to low iron, and stimulation by alternative phospho-donors
675 such as acetyl-phosphate [35-39, 41, 42, 44, 55, 94-99]. The Pho regulon has been well
676 characterized for direct PhoB targets [55] but also shown to have additional indirect
677 targets across various low phosphate media [44]. Co-culture may be an environment
678 conducive to alternative PhoB stimulation by ethanol. The dual requirement of both
679 phosphate limitation and ethanol stimulation for *P. aeruginosa* 5-MPCA production
680 presents a conditionally antagonistic relationship between *P. aeruginosa* and *C. albicans*
681 where the degree to which the organisms cooperate, compete or target each other
682 depends on metabolic cues and the abundance of an essential nutrient. Here we have

683 presented a foundational example of an important and emerging paradigm that seeks to
684 understand how environmental stimuli modulate microbial interactions.
685
686 **Materials and Methods**
687
688 **Strains and growth conditions**
689 Bacterial strains and plasmids used in this study are listed in **Table S3**. Bacteria
690 were maintained on LB (lysogeny broth) with 1.5% agar [100]. Yeast strains were
691 maintained on YPD (yeast peptone dextrose) with 2% agar. Where stated, ethanol (200-
692 proof) was added to the medium (liquid or molten agar) to a final concentration of 1%.
693 Planktonic cultures were grown on roller drums at 37°C for *P. aeruginosa* and at 30°C for
694 *C. albicans*.
695
696 **Construction of in-frame deletions, complementation, and plasmids**
697 Construction of plasmids, including in-frame deletion and complementation
698 constructs, was completed using yeast cloning techniques in *Saccharomyces cerevisiae*
699 as previously described [101] or Gibson assembly [102, 103]. Primers used for plasmid
700 construction are listed in **Table S4**. In-frame deletion and single copy complementation
701 constructs were made using the allelic replacement vector pMQ30 [101]. Promoter fusion
702 constructs were made using a modified pMQ30 vector with *lacZ-GFP* fusion integrating
703 at the neutral *att* site on the chromosome. The *pdtA* promoter region 199 bp upstream of
704 the transcriptional start site (that included a PhoB binding site) was amplified from WT *P.*
705 *aeruginosa* PA14 gDNA using the Phusion High-Fidelity DNA polymerase with primer

706 tails homologous to the modified pMQ30 ATT KI vector containing tandem *lacZ-gfp*
707 reporter genes. All plasmids were purified from yeast using Zymoprep™ Yeast Plasmid
708 Miniprep II according to manufacturer's protocol and transformed into
709 electrocompetent *E. coli* strain S17/λpir by electroporation. Plasmids were introduced
710 into *P. aeruginosa* by conjugation and recombinants were obtained using sucrose
711 counter-selection. Genotypes were screened by PCR and confirmed by sequencing.
712

713 **Co-culture and mono-culture and colony biofilms**

714 Co-cultures were inoculated first with 300 μl of a *C. albicans* culture in YPD grown
715 for ~16 then diluted in dH_2O to OD_{600} = 5, which was bead spread onto YPD plates. *C.*
716 *albican*s mono-cultures were inoculated with 5 μl of the same cell suspension as spots
717 on YPD plates. *C. albicans* cultures were incubated 16 hours at 30°C then 24 hours at
718 room temperature (~23°C). Then, 5 μl of *P. aeruginosa* suspension, prepared from a 5
719 mL culture in LB grown for ~16 then diluted in dH_2O to OD_{600} = 2.5, was spotted on top
720 of the *C. albicans* lawns for co-cultures or onto YPD or MOPS minimal medium for mono-
721 cultures. *P. aeruginosa* cultures were incubated for 16 hours at 30°C. For gradient plates
722 (described below) 500 μl of *C. albicans* suspension was used and *P. aeruginosa* was
723 spotted across as 12 evenly spaced 5 μl spots. All images were taken on a Canon EOS
724 Rebel T6i camera. For visualization of siderophores, pictures were taken under UV light.
725

726 **RNA collection**

727 Total RNA was harvested from *P. aeruginosa* mono-cultures, *C. albicans* mono-
728 cultures and *P. aeruginosa* – *C. albicans* co-cultures grown as described above. All

729 samples were collected as cores from agar plates: cores were taken using a straw, cells
730 were suspended by shaking agar plugs in 1 mL dH_20 on the disrupter genie for three
731 minutes. Cells were spun down and resuspended in 1 mL Trizol and lysed by bead
732 beating with mixed sizes of silicon beads on the Omni Bead Ruptor. Centrifugation
733 induced phase separation and RNA was extracted from the aqueous phase where it was
734 subsequently precipitated out with isopropanol and linear acrylamide. RNA was pelleted,
735 washed with 70% ethanol and resuspended in nuclease-free dH_20 then stored at -80°C.
736 Samples were prepared for sequencing with DNase treatment, ribodepletion and library
737 preparation in accordance with Illumina protocols. Samples were barcoded and
738 multiplexed in a NextSeq run for a total of $4.7x10^8$ reads.
739
740 **RNA-Seq Processing**
741 Reads were processed using the CLC Genomics Workbench wherein reads were
742 trimmed and filtered for quality using default parameters. For co-cultures, reads were first
743 aligned to the *P. aeruginosa* UCPBB_PA14 genome from www.pseudomonas.com.
744 Then, all unaligned reads were aligned to *C. albicans* SC5314 genome Assembly 22 from
745 www.candidagenome.org. For mono-cultures reads were only aligned to their appropriate
746 reference. Results were exported from CLC including total counts, CPM and TPM. R was
747 used for principle component (prcomp, stats library [104]) and consequent plotting
748 (autoplot, ggplot2 [105]) of gene expression TPM data.
749 R was also used for differential gene expression analysis, EdgeR was used to
750 process both *P. aeruginosa* and *C. albicans* gene expression separately [106].
751 Generalized linear models with mixed effect data design matrices were used to calculate

752 fold-changes, p-values and FDRs for each comparison of interest. Volcano plots using
753 EdgeR output (log_2fold-change and -log_{10}(FDR)) were made in R as well (ggplot2 [105]).
754 Pathway over-representation analysis was carried out using *P. aeruginosa*- and *C.*
755 *albicans*-associated KEGG pathways (ADAGEpath [64] and KEGGREST [107]) and
756 calculated using a one-sided hypergeometric test (phyper, stats) with Bonferroni
757 correction for multiple hypothesis testing (p.adjust, stats) [104].
758

759 **Accession Number**

760 Data for our RNA-Seq analysis of *P. aeruginosa* and *C. albicans* gene expression
761 in co-culture has been uploaded to the GEO repository
762 (https://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE148597.
763

764 **eADAGE analysis**

765 We performed an eADAGE analysis in accordance with the workflow published in
766 the R package [64]. Briefly, each gene expression profile in counts per million (CPM) from
767 our RNA-Seq experiment was used to calculate a lower dimensional representation of the
768 data called a signature activity profile. Then, differentially active signatures were identified
769 by linear model. (limma, stats) This resulted in a set of signatures that were significantly
770 different, but which may have been redundant. We applied pareto front optimization of
771 minimal p-value and maximal absolute fold-change to arrive at a set of candidate
772 signatures that exhibit statistically significant differences and less redundancy. Heatmaps
773 show gene expression (CPM) or eADAGE signature activity scaled by feature (gene or

774 signature) and are hierarchically clustered by the complete method using Euclidean
775 distance by sample (ComplexHeatmap [108]).
776
777 **NanoString analysis**
778 NanoString analysis was done using RNA isolated as above (without DNAse
779 treatment) and 100 ng were applied to the codeset PaV5 (sequences for probesets used
780 in this study in **Supplemental Dataset 4**) and processed as previously reported [2].
781 Counts were normalized to the geometric mean of spiked-in technical controls and five
782 housekeeping genes (*ppiD*, *rpoD*, *soj*, *dnaN*, *pepP*, *dapF*). Normalized counts were used
783 for heatmap construction and fold-change calculations.
784
785 **Measurement of β-galactosidase in reporter fusion strains.**
786 For co-culture promoter activity assays, *C. albicans* lawns were grown as
787 described for RNA-Seq. *P. aeruginosa* was inoculated onto two filters placed on the *C.*
788 *albicans* lawns to allow for interaction through diffusible compounds but separation of
789 cells for quantification of promoter activity. *P. aeruginosa* cells were suspended in PBS
790 by disrupter genie and diluted to OD_{600} = 0.05. β-Galactosidase (β-Gal) activity was
791 measured as described by Miller [109]. β-Gal activity was measured in P. aeruginosa WT
792 and normalized to that in Δ*phoB* which acted as a negative control.
793
794 **pNPP**
795 For quantification of AP activity, we used a colorimetric assay using p-Nitrophenyl
796 phosphate (pNPP) (NEB) as a substrate. Briefly, 5 μl of *P. aeruginosa* overnight culture

797 were inoculated onto MOPS 0.7 mM phosphate agar plates with and without 1% ethanol
798 and incubated at 37˚C for 16 hours. Colony biofilms were collected from filters as
799 described for the promoter fusion assays. 100 μl of cell suspension was added to 900 μl
800 of 0.01 M Tris-HCl pH 8 buffer. After the addition of 25 μl 0.1% SDS and 50 μl chloroform
801 cells incubated at 30 ˚ for 10 minutes. 30 μl of the aqueous phase was transferred to a
802 96 well plate containing 15 μl reaction buffer (5 μl 0.5 mM MgCl2 and 10 μl 1 M Tris pH
803 9.5) where 5 μl pNPP was added [36]. After 30 minutes OD_{405} was read on a plate reader
804 (SpectraMax M2) and AP activity units were calculated as $\frac{\Delta A_{405} * ml}{10.67 * min} * \frac{dilution}{A_{600}}$ where 10.67
805 is the extinction coefficient ,normally 18.5, adjusted to path length of the microtiter dish.
806
807 **Gradient plates**
808 Phosphate gradient plates were made similarly to previously reported methods of
809 creating pH gradient plates [110]. For YPD-based phosphate gradients plates used for
810 co-cultures, first 32 mL of molten YPD+10mM potassium phosphate pH6 were poured
811 into a 10 cm square petri dish (Corning, BP124-05) that rested in a custom 3D-printed
812 prop that held the plate slanted at 30º. Once the bottom layer had solidified, the plate was
813 laid flat and 32 mL of molten YPD without phosphate supplementation agar were poured
814 atop. For MOPS-based gradient plates used for *P. aeruginosa* mono-cultures the
815 procedure was the same except the first layer was 32 mL MOPS minimal media with 1
816 mM phosphate and the top layer was 32 mL MOPS minimal medium 0.4 mM phosphate.
817 When needed, BCIP (Sigma-Aldrich #1158002001) (stock solution 60 mg/10 mL DMF)
818 was added to a final molarity of 6 nM.
819

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832

833 **Table 1**. Phenotypes for 5MPCA-accumulation in co-culture *C. albicans* WT by *P.*
834 *aeruginosa* strains and ethanol-induced alkaline phosphatase (AP) activity in
835 monoculture. AP activity was visualized in colonies on agar containing by BCIP. *P.*
836 *aeruginosa* mutants were defective in ppGpp-dependent AlgU and DksA signaling,
837 ethanol catabolism, the kinase KinB and sigma factor VreI, which are known to
838 influence PhoB activity, acetyl-phosphate metabolism and ethanol catabolism.
839

Genotype	*P.a.* 5MPCA on *C. albicans* WT	*P.a.* AP induction by ethanol
WT	Yes	Yes
Δ*phoB*	No	No
Δ*algU*	Yes	Yes
Δ*dksA*	Yes	Yes
Δ*relA*	Yes	Yes
Δ*relA*Δ*spoT*	Yes	Yes
Δ*kinB*	No	Yes
Δ*kinB*+*kinB*	Yes	Yes
Δ*mucB*	Yes	Yes
Δ*vreI*		Yes
Δ*vreR*		Yes
Δ*vreA*		Yes
Δ*vreI*Δ*phoB*		No
Δ*exaA*	No	No
Δ*exaA*+*exaA*	Yes	Yes
acsA::Tn*M*	Yes	No
Δ*ackA*	Yes	Yes
Δ*ackA*Δ*pta*	Yes	Yes
Δ*ackA*Δ*pta*Δ*phz*	No	Yes

840

841 **Figure Legends**

842 **Figure 1. In co-culture of *C. albicans* (*C.a.*) and *P. aeruginosa* (*P.a.*), *C.a.*-**
843 **produced ethanol stimulates *P.a.* to produce 5-MPCA and transcriptional**
844 **responses ensue from both organisms**. A) Co-cultures of *P.a*. wild type (WT) and
845 mutants lacking phenazine biosynthesis operons (Δ*phz1* or Δ*phz2*) were inoculated onto
846 72 h-old lawns of *C.a.* wild type (WT), *C.a. adh1*Δ/Δ and *adh1*Δ/Δ reconstituted with
847 *ADH1* (*adh1*Δ/*ADH1*). The red pigmentation indicates production of the phenazine 5-
848 MPCA by *P.a..* B) Dual RNA-Seq allowed for parallel analyses of *P.a*. (green) and *C.a.*
849 (red or pink) mRNA expression profiles from co-culture lawns to survey the effects of
850 ethanol (green oval) and 5-MPCA (red oval) on gene expression. C) Principle
851 component analysis (PCA) of TPM (transcripts per kilobase per million reads) from
852 transcriptome profiles of *P.a.* grown alone (No *C.a.*), *P.a.* grown with *C.a.* WT, and *P.a.*
853 grown with *C.a. adh1*Δ/Δ. D) PCA of gene expression profiles of *C.a.* WT and *C.a.*
854 *adh1*Δ/Δ grown in mono-culture (No *P.a.*) or co-cultures with *P.a.* WT. E) The
855 expression (z-score of TPM) of genes that differentiate *P.a.* in mono-culture from that
856 grown in co-culture with *C.a.* WT (absolute value of $\log_2$fold-change (logFC) > 1 and
857 false discovery rate (FDR) < 0.05); data for *P.a.* on *C.a*. *adh1*Δ/Δ are also shown. The
858 red bar indicates genes that are significantly different between *C.a.* WT and *adh1*Δ/Δ
859 (logFC >1, FDR < 0.05); the grey bar indicates genes that are not. F) Gene expression
860 (z-score of TPM) of *C.a.* WT and *adh1*Δ/Δ grown in mono-culture or in co-culture with
861 *P.a.*; genes that are significantly different between *C.a.* WT alone or *C.a.* WT with *P.a.*
862 (logFC >1, FDR < 0.05) are shown for all four sample types. Genes that are also
863 significantly different between *C.a.* WT and *adh1*Δ/Δ in the presence of *P.a*. (logFC >1,
864 FDR < 0.05) are indicated by the red bar; the grey bar indicates genes that are not
865 significantly different in this comparison.
866
867
868 **Figure 2. Pathways containing differentially expressed genes in *P. aeruginosa***
869 **and *C. albicans* between co-cultures of *P. aeruginosa* wild type (WT) with *C.***
870 ***albicans* WT or *adh1*Δ/Δ.** DEGs of between *C. albicans* WT and *adh1*Δ/Δ from *P.*
871 *aeruginosa* co-cultures contained over-representations of KEGG pathways for amino
872 acid metabolism, sulfur (S) metabolism, peroxisomal transport, fatty acid beta-oxidation,
873 glycolysis, cation (Fe) import, base excision and mismatch DNA repair. Red indicates
874 higher expression in co-cultures with *C. albicans* WT and blue indicates higher
875 expression in co-cultures with *C. albicans adh1*Δ/Δ. Values indicate $\log_2$fold-change. *C.*
876 *albicans adh1*Δ/Δ has higher expression of glycolysis genes and the production of
877 acetate, which is either secreted or enters into the citric acid cycle (TCA). *P. aeruginosa*
878 DEGs from the same co-cultures were contained over-representations of KEGG
879 pathways for phenazine (PCA, PCN, PYO, 1-OH-P, 5-MCPA) biosynthesis and
880 pyochelin (PCH) biosynthesis pathways. Inset shows increase in siderophore-derived
881 fluorescence of co-cultures of *P. aeruginosa* with *C. albicans adh1*Δ/Δ which is
882 consistent with increased PCH production. P-values are from hypergeometric over-
883 representation tests, FDR corrected.
884

885 **Figure 3. eADAGE analysis reveals a subset of the Pho regulon upregulated in *P.***
886 ***aeruginosa* (*P.a.*) grown on *C. albicans* (*C.a.*) WT compared to that on *C.a.***
887 ***adh1*Δ/Δ.** A) Differentially expressed genes (DEGs) between *P.a.* grown alone or on
888 *C.a*. WT and *C.a. adh1*Δ/Δ for 24 h. Genes that fell within over-represented KEGG
889 pathways (quorum sensing (orange bar), phenazine biosynthesis (red bar) and
890 pyochelin biosynthesis (blue bar)) are indicated. Most DEGs do not belong to any of the
891 three pathways (grey bar). B) Differentially active eADAGE signatures (DASs) for the
892 same samples shown in A. Signatures in which genes annotated as being involved in
893 phenazine biosynthesis (red bar), pyochelin biosynthesis (blue bar), or other KEGG
894 pathways (black bar) are overrepresented are indicated. Signatures that are not over-
895 represented an any KEGG pathways are indicated by the grey bar. Inset shows the fold-
896 change for the expression all of the KEGG pathways that are over-represented among
897 the DASs (# of DASs per KEGG pathway in parentheses); over-representation p-value
898 shown as circle (Supp. Dataset 3). C) DASs with increased activity in transcriptome
899 comparisons of *P.a*. grown on *C.a*. WT compared to on *C.a*. *adh1*Δ/Δ. In addition to
900 DASs with over-representations of pyochelin (blue dots) and phenazine (red dots)
901 biosynthesis, others over-represent the Pho regulon (Node108n, purple) or contain
902 ethanol catabolism genes (N206n, pink). D) The eADAGE signature with the highest
903 increase in activity, Node108neg (N108n, purple), contains many genes with increased
904 expression though not all met the criterion of DEGs individually (logFC > 1, ,FDR <
905 0.05). E) DEGs in *P.a*. grown on *C.a*. WT compared to on *C.a*. *adh1*Δ/Δ with expression
906 levels of PhoB-regulated genes (dark purple) highlighted. F) Network analysis of DEGs
907 suggest groups of DEGs have correlated patterns across eADAGE: phenazine
908 biosynthesis (1) is inversely expressed with the low iron response (2) and coordinately
909 upregulated with the low phosphate response (3) upon exposure to ethanol in co-
910 culture. Other cliques of DEGs participate in shared biological pathways. See table 2 for
911 descriptions of all cliques. G) The Pho Clique (3) contains two clades of DEGs with
912 opposing expression patterns between *P.a.* grown on *C.a.* WT and *C.a*. *adh1*Δ/Δ.
913
914 **Figure 4. *C. albicans* (*C.a.*) WT induced PhoB-regulated genes in *P. aeruginosa***
915 **(*P.a.*) compared to *C.a. adh1*Δ/Δ leading to 5-MPCA production as indicated by red**
916 **pigment formation.** A) Expression of *P.a.* genes involved in phosphate scavenging,
917 phenazine biosynthesis (PHZ), ethanol catabolism (EtOH) and inorganic phosphate
918 transport were measured by NanoString (codesetPAV5) from cells grown with *C.a.*
919 *adh1*Δ/Δ, *C.a.* WT or *C.a.* WT grown on medium with additional 10 mM phosphate.
920 Expression values are normalized to loading controls and housekeeping genes as
921 described in methods. Values are z-scored, scaled by gene. Right-hand barplot shows
922 logFC between *P.a.* WT and *P.a*. Δ*phoB* on *C.a.* WT. The bar is colored red if
923 expression is PhoB-dependent (logFC *P.a*. WT / *P.a.* Δ*phoB* > 1, FDR < 0.05, else
924 grey). * = FDR < 0.05, # = FDR > 0.05. B) Beta-galactosidase activity indicative of
925 expression of a *pdtA-lacZ* promoter fusion in *P.a.* WT in *P.a* grown with *C.a.* WT or *C.a.*
926 *adh1*Δ/Δ in the absence or presence of P_i supplementation, *,p<0.05 by ANOVA (n = 3).
927 C) Red 5-MPCA derivatives produced in co-culture by *P.a* WT, Δ*phoB*, Δ*phoR*, and their
928 complemented derivatives on *C.a.* WT, *adh1*Δ/Δ, and *adh1*Δ/*ADH1*. D) Red 5-MPCA-
929 derivatives produced by *P.a.* WT and *P.a.* Δ*pstB* over a gradient of phosphate
930 concentrations. *P.a.* Δ*pstB* has constitutive PhoB activity. Conversely, *P.a.* WT did not

931 produce 5-MPCA on *Ca. adh1*Δ/Δ at any phosphate concentration, but *P.a.* Δ*pstB*
932 induced a small amount of red pigment independent of the phosphate concentration.
933
934 **Figure 5. Ethanol (EtOH) induced PhoB activity in *P. aeruginosa* (*P.a.*) mono-**
935 **culture.** A) Alkaline phosphatase (AP) activity visualized by blue color derived from
936 cleavage of BCIP in MOPS medium with a gradient of phosphate in the absence and
937 presence of ethanol. The *P.a.* Δ*phz* strain was used to eliminate color differences due to
938 phenazine production. B) AP activity in the absence and presence of ethanol was
939 visualized with BCIP added to MOPS agar (0.7 mM phosphate) for *P.a.* wild type,
940 Δ*phoB* and the Δ*phoB* mutant complemented with a wild-type copy of *phoB* integrated
941 at the native locus. C) AP activity in cells from colony biofilms grown as in B was
942 measured using the colorimetric substrate pNPP for *P.a.* WT, Δ*phoB*, and Δ*pstB*, a
943 strain with constitutive PhoB activity. *,$p<0.01$ by ANOVA ($n \geq 3$). D) Transcripts within
944 the PhoB regulon involved in phosphate scavenging and inorganic phosphate transport
945 and genes involved in phenazine production (PHZ) and ethanol catabolism (EtOH) were
946 measured in cells grown in the absence and presence of ethanol by Nanostring
947 (codeset PAV5). PhoB-regulated genes increased in expression (top section) and
948 ethanol catabolism genes (third section) increase in expression and others decrease
949 (bottom section). Expression values are normalized to loading controls and
950 housekeeping genes as described in methods. Values are scaled by gene. Right-hand
951 barplot shows logFC between *P.a.* WT and *P.a*. Δ*phoB* on MOPS+1%EtOH. The bar is
952 colored red if expression is PhoB-dependent (logFC *P.a*. WT / *P.a.* Δ*phoB* > 1, FDR <
953 0.05, else grey). *,FDR < 0.05, #,FDR > 0.05. E) PhoB activity and ethanol are both for
954 5-MPCA production in response to *C.a.* ethanol. P.a. WT, Δ*phoB* and Δ*pstB* were grown
955 on C.a. WT, or C.a. adh1Δ/Δ in the absence or presence of exogenous ethanol. 5-
956 MPCA production was rescued by the addition of 1% ethanol to a co-culture of *P.a.*
957 Δ*pstB* and, to a lesser extent, *P.a.* WT on *C.a. adh1*Δ/Δ. F) Phosphate (P_i) and ethanol
958 (EtOH) are additive stimuli that promote PhoB-dependent expression of AP and
959 phenazine biosynthesis (PHZ).
960
961 **Figure 6. The *C. albicans* (*C.a.*) *adh1*Δ/Δ has increased expression of the Pho4-**
962 **mediated low phosphate response in co-culture that is inversely correlates with**
963 **PhoB activity in *P. aeruginosa* (*P.a.*).** A) Previously characterized Pho4-regulated
964 genes [86] including a phospholipase and phosphatases (black bars) were more highly
965 expressed in *C.a*. *adh1*Δ/Δ than *C.a.* WT in *P.a*. co-cultures (data shown as z-scores of
966 TPM). Pho4-dependence is shown in the right-hand barplot as $\log_2$FC *C.a*. WT/*C.a.*
967 *pho4*Δ/Δ using data from [86]. B) Analysis of phosphatase activity in *C.a.* WT, *C.a.*
968 *adh1*Δ/Δ, the complemented strain *adh1*Δ/*ADH1* or *pho4*Δ/Δ using the colorimetric
969 phosphatase BCIP substrate in agar. More phosphatase activity was observed in *C.a.*
970 *adh1*Δ/Δ than in strains with *ADH1* in in co-culture with *P.a*. C) The same Pho4-
971 regulated genes as shown in A were also more highly expressed in *C.a. adh1*Δ/Δ than
972 *C.a.* WT in mono-cultures. Right-hand barplot shows Pho4-dependence as in A. D)
973 More phosphatase activity was observed in *C.a. adh1*Δ/Δ than in strains with *ADH1* in in
974 mono-culture. . As predicted, phosphatase activity is not evident in the *C.a*. *pho4*Δ/Δ
975 strain. Phosphatase activity visualized via BCIP as in B. E) Number of CFUs of *P.a*. WT,
976 Δ*phoB* or Δ*phz* after co-culture for 72 h with *C.a.* WT, *C.a. adh1*Δ/Δ *C.a. adh1*Δ/*ADH1*.

977 *P.a.* Δ*phoB* and Δ*phz* had significantly fewer CFUs on *C.a.* strains with high ethanol
978 production (WT and *adh1*Δ/*ADH1*), but not *C.a. adh1*Δ/Δ. F) In the same samples
979 analyzed in A, *C.a*. CFU formation was assessed. *C.a.* WT or *C.a. adh1*Δ/*ADH1* strains
980 had increased fitness in-culture with *P.a*. Δ*phoB* or *P.a*. Δ*phz* compared to with *P.a*. WT.
981 For *C.a. adh1*Δ/Δ, there were no differences in CFU formation when co-cultured with
982 *P.a*. WT, Δ*phoB* or Δ*phz.*
983
984 **Figure 7. *P. aeruginosa* (*P.a.*) PhoB affects both *P.a.* and *C. albicans* (*C.a.*) fitness**
985 **in co-culture through its control of phenazine production and phosphate**
986 **acquisition**. A) Pearson correlation analysis between *P.a.* (green annotations) and *C.a.*
987 (orange annotations) low phosphate-responsive genes from co-cultures of *P.a.* WT with
988 either *C.a.* WT or *adh1*Δ/Δ. Largely inverse relationships between *P.a.* PhoB- and *C.a.*
989 Pho4-regulated genes is apparent. Log_2FC ($p<0.05$) between *P.a*. with either *C.a*. WT
990 or *C.a*. *adh1*Δ/Δ is shown in the right-hand bar plot. Lower half of correlogram shows
991 which correlations are significant (filled circles) and indicates correlation values by color
992 intensity relative to scale. Same species comparisons have white backgrounds and
993 cross-species correlations have grey backgrounds. B) Model of PhoB activity in *P.a.-*
994 *C.a.* co-cultures. PhoB mediates the conditional production of the antagonistic,
995 antifungal phenazine 5-MPCA in response to phosphate and fungal ethanol
996 production.**,$p<0.01$ by ANOVA.
997

998

999 **Supplemental Figure Legend**

1000

1001 **Figure S1. Red pigment formation is dependent on phenazine biosynthesis**
1002 **genes, phenazine transport genes and quorum sensing (QS) pathways in *P.***
1003 ***aeruginosa* (*P.a.*).** A) Co-cultures of *P.a*. wild type (WT) and mutants lacking genes
1004 involved in phenazine biosynthesis were inoculated onto lawns of *C.a.* (WT), *C.a.*
1005 *adh1*Δ/Δ and *adh1*Δ/Δ reconstituted with *ADH1* then incubated for 24 h. *P.a.* 5-MPCA
1006 phenazine biosynthesis (evident by red color) was not observed with the Δ*phzM* but was
1007 still produced by Δ*phzS* and Δ*phzH*. 5-MPCA production was dependent on the
1008 oxidative stress response gene *soxR* and 5-MPCA transport complex *mexGHI-ompD*.
1009 For all *P.a.* strains, 5-MPCA was only produced on *C.a.* with intact *ADH1*. B) Co-
1010 cultures of *P.a*. wild type (WT) and mutants lacking genes involved in quorum sensing
1011 were inoculated onto lawns of *C.a.* (WT), *C.a. adh1*Δ/Δ and *adh1*Δ/Δ reconstituted with
1012 *ADH1* then incubated for 48 h. *P.a.* mutants defective in QS pathways (Δ*lasR*, Δ*rhlR*,
1013 Δ*pqsR*, Δ*pqsA*) form less red pigment that *P.a.* WT on *C.a.* WT and no strains form red
1014 pigment on *C.a. adh1*Δ/Δ. C) Gene expression of *P.a*. LasR (blue), RhlR (orange) and
1015 PqsR (red) regulated genes had heterogenous expression with genes both up and
1016 down regulated. D) Red pigment formation being dependent on QS pathways of RhlR
1017 and PqsR is consistent with integration of QS with PhoB via the integrative quorum
1018 sensing (IQS) pathway in which low phosphate triggers PhoB activity which influences

PqsR and RhlR to act, via their cognate autoinducers PQS and C4-HSL respectively, in a regulatory cascade eventually promoting the transcription of phenazine biosynthesis genes (*phzA1-G1*, *phzA2-G2*) and consequent phenazine carboxylic acid (PCA) production. PhoB and QS have also been reported to effect the expression of phenazine modification genes *phzM, phzS,* and *phzH* necessary for the conversion of PCA to 5-methyl-phenazine-1-carboxylic acid (5-MPCA), pyocyanin, phenazine-1-carboxamide (PCN) and 1-hydroxy-phenazine (1-OH-phenazine) [74].

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Supporting Information

S1 Table. Clustering coefficients for co-culture data.

S2 Table. eADAGE gene-gene network cliques of DEGs from co-culture.

S3 Table. Strains and plasmids used in this study.

S4. Table. Primers used in this study.

S1 Dataset. *P.a*. DEGs in co-culture with *C.a..*

S2 Dataset. *C.a.* DEGS in co-culture with *P.a..*

S3 Dataset. KEGG pathway analyses.

S4 Dataset. Gene sets used throughout the paper.

1418 **Figures**

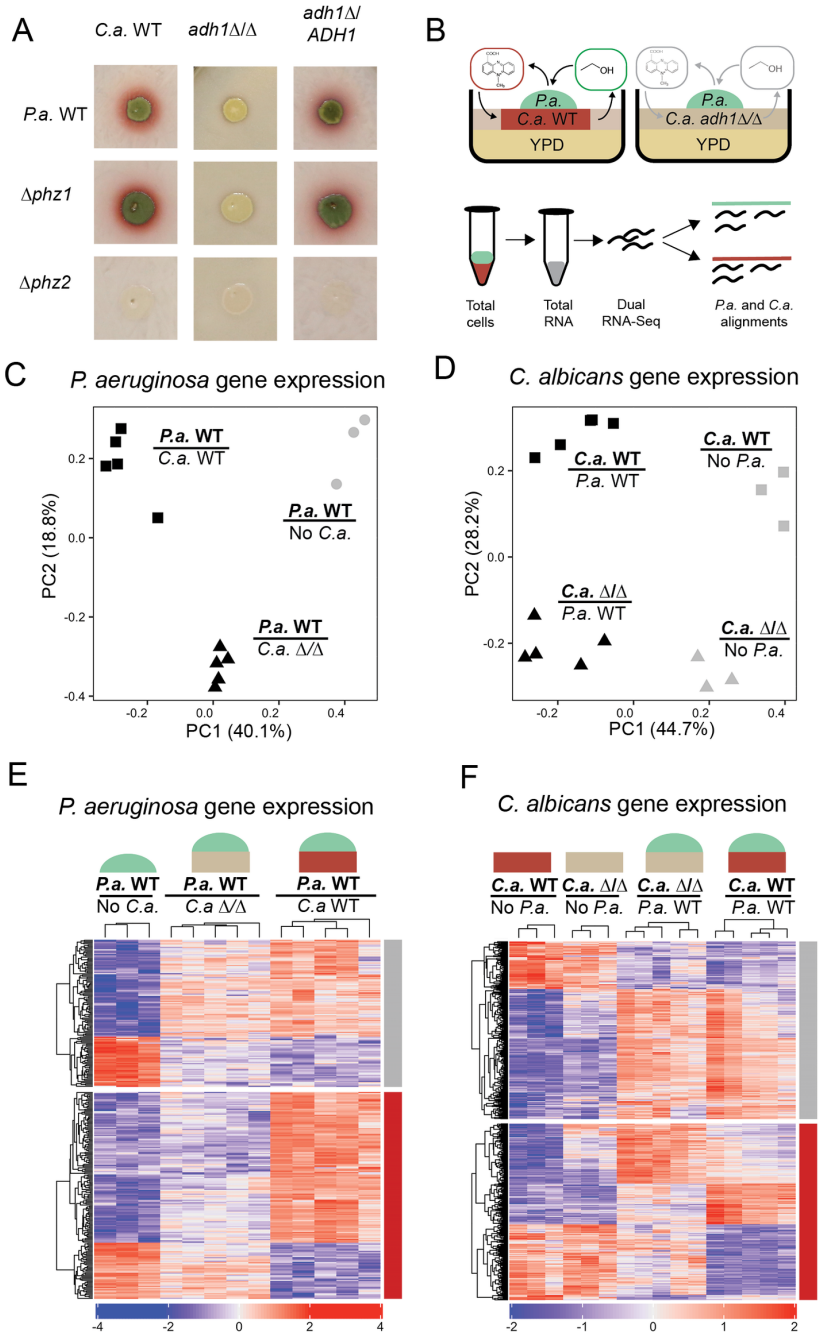

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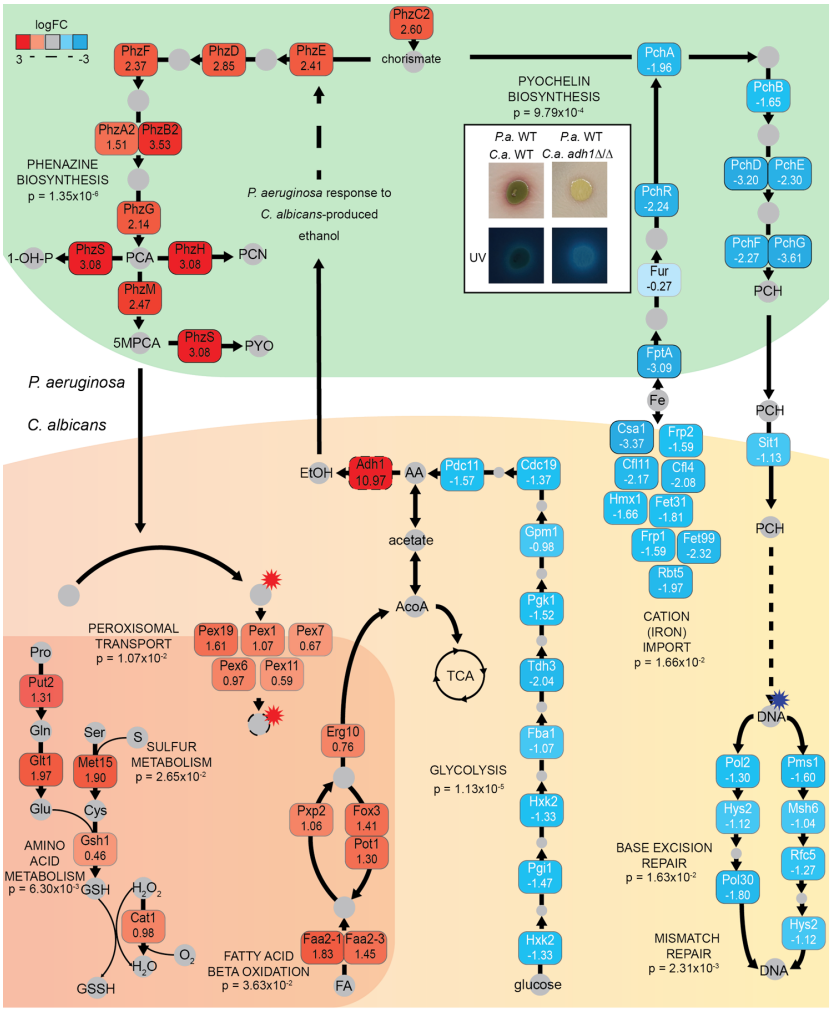
Figure 1. In co-culture of *C. albicans* (*C.a.*) and *P. aeruginosa* (*P.a.*), *C.a.*-produced ethanol stimulates *P.a.* to produce 5-MPCA and transcriptional responses ensue from both organisms. A) Co-cultures of *P.a*. wild type (WT) and mutants lacking phenazine biosynthesis operons (Δ*phz1* or Δ*phz2*) were inoculated onto 72 h-old lawns of *C.a.* wild type (WT), *C.a. adh1*Δ/Δ and *adh1*Δ/Δ reconstituted with *ADH1* (*adh1*Δ/*ADH1*). The red pigmentation indicates production of the phenazine 5-MPCA by *P.a..* B) Dual RNA-Seq allowed for parallel analyses of *P.a*. (green) and *C.a.* (red or pink) mRNA expression profiles from co-culture lawns to survey the effects of ethanol (green oval) and 5-MPCA (red oval) on gene expression. C) Principle component analysis (PCA) of TPM (transcripts per kilobase per million reads) from transcriptome profiles of *P.a.* grown alone (No *C.a.*), *P.a.* grown with *C.a.* WT, and *P.a.* grown with *C.a. adh1*Δ/Δ. D) PCA of gene expression profiles of *C.a.* WT and *C.a. adh1*Δ/Δ grown in mono-culture (No *P.a.*) or co-cultures with *P.a.* WT. E) The expression (z-score of TPM) of genes that differentiate *P.a.* in mono-culture from that grown in co-culture with *C.a.* WT (absolute value of $\log_2$fold-change (logFC) > 1 and false discovery rate (FDR) < 0.05); data for *P.a.* on *C.a*. *adh1*Δ/Δ are also shown. The red bar indicates genes that are significantly different between *C.a.* WT and *adh1*Δ/Δ (logFC >1, FDR < 0.05); the grey bar indicates genes that are not. F) Gene expression (z-score of TPM) of *C.a.* WT and *adh1*Δ/Δ grown in mono-culture or in co-culture with *P.a.*; genes that are significantly different between *C.a.* WT alone or *C.a.* WT with *P.a.* (logFC >1, FDR < 0.05) are shown for all four sample types. Genes that are also significantly different between *C.a.* WT and *adh1*Δ/Δ in the presence of *P.a*. (logFC >1, FDR < 0.05) are indicated by the red bar; the grey bar indicates genes that are not significantly different in this comparison.


logFC
3
-3
PhzC2 2.60
chorismate
PhzE 2.41
PhzD 2.85
PhzF 2.37
PhzA2 1.51
PhzB2 3.53
PHENAZINE BIOSYNTHESIS p = 1.35x10-6
PhzG 2.14
1-OH-P
PhzS 3.08
PCA
PhzH 3.08
PCN
PhzM 2.47
5MPCA
PhzS 3.08
PYO
P. aeruginosa response to C. albicans-produced ethanol
PYOCHELIN BIOSYNTHESIS p = 9.79x10-4
P.a. WT
P.a. WT
C.a. WT
C.a. adh1Δ/Δ
UV
PchA -1.96
PchB -1.65
PchD -3.20
PchE -2.30
PchR -2.24
PchF -2.27
PchG -3.61
Fur -0.27
PCH
FptA -3.09
Fe
P. aeruginosa
C. albicans
PCH
Sit1 -1.13
Csa1 -3.37
Frp2 -1.59
Cfl11 -2.17
Cfl4 -2.08
Hmx1 -1.66
Fet31 -1.81
Frp1 -1.59
Fet99 -2.32
Rbt5 -1.97
CATION (IRON) IMPORT p = 1.66x10-2
EtOH
Adh1 10.97
AA
Pdc11 -1.57
Cdc19 -1.37
acetate
AcoA
TCA
Gpm1 -0.98
Pgk1 -1.52
Tdh3 -2.04
Fba1 -1.07
Hxk2 -1.33
Pgi1 -1.47
Hxk2 -1.33
glucose
GLYCOLYSIS p = 1.13x10-5
PEROXISOMAL TRANSPORT p = 1.07x10-2
Pex19 1.61
Pex1 1.07
Pex7 0.67
Pex6 0.97
Pex11 0.59
Pro
Put2 1.31
Gln
Ser
S
Glt1 1.97
Met15 1.90
SULFUR METABOLISM p = 2.65x10-2
Glu
Cys
Gsh1 0.46
AMINO ACID METABOLISM p = 6.30x10-3
GSH
GSSH
H2O2
Cat1 0.98
O2
H2O
Erg10 0.76
Pxp2 1.06
Fox3 1.41
Pot1 1.30
Faa2-1 1.83
Faa2-3 1.45
FATTY ACID BETA OXIDATION p = 3.63x10-2
FA
DNA
Pol2 -1.30
Pms1 -1.60
Hys2 -1.12
Msh6 -1.04
Rfc5 -1.27
Pol30 -1.80
Hys2 -1.12
BASE EXCISION REPAIR p = 1.63x10-2
MISMATCH REPAIR p = 2.31x10-3
DNA

1449

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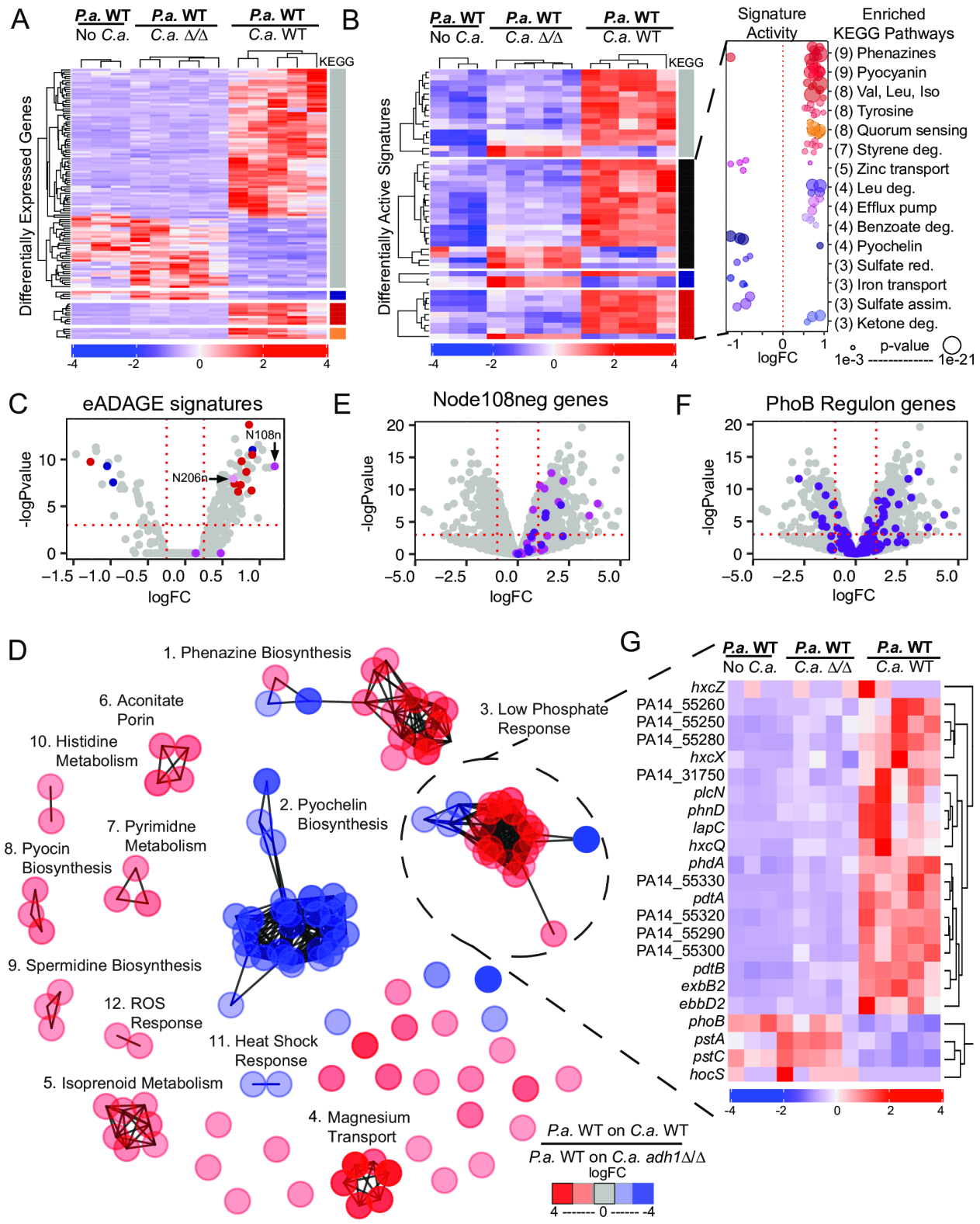
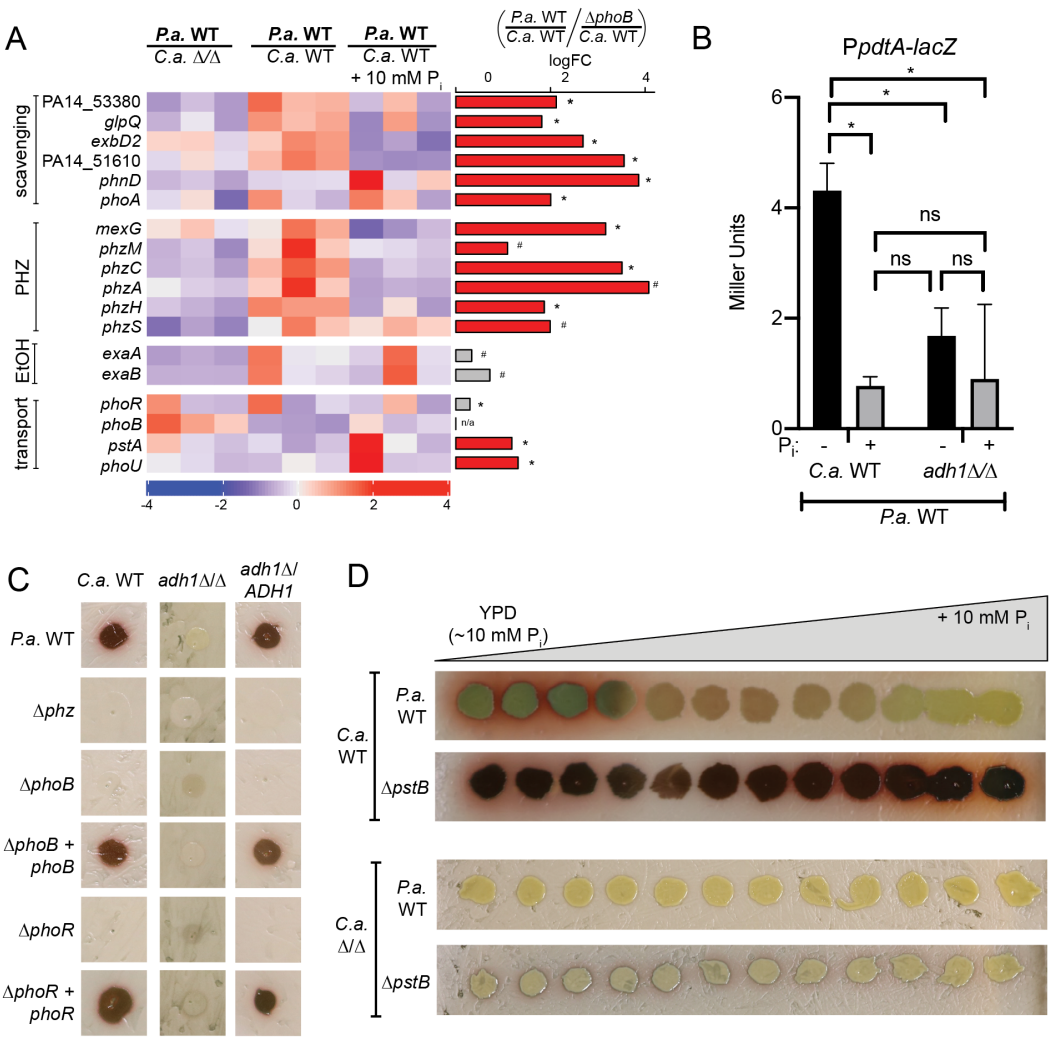
1453 **Figure 2. Pathways containing differentially expressed genes in *P. aeruginosa***
1454 **and *C. albicans* between co-cultures of *P. aeruginosa* wild type (WT) with *C.***
1455 ***albicans* WT or *adh1*Δ/Δ.** DEGs of between *C. albicans* WT and *adh1*Δ/Δ from *P.*
1456 *aeruginosa* co-cultures contained over-representations of KEGG pathways for amino
1457 acid metabolism, sulfur (S) metabolism, peroxisomal transport, fatty acid beta-oxidation,
1458 glycolysis, cation (Fe) import, base excision and mismatch DNA repair. Red indicates
1459 higher expression in co-cultures with *C. albicans* WT and blue indicates higher
1460 expression in co-cultures with *C. albicans adh1*Δ/Δ. Values indicate $\log_2$fold-change. *C.*
1461 *albicans adh1*Δ/Δ has higher expression of glycolysis genes and the production of
1462 acetate, which is either secreted or enters into the citric acid cycle (TCA). *P. aeruginosa*
1463 DEGs from the same co-cultures were contained over-representations of KEGG
1464 pathways for phenazine (PCA, PCN, PYO, 1-OH-P, 5-MCPA) biosynthesis and
1465 pyochelin (PCH) biosynthesis pathways. Inset shows increase in siderophore-derived
1466 fluorescence of co-cultures of *P. aeruginosa* with *C. albicans adh1*Δ/Δ which is
1467 consistent with increased PCH production. P-values are from hypergeometric over-
1468 representation tests, FDR corrected.
1469
1470


A
P.a. WT / No C.a.
P.a. WT / C.a. Δ/Δ
P.a. WT / C.a. WT
KEGG
Differentially Expressed Genes
-4 -2 0 2 4
B
Differentially Active Signatures
Signature Activity
Enriched KEGG Pathways
(9) Phenazines
(9) Pyocyanin
(8) Val, Leu, Iso
(8) Tyrosine
(8) Quorum sensing
(7) Styrene deg.
(5) Zinc transport
(4) Leu deg.
(4) Efflux pump
(4) Benzoate deg.
(4) Pyochelin
(3) Sulfate red.
(3) Iron transport
(3) Sulfate assim.
(3) Ketone deg.
-1 0 1
logFC
p-value
1e-3 1e-21
C
eADAGE signatures
N108n
N206n
-logPvalue
logFC
E
Node108neg genes
F
PhoB Regulon genes
D
1. Phenazine Biosynthesis
2. Pyochelin Biosynthesis
3. Low Phosphate Response
4. Magnesium Transport
5. Isoprenoid Metabolism
6. Aconitate Porin
7. Pyrimidne Metabolism
8. Pyocin Biosynthesis
9. Spermidine Biosynthesis
10. Histidine Metabolism
11. Heat Shock Response
12. ROS Response
G
hxcZ
PA14_55260
PA14_55250
PA14_55280
hxcX
PA14_31750
plcN
phnD
lapC
hxcQ
phdA
PA14_55330
pdtA
PA14_55320
PA14_55290
PA14_55300
pdtB
exbB2
ebbD2
phoB
pstA
pstC
hocS
P.a. WT on C.a. WT
P.a. WT on C.a. adh1Δ/Δ
logFC
4 ------- 0 ------ -4

1471

1472

Figure 3. eADAGE analysis reveals a subset of the Pho regulon upregulated in *P. aeruginosa* (*P.a.*) grown on *C. albicans* (*C.a.*) WT compared to that on *C.a. adh1*Δ/Δ. A) Differentially expressed genes (DEGs) between *P.a.* grown alone or on *C.a*. WT and *C.a. adh1*Δ/Δ for 24 h. Genes that fell within over-represented KEGG pathways (quorum sensing (orange bar), phenazine biosynthesis (red bar) and pyochelin biosynthesis (blue bar)) are indicated. Most DEGs do not belong to any of the three pathways (grey bar). B) Differentially active eADAGE signatures (DASs) for the same samples shown in A. Signatures in which genes annotated as being involved in phenazine biosynthesis (red bar), pyochelin biosynthesis (blue bar), or other KEGG pathways (black bar) are overrepresented are indicated. Signatures that are not over-represented an any KEGG pathways are indicated by the grey bar. Inset shows the fold-change for the expression all of the KEGG pathways that are over-represented among the DASs (# of DASs per KEGG pathway in parentheses); over-representation p-value shown as circle (Supp. Dataset 3). C) DASs with increased activity in transcriptome comparisons of *P.a*. grown on *C.a*. WT compared to on *C.a*. *adh1*Δ/Δ. In addition to DASs with over-representations of pyochelin (blue dots) and phenazine (red dots) biosynthesis, others over-represent the Pho regulon (Node108n, purple) or contain ethanol catabolism genes (N206n, pink). D) The eADAGE signature with the highest increase in activity, Node108neg (N108n, purple), contains many genes with increased expression though not all met the criterion of DEGs individually (logFC > 1, ,FDR < 0.05). E) DEGs in *P.a*. grown on *C.a*. WT compared to on *C.a*. *adh1*Δ/Δ with expression levels of PhoB-regulated genes (dark purple) highlighted. F) Network analysis of DEGs suggest groups of DEGs have correlated patterns across eADAGE: phenazine biosynthesis (1) is inversely expressed with the low iron response (2) and coordinately upregulated with the low phosphate response (3) upon exposure to ethanol in co-culture. Other cliques of DEGs participate in shared biological pathways. See table 2 for descriptions of all cliques. G) The Pho Clique (3) contains two clades of DEGs with opposing expression patterns between *P.a.* grown on *C.a.* WT and *C.a*. *adh1*Δ/Δ.


A
P.a. WT / C.a. Δ/Δ
P.a. WT / C.a. WT
P.a. WT / C.a. WT + 10 mM Pi
(P.a. WT / C.a. WT) / (ΔphoB / C.a. WT)
logFC
scavenging
PA14_53380
glpQ
exbD2
PA14_51610
phnD
phoA
PHZ
mexG
phzM
phzC
phzA
phzH
phzS
EtOH
exaA
exaB
transport
phoR
phoB
pstA
phoU
n/a
B
PpdtA-lacZ
Miller Units
ns
Pi:
C.a. WT
adh1Δ/Δ
P.a. WT
C
C.a. WT
adh1Δ/Δ
adh1Δ/ ADH1
P.a. WT
Δphz
ΔphoB
ΔphoB + phoB
ΔphoR
ΔphoR + phoR
D
YPD (~10 mM Pi)
+ 10 mM Pi
C.a. WT
P.a. WT
ΔpstB
C.a. Δ/Δ

1504

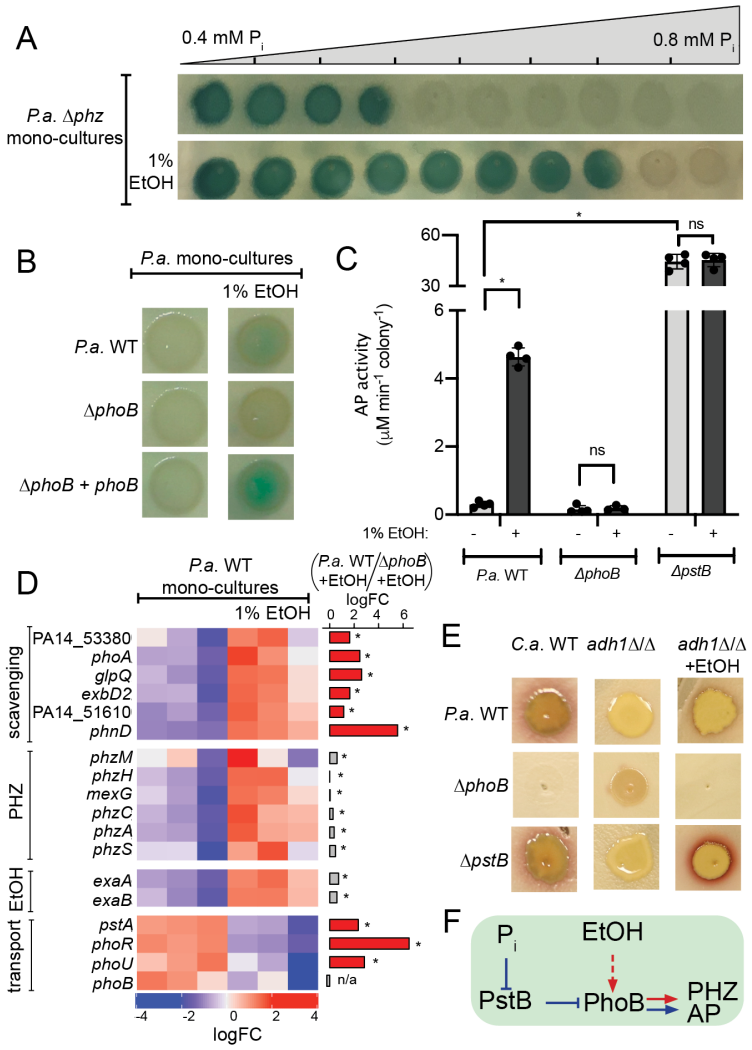
1505

1506

1507 **Figure 4. *C. albicans* (*C.a.*) WT induced PhoB-regulated genes in *P. aeruginosa***
1508 **(*P.a.*) compared to *C.a. adh1*Δ/Δ leading to 5-MPCA production as indicated by red**
1509 **pigment formation.** A) Expression of *P.a.* genes involved in phosphate scavenging,
1510 phenazine biosynthesis (PHZ), ethanol catabolism (EtOH) and inorganic phosphate
1511 transport were measured by NanoString (codesetPAV5) from cells grown with *C.a.*
1512 *adh1*Δ/Δ, *C.a.* WT or *C.a.* WT grown on medium with additional 10 mM phosphate.
1513 Expression values are normalized to loading controls and housekeeping genes as
1514 described in methods. Values are z-scored, scaled by gene. Right-hand barplot shows
1515 logFC between *P.a.* WT and *P.a.* Δ*phoB* on *C.a.* WT. The bar is colored red if
1516 expression is PhoB-dependent (logFC *P.a.* WT / *P.a.* Δ*phoB* > 1, FDR < 0.05, else
1517 grey). * = FDR < 0.05, # = FDR > 0.05. B) Beta-galactosidase activity indicative of
1518 expression of a *pdtA-lacZ* promoter fusion in *P.a.* WT in *P.a* grown with *C.a.* WT or *C.a.*
1519 *adh1*Δ/Δ in the absence or presence of P_i supplementation, *,p<0.05 by ANOVA (n = 3).
1520 C) Red 5-MPCA derivatives produced in co-culture by *P.a* WT, Δ*phoB*, Δ*phoR*, and their
1521 complemented derivatives on *C.a.* WT, *adh1*Δ/Δ, and *adh1*Δ/*ADH1*. D) Red 5-MPCA-
1522 derivatives produced by *P.a.* WT and *P.a.* Δ*pstB* over a gradient of phosphate
1523 concentrations. *P.a.* Δ*pstB* has constitutive PhoB activity. Conversely, *P.a.* WT did not
1524 produce 5-MPCA on *Ca. adh1*Δ/Δ at any phosphate concentration, but *P.a.* Δ*pstB*
1525 induced a small amount of red pigment independent of the phosphate concentration.
1526
1527

1528

1529


A
0.4 mM Pi
0.8 mM Pi
P.a. Δphz mono-cultures
1% EtOH
B
P.a. mono-cultures
1% EtOH
P.a. WT
ΔphoB
ΔphoB + phoB
C
AP activity (μM min-1 colony-1)
1% EtOH:
P.a. WT
ΔphoB
ΔpstB
ns
D
P.a. WT mono-cultures
1% EtOH
(P.a. WT +EtOH / ΔphoB +EtOH)
logFC
scavenging
PA14_53380
phoA
glpQ
exbD2
PA14_51610
phnD
PHZ
phzM
phzH
mexG
phzC
phzA
phzS
EtOH
exaA
exaB
transport
pstA
phoR
phoU
phoB
n/a
logFC
E
C.a. WT
adh1Δ/Δ
adh1Δ/Δ +EtOH
P.a. WT
ΔphoB
ΔpstB
F
Pi
EtOH
PstB
PhoB
PHZ
AP

1530

1531

1532

1533 **Figure 5. Ethanol (EtOH) induced PhoB activity in *P. aeruginosa* (*P.a.*) mono-**
1534 **culture.** A) Alkaline phosphatase (AP) activity visualized by blue color derived from
1535 cleavage of BCIP in MOPS medium with a gradient of phosphate in the absence and
1536 presence of ethanol. The *P.a.* Δ*phz* strain was used to eliminate color differences due to
1537 phenazine production. B) AP activity in the absence and presence of ethanol was
1538 visualized with BCIP added to MOPS agar (0.7 mM phosphate) for *P.a.* wild type,
1539 Δ*phoB* and the Δ*phoB* mutant complemented with a wild-type copy of *phoB* integrated
1540 at the native locus. C) AP activity in cells from colony biofilms grown as in B was
1541 measured using the colorimetric substrate pNPP for *P.a.* WT, Δ*phoB*, and Δ*pstB*, a
1542 strain with constitutive PhoB activity. *,$p<0.01$ by ANOVA ($n \geq 3$). D) Transcripts within
1543 the PhoB regulon involved in phosphate scavenging and inorganic phosphate transport
1544 and genes involved in phenazine production (PHZ) and ethanol catabolism (EtOH) were
1545 measured in cells grown in the absence and presence of ethanol by Nanostring
1546 (codeset PAV5). PhoB-regulated genes increased in expression (top section) and
1547 ethanol catabolism genes (third section) increase in expression and others decrease
1548 (bottom section). Expression values are normalized to loading controls and
1549 housekeeping genes as described in methods. Values are scaled by gene. Right-hand
1550 barplot shows logFC between *P.a.* WT and *P.a*. Δ*phoB* on MOPS+1%EtOH. The bar is
1551 colored red if expression is PhoB-dependent (logFC *P.a*. WT / *P.a.* Δ*phoB* > 1, FDR <
1552 0.05, else grey). *,FDR < 0.05, #,FDR > 0.05. E) PhoB activity and ethanol are both for
1553 5-MPCA production in response to *C.a.* ethanol. P.a. WT, Δ*phoB* and Δ*pstB* were grown
1554 on C.a. WT, or C.a. adh1Δ/Δ in the absence or presence of exogenous ethanol. 5-
1555 MPCA production was rescued by the addition of 1% ethanol to a co-culture of *P.a.*
1556 Δ*pstB* and, to a lesser extent, *P.a.* WT on *C.a. adh1*Δ/Δ. F) Phosphate (P_i) and ethanol
1557 (EtOH) are additive stimuli that promote PhoB-dependent expression of AP and
1558 phenazine biosynthesis (PHZ).
1559
1560

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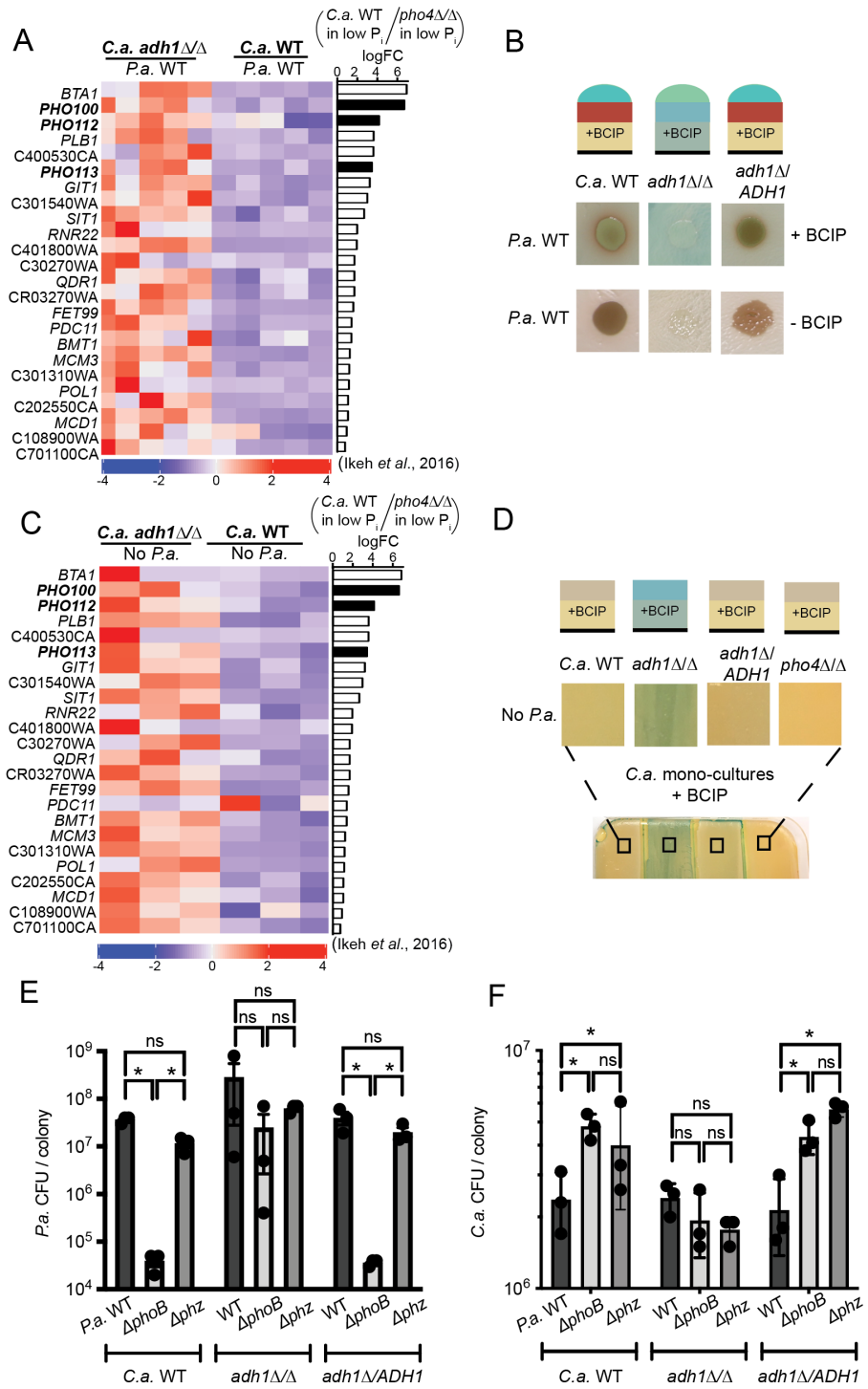
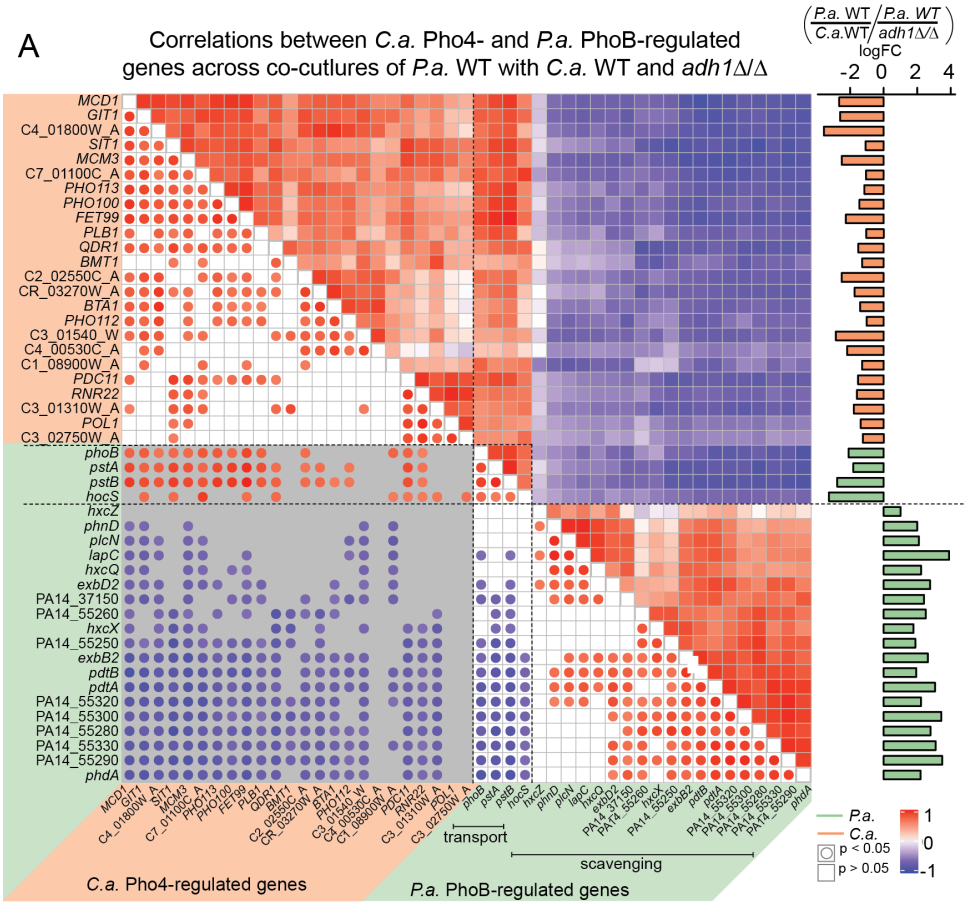
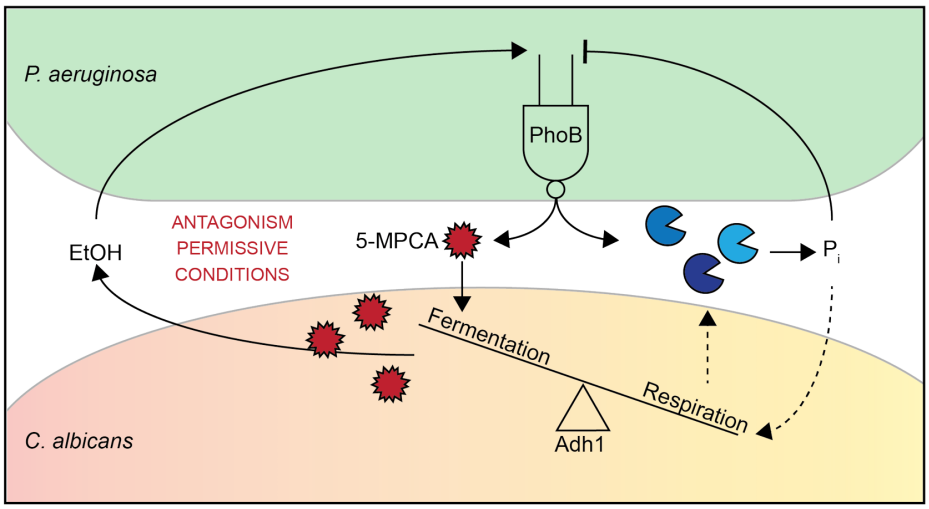

A
C.a. adh1Δ/Δ
P.a. WT
C.a. WT
P.a. WT
(C.a. WT in low Pi / pho4Δ/Δ in low Pi)
logFC
BTA1
PHO100
PHO112
PLB1
C400530CA
PHO113
GIT1
C301540WA
SIT1
RNR22
C401800WA
C30270WA
QDR1
CR03270WA
FET99
PDC11
BMT1
MCM3
C301310WA
POL1
C202550CA
MCD1
C108900WA
C701100CA
(Ikeh et al., 2016)
B
+BCIP
C.a. WT
adh1Δ/Δ
adh1Δ/ ADH1
P.a. WT
+ BCIP
- BCIP
C
C.a. adh1Δ/Δ
No P.a.
C.a. WT
No P.a.
D
pho4Δ/Δ
No P.a.
C.a. mono-cultures + BCIP
E
P.a. CFU / colony
ns
*
P.a. WT
ΔphoB
Δphz
WT
C.a. WT
adh1Δ/Δ
adh1Δ/ADH1
F
C.a. CFU / colony

1563

1564

Figure 6. The *C. albicans* (*C.a.*) *adh1*Δ/Δ has increased expression of the Pho4-mediated low phosphate response in co-culture that is inversely correlates with PhoB activity in *P. aeruginosa* (*P.a.*). A) Previously characterized Pho4-regulated genes [86] including a phospholipase and phosphatases (black bars) were more highly expressed in *C.a*. *adh1*Δ/Δ than *C.a.* WT in *P.a*. co-cultures (data shown as z-scores of TPM). Pho4-dependence is shown in the right-hand barplot as $\log_2$FC *C.a*. WT/*C.a. pho4*Δ/Δ using data from [86]. B) Analysis of phosphatase activity in *C.a.* WT, *C.a. adh1*Δ/Δ, the complemented strain *adh1*Δ/*ADH1* or *pho4*Δ/Δ using the colorimetric phosphatase BCIP substrate in agar. More phosphatase activity was observed in *C.a. adh1*Δ/Δ than in strains with *ADH1* in in co-culture with *P.a*. C) The same Pho4-regulated genes as shown in A were also more highly expressed in *C.a. adh1*Δ/Δ than *C.a.* WT in mono-cultures. Right-hand barplot shows Pho4-dependence as in A. D) More phosphatase activity was observed in *C.a. adh1*Δ/Δ than in strains with *ADH1* in in mono-culture. . As predicted, phosphatase activity is not evident in the *C.a*. *pho4*Δ/Δ strain. Phosphatase activity visualized via BCIP as in B. E) Number of CFUs of *P.a*. WT, Δ*phoB* or Δ*phz* after co-culture for 72 h with *C.a.* WT, *C.a. adh1*Δ/Δ *C.a. adh1*Δ/*ADH1*. *P.a.* Δ*phoB* and Δ*phz* had significantly fewer CFUs on *C.a.* strains with high ethanol production (WT and *adh1*Δ/*ADH1*), but not *C.a. adh1*Δ/Δ. F) In the same samples analyzed in A, *C.a*. CFU formation was assessed. *C.a.* WT or *C.a. adh1*Δ/*ADH1* strains had increased fitness in-culture with *P.a*. Δ*phoB* or *P.a*. Δ*phz* compared to with *P.a*. WT. For *C.a. adh1*Δ/Δ, there were no differences in CFU formation when co-cultured with *P.a*. WT, Δ*phoB* or Δ*phz.*


A
Correlations between C.a. Pho4- and P.a. PhoB-regulated genes across co-cutlures of P.a. WT with C.a. WT and adh1Δ/Δ
logFC
-2 0 2 4
MCD1
GIT1
C4_01800W_A
SIT1
MCM3
C7_01100C_A
PHO113
PHO100
FET99
PLB1
QDR1
BMT1
C2_02550C_A
CR_03270W_A
BTA1
PHO112
C3_01540_W
C4_00530C_A
C1_08900W_A
PDC11
RNR22
C3_01310W_A
POL1
C3_02750W_A
phoB
pstA
pstB
hocS
hxcZ
phnD
plcN
lapC
hxcQ
exbD2
PA14_37150
PA14_55260
hxcX
PA14_55250
exbB2
pdtB
pdtA
PA14_55320
PA14_55300
PA14_55280
PA14_55330
PA14_55290
phdA
transport
scavenging
C.a. Pho4-regulated genes
P.a. PhoB-regulated genes
P.a.
C.a.
p < 0.05
p > 0.05
1
0
-1


B
P. aeruginosa
PhoB
EtOH
ANTAGONISM
PERMISSIVE
CONDITIONS
5-MPCA
Pi
Fermentation
Respiration
Adh1
C. albicans

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1591 **Figure 7. *P. aeruginosa* (*P.a.*) PhoB affects both *P.a.* and *C. albicans* (*C.a.*) fitness**
1592 **in co-culture through its control of phenazine production and phosphate**
1593 **acquisition**. A) Pearson correlation analysis between *P.a.* (green annotations) and *C.a.*
1594 (orange annotations) low phosphate-responsive genes from co-cultures of *P.a.* WT with
1595 either *C.a.* WT or *adh1*Δ/Δ. Largely inverse relationships between *P.a.* PhoB- and *C.a.*
1596 Pho4-regulated genes is apparent. Log_2FC ($p<0.05$) between *P.a*. with either *C.a*. WT
1597 or *C.a*. *adh1*Δ/Δ is shown in the right-hand bar plot. Lower half of correlogram shows
1598 which correlations are significant (filled circles) and indicates correlation values by color
1599 intensity relative to scale. Same species comparisons have white backgrounds and
1600 cross-species correlations have grey backgrounds. B) Model of PhoB activity in *P.a.-*
1601 *C.a.* co-cultures. PhoB mediates the conditional production of the antagonistic,
1602 antifungal phenazine 5-MPCA in response to phosphate and fungal ethanol
1603 production.**,$p<0.01$ by ANOVA.
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1607 **Supplementary Figure**

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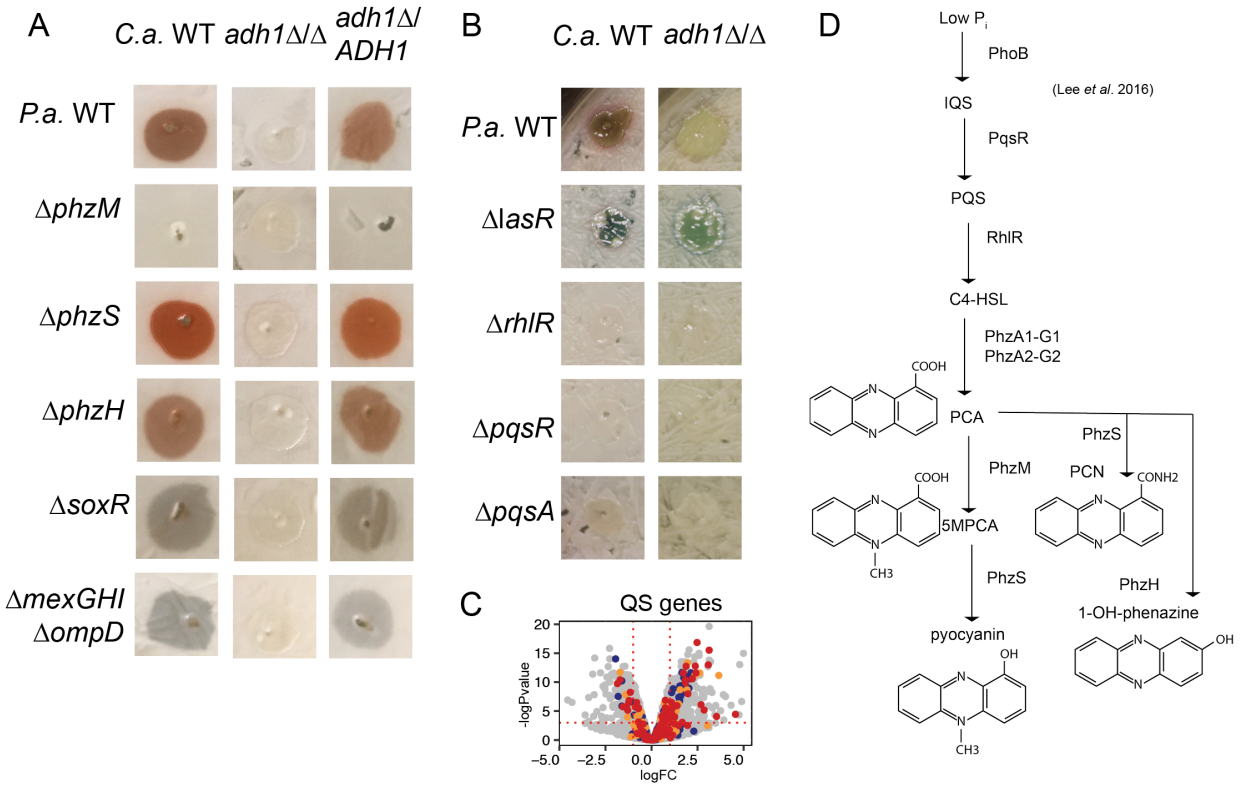

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1611 **Figure S1. Red pigment formation is dependent on phenazine biosynthesis**
1612 **genes, phenazine transport genes and quorum sensing (QS) pathways in *P.***
1613 ***aeruginosa* (*P.a.*).** A) Co-cultures of *P.a*. wild type (WT) and mutants lacking genes
1614 involved in phenazine biosynthesis were inoculated onto lawns of *C.a.* (WT), *C.a.*
1615 *adh1*Δ/Δ and *adh1*Δ/Δ reconstituted with *ADH1* then incubated for 24 h. *P.a.* 5-MPCA
1616 phenazine biosynthesis (evident by red color) was not observed with the Δ*phzM* but was
1617 still produced by Δ*phzS* and Δ*phzH*. 5-MPCA production was dependent on the
1618 oxidative stress response gene *soxR* and 5-MPCA transport complex *mexGHI-ompD*.
1619 For all *P.a.* strains, 5-MPCA was only produced on *C.a.* with intact *ADH1*. B) Co-
1620 cultures of *P.a*. wild type (WT) and mutants lacking genes involved in quorum sensing
1621 were inoculated onto lawns of *C.a.* (WT), *C.a. adh1*Δ/Δ and *adh1*Δ/Δ reconstituted with
1622 *ADH1* then incubated for 48 h. *P.a.* mutants defective in QS pathways (Δ*lasR*, Δ*rhlR*,
1623 Δ*pqsR*, Δ*pqsA*) form less red pigment that *P.a.* WT on *C.a.* WT and no strains form red
1624 pigment on *C.a. adh1*Δ/Δ. C) Gene expression of *P.a*. LasR (blue), RhlR (orange) and
1625 PqsR (red) regulated genes had heterogenous expression with genes both up and
1626 down regulated. D) Red pigment formation being dependent on QS pathways of RhlR
1627 and PqsR is consistent with integration of QS with PhoB via the integrative quorum
1628 sensing (IQS) pathway in which low phosphate triggers PhoB activity which influences
1629 PqsR and RhlR to act, via their cognate autoinducers PQS and C4-HSL respectively, in
1630 a regulatory cascade eventually promoting the transcription of phenazine biosynthesis
1631 genes (*phzA1-G1*, *phzA2-G2*) and consequent phenazine carboxylic acid (PCA)
1632 production. PhoB and QS have also been reported to effect the expression of
1633 phenazine modification genes *phzM, phzS,* and *phzH* necessary for the conversion of
1634 PCA to 5-methyl-phenazine-1-carboxylic acid (5-MPCA), pyocyanin, phenazine-1-
1635 carboxamide (PCN) and 1-hydroxy-phenazine (1-OH-phenazine) [74].
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1638 **Supplementary Tables**

1639 **Table S1**. **Clustering coefficients for co-culture data.** Results of clustering
1640 coefficients (CC) for gene expression and eADAGE signature activity values for
1641 samples grouped by experimental condition. For each sample comparison, random
1642 controls with a comparable number of samples are shown. For all analyses, CC mean
1643 and standard deviation (SD) are shown .

Groups	CC for gene expression	SD	CC for signature activity	SD
CAF2 vs *adh1*Δ/Δ	0.38		0.68	
Random (5v5)	0.14	± 0.07	0.12	± 0.12
Coculture vs Monoculture	0.42		0.53	
Random(10v3)	0.14	± 0.16	0.15	± 0.22
CAF2 vs YPD	0.58		0.80	
Random (5v3)	0.13	± 0.09	0.10	± 0.11

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1647 **Table S2: eADAGE gene-gene network cliques of DEGs from co-culture.** Cliques
1648 from the eADAGE gene-gene subnetwork of genes differentially expressed between
1649 *P.a*. grown on WT and *adh1*Δ/Δ *C.a*.. Cliques were considered as groups of genes (> 1
1650 gene) connected by edges with weights > 0.5 of Pearson correlation in signature
1651 weights in eADAGE. Cliques were numbered arbitrarily and named to represent the
1652 known functional characterizations of genes contained in each clique.
1653

Clique Number	Description	Clique size	Genes
1	Phenazine biosynthesis	17	PA0522, PA3039, PA5383, PA1219, PA1221, PA1220, PA1216, PA1218, *phzA1, phzG2, phzE2, phzC2, phzD2, phzM, phzS, phzB1, phzF2*
2	Pyochelin biosynthesis	29	*fepD, fepB, PA4155, foxI, pvdS, femI, fiuI, femR,* PA5217, PA1301 PA1300, *hasAP*, PA4570, *pchR, fumC1, sodM*, PA4471, *fiuR, pchA, ampO ,pchD, pchB, fptA, pchC, pchE,* PA4220, PA4222, PA4223, *pchF*
3	Phosphate transport and acquisition	23	PA2120, *hocS, phoB, pstA, pstC, plcN,* PA3383, *exbB2, phdA*, *pdtB*, PA0701, PA0699, *hxcX*, l*apA*, exbD2, PA0696, PA0697, PA0698, *hxcQ,* PA0700, *hxcZ*, PA0695, *pdtA*
4	Isoprenoid metabolism	7	*liuD, liuB, mmsB*, PA2557, PA2553, *liuC, liuA*
5	Magnesium transport	6	*mgtA*, PA4826, PA4635, PA4822, PA4824, PA4823
6	Aconitate porin	4	*opdH*, PA0753, PA0752, PA0754
7	Pyrimidine metabolism	3	*dht,* PA0440, PA0439
8	Pyocin Biosynthesis	3	PA0629, PA0630, PA0637
9	Spermidine biosynthesis	3	PA4773, PA4774, PA4782
10	Histidine catabolism	2	*hutH*, PA5096
11	Heat shock response	2	*hscA, fdx2*
12	Reactive oxygen stress response	2	*ahpF, katB*

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1657 **Table S3. Strains and plasmids used in this study.**

Strain	Lab stock#	Strain description	Source
P. aeruginosa			
PA14 WT	DH123	Laboratory reference strain	[1]
PA14 Δ*phoB*	DH3599	Deletion mutant in *phoB*	This study
PA14 Δ*phoB* + *phoB*	DH3600	Native locus complementation of *phoB*	This study
PA14 Δ*phoR*	DH3774	Deletion mutant of *phoR*	[2]
PA14 Δ*phoB* + *phoR*	DH3755	Plasmid-based arabinose inducible over-expression vector of *phoR*	This study
PA14 Δ*pstB*	DH3601	Deletion mutant of *pstB*	This study
PA14 *pstB*::*Tn*M	DH753	Transposon insertion mutant of *pstB*	[3]
PA14 Δ*phz*	DH933	Deletion mutant of *phzA1-G1* and *phzA2-G2*	[4]
PA14 Δ*phzA1*	DH1728	Deletion mutant of *phzA1*	(Dietrich, Price-Whelan et al. 2006)
PA14 Δ*phzA2*	DH1735	Deletion mutant of *phzA2*	(Dietrich, Price-Whelan et al. 2006)
PA14 Δ*phzM*	DH944	Deletion mutant of *phzM*	[5]
PA14 Δ*mexGHI*Δ*ompD*	DH1376	Deletion mutant of *mexGH1 and ompD*	[5]
PA14 Δ*soxR*	DH1377	Deletion mutant of *soxR*	[5]
PA14 Δ*exaA*	DH2256	Deletion mutant of *exaA*	[6]
PA14 Δ*exaA* + *exaA*	DH2677	Native locus complementation of *exaA*	[6]
PA14 WT PpdtA::lacZ-gfp	DH3780	Promoter fusion reported construct of PpdtA chromosomally integrated at the *att* site	This study
PA14 Δ*phoB PpdtA*::*lacZ-gfp*	DH3781	Promoter fusion reported construct of PpdtA chromosomally integrated at	This study

		the *att* site in Δ*phoB* background	
CAF2 WT	DH48	Laboratory reference strain	[7]
CAF2 *adh1*Δ/Δ	DH2236	Homozygous deletion mutant of *ADH1*	[8]
CAF2 *adh1*Δ/Δ + *ADH1*	DH2177	Heterozygous native locus single allele complementation of *ADH1*	[8]
PA14 Δ*ackA*	DH3782	Deletion mutant of *ackA*	[9]
PA14 Δ*ackA*Δ*pta*	DH3783	Deletion mutant of *ackA* and *pta*	[9]
PA14 Δ*ackA*Δ*pta*Δ*phz*	Dh3784	Deletion mutant of *ackA, pta, phzA1-G1* and *phzA2-G2*	[9]
PA14 *exaA*::Tn*M*	DH2130	ethanol catabolic transposon insertion mutant	[10]
PA14 *pqqB*::Tn*M*	DH2131	ethanol catabolic transposon insertion mutant	[10]
PA14 *acsA*::Tn*M*	DH2132	ethanol catabolic transposon insertion mutant	[10]
PA14 Δ*kinB*	DH3778	Deletion mutant of *kinB*	[2]
PA14 ΔkinB + kinB	DH3779	Plasmid-based arabinose inducible over-expression vector of *kinB*	This study
PAO1	DH3283	Laboratory reference strain	[11]
PAO1 Δ*phoB*	DH3284	Deletion mutant of *phoB*	[11]
PAO1 Δ*vreA*	DH3285	Deletion mutant of *vreA*	[11]
PAO1 Δ*vreI*	DH3286	Deletion mutant of *vreI*	[11]
PAO1 Δ*vreR*	DH3287	Deletion mutant of *vreR*	[11]
PAO1 Δ*phoB*Δ*vreR*	DH3288	Deletion mutant of *phoB* and *vreR*	[11]
E. coli			
S17 λpirS	DH71	Mating competent strain used for plasmid conjugation	
Plasmids			
pMQ30		allelic replacement vector, GmR	[12]
phoB complement			This study
pMQ72		Arabinose inducible over-expression plasmid, empty vector	[12]
phoR OE		Arabinose inducible over-expression plasmid, containing *phoR*	This study

pHERD20		Arabinose inducible over-expression plasmid	[13]
kinB OE		Arabinose inducible over-expression plasmid, containing *kinB*	[14]
P*pdtAI-lacZ*		P*pdtA-lacZ* promoter fusion, GmR in S17 *E. coli*	This study
pEX18-Gm		Suicide vector for allelic replacement, GmR	[15]

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1660 **Table S4: Primers used in this study**
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Constructs and sequencing		
Gene/ Feature	**Primer**	**Sequence**
phoB	phoB_KO_UP_FW	ccagggttttcccagtcacgacgttgtaaaacgacggccCCAACGCAACGACCGTCTGGC
	phoB_KO_UP_RV	ccgtccaggggaaacgactccCCTCCAGGCACTCGTAGCCG
	phoB_KO_DWN_FW	CGGCTACGAGTGCCTGGAGGggagtcgtttcccctggacgg
	phoB_KO_DWN_RV	tgtgagcggataacaatttcacacaggaaacagctatgaccGCGGGTGTGGCGTCCAGGC
	phoB_KO_CHK_FW	ccggaacctgttgagcatagccc
	phoB_KO_CHK_RV	ctcctcgacatagacgttgccgc
	phoB_KI_F	atacccgtttttttggg**gaaggagatatacat**ATGGTTGGCAAGACAATCCTCATCGTTG
	phoB_KI_R	tctgtatcaggctgaaaatcttctctcatccgccTCAGCTCTTGGTGGAGAAACGATAGC
	phoB_KI_check_F	cgtcgcacgcaccaaggcg
	phoB_KI_check_R	ccctgttcgtcggcccacc
pstB		
	pstB_KO_UP_FW	agggttttcccagtcacgacgttgtaaaacgacggccGCGCTACAAGGTCCTGGAAGAGC
	pstB_KO_UP_RV	gccgcgaccgccagagccCCGAAGACTACATCACCGGCCG
	pstB_KO_Down_FW	CGGCCGGTGATGTAGTCTTCGGggctctggcggtcgcggc
	pstB_KO_Down_RV	CGGCCGGTGATGTAGTCTTCGGggctctggcggtcgcggc
	pstB_KO_Chk_FW	cgcctgcgcgagaagtacaagg
	pstB_KO_Chk_RV	ctccacgctgaagatcgaagagctg

phoR	phoR_KI_F	catacccgtttttttggg**gaaggagatatacat**ATGCAATCCGTCGTGAACCAAGACTGG
	phoR_KI_R	ttaatctgtatcaggctgaaaatcttctcTCATCCGCCTCACTTCGACGCCTTGCGCTCG
	phoR_KI_check_R	gcggcaacgtctatgtcgaggag
	phoR_KI_check_R	ctggtgcaggcgcagtagctgc
kinB	kinB_KI_F	atacccgtttttttggg**gaaggagatatacat**ATGGAAACCACTTCCGAAAAACAGGGGC
	kinB_KI_R	atctgtatcaggctgaaaatcttctctcatccgccTCATAGGCCGTACTGCTTGCGCTTC
	kinB_KI_check_R	ccgccgaatgcgcggtgacg
	kinB_KI_check_R	gcggcaacgtctatgtcgaggag
pMQ30	pMQ30_seq_mcs_FW	CCTCTTCGCTATTACGCCAGCTGG
	pMQ30_seq_mcs_RV	GCTCACTCATTAGGCACCCCAGG
Reporter fusion	p pdtA 1F	GCGATTGACGGCGGGCGTCGCGATCGCCGGGGCCGCATGACTGCGGATCCCTTCCTGGAAGCTTGCCGTAC
	p prhII 2R	TTGGGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTCATGACGCGAGATTCCTTGGGCGTGTTC
phoB sequencing	FW	cgtcgcacgcaccaaggcg
	RV	ccctgttcgtcggcccacc
pstB sequencing	FW	ggcacggcaccaggtcagc
	RV	cgacgccgcggtaggcg

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1665 **References**

1666

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