RNAseq reveals that *Pseudomonas aeruginosa* mounts medium-dependent competitive responses when sensing diffusible cues from *Burkholderia cenocepacia*

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Abstract

Most habitats on earth are colonized by diverse bacterial communities, offering ample opportunities for inter-species interactions. While competition for space and nutrients might often dominate such interactions, little is known about whether bacteria can sense competitors and mount specific responses to withstand their attacks. The competition-sensing hypothesis proposes that bacteria can do so through nutrient stress and cell damage cues. Here, we conducted a replicated RNAseq-transcriptomic study to test this hypothesis. We exposed *Pseudomonas aeruginosa* to either its own spent medium or to the supernatant of its competitor *Burkholderia cenocepacia*. Compared to controls, we detected significant changes in the transcriptome of *P. aeruginosa* that entail both general responses to spent medium and specific responses to the competitor. Specifically, genes encoding various competitive traits, including the type-VI secretion system, the siderophore pyoverdine, and the toxins phenazines and hydrogen cyanide, were upregulated when exposed to the competitor supernatant. Similarly, several genes coding for stress response and quorum-sensing regulators were overexpressed. Moreover, we found transcriptional responses to vary as a function of iron availability, whereby metabolically more costly responses were launched under iron rich conditions. Altogether, our results reveal nuanced competitive responses of *P. aeruginosa* when exposed to *B. cenocepacia* supernatant, integrating both environmental and social cues.

36 **Introduction**

37 Sequencing technologies have revealed that natural bacterial communities are incredibly diverse, and
38 that myriads of taxa can live in close proximity to one another [1–3]. From an ecological perspective,
39 the living together of completely different species must ultimately lead to intense inter-specific
40 competition for essential resources such as nutrients, space and oxygen [4–6]. This view is supported
41 by the fact that many bacterial species have evolved mechanisms to attack and defend themselves
42 from competitors [7,8]. Defensive strategies include the formation of protective biofilms, motility to
43 move away from competitors or the use of efflux pumps to eject toxic compounds [9–14]. Attacking
44 measures typically involve toxins that are either secreted in the environment, or directly injected into
45 competitor cells via contact-dependent secretion systems [8,15–23].

46 There has recently been much interest in understanding whether and how bacteria can sense
47 the presence of competitors and mount adequate responses [24–31]. This interest roots in the
48 evolutionary thinking that defensive and attacking responses are costly and should only be mounted
49 when required. Cornforth and Foster [24] developed a concept for competition sensing by proposing
50 that bacteria rely on cues such as resource limitation or competitor-induced cell damage, to trigger
51 competitive counter measures such as toxin production and biofilm formation. Moreover, it was
52 proposed that bacteria might be able to sense competitors via their secreted diffusible molecules, such
53 as toxins or quorum-sensing molecules, to mount responses before they physically meet the
54 competitor [25,27].

55 Here we used transcriptomics to test whether *P. aeruginosa* PAO1 [32] changes its gene
56 expression profile when exposed to the supernatant of a competitive species, *B. cenocepacia* H111,
57 containing secreted compounds that could serve as cues for competition sensing. We chose these two
58 bacteria as a model system because both species are opportunistic human pathogens, which can co-
59 infect lungs of cystic fibrosis patients, but potentially also co-exist in natural soil habitats [33–39]. We
60 thus reasoned that *P. aeruginosa* might have evolved ways to sense and respond to diffusible cues
61 from *B. cenocepacia*. To test our hypothesis, we exposed *P. aeruginosa* to spent supernatant from *B.*

62 *cenocepacia* cultures mixed with fresh medium and compared its transcriptome to conditions where
63 it grew in fresh medium alone or when exposed to its own supernatant (Fig. 1). We carried out this
64 setup in three-fold replication under both iron-rich and iron-limited conditions. We then used the
65 replicated transcriptomic data (n = 18) to perform the following main analyses. First, we compared the
66 transcriptomes of *P. aeruginosa* growing in iron-rich versus iron-limited CAA medium without
67 supernatant supplementations to validate whether genes known to be iron-regulated respond to our
68 treatment in the expected way. Second, we conducted a global analysis to quantify the number and
69 type of differentially up- and downregulated genes across the supernatant treatments in the two
70 nutrient environments. Third, we focused on specific gene classes encoding competitive traits and
71 regulators that could potentially be involved in competition sensing and tested whether they are
72 differentially expressed when *P. aeruginosa* is exposed to the supernatant of *B. cenocepacia*.
73
74
75 **Results**
76 ***P. aeruginosa* transcriptomes segregate in response to supernatant treatment and iron availability**
77 We grew *P. aeruginosa* in 100% fresh CAA medium (SN.No) and in 70% fresh CAA medium
78 supplemented with either 30% of its own spent supernatant (SN.Pa) or 30% of the spent supernatant
79 of its competitor *B. cenocepacia* (SN.Bc). The three growth treatments were carried out under both
80 iron-rich and iron-limited conditions (Fig. 1). Across the six conditions and the three replicates per
81 condition, we could map between 4 595 376 and 8 827 668 reads to the PAO1 genome (Table S1).
82 Based on these reads, we conducted a principal component analysis (PCA) using the rlog transformed
83 and normalized raw counts data [40]. We found that the first three principal components (PC)
84 explained 75.2 % of the total variance observed (Fig. 2A+B). While PC1 reveals a clear separation
85 between the cultures growing under iron-rich versus iron-limited conditions, PC3 shows segregation
86 between cultures growing in 100% CAA medium and cultures that received spent supernatant in
87 addition to 70% CAA. We further observed that the triplicates of the same treatment clustered

88 together, demonstrating that our independent biological replicates yielded reproducible results. The
89 strong clustering of replicates and the distinct differences in the transcriptomes between iron-rich and
90 iron-limited conditions also emerged in a Euclidean distance matrix analysis (Fig. 2C).
91
92 **Expression of siderophore genes and other iron-regulated genes respond to iron limitation**
93 To validate our transcriptomic data, we tested whether genes known to be regulated by iron
94 availability responded in the expected way in our conditions where bacteria grew in plain CAA medium
95 (iron-limited versus iron-rich) without the addition of supernatants (Fig. 3). We first focused on the
96 genes involved in the synthesis, secretion and regulation of the two iron-scavenging siderophores
97 pyoverdine and pyochelin [41–43]. As expected, we observed that all the siderophore genes were
98 significantly upregulated under iron limitation. The expression of *toxA* and *prpL* (*piv*), encoding
99 exotoxinA and protease IV, which are known to be induced in the presence of pyoverdine [44,45], were
100 also significantly upregulated under iron limitation. Next, we focused on two genes encoding the
101 superoxide dismutase SodB (iron-dependent) and SodM (manganese-dependent). We observed that
102 *sodB* is downregulated, whereas *sodM* is upregulated under iron limitation, a pattern that is in line
103 with the transcriptional switch described by Bollinger et al. [46]. Finally, we examined the genes
104 involved in hydrogen cyanide synthesis, which is controlled by the ANR regulator featuring an iron-
105 sulfur cluster [47,48]. Consistent with previous work, we found that *anr* expression itself was not
106 affected by iron availability, but the expression of *hcnABC* decreased under iron limitation. These
107 control analyses confirm that our RNA-Seq analysis yields reliable results. Finally, we also measured
108 the production of the siderophore pyoverdine over time as a key phenotype affected by iron
109 availability. Consistent with our transcriptomic data, we found higher levels of pyoverdine production
110 in iron-limited compared to iron-rich conditions (Fig. S2). In line with previous work, we further
111 observed that *P. aeruginosa* down-scaled *de novo* pyoverdine production when growing in its own
112 supernatant, which already contains pyoverdine [49].
113

114 ***P. aeruginosa* shows multiple distinct changes in gene expression when exposed to the supernatant**
115 **of *B. cenocepacia***

116 Next, we examined differential gene expression of *P. aeruginosa* between our three growth conditions
117 (SN.No, SN.Pa and SN.Bc), separately for iron-rich and iron-limited conditions (Fig. 4). We considered
118 a gene to be differentially expressed with log2-fold change values > 1 or < -1 and with FDR-adjusted p-
119 values < 0.05. While 5570 open reading frames were originally annotated in the *P. aeruginosa* PAO1
120 genome [50], this number has gone up to 5713 as of today, according to the *Pseudomonas* Genome
121 Database (pseudomonas.com) [51]. Our RNA-Seq analysis revealed that > 98% of these predicted
122 genes were expressed (Table S2). Across all pairwise comparisons, between 5.7% and 10.7% of the
123 genes were differentially expressed, of which on average 3.3% and 4.4% were up- and downregulated,
124 respectively (Table S2). The Venn diagrams in Fig. 4A+B show the number of differentially expressed
125 genes that are unique or shared across the growth conditions.

126 We then compared the gene expression profile of *P. aeruginosa* cells grown with *B.*
127 *cenocepacia* supernatant (SN.Bc) to the no (SN.No) and own (SN.Pa) supernatant condition. We had
128 four comparisons in total (SN.Bc vs. SN.No and SN.Bc vs. SN.Pa in both iron-limited and iron-rich
129 medium), and we show the MA plots (Fig. 4C-F), a functional classification of the differentially
130 regulated genes (Fig. 4G-J), and the top 50 up- and downregulated genes (Table S3-S6) for all four
131 comparisons. We observed more gene categories to be significantly up- or downregulated in the SN.Bc
132 vs. SN.No comparison than in the comparison between the two supernatant conditions (SN.Bc vs.
133 SN.Pa). This finding agrees with our observation that the SN.Bc and SN.Pa conditions are close to one
134 another in the PCA plot (Fig. 2) and that only a relatively small number of genes were differentially
135 regulated genes in this comparison (Fig. 4 A+B).

136 When focusing on the SN.Bc vs. SN.No comparison (showing more pronounced differences),
137 we found four defined gene categories to be significantly upregulated under iron-rich conditions (Fig.
138 4G): (E) amino acid transport and metabolism, (I) lipid transport and metabolism, (P) inorganic ion
139 transport and metabolism, and (Q) secondary metabolite biosynthesis transport and catabolism.

140 Especially the differential regulation of secondary metabolite genes is interesting as they often encode
141 competitive traits. Indeed, the table of the top 50 differentially regulated genes (Table S3) features
142 secondary metabolite genes involved in pyoverdine, rhamnolipid, phenazine and type-VI secretion
143 system (T6SS) synthesis. Under iron-limited conditions, seven gene categories were significantly
144 upregulated (Fig. 4I). The categories predominantly involve metabolism: amino acids (E), nucleotides
145 (F), carbohydrates (G), coenzymes (H), lipids (I); but also energy conversion (C) and transcription (K).
146 These results suggest that *P. aeruginosa* accelerates metabolic activities as a major competitive
147 response against *B. cenocepacia* under iron-limiting conditions.

148 Beyond these global analyses, two observations in Tables S3-S6 caught the eye. First, genes of
149 the PA0878-PA0883 cluster were among the topmost upregulated genes in iron-rich conditions (Tables
150 S3 and S5). These genes encode enzymes involved in the degradation of mesaconate and itaconate
151 [52,53]. The two acids show antimicrobial activities and are produced by macrophages but also certain
152 bacteria through the glutamate metabolism pathway [54,55]. The upregulation of these genes could
153 thus represent a defensive response against an unknown antibacterial compound in the supernatant.
154 Second, genes of the clusters PA5081-PA5086 and PA1343-1334 were among the topmost upregulated
155 genes in iron-limited conditions (Tables S4 and S6). These genes encode proteins involved in the
156 utilization of D-glutamic acid (D-Glu) and D-glutamine (D-Gln), two essential components of the
157 bacterial cell wall [56]. Their upregulation could potentially be a response to cell wall damage induced
158 by compounds secreted by *B. cenocepacia*.

159

160 **_P. aeruginosa_ upregulates competitive traits when exposed to the supernatant of _B. cenocepacia_ in**
161 **a medium-specific manner**

162 We further focused on gene clusters encoding traits that are known to be involved in inter-species
163 interactions and tested whether they are differentially regulated upon exposure to *B. cenocepacia*
164 supernatant. Our list of 407 genes covers a broad spectrum of putatively competitive traits, including
165 secretion systems, efflux pumps, siderophores, toxins, motility, adhesion and biofilm components (Fig.

166 5 + Table S7). Numbers and names of all genes together with their log2-fold changes and adjusted p-
167 values are listed in Table S8 (SN.Bc vs. SN.No) and Table S9 (SN.Bc vs. SN.Pa). We tested whether gene
168 clusters were significantly up- or downregulated. We argue that relevant gene clusters should be
169 differentially regulated in both comparisons to be considered as specific responses of *P. aeruginosa* to
170 the supernatant of *B. cenocepacia*. Conversely, gene clusters that are only upregulated in the SN.Bc vs.
171 SN.No, but not in the SN.Bc vs. SN.Pa comparison more likely reflect general responses to spent media.
172 In iron-rich conditions, gene clusters encoding T6SS were significantly upregulated in both
173 comparisons (Fig. 5A+B & Table S10). The T6SS is a speargun-type of weapon deployed against other
174 gram negatives [22]. *P. aeruginosa* has three T6SS clusters and a closer inspection of the data revealed
175 that only two of the three clusters (HSI-I and HSI-II) were significantly upregulated (Table S8+S9).
176 Furthermore, the gene cluster encoding synthesis and transportation of the siderophore pyoverdine
177 was also significantly upregulated in both comparisons (Fig. 5A+B & Table S10). Pyoverdine can induce
178 iron starvation in competitors like *B. cenocepacia* that lack the receptor for pyoverdine uptake [29]
179 and increased production of this siderophore can be considered a competitive response [57]. In
180 contrast, the gene cluster encoding efflux pumps and phenazines were significantly downregulated in
181 both comparisons (Figure 5A+B). While efflux pumps can be used to expel toxic compounds from the
182 bacterial cell [14], phenazines are broad-spectrum toxins that are also involved in redox reactions [58].
183 In iron-limited conditions, the observed gene regulatory responses of *P. aeruginosa* to *B.*
184 *cenocepacia* supernatant were completely different. Here, the gene clusters encoding phenazines and
185 hydrogen cyanide, two groups of bacterial toxins, were significantly upregulated in both comparisons
186 (Figure 5C+D & Table S10). Moreover, several gene clusters were significantly downregulated in both
187 comparisons, including the ones encoding the T1SS, synthesis and transportation of the siderophore
188 pyochelin, the production of pyocin toxins, and the production of motility (flagella, rhamnolipids, pili)
189 as well as biofilm structures (exopolysaccharides) (Figure 5A+B & Table S10). Taken together, our
190 results indicate that *P. aeruginosa* adjusts its competitive strategy according to iron availability.
191

192 **General stress response, quorum sensing, and two-component systems are involved in competition**
193 **sensing**

194 Finally, we focused on potential regulatory elements guiding competition sensing (Table S7-S9). We
195 followed Cornforth & Foster [24] who highlighted three potential competition sensors: (i) general
196 stress response components, (ii) quorum-sensing systems, and (iii) two-component signaling systems.
197 The three categories are not mutually exclusive. Here, we keep all the two-component signaling
198 systems together, even though some of them (e.g. *gacS*) are involved in the general stress response
199 [59,60]. In Fig. 6, we plot the log2-fold changes of 93 genes belonging to these three classes in *P.*
200 *aeruginosa*. Tags and names of all genes together with their log2-fold changes and adjusted p-values
201 are listed in Table S8 (SN.Bc vs. SN.No) and Table S9 (SN.Bc vs. SN.Pa). We contrasted the log2-fold
202 changes of the comparison SN.Bc vs. SN.No against the comparison SN.Bc vs. SN.Pa to identify stress
203 response and regulatory genes that were significantly up (+) or down (-) regulated in both comparisons.

204 In iron-rich conditions (Fig. 6A), we found nine such genes, including the general stress
205 response components *rpoS* (+) and *sodB* (-); the quorum-sensing genes *rhlR* (-), *rhlI* (-) and *lasI* (-); and
206 the two-component signaling systems *retS* (+), *narX* (+), *dctB* (+), and *parS* (-). In iron-limited conditions
207 (Fig. 6B), we found another set of three elements that were differentially regulated when *P. aeruginosa*
208 was exposed to the supernatant of *B. cenocepacia*, namely *pqsA* (+), *ercS* (+), and PA3271 (+). Our
209 analysis identifies several candidate regulatory elements that could steer competition sensing, but we
210 also find that the candidate elements differ between iron-rich and iron-limited conditions.

211

212 **Discussion**

213 Bacteria commonly live in diverse communities, be it in environmental habitats, in infections or in host-
214 associated microbiomes [1,2,61]. It has increasingly been recognized that bacteria interact in these
215 communities, with interactions covering the entire range of the social spectrum, from cooperation to
216 commensalism to competition [4,5]. While competition for space and nutrients seems ubiquitous, the
217 question is whether bacteria preemptively deploy competitive traits, or whether they have evolved

218 mechanisms to sense competitors and to mount responses when needed. The latter scenario is
219 captured by the competition-sensing hypothesis [24], proposing that bacteria can use cues such as
220 nutrient stress and competitor-induced cell damage to launch competitive responses. Here we tested
221 this hypothesis and asked whether competition sensing is detectable at the transcriptional level in *P.*
222 *aeruginosa* exposed to either its own spent medium or the supernatant of its competitor *B.*
223 *cenocepacia*. Our results reveal large-scale changes in the transcriptome of *P. aeruginosa*, entailing
224 both general responses to spent medium and specific responses to the competitor. Specifically, we
225 found genes for several competitive traits, including the T6SS, pyoverdine, phenazines, hydrogen
226 cyanide, and putative antibacterial (e.g. itaconate/mesaconate) degrading enzymes to be upregulated
227 upon exposure to the competitor supernatant. We further identified several genes encoding
228 regulatory elements (general stress response, quorum sensing and two-component signaling systems)
229 that are increasingly expressed with competitor supernatant. Moreover, transcriptional and
230 competitive responses varied as a function of iron availability. Altogether, our results support the
231 competition-sensing hypothesis by revealing nuanced responses of *P. aeruginosa* to *B. cenocepacia*
232 supernatant, integrating both environmental and social information.

233 Transcriptomic studies, such as ours, reveal correlations between experimental treatments
234 and shifts in gene expression patterns. They do not necessarily allow to draw direct conclusions of how
235 exactly competition sensing works and whether changes observed are indeed caused by competitors
236 and reflect evolutionary adaptations. Furthermore, transcriptional analysis does not take translational
237 regulations at the protein level into account. Nonetheless, our study revealed several notable shifts in
238 gene expression, which we wish to discuss in the context of the competition-sensing hypothesis at
239 three different levels: (i) responses to own spent medium versus supernatant of competitor; (ii) iron-
240 dependent responses; and (iii) regulatory elements that could steer competition sensing.

241 The competition-sensing hypothesis postulates that bacteria can sense competitors in two
242 different ways: indirectly via the sensing of nutrient limitation or directly via cell damage induced by
243 the competitor itself or its secreted molecules. Our study design allows us to partially differentiate

244 between the two scenarios. Specifically, the SN.Bc vs. SN.No comparison captures both nutrient
245 limitation (30% spent medium) and cell damage (via diffusible molecules), whereas the SN.Bc vs. SN.Pa
246 comparison filters out limitations for basic nutrients such as carbon and nitrogen, as both treatments
247 entail spent media. Given this setup, we identified genes of four competitive traits that were
248 upregulated in both comparisons, suggesting that these changes reflect direct responses to the *B.*
249 *cenocepacia* supernatant. Among the upregulated genes in iron-rich conditions, there were two T6SS
250 clusters (HSI-I and HSI-II) and genes encoding the synthesis/transportation machinery of the
251 siderophore pyoverdine (Fig. 5A+B), whereas in iron-limited conditions genes encoding the synthesis
252 of phenazines and hydrogen cyanide were upregulated (Fig. 5C+D). T6SS, phenazines and hydrogen
253 cyanide are well-known broad-spectrum weapons of *P. aeruginosa* against competitors [8]. Pyoverdine
254 can also be a competitive agent since this strong siderophore can withhold iron from competitors such
255 as *B. cenocepacia* [57,62]. There were also changes in gene expression that can primarily be attributed
256 to nutrient limitation because they were only upregulated in the SN.Bc vs. SN.No comparison. The
257 affected genes encode the synthesis/transportation machinery of the siderophore pyochelin under
258 iron-rich condition (Fig. 5A), and several classes of metabolic genes under iron-limited conditions (Fig.
259 4I). In the light of the competition-sensing hypothesis, these patterns suggest that both nutrient
260 limitation and competitor-induced cues have likely contributed to the observed gene expression
261 changes.

262 We found that the putative competition-sensing responses in *P. aeruginosa* markedly differed
263 between iron-rich and iron-limited conditions. Under iron-rich conditions, we observed the
264 upregulation of T6SS and pyoverdine genes (Fig. 5B), whereas phenazines and hydrogen cyanide genes
265 were upregulated under iron-limited conditions (Fig. 5D). We propose that these differential responses
266 are the result of the different production costs associated with the respective competitive traits. T6SS
267 is a complex structure comprising many different proteins and is likely costly to produce [63].
268 Pyoverdine is also costly to produce since it involves a complex non-ribosomal peptide synthesis
269 machinery that needs to be built up [64]. Conversely, phenazines and hydrogen cyanide are relatively

270 simple molecules and only few enzymes are required for their synthesis, which means that they are
271 comparatively cheap to make [58,65]. Thus, our finding suggests that *P. aeruginosa* invests into costly
272 competitive traits in nutrient (e.g. iron) rich environments, whereas the bacteria switch to cheaper
273 competitive traits when essential nutrients, such as iron, are limited. The idea that *P. aeruginosa*
274 follows a cost-saving strategy is further supported by our observation that genes for several
275 competitive traits are downregulated (T1SS, pyochelin, pyocins, motility, biofilm formation) under
276 iron-limited conditions (Fig. 5C+D). Conversely, we observed that several classes of metabolic genes
277 were significantly upregulated under iron-limited conditions (Fig. 4I). Accelerated metabolic activities
278 is a form of nutrient competition, and our data thus indicate that *P. aeruginosa* rather invests in
279 nutrient than in costly interference competition mechanisms when iron is limited.

280 The competition-sensing hypothesis predicts that general stress response and regulators
281 detecting nutrient limitation and cell damage should be involved in the process [24,30]. We indeed
282 found that several genes involved in the general stress response [66,67] and two-component signaling
283 systems [59,60,68] were significantly differentially regulated in both the SN.Bc vs. SN.No and SN.Bc vs.
284 SN.Pa comparisons [iron-rich condition: *rpoS*(+), *sodB*(-), *retS*(+), *narX*(+), *dctB*(+), *parS*(-); iron-limited
285 condition: *ercS*(+), PA3271(+); Fig. 6]. Here, we discuss three candidates, RpoS, RetS, and ParS, where
286 links to competition-sensing may exist. RpoS is a sigma factor that mediates the general stress
287 response in *P. aeruginosa* and other bacteria [66,67]. It is involved in the regulation of protective
288 measures against numerous environmental stressors like high temperature, hyperosmolarity and
289 oxidative stress. Our finding that this key regulator is significantly upregulated in response to
290 competition shows that our spent supernatant treatments induced significant stress in *P. aeruginosa*.
291 ParS-ParR is a two-component system that reacts to cell wall damage induced by molecules such as
292 polymyxin B and colistin [69,70]. Detecting cell wall damage was highlighted as one of the most likely
293 trigger of a competition-sensing response [24], which seems to be corroborated by our finding. The
294 ParS-ParR system works both as activator and repressor [70], such that the observed down-regulation
295 of *parS* could trigger the expression of an otherwise repressed trait. However, it remains unclear

296 whether the ParS-ParR system is a repressor to any of the upregulated traits in our study. Finally, RetS
297 is a repressor of the GacA/S two-component regulatory system [71]. As such, it represses biofilm
298 formation and activates T6SS synthesis [26]. The latter matches our finding that genes encoding the
299 T6SS were significantly upregulated. RetS is induced in response to cell lysis by kin cells and is thus a
300 sensor for cell death, which is (similar to ParS) in support of the idea that cell damage forms the basis
301 of competition sensing.

302 In contrast to iron-rich conditions, we found few stress-related regulatory candidates that
303 could be involved in competition sensing under iron-limited conditions (Fig. 6B). Instead, we found
304 that several genes of the PQS quorum-sensing system were significantly upregulated, particularly in
305 the SN.Bc vs. SN.Pa comparison. The PQS quorum-sensing system directly controls the expression of
306 phenazines and hydrogen cyanide. The increased activity of PQS genes thus matches our observation
307 that phenazine and hydrogen cyanide genes were overexpressed. Interestingly, quorum sensing was
308 proposed to feed into competition sensing cascades [24,72] and our finding now lend support to this
309 hypothesis. How the connection between quorum- and competition-sensing works yet remains an
310 open question.

311 In summary, our study adds to the growing body of evidence that bacteria can sense
312 competitors and mount specific responses to fight and withstand them [24,29–31]. Our findings
313 further indicate that *P. aeruginosa* reacts to both nutrient limitation and compounds secreted by the
314 competitor *B. cenocepacia*. Another relevant insight is that the transcriptional responses observed are
315 not uniform but highly specific to iron (nutrient) availability. Our study opens several new questions to
316 be addressed in future research. For example, it would be interesting to test whether the same
317 competitive traits are upregulated when *P. aeruginosa* interacts directly with *B. cenocepacia* and not
318 just via molecules in the supernatant. Another key point is whether *P. aeruginosa* can discriminate
319 between different competitors and adjust its competitive response accordingly. At the ecological level,
320 one might wonder how competition sensing affects species dynamics. Is competition-sensing required
321 to displace a competitor or simply useful to defend its niche? These questions reveal that we are only

322 at the beginning of understanding whether competition-sensing is a key modulator of bacterial
323 interactions and whether the transcriptional changes observed in our study have at least partly evolved
324 for the purpose of sensing competitors.
325
326 **Methods**
327 ***Bacterial strains and media***
328 For all our experiments, we used the standard laboratory *P. aeruginosa* strain PAO1 (ATCC 15692) and
329 *B. cenocepacia* strain H111 (LMG 23991), an isolate from a cystic fibrosis patient [73,74]. Overnight
330 cultures were grown in lysogeny broth (LB). All gene-expression experiments were conducted in
331 casamino acids (CAA) medium (per 1 liter: 5 g casamino acids; 1.18 g $K_2HPO_4 \cdot 3H_2O$; 0.25 g $MgSO_4 \cdot 7H_2O$)
332 supplemented with 20 mM $NaHCO_3$ and 25 mM HEPES buffer. We induced strong iron limitation by
333 adding 100 µg/ml of the natural iron chelator human apo-transferrin. To create iron rich medium, we
334 omitted transferrin from the above recipe and added 20 µM $FeCl_3$ instead. All chemicals for the
335 bacterial culture media were purchased from Sigma-Aldrich, Switzerland.
336
337 ***Supernatant preparation from** P. aeruginosa **and** B. cenocepacia **cultures***
338 To generate spent supernatants, we first grew cultures of *P. aeruginosa* and *B. cenocepacia* in LB
339 overnight at 37°C in an orbital shaker at 220 rpm. Overnights were then centrifuged at 7500 rpm for 2
340 minutes at room temperature, and cell pellets washed twice with 0.8% NaCl to remove spent LB
341 medium. We then adjusted resuspended bacteria to an optical density of 1 at 600 nm (OD_{600}) in 0.8%
342 NaCl. Subsequently, we inoculated 200 µl of the bacterial suspensions (*P. aeruginosa* or *B.*
343 *cenocepacia*) in 500 ml Erlenmeyer flasks containing either iron-limited or iron-rich CAA medium
344 (150 ml) and incubated the cultures at 37°C in an orbital shaker at 220 rpm. We had three biological
345 replicates for each species and iron treatment. We followed the growth of the *P. aeruginosa* and *B.*
346 *cenocepacia* monocultures by monitoring the OD_{600} over time in a spectrophotometer (Ultrospec 2100
347 pro, Amersham Biosciences). After reaching stationary phase (iron-limited medium: *P. aeruginosa* at

348 46 h; *B. cenocepacia* at 48 h; iron-rich medium: *P. aeruginosa* and *B. cenocepacia* at 17 h), the cultures
349 were distributed to sterile 50 ml tubes (Sarstedt) and centrifuged at 8000 rpm for 2 minutes at room
350 temperature. Supernatants were decanted into fresh 50 ml tubes (Sarstedt) and then sterilized using
351 PES membrane filters with low protein adsorption (0.22 µm cutoff) and stored in new 50 ml sterile
352 tubes at -80°C until further usage.
353
354 ***Experimental design of transcriptome study***
355 For the main transcriptome experiment, we cultured *P. aeruginosa* under six different growth
356 conditions in three-fold replication (18 cultures in total) (Fig. 1). We had one additional replicate per
357 growth condition that we used solely to monitor growth (Fig. S1). This is important to ensure that
358 experimental cultures are harvested in the mid- to late-exponential phase prior to nutrient limitation
359 (Fig. S1). Our main treatment consisted of *P. aeruginosa* exposed to 30% of *B. cenocepacia* supernatant
360 mixed with 70% fresh CAA medium. 30% supernatant reflects a substantial exposure of *P. aeruginosa*
361 to *B. cenocepacia*, without affecting the growth of *P. aeruginosa* (Fig. S1). As control treatments, we
362 grew *P. aeruginosa* in either 100% fresh CAA medium, or in 70% CAA medium supplemented with 30%
363 *P. aeruginosa* supernatant. All three treatments were carried out under iron-limited and iron-rich
364 conditions with the supernatants being generated under the same iron treatment. We first prepared
365 two starter cultures of *P. aeruginosa* by inoculating 20 ml LB medium with 200 µl of a *P. aeruginosa*
366 overnight culture in 100 ml Erlenmeyer flasks. These starter cultures were incubated at 37°C in an
367 orbital shaker at 220 rpm. Once the cultures reached the late exponential growth phase, we adjusted
368 their OD_{600} to 1, and used one of the two starter cultures to either inoculate the 12 iron-limited or the
369 12 iron-rich replicates. The final medium volume was 200 ml in 1l Erlenmeyer flasks, and the initial
370 inoculum was $OD_{600} = 1x10^{-2}$ (corresponding approximately to 9.10^6 cells/ml). Each replicate of the *P.*
371 *aeruginosa* and *B. cenocepacia* supernatant treatment contained a supernatant from an individually
372 grown *P. aeruginosa* or *B. cenocepacia* monoculture under the respective iron condition. For culture
373 harvesting, we followed the protocol by Pessi et al 2007 [75]. In brief, once the cultures reached the

374 late exponential phase, we transferred them to ice and distributed them to 50 ml tubes (Greiner)
375 containing 5 ml ice cold stop solution (10% phenol buffered with Tris-HCl to pH8 (Sigma-Aldrich,
376 Switzerland) and 90% ethanol). We mixed the suspension by manual inversion of tubes followed by
377 centrifugation at 4°C for 5 minutes at 9000 rpm. We discarded the supernatant and flash-froze the cell
378 pellet in liquid nitrogen, which was then stored at -80°C.
379
380 ***RNA isolation, library preparation and RNA Sequencing***
381 We extracted the total RNA from *P. aeruginosa* cell pellets using a modified hot acid phenol protocol
382 (see [75,76] for detailed protocols), followed by the removal of short RNAs, including the 5S ribosomal
383 RNA (5S rRNA), with the RNAeasy MiniKit (Qiagen). We completely removed the genomic DNA (gDNA)
384 with RQ1 RNase-Free DNase (Promega), purified the RNA with the RNAeasy MiniKit (Qiagen) and the
385 absence of gDNA was confirmed by a 40-cycles PCR using primers for the *rpoD* gene (rpoD_fwd:
386 5'CTGATCCAGGAAGGCAACAT; rpoD_rev: 5'TGAGCTTGTTGATCGTCTCG). The quality of the mRNA was
387 analyzed by measuring the rRNA integrity through capillary electrophoresis in a Bioanalyzer 2100
388 (Agilent) using the Agilent RNA 6000 Nano Chips. We used 150 ng of RNA for cDNA synthesis and library
389 preparation with the Encore® Complete Prokaryotic RNA-Seq DR Multiplex System from NuGEN
390 (NuGEN Tecan, San Carlos, USA). During the cDNA synthesis, most of the ribosomal RNA in the samples
391 was eliminated with the use of the insert-Dependent Adaptor Cleavage (lnDAC) technology to selective
392 priming. The resulting cDNA was fragmented into 200 bp fragments with a Covaris S220 AFA system
393 prior to the library preparation. For library preparation, we followed the protocol described by Lardi
394 et al. [77]. We analyzed the concentration, quality and size distribution of the libraries with capillary
395 electrophoresis using D1000 ScreenTapestation from Agilent (size range 100-800 bp) (Agilent
396 Technologies, Santa Clara, USA). Single-end 70 base-pair sequencing of the libraries was performed on
397 an Illumina HiSeq2500 machine at the Functional Genomics Center Zurich.
398
399 ***Analysis of differential gene expression***

400 Illumina-generated sequencing reads were processed and mapped to the *P. aeruginosa* PAO1 genome
401 sequence [50] from the *Pseudomonas* Genome Database (www.pseudomonas.com) [51] using the CLC
402 Genomics Workbench v7.0 (QIAGEN CLC bio, Aarhus, Denmark). We only considered transcripts that
403 could be unambiguously mapped (allowing up to two mismatches per read) to the *P. aeruginosa*
404 genome. We excluded genes with zero read in all the compared conditions from further analysis. The
405 RNA-seq raw data files of all analyzed samples are accessible through the GEO Series accession number
406 GSE224821.

407 We used the Bioconductor package DESeq2 version 1.6.3 [40] in the R environment version
408 4.1.1 [78] for the analysis of differential gene expression. We used a principal component analysis
409 (PCA) and a pairwise Euclidean distance analysis between all replicates to test whether our biological
410 replicates cluster and whether there is segregation according to treatments. For this analyses, we
411 included all expressed genes and used the regularized logarithm (rlog) transformation (which also
412 normalizes for library size) from the DESeq2 package to increase the similarity of variances of genes
413 with different read counts [40]. We used the false discovery rate (FDR) test by Benjamini and Hochberg
414 (1995) [79] to adjust p-values to account for multiple comparisons, and considered genes to be
415 differentially expressed when the adjusted p-value was < 0.05 and the log2-fold change was > 1 or $< -$
416 1. For some genes no p-value could be assigned. That is the case when one of the three replicates
417 constitutes an outlier, which is determined by the Cook's cutoff. In addition, only genes with large
418 enough counts (depending on the basemean and variance of the expression of the single gene across
419 the respective three replicates) generate sufficient statistical power to yield significant fold changes.
420 To analyze the gene expression changes of *P. aeruginosa* in response to the supernatant of its
421 competitor *B. cenocepacia*, we compared the treatment that involve this supernatant (i.e. SN.Bc) with
422 control treatments (i.e. vs. SN.No or SN.Pa) either in iron-rich or iron-limited conditions. Firstly, we
423 studied global changes in gene expression. Then, all the mapped genes were assigned to global
424 functional categories defined according to the COG database [80,81]. By using this database, some
425 genes have been placed in several categories while other have not been assigned at all. We used the

426 Fisher's Exact Test for count data to compare the number of all the expressed genes with the number
427 of the significantly up- or downregulated genes to highlight the functional categories in which genes
428 are differentially expressed between our conditions of interest. Secondly, we focused on 500 specific
429 genes encoding competitive traits (i.e., secretion systems, efflux pumps, siderophores, toxins, motility,
430 adhesion, biofilm formation) and regulators that could be potentially involved in competition sensing.
431 For the competitive traits, we compared the mean of the log2-fold changes for each gene classes with
432 the theoretical value of zero using a t-test to highlight the traits that are globally differentially
433 expressed in *P. aeruginosa* upon exposure to *B. cenocepacia* supernatant. Because the regulators can
434 be activators or inhibitors, we sought to identify regulatory genes that were significantly up- or
435 downregulated in our comparisons of interest (SN.Bc vs. SN.No and SN.Bc vs. SN.Pa).
436

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443

444 **Competing interests**

445 The authors declare no competing interests.
446

447 **Literature**

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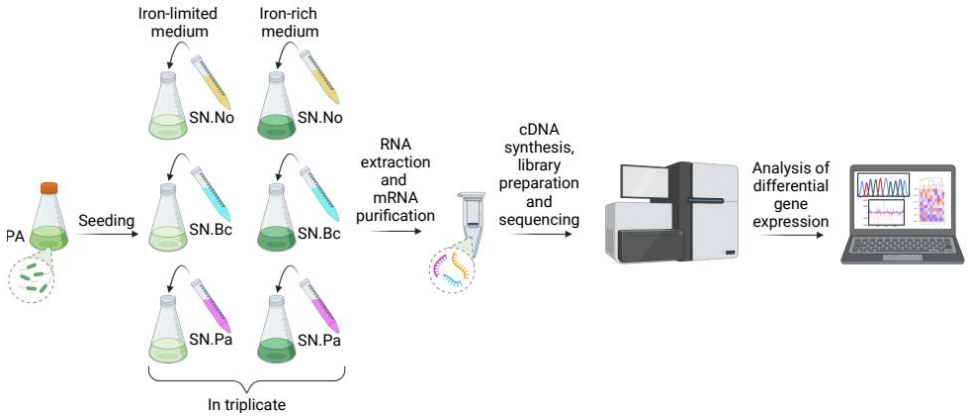
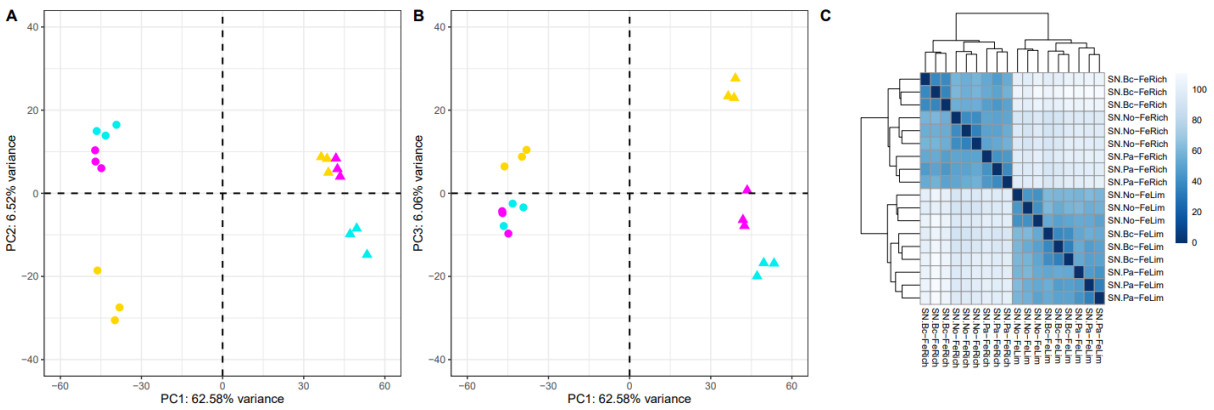
649

650 **Figures**



652 **Fig 1. Experimental design and workflow of our transcriptomic study.** Starter cultures of *P. aeruginosa* PAO1 were grown under standardized conditions, harvested upon reaching the late exponential phase, 654 and prepared to seed the experimental treatments in triplicates. We had three main treatments under both iron-limited and iron-rich conditions. Yellow (SN.No): *P. aeruginosa* grows in 100% fresh casamino 656 acid (CAA) medium. Cyan (SN.Bc): *P. aeruginosa* grows in 70% fresh CAA + 30% of spent supernatant of its competitor *B. cenocepacia* H111. Magenta (SN.Pa): *P. aeruginosa* grows in 70% fresh CAA + 30% 658 of its own spent supernatant. Upon reaching the late-exponential growth phase, cells were pelleted and their RNA extracted and processed for transcriptomics analysis (RNA preparation and cDNA 660 synthesis, library preparation, sequencing and gene expression analyses).



662

Fig. 2 Principal component and Euclidean distance analyses reveal separation between experimental 664 conditions and clustering between biological replicates of the same condition. (**A**) + (**B**) The first three principal components (PC) on all transcriptomics read explain 75.2 % of all the variation observed in

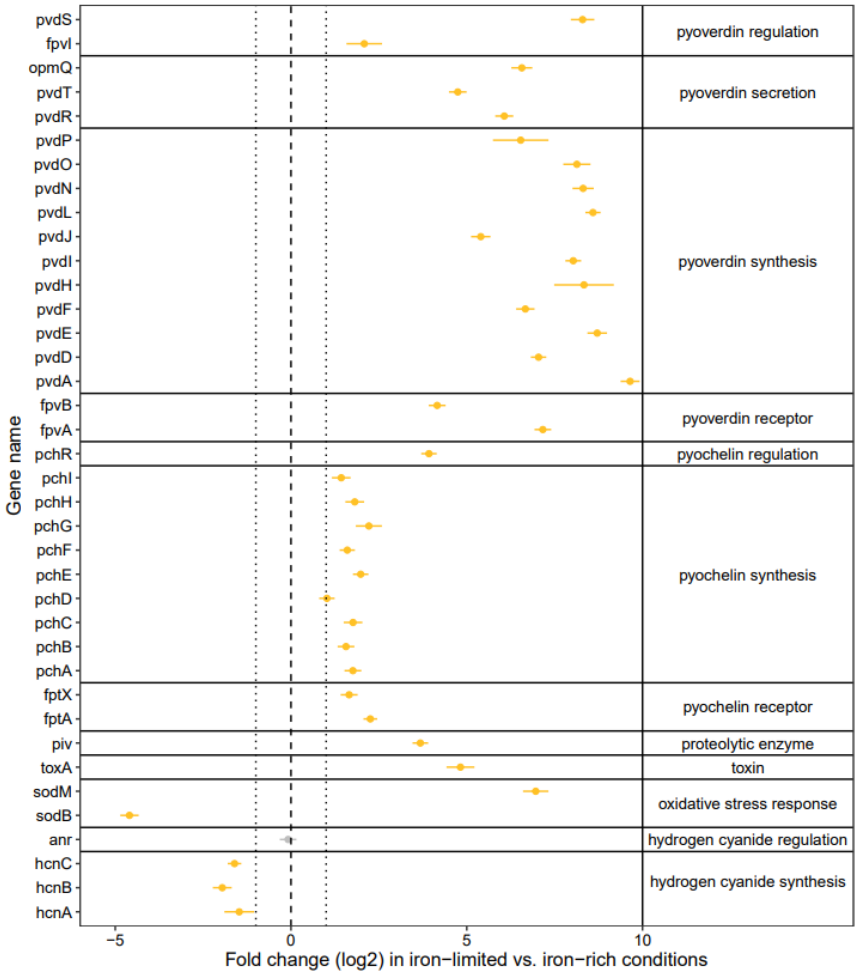
666 the data set. Circles and triangles depict replicates grown under iron-limited and iron-rich conditions, respectively. Yellow symbols: *P. aeruginosa* grown in 100% fresh casamino acid (CAA) medium

668 (SN.No). Cyan symbols: *P. aeruginosa* grown in 70% fresh CAA + 30% of spent supernatant of its competitor *B. cenocepacia* H111 (SN.Bc). Magenta symbols: *P. aeruginosa* grown in 70% fresh CAA +

670 30% of its own spent supernatant (SN.Pa). (**C**) Pairwise Euclidean distance analysis between all replicates, shown as a heatmap covering the scale from 0 (absolute identity, dark blue) to the

672 maximum possible discrepancy (white). PC and Euclidean distance analyses both reveal a clear separation between replicates grown in iron-limited (FeLim) versus iron-rich (FeRich) conditions and a

674 clustering of the three replicates grown under the same supernatant condition.



676

Fig. 3 Gene expression analysis reveals that genes known to be iron regulated respond to iron

678 **limitation.** Log2-fold change (iron-limited versus iron-rich CAA medium) in the expression of a set of

representative genes known to be regulated in response to iron availability. The data shown are from
680 the SN.No conditions where *P. aeruginosa* was grown in 100% CAA medium without supernatants, supplemented with either no iron or 20 µM $FeCl_3$. Data represent the means and standard deviations
682 from the three biological replicates. Yellow and grey dots depict genes that are significantly and not-significantly differently regulated, respectively (FDR-$p_{adj} < 0.05$). Genes are grouped according to the
684 functional unit they belong to.

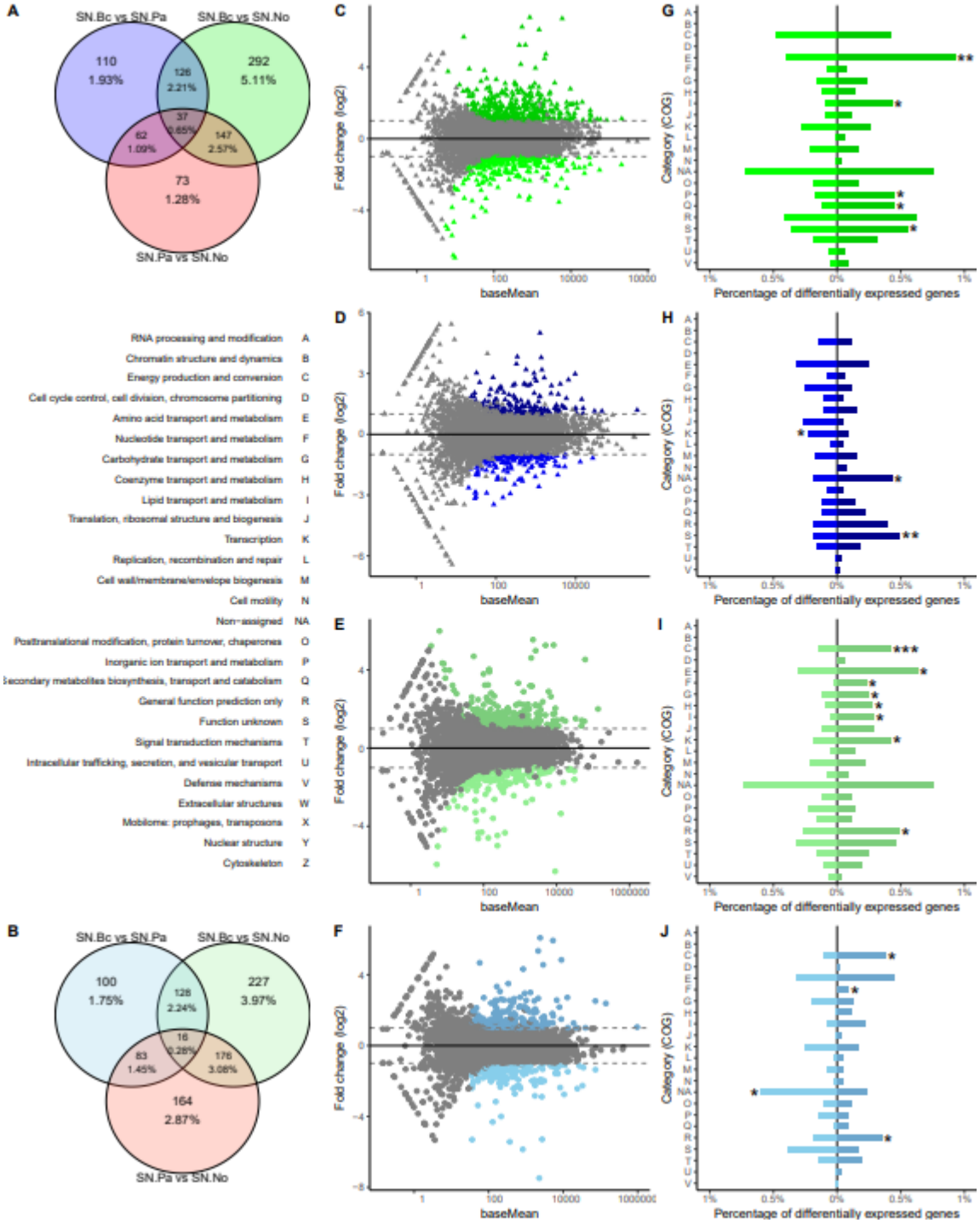


Fig. 4 Global differential gene expression analysis of *P. aeruginosa* in response to the exposure to spent supernatant of its competitor *B. cenocepacia*. Transcriptomic analysis in triplicates were carried out for three different conditions: *P. aeruginosa* growing in (i) 100% casamino acid (CAA) medium (SN.No), (ii) 70% CAA + 30% of spent supernatant of *B. cenocepacia* H111 (SN.Bc), and (iii) 70% CAA + 30% of its own spent supernatant (SN.Pa). Differential gene expression analysis were performed for all three pairwise comparisons (green: SN.Bc vs. SN.No; blue: SN.Bc vs. SN.Pa; red: SN.Pa vs. SN.No),

separately for iron-rich (intense shadings) and iron-limited (faint shadings) growth conditions. Venn diagrams show the number of genes that are differentially expressed in one, two or all the comparisons for iron-rich (**A**) and iron-limited (**B**) conditions. (**C-F**) MA-plots depicting the number of significantly differentially regulated genes as a function of basemean values (FDR-p_{adj} < 0.05 and log2-fold change > |1|) for the comparisons involving SN.Bc. (**G-J**) Percentage of differentially regulated genes according to their functional (COG) classes for the comparisons involving SN.Bc. Asterisks indicate functional classes that are significantly up- or down-regulated based on the Fisher's Exact Test for count data comparing the number of all the expressed genes with the number of the significantly up- or downregulated genes. * p < 0.05, ** p < 0.01, *** p < 0.001.

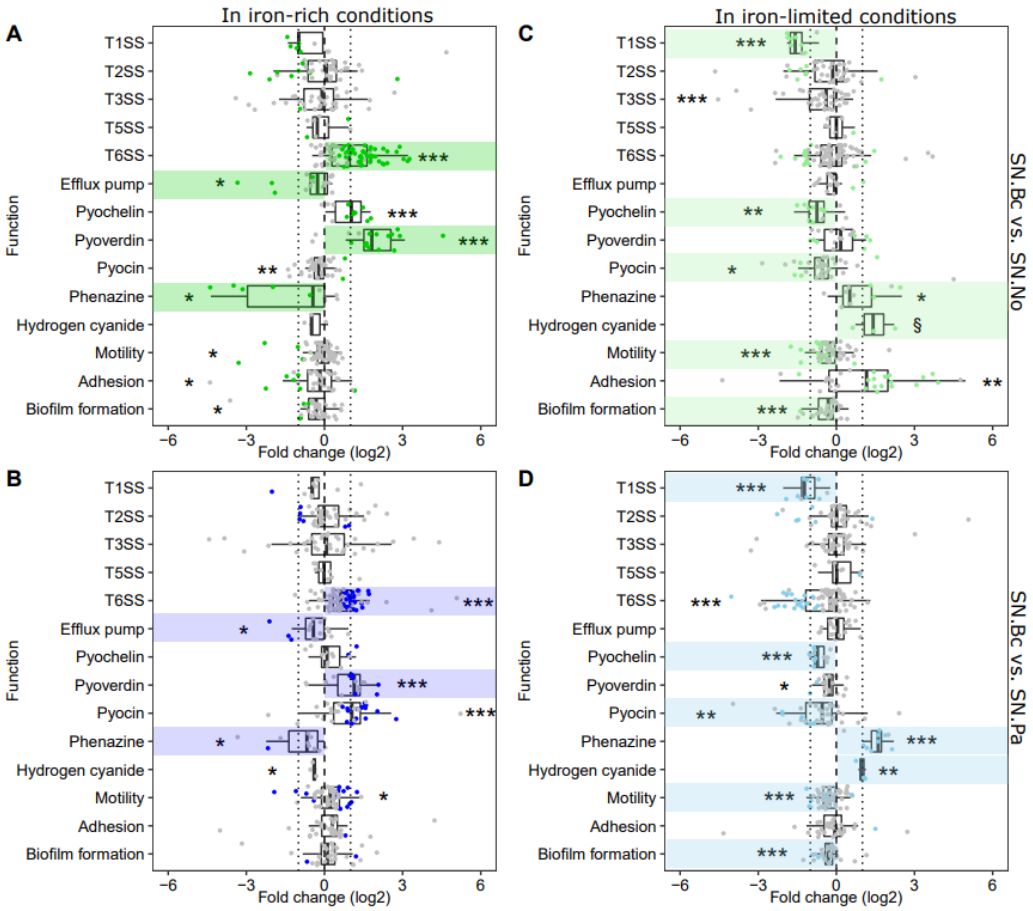
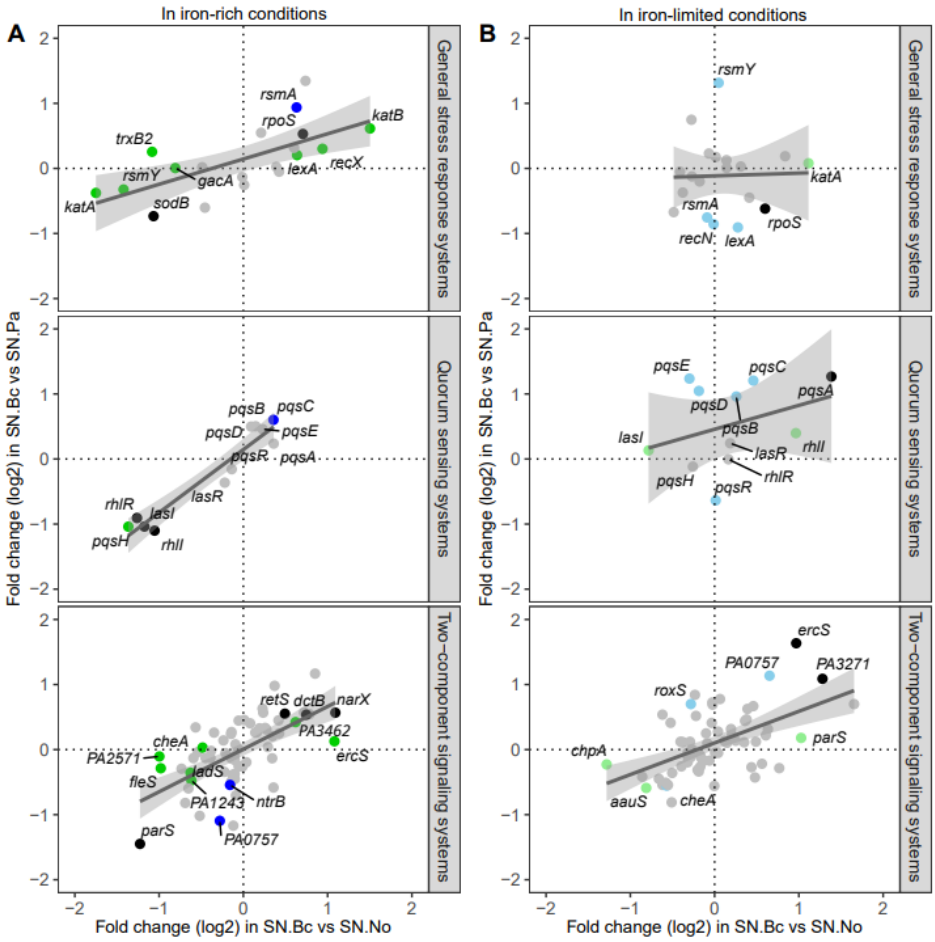


Fig. 5 *P. aeruginosa* increases the expression of certain gene clusters encoding competitive traits when exposed to *B. cenocapacia* supernatant in a medium-specific way. Transcriptomics analysis in triplicates were carried out for three different conditions: *P. aeruginosa* growing in (i) 100% casamino

acid (CAA) medium (SN.No), (ii) 70% CAA + 30% of spent supernatant of *B. cenocepacia* H111 (SN.Bc), and (iii) 70% CAA + 30% of its own spent supernatant (SN.Pa). Data shows the log2-fold change in the gene expressions for gene clusters encoding competitive traits for two comparisons (green: SN.Bc vs. SN.No; blue: SN.Bc vs. SN.Pa), separately for iron-rich (**A+B**) and iron-limited (**C+D**) growth conditions. Each dot represents an individual gene within a cluster. Colored and grey dots depict genes that are significantly and not-significantly differently regulated at the single gene level, respectively (FDR-p_{adj} < 0.05). Asterisks indicate gene clusters that are, as a group, significantly up- and downregulated, according to a t-test conducted using zero as the theoretical mean (* p < 0.05, ** p < 0.01, *** p < 0.001). § All individual genes in this group are significantly upregulated, but only marginally so when treated as a group. This is the result of the small number of genes (n=3) in this category and thus due to low statistical power. The dotted vertical lines represent log2-fold change = |1|.



720 **Fig. 6 Differential expression of regulatory systems and their potential involvement in competition sensing.** Panels show, from top to bottom, the log2-fold changes of genes involved in the general stress
722 response, quorum-sensing, and two-component signaling systems. Log2-fold gene expression changes in the comparison SN.Bc vs. SN.No are plotted against the comparison SN.Bc vs. SN.Pa for iron-rich (**A**)
724 and iron-limited (**B**) growth conditions. Each dot depicts a regulatory gene of the corresponding class. Black dots = genes significantly differently regulated in both comparisons; grey dots = genes not
726 significantly differentially regulated in neither of the two comparisons; green dots = genes significantly differentially regulated in the SN.Bc vs. SN.No comparison; blue dots = genes not significantly
728 differentially regulated in the SN.Bc vs. SN.Pa comparison. Significance is based on FDR-$p_{adj} < 0.05$. The solid lines and shaded areas are regression lines and 95% confidence intervals respectively.

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