

1 Visualization of mRNA Expression in *Pseudomonas aeruginosa* Aggregates  
2 Reveals Spatial Patterns of Fermentative and Denitrifying Metabolism

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9 **Abstract:**

10 Gaining insight into the behavior of bacteria at the single cell level is important given that  
11 heterogeneous microenvironments strongly influence microbial physiology. The hybridization  
12 chain reaction (HCR) is a technique that provides *in situ* molecular signal amplification, enabling  
13 simultaneous mapping of multiple target RNAs at small spatial scales. To refine this method for  
14 biofilm applications, we designed and validated new probes to visualize expression of key  
15 catabolic genes in *Pseudomonas aeruginosa* aggregates. In addition to using existing probes for  
16 the dissimilatory nitrate reductase (*narG*), we developed probes for a terminal oxidase (*ccoN1*),  
17 nitrite reductase (*nirS*), nitrous oxide reductase (*nosZ*), and acetate kinase (*ackA*). These probes  
18 can be used to determine gene expression levels both in liquid culture and in biofilms. Using  
19 these probes, we quantified gene expression across oxygen gradients in aggregate populations  
20 grown using the agar block biofilm assay (ABBA). We observed distinct patterns of catabolic  
21 gene expression, with upregulation occurring in particular ABBA regions both within individual  
22 aggregates and over the aggregate population. Aerobic respiration (*ccoN1*) showed peak  
23 expression under oxic conditions, whereas fermentation (*ackA*) showed peak expression in the  
24 anoxic cores of high metabolic activity aggregates near the air-agar interface. Denitrification  
25 genes *narG*, *nirS*, and *nosZ* showed peak expression in hypoxic and anoxic regions, although *nirS*  
26 expression was much stronger in anoxic environments compared to other denitrification genes.  
27 These results reveal that the microenvironment correlates with catabolic gene expression in  
28 aggregates, and demonstrate the utility of HCR in unveiling cellular activities at the microscale  
29 in heterogeneous populations.

30

## 31 **Importance:**

32 To understand bacteria in diverse contexts we must understand the variations in behaviors and  
33 metabolisms they express spatiotemporally. Populations of bacteria are known to be  
34 heterogeneous, but the ways this variation manifests can be challenging to characterize due to  
35 technical limitations. By focusing on energy conservation, we demonstrate that HCR v3.0 can  
36 visualize nuances in gene expression, allowing us to understand how metabolism in  
37 *Pseudomonas aeruginosa* biofilms responds to microenvironmental variation at high spatial  
38 resolution. We validated probes for four catabolic genes: a constitutively expressed oxidase,  
39 acetate kinase, nitrite reductase, and nitrous oxide reductase. We showed that the genes for  
40 different modes of metabolism are expressed in overlapping but distinct subpopulations  
41 according to oxygen concentrations in a predictable fashion. The spatial transcriptomic  
42 technique described here has the potential to be used to map microbial activities across diverse  
43 environments.

44

## 45 **Introduction:**

46 Even in an isogenic population, bacteria can display a range of different phenotypes  
47 (Ackerman, 2015; Evans, 2020). This phenotypic variation can form along microenvironmental  
48 gradients (Lara, 2006) but has also been observed in well-mixed cultures (Thattai, 2004; Kopf,  
49 2015). Heterogeneity in gene expression allows cells to fit different ecological niches and  
50 survive in ecosystems with fluctuating conditions, increasing the overall fitness of the  
51 population (Thattai, 2004; Smits, 2006; Martins, 2015). Phenotypic heterogeneity is relevant in  
52 a variety of contexts, from rhizosphere colonization (Broek, 2005) to metabolic engineering for  
53 optimal biosynthesis of fuels and medicines (Xiao, 2016). In chronic infections, phenotypic  
54 variation shields pathogens from antibiotics, allowing subpopulations with distinct metabolic  
55 activities to survive rounds of treatment (Michiels, 2016; Fisher, 2017) and contribute to the  
56 development of antibiotic resistance (Cohen, 2013).

57 While many technologies exist to study gene expression at the population (Guell, 2011)  
58 and single cell (Kuchina, 2020) levels, few allow the mRNA output of individual bacteria to be

59 observed while maintaining the microenvironmental context and without genetically modifying  
60 the cells (e.g. use of genetically-encoded fluorescent proteins). Recently, a spatial  
61 transcriptomic method was introduced that enables visualization of hundreds of transcripts at a  
62 time in single cells (Dar, 2021), yet this method requires specialized instrumentation and  
63 analysis software, providing a barrier to widespread usage. By contrast, the hybridization chain  
64 reaction (HCR) is a more accessible technology that permits single cell transcript visualization of  
65 a smaller number of genes using standard instrumentation (Choi, 2018; Trivedi, 2018). HCR is a  
66 form of fluorescence *in situ* hybridization (FISH), where the binding of fluorescent probes to a  
67 target RNA of interest results in the binding of additional fluorescent probes, thus amplifying  
68 the signal (Choi, 2018). The technology first was developed in eukaryotes (Dirks, 2004), but has  
69 been used in bacteria to image rRNA for the purposes of species identification and growth rate  
70 assessment in mixed communities (Nikolakakis, 2015; Depas, 2016), as well as to visualize gene  
71 expression in heterogeneous populations (Jorth, 2020).

72 Building on these studies, we reasoned that HCR was well suited to enabling a  
73 systematic exploration of the variation in catabolic activities within aggregate biofilms of the  
74 model biofilm forming bacterium *Pseudomonas aeruginosa*. One environmental parameter that  
75 has profound effects on the metabolic state of bacteria is oxygen. For example, many infection  
76 sites have large regions of hypoxia, including those found in skin wounds, lungs, and the gut  
77 (Palmer, 2007; Hong 2014), Additionally, opportunistic pathogens like *P. aeruginosa* can form  
78 aggregate biofilms during infection, (Grant, 2013; Zhao, 2013; Depas, 2016), and biofilms  
79 produce their own oxygen gradients, with biofilm-interior populations experiencing oxygen  
80 limitation (Stewart, 2003). This has devastating consequences for human health because  
81 commonly used antibiotics such as tobramycin and ciprofloxacin are ineffective at killing  
82 oxygen-limited *P. aeruginosa* at the center of biofilms, while drugs that target anaerobic  
83 metabolism specifically kill oxygen-limited populations (Walters, 2003; Spero, 2018).  
84 Accordingly, antibiotic treatments must take the microenvironment and the phenotypic  
85 heterogeneity experienced by opportunistic pathogens in the host into account to be successful  
86 (Spiro, 2012; Cook, 2015). Before we can rationally design better treatments, we must improve

87 our understanding of the range of metabolic states that exist in biofilm populations  
88 spatiotemporally (O'Toole, 2021).

89 In the absence of oxygen, *P. aeruginosa* cells utilize different strategies to conserve  
90 energy, ranging from oxidative to substrate-level phosphorylation (Schobert, 2010). Because  
91 chronic infections are often oxidant limited, how *P. aeruginosa* responds to electron acceptor  
92 availability is expected to lead to metabolic differentiation within these environments, which  
93 partly underpins its antibiotic tolerance and resistance (Ciofu, 2019). Under anoxic conditions,  
94 *P. aeruginosa* can use nitrate and other N-oxides as alternative terminal electron acceptors to  
95 oxygen by performing denitrification (Arat, 2015). The nitrate levels in chronic surgical wound  
96 and cystic fibrosis lung infections are high enough to support denitrification in some regions,  
97 while the oxygen levels are low enough to induce denitrification (Palmer, 2007; Line, 2014;  
98 Turner, 2014). In the absence of nitrate, *P. aeruginosa* may survive under anoxic conditions for  
99 long periods by performing pyruvate fermentation, in which pyruvate is converted to lactate  
100 and acetate (Eschbach, 2004), or by fermenting arginine (Vander Wauven, 1984) or using  
101 phenazines to facilitate glucose fermentation (Glasser, 2014). Aerobic, denitrifying, and  
102 fermentative metabolisms are all relevant to bacterial populations in chronic infection  
103 environments.

104 If we are to use HCR to map these metabolisms within biofilm aggregates, it is important  
105 to interpret any gene expression patterns we might see within an ecophysiological regulatory  
106 framework. In *P. aeruginosa*, the primary respiratory oxidase under highly aerobic conditions is  
107 *cbb<sub>3</sub>-1*, encoded by *cco1* (Jo, 2014). *cco1* is constitutively expressed when oxygen is replete but  
108 slightly downregulated in stationary phase (Alvarez-Ortega, 2007; Arai, 2011). Denitrification is  
109 catalyzed by a series of reductases, in particular *nar*, *nir*, *nor*, and *nos* (Arai, 2011; Arat, 2015).  
110 The main regulatory proteins that govern the expression of these genes are Anr, Dnr, NarXL,  
111 and NirQ (Figure 1). Under anoxic conditions, Anr activates Dnr, a regulatory protein that is  
112 downregulated by phosphate availability, which activates NirQ. Where nitrate is available and  
113 oxygen is limited, Anr additionally activates NarXL which in turn amplifies the activation of Dnr  
114 and NirQ. The nitrate reductase gene, *nar*, is upregulated by NarXL, and this upregulation is  
115 amplified by Dnr in the presence of nitric oxide (Arat, 2015). The nitrite reductase gene, *nir*, is

116 highly upregulated under anoxic conditions and has higher differential expression from oxic to  
117 anoxic conditions than *nar* (Palmer, 2007; Alvarez-Ortega, 2007; Arai, 2011; Arat, 2015). *nir* is  
118 slightly upregulated by Anr, causing a small amount of nitrite to be converted to nitric oxide,  
119 which triggers Dnr and increases the upregulation of *nir* in a feedback loop (Kuroki, 2014). NirQ  
120 and Dnr tightly coregulate *nir* with the nitric oxide reductase *nor* in response to nitric oxide  
121 availability, preventing nitric oxide from accumulating (Arat, 2015). The nitrous oxide reductase,  
122 *nos*, is upregulated by Dnr in response to nitric oxide availability (Arat, 2015). In contrast, the  
123 acetate kinase gene, *ackA*, responsible for ATP synthesis during pyruvate and phenazine-  
124 mediated glucose fermentation, is induced by the regulatory proteins Anr and IHF (Integration  
125 host factor).

126 We targeted the expression levels of *cco1*, *narG*, *nirS*, *nosZ* and *ackA* mRNA molecules to  
127 investigate the spatiometabolic patterning of catabolic gene expression within and across *P.*  
128 *aeruginosa* aggregate biofilm populations. Our goal was to use a finite set of genes to identify  
129 the range of metabolic states adopted by multicellular aggregates under oxidant-limited  
130 conditions. Here we show that HCR 3.0 not only enables spatiometabolic mapping *in situ*, but  
131 that it allows us to infer the metabolic state and environmental conditions that single cells  
132 within these heterogeneous populations are experiencing.

133

## 134 **Results:**

### 135 **HCR Probes are Specific for their mRNA Targets**

136 To validate the probes designed for this study, we tested their specificity using WT and  
137 mutant strains in which the target gene had been cleanly deleted. To test *narG* and *nirS* probes,  
138 we grew WT,  $\Delta narGHJI$ , and  $\Delta nirS$  strains aerobically in LB supplemented with 40 mM nitrate to  
139 late exponential phase to achieve oxygen limitation and activate Anr. To test *ccoN1* probes, we  
140 grew WT and  $\Delta ccoN1$  strains aerobically in the same medium to early exponential phase to  
141 achieve highly oxygenated conditions. To test *nosZ* probes, we grew WT and  $\Delta nosZ$  strains  
142 anaerobically in the same medium to mid-exponential phase to achieve denitrifying  
143 metabolism. We were unable to observe *ackA* signal in any of the liquid culture conditions we  
144 tested, so to test *ackA* probes, WT and  $\Delta ackA$  were grown in the agar biofilm block assay

145 (ABBA) for 12 hours in LB medium and 0.5% agar, supplemented with 40 mM nitrate. Briefly, in  
146 ABBA, *P. aeruginosa* cells are suspended in an agar medium that is allowed to solidify. Cells  
147 then grow as aggregate biofilms suspended in this agar matrix, and nutrient gradients are  
148 known to develop within individual aggregates as well as across the population of aggregates  
149 (see below).

150 The *narG*, *nirS*, *nosZ*, and *ccoN1* probes showed an average mean intensity per cell that  
151 was an order of magnitude higher in the wildtype compared to the deletion strains, indicating  
152 our probe sets are specific for each target mRNA (Figures 2A, 2C). Additionally, the *ackA* probe  
153 showed significantly higher average mean intensity per ABBA aggregate compared to the  
154 deletion strain (Figures 2B, 2D). In some of the deletion mutant controls, particularly for the  
155 *nosZ* and *ccoN1* probe sets, we occasionally observed fluorescent puncta that did not overlap  
156 with cells and appeared qualitatively different from the signal observed in the positive control.  
157 We therefore assume that these puncta were due to non-specific binding to the slide and not  
158 biologically significant.

159

## 160 **Metabolite Gradients are Readily Established in ABBA**

161 To explore the phenotypic heterogeneity of *P. aeruginosa* wildtype PA14, we used HCR  
162 to visualize the localization of *narG*, *nirS*, *nosZ*, *ccoN1*, and *ackA* expression across biofilm  
163 aggregates grown in the ABBA. Within ABBA, cells generate oxygen and nitrate gradients over  
164 the course of a few hours. We used a thresholded 16S rRNA intensity value to define aggregate  
165 boundaries and to control for baseline metabolic activity, and quantified mRNA and rRNA  
166 signals within this mask. *P. aeruginosa* aggregates were grown in LB medium with 0.5% agar,  
167 supplemented with 40 mM nitrate. We chose these conditions to capture the effects of oxygen  
168 and nitrate gradients on the expression of our genes of interest. Physiological levels of nitrate in  
169 infected cystic fibrosis lung samples and chronic wounds are ~400  $\mu$ M (Palmer, 2007;  
170 Bernatchez, 2013), but we chose a higher concentration of nitrate to ensure that it would still  
171 be available after 12 hours of growth.

172 In an ABBA, an oxygen gradient develops, and the oxygen minimum migrates upwards  
173 towards the air-agar interface over the course of the incubation as cell density increases, and

174 thus oxygen availability decreases (Figure 3A). The exact depths at which oxygen is minimal vary  
175 between agar blocks, but the pattern is conserved. A steep oxygen gradient occurs across the  
176 first ~100 microns directly below the surface, and regions deeper than ~250  $\mu\text{m}$  are uniformly  
177 oxygen starved. Accordingly, deeper aggregates in the anoxic zone will have experienced anoxia  
178 for longer than the aggregates above them. As aggregates begin experiencing hypoxia, cells will  
179 upregulate denitrification genes and reduce nitrate, such that a similar nitrate gradient is also  
180 expected to develop. The deepest aggregates are likely the first to begin consuming nitrate in  
181 their local environment, so deeper aggregates likely have less access to nitrate at this time  
182 point. As the denitrification pathway proceeds, nitric oxide is generated (Williams, 1978), so a  
183 greater amount of nitric oxide is expected at lower depths.

184         Given these conditions and the regulatory architecture controlling expression of these  
185 genes (Figure 1), we predicted that *narG* would be expressed in regions with high nitrate and  
186 low oxygen concentrations, *nirS* would be expressed in regions with high nitrate and/or nitric  
187 oxide and low oxygen concentrations, and *nosZ* would be expressed in regions with nitric oxide  
188 present and low oxygen concentrations. We expected *ccoN1* to be expressed most highly in  
189 regions with high oxygen and *ackA* to be expressed in regions that are limited for both oxygen  
190 and nitrate.

191

## 192 **Metabolic Genes Show Distinct Expression Patterns within ABBAs**

193         The oxidase *ccoN1* was maximally expressed in the oxic region (0-50  $\mu\text{m}$  from the air-  
194 agar interface at the top of the ABBA blocks), and steeply decreased in expression deeper into  
195 the block along the oxygen gradient which is the same pattern shown by the 16S rRNA signal  
196 (Figure 3B). In the larger aggregates closer to the surface, signal for *ccoN1* and 16S rRNA was  
197 higher along the aggregate exterior than in the centers, while signal for the *ackA* gene was  
198 highly expressed in the interior (Figures 3B,3C, Figure S1). Elevated signal for the denitrification  
199 genes was not detected in this region.

200         In the region below the oxic portion of our experimental system (>50  $\mu\text{m}$  from the air-  
201 agar interface at the top of the ABBA blocks), rRNA, *ccoN1* and *ackA* expression was reduced,  
202 and expression of the denitrification genes were elevated, correlating with low oxygen

203 concentrations that decreased with depth across this region (Figure 3A, 3B, 3C) All three  
204 denitrification genes were expressed at their maximal levels in aggregates in this region. The  
205 nitrate reductase *narG* was upregulated under hypoxic conditions in the 40 mM nitrate  
206 samples, peaking in average mean intensity per aggregate at a depth of 100-150  $\mu\text{m}$  from the  
207 air-agar interface (Figure 3B, 3C). *narG* signal was restricted to the interior of aggregates,  
208 where oxygen availability is expected to be reduced compared to cells on the aggregate  
209 exterior (Figure 3B, Figure S1). The nitrite reductase *nirS* was upregulated under hypoxic and  
210 anoxic conditions, peaking between 100-200  $\mu\text{m}$  from the surface. The nitrous oxide reductase  
211 *nosZ* was expressed in cells in the cores of some aggregates in the hypoxic and anoxic regions,  
212 but was not uniformly expressed by aggregates at any depth (Figure 3C). Oxygen levels reached  
213 their minimal levels between 150-250  $\mu\text{m}$  from the air-agar interface (Figure 3A).

214 Oxygen levels were uniformly low below 250  $\mu\text{m}$  from the air-agar interface. All genes  
215 measured showed reduced expression with depth across this anoxic region, likely due to  
216 oxidant limitation, although *nirS* expression was elevated in the cores of aggregates at all  
217 depths. *nirS* signal was restricted to the interior of aggregates in the hypoxic zone, but was  
218 expressed across entire aggregates further into the anoxic zone (Figure 3B, Figure S1).  
219 Appreciable *narG* signal was not detected in the deepest anoxic region (Figure 3C). Data from  
220 two additional replicates of this experiment (mRNA and rRNA ABBA images with quantification)  
221 can be found in Figures S2-S7.

222

### 223 **Differentially Regulated Metabolic Genes show Different Expression Patterns than rRNA**

224 By comparing the rRNA and mRNA signal per aggregate in each sample, we can detect  
225 where the mRNA signal diverges from the basal metabolic rate, reflected by the rRNA signal.  
226 The *ccoN1* signal linearly and positively covaried with the rRNA signal across depths (Figure 4).  
227 No other genes showed this pattern. All three denitrification genes showed maximal expression  
228 in regions where the rRNA signal was  $\leq$  half the maximum, corresponding to the hypoxic  
229 transition region of the agar block. The *nirS* signal showed the highest negative covariance with  
230 the rRNA signal, with maximal expression occurring in aggregates with one-third the maximum  
231 rRNA signal. The *ackA* signal showed positive, but non-linear, covariance with the rRNA signal,

232 showing the highest mRNA signal in the aggregates at the surface with the highest rRNA signal,  
233 and linearly covarying throughout the oxic and hypoxic regions before a steep dropoff of mRNA  
234 signal in the anoxic region. The rRNA and *ccoN1* signals were qualitatively highest at the  
235 aggregate edges, while the *narG*, *nirS*, and *nosZ* signals were highest in the aggregate cores  
236 (Figure S1), likely indicating a radial oxygen gradient from the edges to the cores of individual  
237 aggregates. The *ackA* signal was heterogeneously expressed in some cells throughout  
238 aggregates.

239

#### 240 **Discussion:**

241 In this work, we developed and validated a set of HCR probes targeting *mRNA* molecules  
242 for key denitrification, aerobic respiration, and fermentation genes in *P. aeruginosa*. This  
243 targeted probe set permits mapping of the metabolic states of cells in aggregate biofilms at  
244 high resolution across microenvironments characterized by steep oxygen and nitrate gradients.  
245 Our results reveal distinct patterns of catabolic gene expression within these populations,  
246 underscoring that metabolic activity can be heterogeneous over small spatial scales. The  
247 demonstration that HCR can be applied to chart catabolism within a reductionist laboratory  
248 biofilm sets the stage for future studies of microbial responses to dynamic microenvironments  
249 in more complex contexts. Importantly, HCR permits the study of gene expression dynamics in  
250 complex wildtype populations without the need for generating reporter strains; though  
251 developed here for a model organism, this capability has obvious advantages for the study of  
252 strains that are not yet genetically tractable.

253 Our results show that the relationship between *ccoN1* and rRNA signals for entire  
254 aggregates is linear and positive, indicating that changes in *ccoN1* expression reflect changes in  
255 the metabolic rate on the population level (DePas, 2016). This is consistent with previous  
256 studies showing *ccoN1* as constitutively expressed (Jo, 2017). Most genes in Gram-negative  
257 bacteria such as *P. aeruginosa* show basal levels of gene expression even outside the  
258 upregulation condition (Volke, 2020) so the expression pattern shown by *ccoN1* represents the  
259 null hypothesis of what expression should look like if the gene is not regulated by  
260 environmental conditions. If gene expression patterns diverge from the pattern shown by

261 *ccoN1* and rRNA, we can infer that they are upregulated and downregulated based on  
262 additional conditions other than the metabolic rate.

263 We observed that expression of the genes encoding the denitrification machinery in *P.*  
264 *aeruginosa* is spatially stratified across the ABBA system after 12 hours. Previous studies have  
265 demonstrated that denitrification in liquid cultures occurs in successive steps over time  
266 (Williams, 1978). Recently this sequential pattern was observed in structured communities of *P.*  
267 *aeruginosa* for *narG* and *nirS* using reporter strains (Smirga, 2021), revealing maximal  
268 expression of these genes moved outward from the center of particles suspended in bulk  
269 medium across time, suggesting aggregates progressively extinguished local oxygen, nitrate,  
270 and nitric oxide concentrations. Our findings confirm this spatial pattern in *P. aeruginosa*,  
271 showing that at a single time point maximal *narG* expression occurs closer to the air-agar  
272 interface than maximal *nirS* and *nosZ* expression, as oxygen limitation and nitrate limitation  
273 increase with depth. Furthermore, we were able to visualize this spatial pattern in individual  
274 aggregates. In aggregates between 50-150  $\mu\text{m}$  from the air-agar interface, *narG* expression  
275 occurs closer to the exterior of individual aggregates compared to *nirS* and *nosZ* expression, and  
276 *nirS* expression is present across entire aggregates at greater, mostly anoxic depths but limited  
277 to the cores of aggregates in hypoxic conditions.

278 Despite both being upregulated by Anr, fermentation and denitrification genes showed  
279 distinct expression patterns. The fermentation gene *ackA* was upregulated in the cores of the  
280 aggregates closest to the air-agar interface where oxygen concentrations are higher, while the  
281 denitrification genes were upregulated in the cores of the aggregates in the hypoxic regions.  
282 Both types of metabolism mark hypoxic/anoxic conditions, but they are regulated in response  
283 to distinct nitrate and nitric oxide conditions. Given that *ackA* is expressed in the absence of  
284 nitrate (Eschbach, 2004), the pattern of the denitrification and fermentation genes suggests  
285 that the cores of the aggregates at the top of the agar blocks may be conserving energy by  
286 fermentation, due to a microenvironmental regulatory cue. Notably, the denitrification genes  
287 are directly upregulated by Dnr, which is activated by nitric oxide, while *ackA* is directly  
288 upregulated by Anr, which is deactivated by nitric oxide. We can infer from this regulatory  
289 relationship that nitric oxide signaling is occurring in the lower aggregates but not in the upper

290 aggregates at this time point. Direct measurement of N-oxide concentrations in the ABBA  
291 system is important for understanding the relationship between microenvironmental  
292 conditions and metabolic state, and will be the focus of future experiments.

293         Going forward, this set of probes may be expanded and multiple high-signal probes may  
294 be used to determine correlation and anticorrelation more precisely. HCR may be used in two  
295 directions: read-out, in which specific regions of a sample are queried to learn which genes are  
296 co-expressed within them, and read-in, in which knowledge of genes whose co-expression is  
297 already understood is leveraged to infer something about the microenvironment of the region  
298 in which they are expressed (Trivedi, 2018). The former application allows us to choose a  
299 specific context in space and time and see which genes are being expressed without prior  
300 knowledge, whereas the latter approach allows us to apply our knowledge of gene regulation to  
301 infer something about the microenvironment—such as we did in this study to dissect the  
302 microenvironmental conditions of ABBA subpopulations. In the future, the probes developed in  
303 this study may be applied to observe metabolisms and infer potential microenvironmental  
304 conditions experienced by *P. aeruginosa* in clinical samples, opening the doors for greater  
305 insight into the metabolic processes sustaining cells in infections.

306

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308

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318

319

320 **Materials and Methods:**

321

322 **Microbial strains and growth conditions**

323 *P. aeruginosa* strains UCBBP- PA14 (wild type (WT)) and isogenic  $\Delta narGHJ$  (Spero, 2018),  $\Delta ccoN1$  (Jo,  
324 2017),  $\Delta ackA$  (Glasser, 2014),  $\Delta nirS$  and  $\Delta nosZ$  (Wilbert, 2021) were routinely grown in 5 mL Luria  
325 Bertani (LB) broth supplemented with 40 mM KNO<sub>3</sub> shaking at 37°C. Aerobic cultures were incubated at  
326 37°C shaking at 250 rpm unless described otherwise. Anaerobic cultures were incubated in an anaerobic  
327 chamber at 37°C without shaking.

328

329 **HCR v3.0 probe validation**

330 WT and deletion mutant cultures were grown overnight from freezer stocks in LB broth supplemented  
331 with 40 mM KNO<sub>3</sub>. For the *ackA* mutant, cells were grown in the agarose biofilm block assay (ABBA) as  
332 described below. For other mutants, 20  $\mu$ L overnight culture was inoculated into 5 mL of pre-warmed  
333 and degassed media and incubated under conditions meant to induce upregulation of the target gene.  
334 For *narG* and *nirS*, cultures were grown aerobically as described above for 4 hours to an approximate  
335 optical density at 500 nm of 1.1. For *ccoN1*, cultures were grown aerobically as described above for 2.5  
336 hours to an approximate optical density at 500 nm of 0.2. For *nosZ*, wildtype and deletion mutant  
337 cultures were grown anaerobically for 2.5 hours, to an approximate optical density at 500 nm of 0.2. 1  
338 mL of culture was pelleted and cells were fixed by resuspending in 1 mL of 4% paraformaldehyde in  
339 phosphate-buffered saline (PBS) pH 7.2, and incubating resuspensions at 25°C for 90 minutes. After  
340 fixing, cells were pelleted, washed with PBS, resuspended in 1mL of a 1:1 ethanol:PBS solution, and  
341 stored at -20C until HCR 3.0 experiments were performed.

342

343 Prior to performing HCR, cell suspensions were washed in PBS and treated with 1 mg/ml lysozyme  
344 (catalog number L6876; Sigma) in 10 mM Tris-HCl (pH 7.6) for 1 h at 37°C with shaking. After these  
345 treatments, cells were washed with PBS, resuspended in 1mL of a 1:1 ethanol:PBS solution. Cell  
346 solutions were suspended in hybridization buffer for 1 hour at 37°C. Probe solutions were prepared,  
347 containing 5 nM of appropriate HCR v3.0 initiator odd and even probe pairs in filter-sterilized  
348 hybridization buffer. 20  $\mu$ L of probe solutions (Integrated DNA Technologies) were added to each cell  
349 solution. *Pseudomonas aeruginosa* rRNA and *narG* mRNA sequences were targeted using target  
350 sequences previously used in Jorth, 2020. Probes specific to *nirS*, *nosZ*, *ackA*, and *ccoN1* were designed  
351 in the study, and the target sequences are included in Table S1. Solutions were hybridized overnight at

352 37°C, then washed twice with 200  $\mu$ L probe wash buffer at 37°C. Cells were then resuspended in 40  $\mu$ L  
353 amplification buffer and pre-amplified for 30 minutes, while fluorescent hairpin probes (Molecular  
354 Technologies) were thawed, each denatured in separate tubes in a thermal cycler for 90s at 95°C and  
355 allowed to cool for at least 30 m at 25°C in the dark.

356  
357 Hairpins with Alexa Fluor 488 were used for the rRNA probes, and hairpins with Alexa Fluor 647 were  
358 used for the mRNA probes. The B1 initiator/hairpin system from Molecular Technologies was used for  
359 rRNA, while B2 was used for *nosZ* and *ccoN1* mRNA, B3 was used for *narG* mRNA, and B4 was used for  
360 *nirS* and *ackA* mRNA. 25  $\mu$ L of each hairpin were suspended in 20  $\mu$ L of amplification buffer and added to  
361 the cell solutions, and incubated overnight in the dark at 25°C. The cells were then washed three times  
362 in 200  $\mu$ L 5X SSCT, before being resuspended in 20  $\mu$ L 5X SSCT and spotted onto a microscope slide  
363 (catalog number: 3039-002; ThermoFisher) and allowing to dry overnight in the dark at 4°C. Finally,  
364 slides were washed with ice-cold ~0°C ultra-purified water, then with -20°C ethanol, and slides were  
365 allowed to air-dry at 25°C in the dark. Each dried cell spot was then covered with 2.5 ml ProLong Gold  
366 Antifade Mountant (catalog number: P36930; ThermoFisher), slides were incubated for 48 hours in the  
367 dark at 25°C before being stored, and imaged using a Nikon Eclipse Ti2 fluorescence microscope at 40x  
368 magnification with 50% laser power and 1 second exposure for the fluorescent channels. Image analysis  
369 was performed using FIJI. Phase images were thresholded to produce a mask and detect cells, then the  
370 marked cells were thresholded by rRNA signal to eliminate non-bacterial debris from the dataset, then  
371 mean intensity was determined for each labeled cell. Quantitative graphs were generated using bokeh.

372  
373 Hybridization, probe wash, and amplification buffers were acquired from Molecular Technologies  
374 (Caltech, Pasadena, CA).

375  
376 **ABBA Preparation:**

377 ABBA samples were prepared as described previously (Jorth, 2020). Two sequential overnight cultures of  
378 wildtype *P. aeruginosa* PA14 were grown in the same liquid media as the final ABBA, shaking  
379 aerobically at 37°C, then diluted to OD<sub>500</sub> .001 in molten LB supplemented with nitrate depending on  
380 experimental conditions and 0.5% noble agar. The agar suspension was transferred to chambered cover  
381 glass slides and allowed to cool for 10 minutes, before being transferred to a humidified chamber and  
382 incubated at 37°C for 12 hours. After 12 hours, blocks were carefully removed from their chambers  
383 using a sterilized metal spatula into pre-chilled 1 mL of 4% paraformaldehyde in PBS + ProtectRNA and

384 incubated for 24 hours at 4°C. Blocks were washed three times for 30 minutes each in 1 mL PBS, then  
385 incubated for 1 hour in 1 mg/mL lysozyme in 10 mM Tris HCl at 37°C to lyse cell walls, then washed  
386 again three times for 30 minutes each in 1 mL PBS and stored in 1 mL PBS. Blocks were checked on a  
387 microscope for proper growth patterning (large aggregates at the top and smaller ones in the bottom,  
388 for wildtype in LB+40 mM nitrate) prior to continuing. For each condition and each gene target, three  
389 biological replicates were performed, in addition to one blank (no cell inoculation) per condition.

390

#### 391 **ABBA HCR:**

392 For each sample, 500 µL of hybridization buffer was mixed with 1 µL even and odd HCR initiator probes  
393 (2 µM stock) for 4 nM ultimate concentration. Each block was incubated in the buffer overnight at 37°C,  
394 then washed three times for 2 hours in the 1 ml pre-warmed wash buffer at 37°C. Each sample was then  
395 combined with 250 µL of preheated amplification buffer and 5 µL snap-cooled fluorescent hairpins (3  
396 µM stock) for 60 nM final concentration. Samples were incubated overnight in the dark at room  
397 temperature, then washed for 6 hours in 1 mL 5X SSCT at room temperature in the dark, then  
398 transferred to 500 µl PBS and stored at 4°C in the dark until imaging using confocal fluorescence  
399 microscopy. Imaging was performed on a Zeiss LSM 800 in the Caltech Bioimaging Facility. Agar blocks  
400 were mounted on a glass-bottomed dish with the original air-agar interface oriented toward the glass  
401 bottom. Both the Alexa Fluor 488 rRNA and Alexa Fluor 647 mRNA channels were imaged  
402 simultaneously, with a laser power of .2% and a gain of 650 for the 488 channel, and a laser power of 2%  
403 and a gain of 750 for the 647 channel. At least four image fields were imaged per sample using a 10x  
404 objective with 2x zoom, for a final image field of 319.45 x 319.45 microns. For each image field, a 100  
405 slice z-stack was imaged with an interslice distance of 6.24 microns. Images were framed such that the  
406 surface, which appears visible as a plane of rRNA signal due to a lawn of cells growing on the agar  
407 surface, was within 4 slices of the top of the image. Quantification of fluorescence signal per aggregate  
408 was performed using Imaris v9.5.1. A batch pipeline was created to segment aggregates. The 488 signal  
409 intensity of each Z plane was normalized, and then aggregates were segmented based on the  
410 thresholded 488 signal, with the threshold chosen manually per imaging date. The normalized signal was  
411 only used for the purpose of segmentation, and the mean signal intensity values reported here are  
412 based on the raw signal rather than the normalized signal. The mean 488 and 647 mRNA signal intensity,  
413 as well as aggregate size, was calculated and exported per aggregate. Depth was defined as distance  
414 from the top of the image, and images were cropped for analysis such that the surface was at the top

415 slice using native Imaris functions. Quantitative graphs were generated using seaborn and bokeh. Raw  
416 images were displayed using FIJI.  
417

418 **Figure Legends**

419 **Figure 1: Regulatory Diagram of Target Genes** This diagram was compiled from a literature  
420 search of transcriptomics reviews in *P. aeruginosa*. (Eschbach, 2004; Arai, 2005; Schreiber,  
421 2007; Arai, 2011; Arat, 2015)

422 **Figure 2: mRNA Probes Are Target-Specific** (A) Micrographs of Single Cell Deletion Validation  
423 Controls for mRNA Probes. (B) Micrographs of Aggregate Deletion Validation Controls for mRNA  
424 probes. Images reflect the region 50-100  $\mu\text{m}$  from the air-agar interface. (C) Quantification of  
425 mean mRNA probe intensities for single cells grown in liquid culture of wildtype and deletion  
426 mutants for target genes under upregulating growth conditions. Mean intensity was 10-fold  
427 higher in the wildtype than in the deletion for all probe sets. Each boxplot summarizes  
428 approximately 10 images per replicate. Three replicates were performed per condition. (D)  
429 Quantification of mean mRNA probe intensities for aggregates grown in agar blocks wildtype  
430 and deletion mutants for *ackA* under upregulating growth conditions. Mean intensity was 2-  
431 fold higher in the wildtype, and each boxplot summarizes 3-5 images per replicate. Three  
432 replicates were performed per condition.

433 **Figure 3: Metabolic Genes Show Distinct Patterns Across 3D Oxygen Gradients** (A) Mean  
434 oxygen levels of ABBA samples grown with LB+40 mM Nitrate for 12 hours. The dark line is the  
435 mean, while the shading represents the standard deviation of 8 biological replicates grown  
436 under identical conditions. (B) 3D Micrographs of Probe Signal in LB + 40 mM Nitrate ABBA.  
437 Each image represents a 50 micron slice of agar, compiled from 8 individual z-slices with an  
438 interslice distance of 6.24 microns, viewed from the top of the block, with each sequential  
439 image from the top of the figure representing the section directly below the slice above it. rRNA  
440 signal is colored cyan, while mRNA signal is colored magenta. (C) Mean mRNA Channel Intensity  
441 per aggregate (x axis) plotted by depth. Each plot represents four images of one replicate each  
442 of an experimental and control condition, and each point represents one aggregate. Filled  
443 points represent the experimental condition, while the white circles represent a control  
444 condition where only rRNA probes were used.

445 **Figure 4: Metabolic Genes Show Distinct Patterns Compared to rRNA Expression** Mean mRNA  
446 intensity per aggregate (x) plotted against mean rRNA intensity per aggregate (y) colored by

447 depth. Each plot represents four z-stacks of one replicate, and each point represents one  
448 aggregate. Large points represent the experimental condition, and small points represent the  
449 control condition without mRNA probes.

450

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