1 Phenylacetyl-CoA, not phenylacetic acid, mediates quorum sensing-regulated virulence in
2 *Burkholderia cenocepacia*
3
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17 Running Head: Phenylacetyl-CoA inhibits LuxIR-type quorum sensing
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21 Abstract word count: 244
22 Importance word count: 150
23 Text word count: 6,373
24

Abstract

In many bacteria, phenylalanine and other aromatic compounds are catabolized through phenylacetic acid (PAA) degradation. In this pathway, PAA is converted to phenylacetyl-CoA (PAA-CoA) by a ligase, PaaK, and then epoxidized by a multicomponent monooxygenase, PaaABCDE, before further degradation to the TCA cycle. As *Burkholderia cenocepacia paaABCDE* mutants release PAA and are attenuated for quorum sensing-regulated virulence in rat and nematode models of infection, overproduction of PAA in *B. cenocepacia* was linked to the regulation of the LuxIR-like quorum sensing system, CepIR. To further investigate this link, we created knockout mutants of the first step of the pathway (PAA-CoA synthesis by PaaK) and characterized them in comparison to wild type and PaaABCDE mutant strains using liquid chromatography/mass spectrometry (LC-MS/MS) and phenotypic assays. Both mutants released high levels of PAA. However, only the PaaABCDE mutant accumulated PAA-CoA intracellularly, had decreased virulence in *Caenorhabditis elegans,* and was defective for exoprotease activity. Conversely, the PaaK mutant did not accumulate PAA-CoA and was virulent, linking PAA-CoA to the attenuation of virulence in *B. cenocepacia*. While transcriptional analysis showed no evidence of *cepIR* downregulation by PAA-CoA, the attenuated pathogenicity of a *cepR* mutant was dependent on the presence of PAA-CoA in PAA pathway mutants, as it drastically changed to a virulent phenotype upon deletion of *paaK* but not when *paaABCDE* was deleted. Our results demonstrate the role of PAA-CoA in quorum sensing-regulated virulence and are consistent with a virulence regulatory mechanism that is evident when CepR is not active.

48 **Importance**

49 The opportunistic pathogen *Burkholderia cenocepacia* uses a chemical signal process called
50 quorum sensing to produce virulence factors. In *B. cenocepacia*, quorum sensing relies on the
51 presence of the transcriptional regulator CepR, which upon binding quorum sensing signal
52 molecules, activates virulence. In this work, we have found that even in the absence of CepR, *B.*
53 *cenocepacia* can elicit a pathogenic response if phenylacetyl-CoA, an intermediate of the
54 phenylacetic acid (PAA) degradation pathway, is not produced. Instead, accumulation of
55 phenylacetyl-CoA attenuates pathogenicity. Therefore, we have discovered that triggering of
56 virulence is possible in the absence of quorum sensing, challenging the classical view of
57 activation of virulence by quorum sensing. Our work provides new insight into the relationship
58 between metabolism and virulence in opportunistic bacteria. We propose that, in the event that
59 quorum sensing signaling molecules cannot accumulate to trigger a pathogenic response, a
60 metabolic signal can still activate virulence in *B. cenocepacia*.

61

62 **Introduction**

63 Intercellular communication in bacteria, or quorum sensing (QS), relies on the production
64 and detection of signalling molecules to regulate gene expression in a cell-density dependent
65 manner (1, 2). In many Gram-negative bacteria, LuxI homologs synthesize *N*-acyl homoserine
66 lactones (AHLs) as signalling molecules. These small diffusible molecules accumulate
67 extracellularly as the population increases until the concentration is sufficient for AHLs to return
68 into the cell where they bind LuxR-type transcriptional regulators. Complexing of the AHL to
69 the LuxR homolog results in a conformational change that alters the ability of the transcriptional

70 regulator to bind DNA and regulate gene expression (3). Many of the genes regulated by QS
71 control expression of virulence traits, which are important for bacteria to establish infection (4).
72 A bacterium with a LuxIR-type QS system is *Burkholderia cenocepacia*, a member of the
73 *Burkholderia cepacia* complex (5). The strain *B. cenocepacia* K56-2, isolated from the sputum
74 of a cystic fibrosis patient, has two complete LuxIR-type QS systems, CepIR and CciIR, and one
75 orphan transcriptional regulator, CepR2 (5, 6). The regulons of CepR and CciR have been
76 examined and a large number of the genes that are regulated are linked to virulence (7). While
77 CepR and CciR have separate regulons, certain genes are regulated by both in a reciprocal
78 manner (7). CepR positively regulates gene expression, whereas CciR is responsible for negative
79 gene regulation (7). While these two systems regulate certain genes in a reciprocal manner,
80 rather than hierarchically like those of *Pseudomonas aeruginosa,* the transcription of both *cepI*
81 and *cciIR* is dependent on CepR and in turn CciR negatively regulates the expression of *cepI* (7,
82 8). CepR and CciR both positively regulate their canonical autoinducer synthase and negatively
83 regulate their own transcription (8, 9). CepI mainly produces *N*-octanoyl homoserine lactone
84 (C8-HSL) with slight production of *N*-hexanoyl homoserine lactone (C6-HSL), whereas CciI
85 synthesizes C6-HSL with slight secondary production of C8-HSL (8, 10). CepR2 lacks a cognate
86 AHL synthase and has been shown to regulate known QS-regulated genes independent of a
87 signalling molecule (11).
88 Several lines of evidence point to metabolic adaptation as an important component of
89 bacterial virulence regulation (12–15) and the relationship between phenylacetic acid (PAA)
90 degradation and virulence in *B. cenocepacia* is the focus of this study. *B. cenocepacia* can use
91 the PAA degradation pathway to catabolize diverse aromatic compounds, such as phenylalanine,
92 to components of the three carboxylic acid (TCA) cycle (16). Based on the PAA degradation

93 pathway elucidated in *Escherichia coli* K12*,* PAA is converted to phenylacetyl-Coenzyme A
94 (PAA-CoA) by the action of a phenylacetyl-CoA ligase*,* PaaK (16). The conversion to PAA-
95 CoA by PaaK can be reverted to PAA by the action of PaaI, a thioesterase, to prevent
96 accumulation should downstream steps be disrupted (17). Next, PAA-CoA is epoxidized by the
97 multicomponent monooxygenase, PaaABCDE and then further degraded into succinyl-CoA and
98 acetyl-CoA (16).

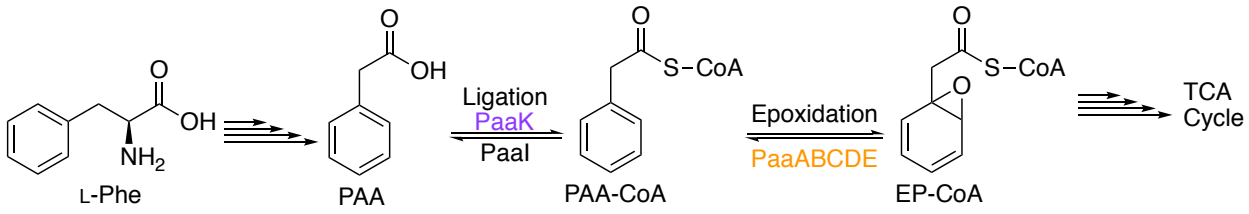
99 We previously found that CepIR-regulated virulence traits and *cepI* and *cepR* promoter
100 activity were downregulated in a *B. cenocepacia* mutant of the *paaABCDE* operon that released
101 PAA (18). We also demonstrated that PAA could be produced to detectable levels in wild type *B.*
102 *cenocepacia* K56-2 under certain conditions, suggesting a potential physiological role of the
103 regulation of virulence by this molecule (19). However, the PaaABCDE mutant is still able to
104 degrade PAA to PAA-CoA. Therefore, the metabolite responsible and the mechanism of the
105 regulation of virulence by accumulated PAA-metabolites is not known. In this work, we further
106 characterized mutants of the PAA degradation pathway to show that the accumulation of PAA-
107 CoA, but not PAA, is responsible for the attenuation phenotype. While we found no evidence of
108 a direct effect of PAA-CoA on CepI activity or the formation of CepR:C8-HSL complexes, our
109 results are consistent with a model in which a parallel mechanism that uses PAA-CoA as a
110 regulatory ligand, competes with the CepIR complex to regulate virulence. We propose that
111 PAA-CoA acts as a co-repressor that binds to a yet unidentified transcriptional regulator,
112 competitively inhibiting the CepIR QS regulation.

113

114 **Results**

115 *Creation and phenotypic characterization of a* paaK *double mutant*

116 Previously, we demonstrated a link between the attenuation of *B. cenocepacia* K56-
117 2Δ*paaABCDE* and the accumulation of PAA (18). However, the interruption of the pathway at
118 this step could also result in an accumulation of PAA-CoA because PAA is converted to PAA-
119 CoA by a PaaK ligase (Fig. 1). To determine the role of each of these molecules in the
120 attenuation of virulence, we characterized a *paaK* mutant that should only produce PAA and
121 compared it to the *paaABCDE* mutant. In K56-2 there are two similar but not identical
122 phenylacetyl-CoA ligases, PaaK1 and PaaK2 (20). We created marker-less deletion mutants of
123 BCAL0404 (*paaK1*) and BCAM1711 (*paaK2*). Knockout mutants of the other *paaK* were then
124 made in each background resulting in two independently made double knockouts, which we have
125 named K56-2Δ*paaK1*Δ*paaK2* and K56-2Δ*paaK2*Δ*paaK1*.



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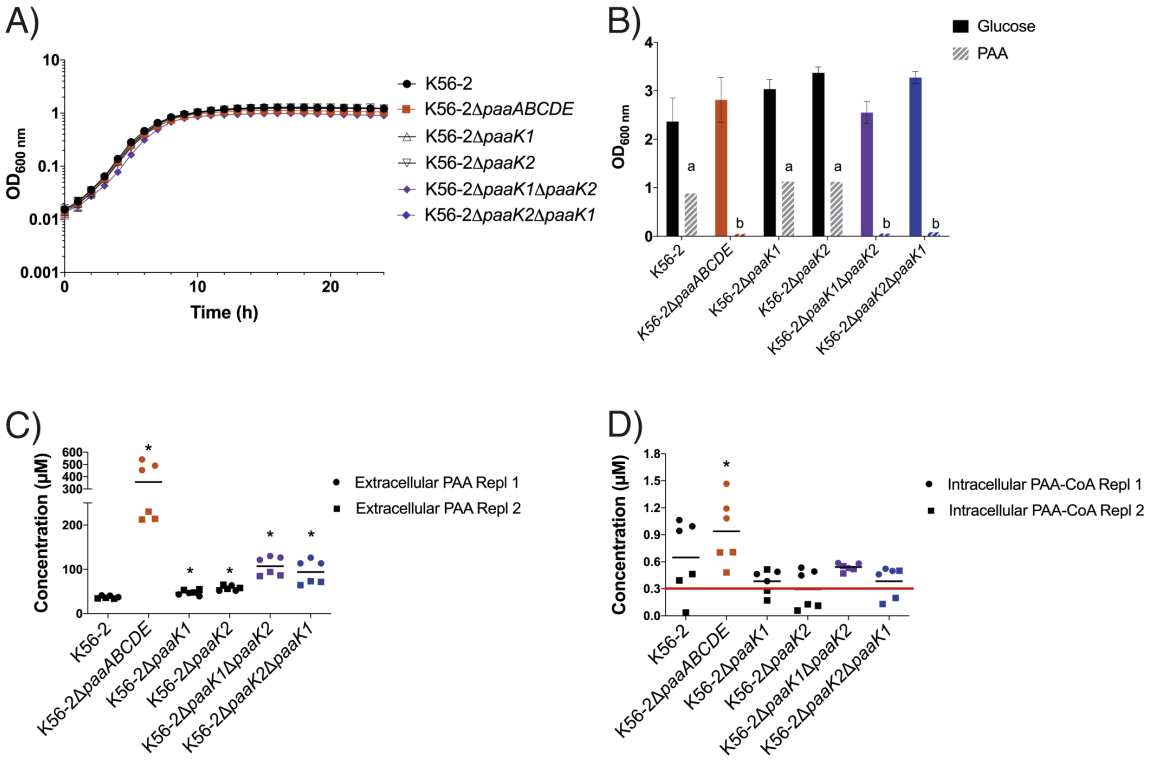
127 **Fig. 1: The proposed phenylacetic acid degradation (PAA) pathway in *B. cenocepacia.***
128 Based on the pathway described in *E. coli* and *Pseudomonas* by Teufel *et al.* (2010),
129 phenylalanine (Phe) is degraded to PAA and the first step of the pathway is the conversion of
130 PAA to phenylacetyl-CoA (PAA-CoA) by the phenylacetyl-CoA ligase, **PaaK** (shown in
131 purple). The phenylacetyl-CoA thioesterase, **PaaI,** can reversibly convert PAA-CoA back to
132 PAA to prevent toxic accumulation of the epoxide if the downstream pathway is blocked. PAA-
133 CoA is epoxidized by the ring 1,2 phenylacetyl-CoA epoxidase, **PaaABCDE** (shown in orange),
134 forming ring-1,2-epoxyphenylacetyl-CoA or epoxide (EP-CoA). The epoxide is further degraded
135 to components of the TCA cycle, acetyl-CoA and succinyl-CoA.
136

137 To confirm the interruption of the PAA pathway, wild type K56-2 and mutants were
138 grown on LB media and M9 minimal medium with glucose or PAA as a sole carbon source. No
139 growth defect was observed in LB or glucose (Fig. 2 A and B). Both *paaK* single knockout
140 mutants were also able to grow on PAA as a sole carbon source to wild type levels, indicating
141 that PAA can be used as a substrate for both ligases (Fig. 2B). While mutants were able to grow
142 normally on glucose as a sole carbon source, the *paaK* double knockout mutants (K56-
143 2Δ*paaK1*Δ*paaK2* and K56-2Δ*paaK2*Δ*paaK1*) were unable to grow on PAA (Fig. 2B). Growth
144 was complemented with a plasmid containing a rhamnose-inducible *paaK2* (BCAM1711) (Fig.
145 S2) confirming that the interruption of the pathway in these mutants was caused by the deletion
146 of *paaK*.
147 The PaaABCDE mutant has been shown to accumulate extracellular PAA due to the
148 interruption of the pathway (18) but the concentration of intracellular PAA and PAA-CoA was
149 unknown. Intracellular and extracellular concentrations of both PAA and PAA-CoA were
150 measured with ion pair reversed phase ultra-high performance liquid chromatography tandem
151 mass spectrometry (IP-RP-UHPLC-MS/MS) using the differential method (21–23). As expected,
152 mutants with complete interruption of the pathway (Δ*paaABCDE,* Δ*paaK1*Δ*paak2,* and
153 Δ*paaK2*Δ*paak1*) accumulated more PAA than wild type (Fig. 2C). PAA was released
154 extracellularly (Fig. 2C, Fig. S3A), whereas PAA-CoA accumulated intracellularly (Fig. 2D, Fig.
155 S3B). The only mutant strain that accumulated PAA-CoA was Δ*paaABCDE* (Fig. 2D).



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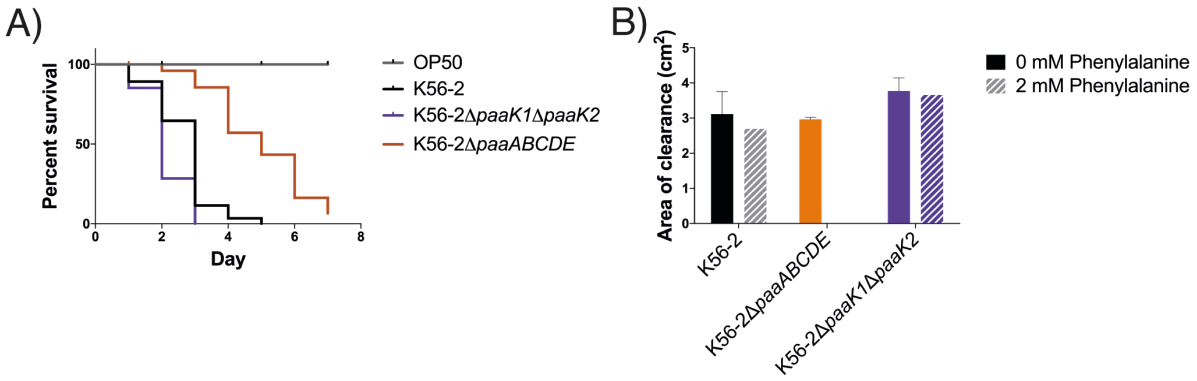
157 **Fig. 2: Characterization of the growth and metabolite production of *B. cenocepacia* PAA**
158 **pathway mutants. A)** *B. cenocepacia* K56-2 PAA pathway mutants exhibited no growth defects
159 when grown on LB media. **B)** *B. cenocepacia* K56-2 PAA pathway mutants were grown on M9
160 with 25 mM Glucose as the sole carbon source or M9 with 5 mM PAA as the sole carbon source
161 for 48 hours and the OD_{600} was measured. Mutants with full interruption of the pathway
162 (*ΔpaaABCDE*, *ΔpaaK1ΔpaaK2,* and *ΔpaaK2ΔpaaK1*) were unable to grow on PAA as a sole
163 carbon source. Single deletion mutants of the *paaK* ligases (*ΔpaaK1* and *ΔpaaK2*) grew to wild
164 type levels on PAA indicating that the pathway is not interrupted in these strains. Error bars
165 represent SD for three biological replicates. b represents p values ≤ 0.05 as determined by
166 student's T-test (two tailed) when compared to growth on PAA of K56-2. **C)** IP-RP-UHPLC-
167 MS/MS revealed that PAA is released extracellularly in the *paaABCDE* and *paaK* knockout
168 mutants. PAA did not accumulate in the wild type and *paaK* single knockout strains where the
169 PAA pathway is still functional. **D)** PAA-CoA was present at detectable limits in all of the

170 strains, but PAA-CoA accumulates solely in the *paaABCDE* operon knockout mutant. For all IP-
171 RP-UHPLC-MS/MS two biological replicates with three technical replicates each were
172 performed. An Asterix denotes $p \leq 0.05$ as determined by student's T-test (two tailed) when
173 compared to K56-2.
174



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176 **Fig. 3: The Δ*paaK1*Δ*paaK2* mutant has wild type levels of virulence in *C. elegans* and**
177 **proteolytic activity whereas the Δ*paaABCDE* mutant is attenuated. A)** Slow killing assays
178 with *C. elegans* over a period of seven days show that the Δ*paaK1*Δ*paaK2* mutant kills *C.*
179 *elegans* in a similar time frame as wild type, whereas the nematodes exposed to the Δ*paaABCDE*
180 mutant have increased survival. The increased killing ability of the Δ*paaK1*Δ*paaK2* mutant
181 compared to wild type was determined as significant according to the log-rank test. Three
182 biological replicates were performed. **B)** Proteolytic activity was measured as the area of the
183 zone of clearance (excluding colonies) on agar containing 2% skim milk with or without the
184 addition of 2 mM of phenylalanine. The error bars represent the SD of three independent
185 experiments.
186

187 *PAA-CoA has no effect on CepR and CepI function* in vitro

188 The above findings suggest that a mutant that releases PAA and accumulates PAA-CoA
189 is attenuated for virulence in *C. elegans* and has decreased proteolytic activity whereas a mutant
190 that only releases PAA is not attenuated for these QS-regulated virulence traits. Since the
191 accumulation of PAA-CoA is the major difference between these strains we hypothesized that
192 PAA-CoA or a derivative was responsible for attenuating these traits. Because the CepIR QS
193 system is required for full virulence of *B. cenocepacia* K56-2 in *C. elegans*, while the CciIR QS
194 system is not (25), we focused on the effect of PAA-CoA on the CepIR QS system. In addition,
195 Δ*paaABCDE* had decreased *cepI* and *cepR* promoter activity and AHL signaling (18).
196 Specifically, we asked whether PAA-CoA could: 1) inhibit the formation of CepR:AHL
197 complexes; 2) inhibit the enzymatic activity of CepI; and/or 3) indirectly affect an upstream
198 regulator.
199 To address the first question, we built a reporter system similar to those commonly used
200 to identify quorum sensing inhibitors (26). *E. coli* W14 was selected as our reporter strain as it
201 lacks the PAA pathway genes (27, 28) ensuring the exogenously added PAA-related compounds
202 would not be degraded. Using arabinose-inducible expression of *cepR* (BCAM1868) we created
203 a reporter strain that responds to CepR:AHL complexes by activating the *cepI* promoter which
204 controls the expression of luminescence genes *luxCDABE* (Fig. 4A). Expression of luminescence
205 is decreased in the presence of an inhibitor of the formation of CepR:AHL complexes. However,
206 PAA-CoA cannot be added exogenously to cultures because it is too large to cross the
207 membrane. Therefore, we created a plasmid that contains the *E. coli* W *paaK* ligase gene
208 (ECW_RS07845) under the control of a rhamnose-inducible promoter. In the presence of
209 rhamnose, PaaK converts exogenously added PAA to PAA-CoA, as confirmed by IP-RP-
210 UHPLC-MS/MS (Fig. 4B). To ensure that any effects we saw were not due to an inhibition of

211 the arabinose-inducible promoter or luminescence itself we created two control strains that

212 contain either the arabinose-inducible P_{BAD} promoter or a constitutive promoter, *dhfr*, controlling

213 the *luxCDABE* genes.

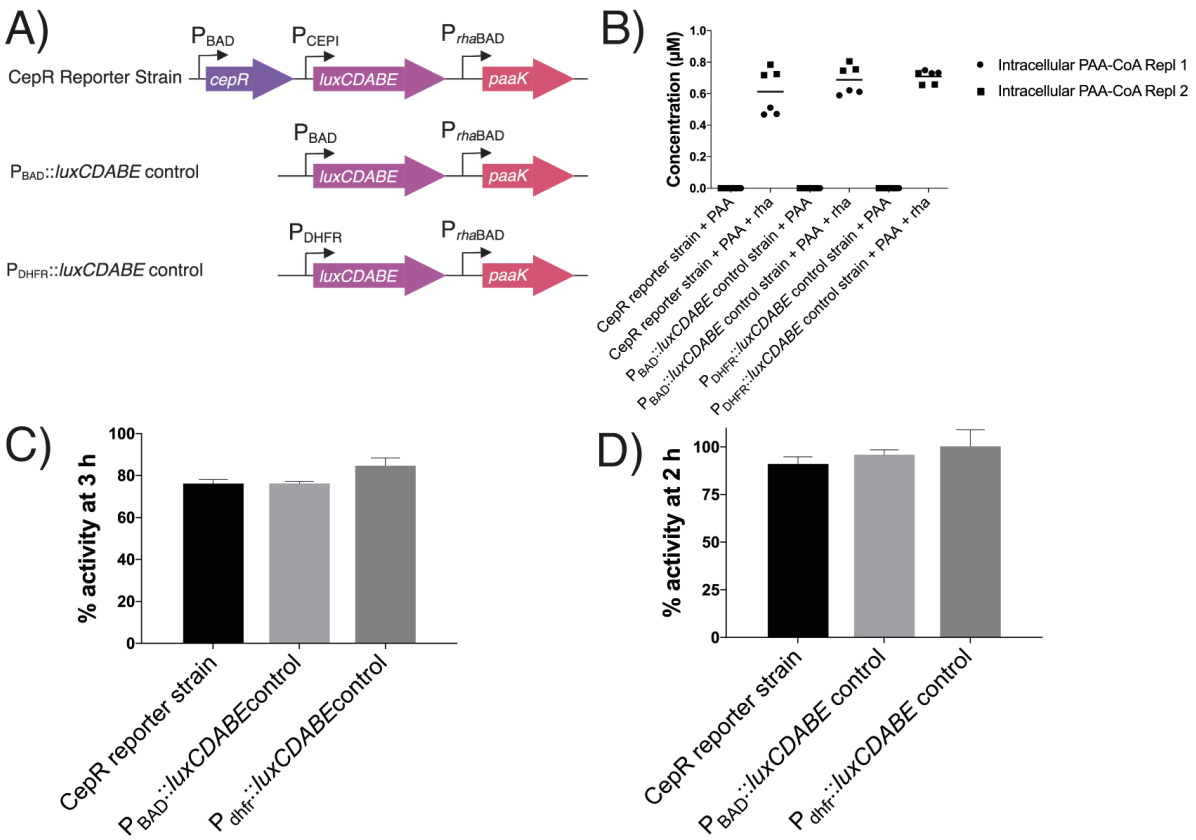
214 Using this reporter system (W14/pTL7/pTL5/pTL22 referred to from here on as CepR

215 reporter strain, Fig. 4A) we observed that although there was inhibition of luminescence in the

216 presence of PAA-CoA, the level of inhibition was similar to that of the control strains (Fig. 4C).

217 Similarly, PAA itself also had no effect on the system (Fig. 4D). In summary, these results show

218 that PAA-CoA and PAA do not directly affect CepI activity or CepR:C8-HSL formation.



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220 **Fig. 4: Neither PAA nor PAA-CoA affect the formation of CepR:C8-HSL complexes. A)**

221 The CepR reporter strain and control strains used. Regulatory genes *araC* (for P_{BAD}) and *rhaSR*

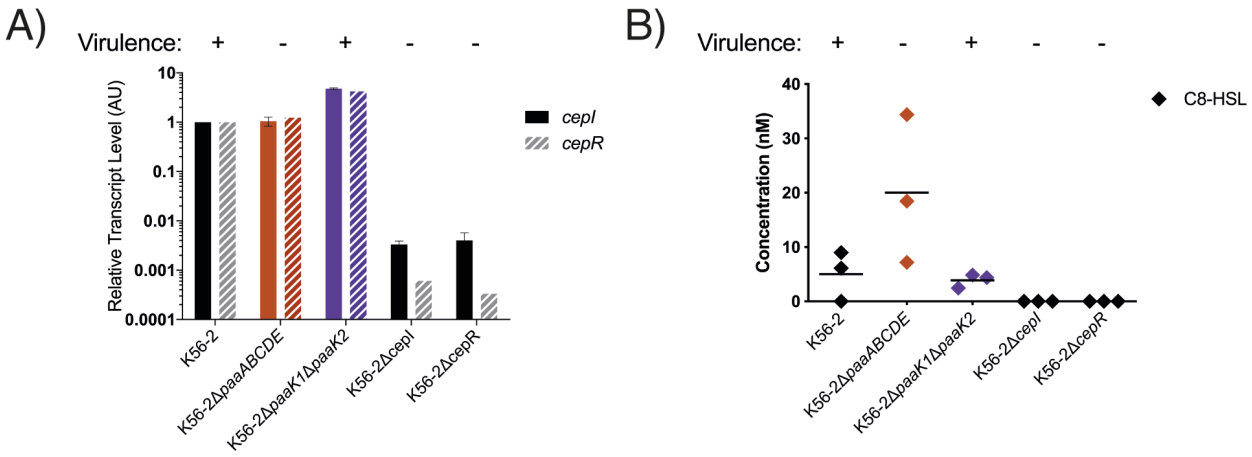
222 (for P_{rhaBAD}) were excluded from the figure for simplicity. **B)** In the presence of 0.01% rhamnose

223 (rha), 0.5 mM of PAA was converted to PAA-CoA by the PaaK ligase in the CepR reporter
224 strain. **C)** PAA-CoA had no effect on the CepR reporter strain compared to that of the effect on
225 control strains. **D)** PAA itself was tested at various concentrations from 0.1-1mM (0.1 mM
226 shown here) and no effect was observed when compared to the control strains. The error bars
227 represent the SD of three independent experiments.
228
229 To analyze if PAA or PAA-CoA inhibits the enzymatic activity of CepI, we used
230 recombinant CepI in the presence of dichlorophenolindophenol (DCPIP) to
231 spectrophotometrically measure the activity of CepI (29). CepI catalyzes the formation of C8-
232 HSL from octanoyl-Acyl Carrier Protein (C8-ACP) and *S*-adenosyl methionine (SAM). During
233 this reaction DCPIP is reduced resulting in a colour change that can be used to measure activity
234 of CepI. Enzyme activity showed no change in the presence of 200 μM of PAA-CoA or PAA
235 (Table 1), suggesting that neither molecule directly inhibits the activity of CepI.
236
237 **Table 1: CepI activity is not inhibited by the presence of PAA or PAA-CoA.**

Conditions	Enzyme activity ± SD (min^{-1})
C8-ACP + SAM	3.46 ± 0.12
C8-ACP + SAM + 200 μM PAA	3.29 ± 0.21
C8-ACP + SAM + 200 μM PAA-CoA	3.04 ± 0.29

238
239 As our data did not show a direct effect of PAA-CoA on the CepIR QS system but *cepI*
240 and *cepR* promoter activity was decreased in the PaaABCDE mutant (18), we hypothesized that
241 PAA-CoA may indirectly affect an upstream regulator of the CepIR QS system. If this was the
242 case, we predicted that the differences in pathogenicity between the Δ*paaABCDE* and the
243 Δ*paaK1*Δ*paaK2* mutant strains would be explained by differential expression of *cepI* and *cepR*.

244 Using stationary phase cultures, we measured the transcript levels of *cepI* and *cepR* and the
245 levels of C8-HSL in the PAA pathway mutants and compared it to wild type. Surprisingly, the
246 Δ*paaABCDE* and the Δ*paaK1*Δ*paaK2* mutants had wild type levels of *cepI* and *cepR*
247 transcription (Fig. 5A and Fig. S5A) despite the striking differences in their virulence
248 phenotypes and contradicting our previous results where reporter systems measured decreased
249 *cepI* and *cepR* promoter activity and decreased AHL signaling (18). This suggested that the
250 attenuation of the Δ*paaABCDE* mutant is independent of the transcription of the CepIR QS
251 system. To confirm the wild type transcription levels of our mutants, we measured the levels of
252 AHL molecules in each strain. Similar to the transcription, the levels of C8-HSL, taken from
253 stationary phase samples did not correlate with the decreased virulence of our PAA pathway
254 mutants (Fig. 5B and Fig. S5B). However, both Δ*cepI* and Δ*cepR* had no measurable C8-HSL
255 which is consistent with their lack of virulence (Fig. 5B).



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257 **Fig. 5: Transcription of *cepI* and *cepR* and C8-HSL production is not decreased in any of**
258 **the PAA pathway mutants. A)** RT-qPCR shows that *cepI* and *cepR* gene expression is not
259 decreased compared to wild type in the PAA pathway mutants. **B)** C8-HSL levels were not
260 decreased in any of the mutants, but C8-HSL levels were higher in the Δ*paaABCDE* mutant.

261 Virulence of strains in both *C. elegans* assays and exoprotease activity assays is denoted above
262 the graphs per the results from Fig. 3.
263
264 *The CepR-independent virulence of PaaK knockout mutants is mediated by the presence/absence*
265 *of PAA-CoA*
266 Transcription of *cepI* and *cepR* is not decreased in PAA pathway mutants and C8-HSL is
267 present in the mutants yet the virulence factors attenuated in the Δ*paaABCDE* mutant
268 (pathogenicity in *C. elegans* and exoprotease activity) are known to be under the control of the
269 CepIR system. To further understand how the PAA pathway may regulate QS related virulence
270 without affecting *cepIR* transcription, we created marker-less deletions of *cepI* and *cepR* in the
271 PAA pathway deletion mutant backgrounds. We reasoned that if PAA-related regulation of
272 virulence was completely independent of CepI and CepR activity, then deletion of *cepI* and *cepR*
273 in a Δ*paaABCDE* background should show an additive attenuation effect. Similarly, deletion of
274 *cepI* and *cepR* in a Δ*paaK1*Δ*paaK2* mutant background should show the same degree of
275 attenuation as the *cepI* and *cepR* deletions in a wild type background. Once we had created
276 knockout mutants of *cepI* and *cepR* for each PAA pathway mutant in parallel with *cepI* and *cepR*
277 deletions in wild type K56-2, we analyzed their growth and found no defect when grown in LB
278 media over 24 hours (Fig. S6). Next, we used virulence assays to determine if the CepIR QS
279 system was needed for the regulation of their QS. The virulence of the *cepI* and *cepR* knockout
280 mutants in wild type and PAA mutant backgrounds was compared in slow killing assays (Fig. 6A
281 and B, Fig. S7A and B). Both *cep* QS mutants in the Δ*paaABCDE* background were more
282 attenuated for virulence than Δ*paaABCDE* (Fig. S7A). However, they failed to show an additive
283 effect (Fig. S7A). Moreover, while the Δ*paaK1*Δ*paaK2*Δ*cepI* mutant was as attenuated as Δ*cepI*,

284 Δ*paaK1*Δ*paaK2*Δ*cepR* was not, showing that the pathogenic phenotype was independent of
285 CepR (Fig. 6A and B). This was mirrored in the Δ*paaK2*Δ*paaK1*Δ*cepR* mutant (Fig. S7B). This
286 is surprising considering the *cepR* deletion in wild type and the *paaABCDE* mutant has a severe
287 attenuation (Fig. S7A).

288 Corroborating the results of the *C. elegans* assay, Δ*paaK1*Δ*paaK2*Δ*cepR* had wild type
289 levels of proteolytic activity whereas the Δ*paaK1*Δ*paaK2*Δ*cepI* mutant had none (Fig. 6C and
290 Fig. S7C). The only difference between the Δ*paaK1*Δ*paaK2*Δ*cepR* mutant and the *cepR* mutant
291 is the presence of PAA. If PAA was responsible for the elimination of the *cepR* attenuation, the
292 Δ*paaABCDE*Δ*cepR* mutant should be pathogenic but our results show it is not. Therefore, we
293 conclude that it is the lack of PAA-CoA which causes the increase in pathogenicity in
294 Δ*paaK1*Δ*paaK2*Δ*cepR*, confirming the link between PAA-CoA and CepR mediated QS.

295 To further understand the virulence of the Δ*paaK1*Δ*paaK2*Δ*cepR* mutant, we examined
296 the expression levels of *cepI* and *cepR* and the levels of C8-HSL in the mutants. While
297 transcription of *cepR* is abolished in Δ*cepI*, it is restored to wild type levels when the deletion of
298 *cepI* occurs in a Δ*paaK1*Δ*paaK2* or Δ*paaABCDE* background (Fig. 6D). Therefore PAA, which
299 is present in both PAA pathway mutants, restores *cepR* transcription but not pathogenicity,
300 because there is no CepI and no C8-HSL (Fig. 6F). When *cepI* transcript levels were analyzed in
301 Δ*cepR* in wild type and PAA degradation mutant backgrounds, the transcription of *cepI* was
302 downregulated in the CepR mutant, as expected. Similarly, *cepI* transcription was reduced in the
303 attenuated Δ*paaABCDE*Δ*cepR* and the pathogenic Δ*paaK1*Δ*paaK2*Δ*cepR* (Fig. 6E). The lack of
304 C8-HSL in both the Δ*paaK1*Δ*paaK2*Δ*cepI* and Δ*paaK1*Δ*paaK2*Δ*cepR* (Fig. 6F) mutants
305 suggests the pathogenicity of the Δ*paaK1*Δ*paaK2*Δ*cepR* mutant is independent of *cepI*. This was
306 mirrored in the Δ*paaK2*Δ*paaK1*Δ*cepR* mutant (Fig. S8).

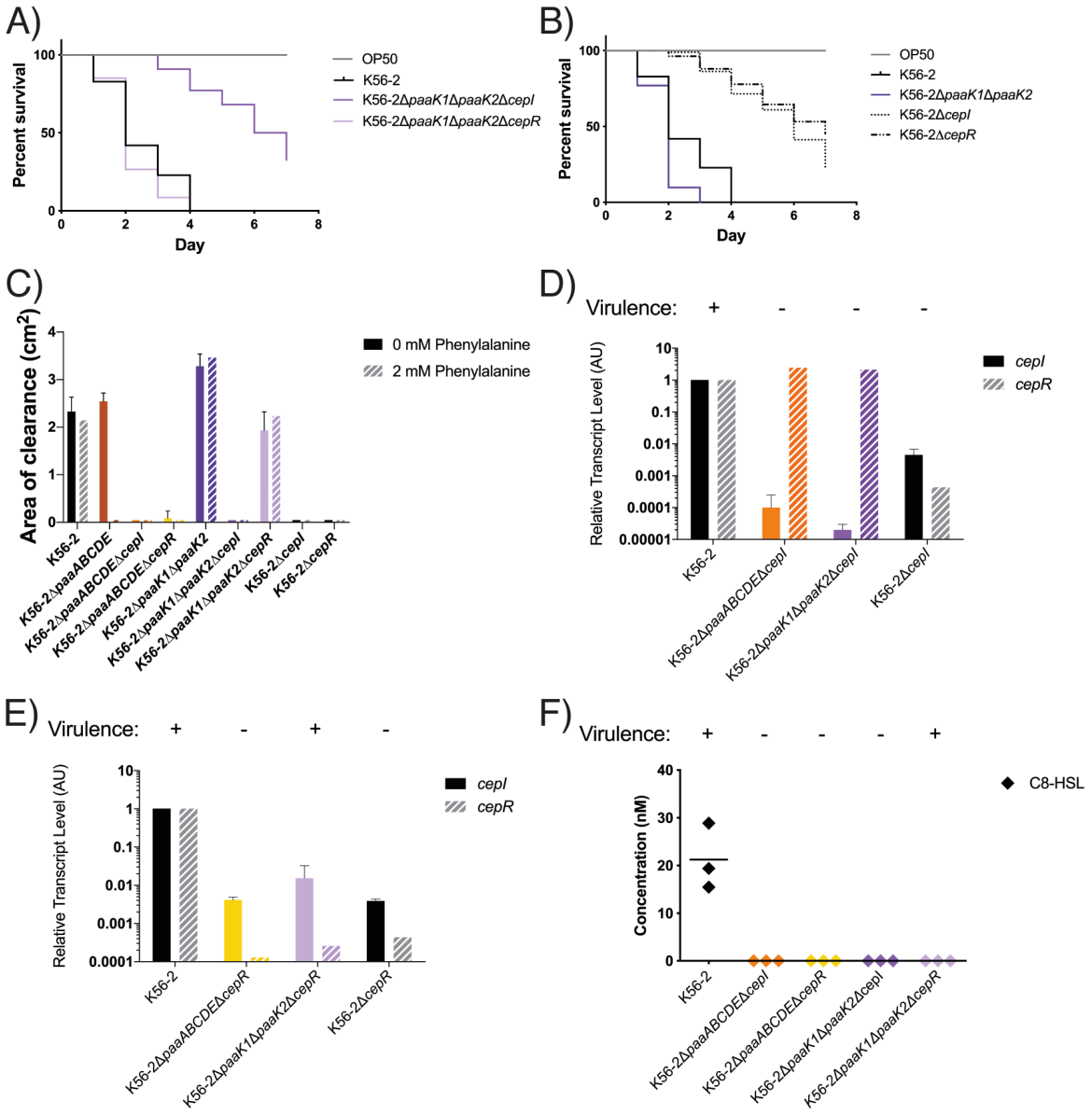


Fig. 6: The virulence of Δ*paaK1*Δ*paaK2*Δ*cepR* is independent of the CepIR QS system. A) The *cepI* and *cepR* mutations in a Δ*paaK1*Δ*paaK2* mutant have opposing levels of virulence. Δ*paaK1*Δ*paaK2*Δ*cepR* is as virulent as wild type whereas Δ*paaK1*Δ*paaK2*Δ*cepI* is attenuated for virulence in *C. elegans*. **B)** *cepI* and *cepR* mutants have similar levels of attenuation in *C. elegans*. Figures **A)** and **B)** are representative of three biological replicates. **C)** The Δ*paaK1*Δ*paaK2*Δ*cepR* mutant has wild type levels of proteolytic activity with or without phenylalanine whereas the Δ*cepR* mutant has decreased proteolytic activity. Proteolytic activity

315 was measured as the area of the zone of clearance (excluding colonies) on agar containing 2%
316 skim milk with or without the addition of 2 mM of phenylalanine. **D)** *cepR* transcription is
317 restored to wild type levels in Δ*paaABCDE* and Δ*paaK1*Δ*paaK2*Δ*cepI* indicating that PAA
318 restores *cepR* transcription. **E)** *cepI* is downregulated in all of the Δ*cepR* mutants. **F)** C8-HSL
319 levels were measured and none of the QS mutants had detectable levels of C8-HSL. The error
320 bars represent the SD of three independent experiments.
321
322 Deletions of the *cepI* and *cepR* genes in the PAA pathway mutant backgrounds allowed
323 us to determine that the PAA pathway has an effect on CepIR-regulated virulence that is evident
324 when CepI or CepR is absent. In this context, our results suggest that the absence of PAA-CoA,
325 or a derivative of PAA-CoA, permits an alternative signalling pathway to activate virulence in
326 the Δ*paaK1*Δ*paaK2*Δ*cepR* mutant. The opposite effects of the PaaK and PaaABCDE mutants
327 with regards to pathogenicity and the striking differences that *paaK* and *paaABCDE* gene
328 deletions have in the *cepR* mutant background suggests that PAA-CoA, or a derivative, is the
329 central molecule implicated in this alternative signalling pathway.
330
331 **Discussion**
332 In *B. cenocepacia*, the PAA pathway has been linked to the regulation of virulence. The
333 attenuation of CepIR QS-regulated virulence traits in PAA pathway mutants was thought to be
334 due to the accumulation of PAA in these mutants (18). PAA has also been shown to regulate the
335 virulence of other Gram-negative bacteria, *Acinetobacter baumannii* (30) and *Pseudomonas*
336 *aeruginosa* (31, 32), two Gram-negative bacteria that also use LuxIR-type QS systems to
337 regulate their virulence (33, 34). Previously, we demonstrated that the release of PAA in a *B.*

338 *cenocepacia* mutant of the PAA pathway resulted in the inhibition of CepIR-regulated QS, a
339 LuxIR-type system (18). However, the Δ*paaABCDE* mutant has the genes necessary to convert
340 PAA to PAA-CoA (Fig. 1). Therefore, PAA or PAA-CoA could be the molecule responsible for
341 the inhibition of CepIR-regulated QS.

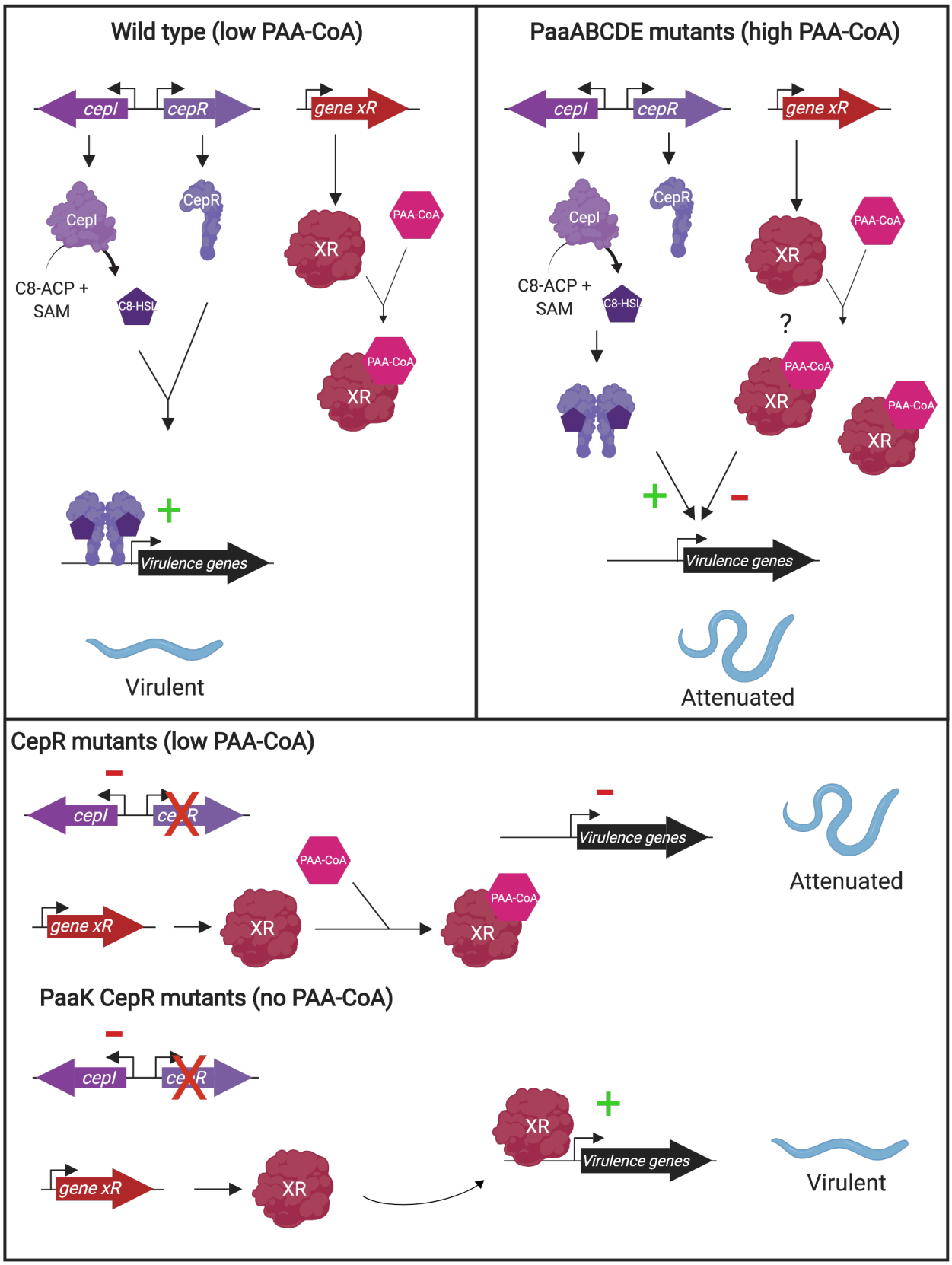
342 To determine the molecule responsible, we initially created a mutant unable to
343 accumulate PAA-CoA. Unlike the Δ*paaABCDE* mutant, the PaaK knockout mutant was as
344 virulent as wild type, suggesting that the attenuated virulence was due to the accumulation of
345 PAA-CoA, or a derivative. Another difference between the PaaK and the PaaABCDE mutant is
346 that the PaaK knockout mutant released less PAA than the PaaABCDE mutant. However, the
347 intracellular concentrations of PAA were similar and detected at levels very close to, or lower
348 than, the smallest standard used for the calibration curve (8 μM). We believe that differences in
349 intracellular PAA levels should have been found if PAA affected the regulation of virulence.

350 We hypothesized CepIR-regulated QS inhibition was achieved by one of three
351 mechanisms: 1) direct inhibition of the enzymatic activity of CepI or its products; 2) inhibition of
352 the formation of CepR:C8-HSL complex; or 3) an indirect effect on an upstream regulator. Using
353 an *E. coli* reporter system that can't metabolize PAA, we examined the ability of PAA-CoA to
354 act as a direct inhibitor of the CepIR QS system. We were able to observe strong but unspecific
355 inhibition of luminescence by PAA and PAA-CoA, highlighting the importance of control strains
356 when reporter systems are used to identify QS inhibitors: QS-regulated phenotypes often depend
357 on other factors; and reporter strains using bioluminescence can be affected by the overall
358 metabolic activity of the cell (26). In our system, the control strains had either the arabinose-
359 inducible P_{BAD} promoter or the *dhfr* constitutive promoter controlling the expression of
360 *luxCDABE* luminescence genes. First, in the presence of higher concentrations of PAA (above 1

361 mM) the expression of luminescence by the constitutive promoter was inhibited suggesting a
362 general effect on luminescence. Second, the concentration of rhamnose used to express *paaK,*
363 0.01% w/v, inhibited the arabinose-inducible promoter. With these control strains we were able
364 to minimize the general effects on the reporter system and determine that neither PAA or PAA-
365 CoA have a direct effect on the formation of CepR:C8-HSL complexes or on CepI activity.
366 Interestingly, we found that the attenuation of virulence in Δ*paaABCDE* did not
367 correspond to decreased *cepI* and *cepR* transcription or C8-HSL production. In fact, the
368 concentration of C8-HSLs present in the strains did not correlate to the pathogenicity of the PAA
369 pathway mutants. While one of the pathogenic *paaK* knockout mutants had wild type levels of
370 C8-HSL, the independently made mutant had a similar concentration to that of the Δ*paaABCDE*
371 mutant despite their opposing pathogenicity phenotypes. Based on the current knowledge of the
372 CepIR QS system, transcription of *cepI* relies on CepR and CepR is negatively autoregulated (9).
373 This is in agreement with our findings that the transcription of *cepI* is dependent on CepR and
374 that *cepR* is downregulated in the absence of *cepI.* There was no detectable C8-HSL in either of
375 the *cepI* and *cepR* deletion strains supporting the interdependence of these two genes. Therefore
376 the lack of downregulation of either *cepI* or *cepR* in the Δ*paaABCDE* mutant was quite
377 surprising because this contradicted the results from our plasmid-based reporter systems that
378 showed decreased *cepI* and *cepR* promoter activity and decreased AHL signalling (18). To
379 investigate this discrepancy in results between the different methods, we introduced the plasmids
380 with P_{cepI}::*luxCDABE* or P_{cepR}::*luxCDABE* to the Δ*paaK1*Δ*paaK2* and Δ*paaK2*Δ*paaK1* mutants
381 and found decreased *cepI* and *cepR* promoter activity almost identical to that of the Δ*paaABCDE*
382 mutant with the same plasmid based reporter system (data not shown). This suggests that the
383 PAA present in both these mutants might have a general effect on the reporter system itself. We

384 thought that PAA may affect the copy number of the plasmid or affect the metabolism of the
385 cells in such a way that affects plasmid-based reporter strains. Previously PAA has been shown
386 to affect the copy number and increase the frequency of plasmid loss from an *E. coli* strain (35).
387 The concentration of PAA used in that assay was higher than what we measured with IP-RP-
388 UHPLC-MS/MS for these strains, but the effect of PAA on plasmid copy number in *B.*
389 *cenocepacia* has not been studied. These reporter strains also use bioluminescence, which
390 requires large amounts of energy, so any changes in metabolic activity could be reflected in the
391 reporter strain and misinterpreted without the use of proper controls (26).
392 To determine if the attenuated virulence of Δ*paaABCDE* could be independent of CepI
393 and CepR activity we created *cepI* and *cepR* mutants in the PAA pathway mutant backgrounds.
394 If PAA-related regulation of virulence was independent of CepI and CepR activity, then we
395 would see an additive effect on attenuation. Whereas, in a *paaK* knockout mutant background we
396 expected to see a virulence phenotype similar to that of the same deletion in a wild type strain. In
397 the Δ*paaABCDE* QS mutants we saw no evidence of an additive effect as the QS mutations were
398 dominant, leading to the same pathogenicity as a wild type Δ*cepI* or Δ*cepR* mutant. Because the
399 *cepI/R* mutations in Δ*paaABCDE* did not have an additive effect, this suggests the mechanism of
400 attenuation involves the CepIR QS system. In the *paaK* knockout background the QS mutations
401 had opposing effects. While a *cepI* deletion resulted in the expected attenuated virulence, a *cepR*
402 deletion had wild type levels of virulence. Transcription of *cepI* and *cepR* in these mutants
403 showed that PAA pathway mutants with a *cepI* deletion had increased transcription of *cepR*.
404 Therefore, the presence of PAA, present in both PAA pathway mutants, can restore the
405 transcription of *cepR* to wild type levels. Based on our findings, we propose that PAA-CoA
406 exerts its effect by forming a complex with a non-canonical transcriptional regulator XR that

407 competes with CepR:C8-HSL for binding in virulence gene regulatory elements (Fig. 7). In this
408 context, XR would increase virulence while XR:PAA-CoA would not. In wild type (Fig. 7, left
409 panel) *cepI* and *cepR* are transcribed and CepR complexes with C8-HSL and regulates virulence.
410 In conditions where the PAA pathway is functional PAA-CoA does not accumulate to levels that
411 would inhibit virulence. In the PaaABCDE mutant background, where PAA-CoA is abundant,
412 the XR:PAA-CoA complexes displace CepR:C8-HSL complexes. This competition results in
413 attenuated virulence in *C. elegans* (Fig. 7, right panel) and would be diminished by the addition
414 of C8-HSL as we determined previously (18). In the PaaABCDE QS mutants XR:PAA-CoA has
415 no CepR:AHL complexes to displace so the virulence is the same as a QS mutant in a wild type
416 background. CepR mutants have decreased *cepI* transcription so no CepR:C8-HSL complexes
417 are formed resulting in attenuated virulence (Fig. 7, bottom panel). This model would also
418 explain the CepR-independent virulence of Δ*paaK1*Δ*paaK2*Δ*cepR*. In the absence of both *cepR*
419 and PAA-CoA, XR is able to activate certain virulence genes (Fig. 7, bottom panel). We were
420 unable to detect differences between the levels of PAA-CoA in wild type and PaaK mutants as
421 the amounts of PAA-CoA were very close to the limit of detection of our method. However, it is
422 reasonable to assume that wild type can produce PAA-CoA while a PaaK mutant cannot.
423 Therefore, we propose that, in the absence of PAA-CoA, XR positively regulates the virulence
424 genes. This alternative regulatory system does not require C8-HSL to activate XR as C8-HSL
425 was absent in all of the PAA pathway QS mutants. We propose that this alternative pathway may
426 be relevant in environmental conditions where quorum sensing signals do not accumulate but a
427 virulent response is necessary.


Wild type (low PAA-CoA)
cepI
cepR
gene xR
CepI
CepR
XR
PAA-CoA
C8-ACP +
SAM
C8-HSL
XR
PAA-CoA
+
Virulence genes
Virulent
PaaABCDE mutants (high PAA-CoA)
cepI
cepR
gene xR
CepI
CepR
XR
PAA-CoA
C8-ACP +
SAM
C8-HSL
?
PAA-CoA
XR
PAA-CoA
XR
+
-
Virulence genes
Attenuated
CepR mutants (low PAA-CoA)
-
cepI
cepR
-
Virulence genes
Attenuated
PAA-CoA
gene xR
XR
PAA-CoA
XR
PaaK CepR mutants (no PAA-CoA)
-
cepI
cepR
XR
+
gene xR
XR
Virulence genes
Virulent

428

429 **Fig. 7: Model of the role of the PAA pathway in the regulation of CepIR quorum sensing-**
430 **controlled virulence.** Our model suggests that PAA-CoA acts as a co-repressor, with an as yet
431 unidentified transcriptional regulator (XR). In wild type CepIR QS dominates the regulation of
432 virulence due to the lower concentration of PAA-CoA (top left panel). Whereas in Δ*paaABCDE*
433 the levels of PAA-CoA are high and the XR:PAA-CoA complex can compete with CepR:C8-
434 HSL complexes to repress virulence, resulting in an attenuated virulence compared to wild type
435 (top right panel). CepR mutants in wild type don't have CepR:C8-HSL complexes to regulate
436 virulence and are therefore attenuated for virulence (bottom panel). However, in a PaaK
437 background CepR mutants are virulent. The absence of PAA-CoA in these mutants in
438 combination with the lack of CepR create the perfect conditions for XR to positively regulate
439 virulence genes.
440
441 Normally Δ*cepI* mutants have downregulation of *cepR*, but as we observed in RT-qPCR
442 of the PAA pathway mutants with Δ*cepI* there was increased *cepR* transcription (Fig. 6A)
443 suggesting that cepR transcription can be increased even in the absence of AHL molecules.
444 However, both these mutants are attenuated in pathogenicity so the presence of *cepR* in these
445 mutants may prevent the action of the alternate transcriptional regulator that bypasses the CepIR
446 system.
447 When taken together, the results presented here suggest that *B. cenocepacia* is able to
448 modulate QS-controlled gene expression in the absence of the CepIR QS system through the
449 PAA catabolic pathway. *B. cenocepacia* might use precursors of PAA as carbon sources to
450 support growth in certain environmental conditions. The CepIR QS system has been the most
451 studied QS system in *B. cenocepacia* due to its role in pathogenicity and its position in the

452 hierarchy but our results suggest that the regulation of QS in *B. cenocepacia* is still not fully
453 understood and that an, as yet, unidentified transcriptional regulator may regulate the virulence
454 in the absence of the CepIR QS system. Efforts to identify this putative transcriptional regulator
455 are ongoing. While this work contributes to our knowledge of the role of the PAA pathway in the
456 regulation of QS-related virulence more work is needed to fully understand the complexity that is
457 PAA degradation during *B. cenocepacia* natural interactions with their diverse hosts.
458

459 **Materials and Methods**

460 *Strains, media preparation and growth conditions*

461 Bacterial strains, listed in Table S1, were grown at 37°C with agitation at 230 rpm in
462 Lysogeny Broth (LB) medium, unless specified. All cultures were started from isolated colonies
463 grown 48 hours on LB agar at 37°C, unless specified. Plasmids used are listed in Table S2. For
464 *E. coli* strains the following antibiotics and concentrations were used: Ampicillin (100 µg/ml;
465 200 µg/ml for W14 strain); Chloramphenicol (30µg/ml), Kanamycin (30 µg/ml; 40 µg/ml for
466 MM290 strain), Trimethoprim (50 µg/ml; 100 µg/ml for W14 strain), Tetracycline (20 µg/ml).
467 For *B. cenocepacia* strains the following antibiotics and concentrations were used: Trimethoprim
468 (100 µg/ml), Tetracycline (100 µg/ml), Gentamycin (50 µg/ml). Stock solutions of C8-HSL
469 (Sigma Aldrich), were suspended in dimethyl sulfoxide (DMSO) and stored at -20°C. For assays
470 with exogenous addition of C8-HSL cultures were grown in LB supplemented with 50 mM 3-(*N*-
471 morpholino)propanesulfonic acid (MOPS) to prevent pH-dependent lactonolysis (36). Stock
472 solutions of L-arabinose (Alfa Aesar, Fisher Scientific) and L-rhamnose (Sigma Aldrich) were
473 prepared at a concentration of 20% (w/v) in deionized water and filter sterilized. Stock solutions
474 of phenylalanine and PAA (Sigma Aldrich) were prepared at 100 mM in deionized water, filter

475 sterilized and stored at room temperature. Stock solutions of PAA-CoA (Sigma Aldrich) were
476 prepared at 3.5 mM in DMSO and stored at -80°C.
477
478 *Molecular biology techniques*
479 Genetic manipulation of *B. cenocepacia* K56-2 was performed via tri-parental matings
480 with *E. coli* MM290/pRK2013 as a helper strain. Plasmids generated were maintained in *E. coli*
481 SY327λpir or DH5α Z-competent cells depending on their origin of replication. PCRs were
482 performed with an Eppendorf Mastercycler ep gradient S thermocycler with either HotStar
483 HiFidelity *Taq* polymerase (Qiagen) or *Taq* DNA polymerase (Qiagen). Conditions were
484 optimized for each primer set; primers used are listed in Table S3. Restriction enzymes and T4
485 DNA ligase (New England Biolabs) were used as recommended by the manufacturer. PCR
486 products and digested DNA were purified by QIAquick Gel or PCR purification kits (Qiagen)
487 and plasmids were isolated using QIAprep Miniprep kit (Qiagen).
488
489 *Construction of unmarked deletion mutants in B. cenocepacia*
490 Unmarked deletion mutants were created using the system developed by Flannagan *et al*.
491 (37). Flanking regions of the target gene were synthesized as a custom gene fragment with *Xba*I
492 and *Sma*I restriction sites (Integrated DNA Technologies, Inc), see Table 4 for sequences. The
493 fragment with regions flanking BCAL0404 (*paaK1*) and pGPI-*Sce*I were digested and ligated
494 together to create pTL17 and pTL18 was created by insertion of the fragment with regions
495 flanking BCAM1711 (*paaK2*). Each plasmid was introduced into wild-type K56-2 by tri-parental
496 mating and were integrated into the genome by homologous recombination. Colonies were
497 picked onto LB medium supplemented with trimethoprim and onto *Burkholderia cepacia*

498 selective agar (BCSA). Conjugates with a dull phenotype on BCSA were selected. The
499 integration of the suicide plasmids was confirmed with primers 900/901for pTL17 and primers
500 902/903 for pTL18. Then, pDAI-*Sce*I, expressing I-*Sce*I endonuclease, was introduced into K56-
501 2::pTL17 and K56-2::pTL18 by tri-parental mating. The double-stranded break caused by the I-
502 *Sce*I endonuclease triggered a recombination event that resulted in a reversion of the conjugate to
503 a wild type allele or a deletion of the fragment between the two flanking regions. Potential
504 conjugates were tetracycline resistant and trimethoprim sensitive. The deletion event was
505 confirmed by PCR using primer set 980/981 for *paaK1* and 982/983 for *paaK2*. Positive clones
506 were cured of the pDAI-*Sce*I plasmid by sequential sub-culturing in LB medium. Loss of the
507 plasmid was confirmed by the loss of tetracycline resistance using the replica plate method and
508 PCR with primer set 920/921 for the absence of pDAI-*Sce*I.
509 Once cured, the mutants were tested for growth defects by washing overnight cultures
510 twice in PBS, diluting to an OD_{600} of 0.04 in 200 µl of LB in a clear 96-well plate in triplicate.
511 Plates were incubated at 37 °C in a Biotek Synergy II plate reader with continuous shaking for
512 24 hours with OD_{600} measured every hour. Deletions of *cepI* and *cepR* were performed in the
513 wild type and PAA pathway mutant backgrounds using the pDelCepI and pDelCepR plasmids
514 created by Aubert *et al.* (38). Integration of the plasmids was confirmed using primer set
515 1082/154 for *cepI* deletions and 1080/154 for *cepR*. pDAI-*Sce*I was introduced and potential
516 conjugates were selected on appropriate antibiotics and the deletion event was confirmed by
517 PCR with primer sets 1112/1113 and 1080/1081 for *cepI* and *cepR*, respectively. Positive clones
518 were cured of the pDAI-*Sce*I and screened for the loss of the plasmid as described above.
519
520

521 *Plasmid construction*

522 To create pKF2, *paaK2* (BCAM1711) was amplified from genomic *B. cenocepacia* K56-
523 2 DNA using primers 1055/1056and restricted with *Xba*I and *Nde*I before ligation into similarly
524 digested pSCrhaB2 (39). The plasmid construct was verified using primers 1055/848. To create
525 the *E. coli* reporter system, *cepR* (BCAM1868) was amplified using primers 703/704 and the
526 amplicon and pBAD30 were restricted with *Eco*RI and *Xba*I and then ligated to form pTL5. To
527 construct a plasmid expressing the *luxCDABE* operon, the *cepI* promoter region including the
528 first 15 codons of *cepI* was amplified and an in-frame stop codon was added using primers 712
529 and 713. Plasmid pBBRlux and the amplicon were digested with *Spe*I and *Bam*HI, ligation then
530 resulted in pTL7. Plasmids pTL12 and pTL13 were created in the same way with primers
531 768/769 for the P_{BAD} promoter and regulatory *araC* and primers 770/771 for the *dhfr* promoter
532 region of pTnMod-STp. To create a plasmid that expressed PaaK and was compatible with the
533 reporter strain we had to use the pSC101 ori. pTL9 was created by digesting pTnMod-STp with
534 *Bgl*II to remove the Tn*5* transposase, then the rhamnose inducible promoter and its regulatory
535 genes were added to create pTL21 using primers 724 and 947. Using primers 725 and 726 the
536 *paaK* ligase from *E. coli* W was amplified from pAAD (27) and restriction cloned into pTL21 to
537 create pTL22.

538

539 *Growth of* B. cenocepacia *strains on various carbon sources*

540 One ml of culture from a five ml overnight culture in LB with appropriate antibiotic was
541 washed in PBS twice and the OD_{600} was adjusted to 0.08 in 1 ml of M9 with 25 mM of Glucose
542 or M9 with 5 mM PAA. 100 µl was aliquoted to each well of a clear 96-well plate with 100 µl of
543 appropriate media for a starting OD_{600} of 0.04 in triplicate. Plates were incubated at 37°C with

544 aeration at 230 rpm and the OD_{600} was read after 48 hours of incubation. OD_{600} values were
545 converted to 1-cm-path-length by prior calibration with a GeneQuant™ III 4283, version
546 4283V1.6.
547
548 *Quantification of intracellular and extracellular levels of PAA and PAA-CoA by ion pair*
549 *reversed phase ultra high performance liquid chromatography tandem mass spectrometry (IP-*
550 *RP-UHPLC-MS/MS)*
551 *B. cenocepacia* cultures were diluted 1:100 into 10 ml of LB broth in a 50 ml Erlenmeyer
552 flask and incubated at 37°C with agitation at 230 rpm for 6 hours until late exponential phase.
553 Using the differential method (21, 22), 1 ml of culture was immediately transferred into weighed
554 tubes containing 5 ml of 60% (v/v) methanol in water held at -40°C in an acetonitrile/dry-ice
555 bath, vortexed and returned to -40°C. Two ml of culture was drawn into a syringe, equipped with
556 a 0.45 µm filter, and 1 ml of filtrate was immediately transferred into 5 ml of 60% (v/v)
557 methanol in water held at -40°C in an acetonitrile/dry-ice bath, vortexed and returned to -40°C.
558 Tubes were weighed and 500 µl was transferred to a fresh tube and held at -40°C. 5 ml of 75%
559 (v/v) ethanol was boiled at 95°C for 4 minutes and then poured into the tube with 500 µl of
560 sample, mixed and placed at 95°C for 3 minutes. Extracted samples were then put back at -40°C
561 and evaporated in a speedvac (Savant SC110 with refrigerated condensation trap RT100) until
562 dry. Care was taken to ensure that the samples were not overdried. All measurements were
563 carried out using IP-RP-UHPLC-MS/MS as described previously (23) with slight modifications.
564 Briefly, no splitter or make up flow was used and the chromatographic separation was performed
565 on a narrower column (1x100 mm) with a lower eluant flow (0.075 ml/min).

566 For the *E. coli* W14 CepR reporter assay strains, cultures were diluted 1:100 in 5 ml of
567 LB supplemented with 50 mM MOPS and appropriate antibiotics and grown at 30°C, 230 rpm to
568 OD_{600} ~ 0.3. Cultures were provided 0.5 mM PAA and 0.01% rhamnose to induce the expression
569 of *paaK* to convert PAA to PAA-CoA. Cultures were incubated again until an OD_{600} of 0.6 and
570 diluted back to OD_{600} 0.3 before incubation for 1h30 minutes to simulate the conditions of the
571 reporter assay. Samples were immediately prepared using the differential method described
572 above.
573
574 *Slow killing assays in Caenorhabditis elegans*
575 Worms were propagated and maintained on nematode growth medium (NGMI) seeded
576 with a lawn of *E. coli* OP50 at 16°C. Slow killing assays were performed as described previously
577 (18). Sixty-millimetre Petri plates filled with 10 ml of NGMII were plated with 50 µl of a
578 stationary-phase culture adjusted to an OD_{600} of 1.7. Plates were incubated overnight at 37°C to
579 allow formation of a bacterial lawn. 20 to 40 hypochlorite-synchronized *C. elegans* DH26
580 nematodes at L4 larval stage were inoculated onto each plate and subsequently incubated at 25°C
581 for the duration of the assay. Worms were scored as live or dead for a period of 7 days.
582
583 *Exoprotease assays*
584 Cells from an overnight culture were washed with PBS twice and adjusted to an OD_{600} of
585 1.0. Three microliters of culture were spotted on 2% skim milk agar plates or 2% skim milk agar
586 supplemented with 2 mM phenylalanine. Plates were incubated at 37°C for 48 hours. Plates were
587 prepared in duplicate and three biological replicates were performed. Proteolytic activity was
588 measured as a zone of clearing surrounding the colony. Images were taken at 48 hours and the

589 area of the zone of clearance was analyzed using ImageJ software (40). A scale was applied to
590 the images and a threshold analysis was used to determine the zone of clearance. The area of this
591 zone was then calculated (minus the area of the colony).
592
593 *CepI enzymatic assay*
594 CepI enzyme activity was determined spectrophotometrically by measuring *holo*-ACP
595 formation with dichlorophenylindophenol (DCPIP; = 19100 M-1 cm-1) using the assay
596 described previously (29, 41). Reaction mixtures contained 50 mM 4-(2-hydroxyethyl)-1-
597 piperazineethanesulfonic acid (HEPES) pH 7.5, 0.005% Nonidet P-40, 0.13 mM DCPIP, 70 µM
598 C8-ACP, 4 µM CepI. After 10 minutes of pre-incubation the reaction was initiated by addition of
599 40 µM S-adenosyl methionine. The compounds were assayed in triplicate at a concentration of
600 200 µM.
601
602 *Luminescence assays with CepR reporter strain*
603 The reporter assay was performed based on the assay by McInnis and Blackwell (42)
604 with slight modifications. Briefly, overnight cultures were prepared from glycerol stocks in LB
605 with appropriate antibiotics and incubated overnight at 30°C with agitation at 230 rpm. The
606 culture was diluted 1:100 into 5 ml fresh LB medium supplemented with 50 mM MOPS and
607 appropriate antibiotics. This subculture was incubated at 30°C with shaking at 230 rpm until it
608 reached an optical density of 0.6. One hundred microliters of culture were plated into wells with
609 100 µl of appropriate media with C8-HSL, PAA and arabinose as needed, resulting in a starting
610 OD_{600} of 0.3. For the reporter strain with pTL22 (rha-inducible *paaK*) the culture was grown and
611 subcultured as described above. This subculture was incubated at 30°C with shaking at 230 rpm

612 until it reached an optical density of ~0.3. At this time 0.5 mM PAA and 0.01% rhamnose were
613 added to induce *paaK* expression resulting in the conversion of PAA to PAA-CoA. The cultures
614 were incubated at 30°C, 230 rpm until an OD_{600} of 0.6. One hundred microliters of culture were
615 plated into wells with 100 µl of appropriate media with C8-HSL, PAA, and arabinose and
616 rhamnose as needed, resulting in a starting OD_{600} of 0.3. Arabinose and rhamnose were used at
617 final concentrations of 0.2% (v/v) and 0.01% (v/v), respectively. C8-HSL was used at a final
618 concentration of 20 nM, which was the EC_{50} of C8-HSL for the conditions of this assay as
619 determined using a dose response curve (Supplemental Fig. S1). PAA was tested at several
620 concentrations (100 µM, 500 µM, 1 mM and 5 mM) and PAA was added at 500 µM in the
621 presence of 0.01% rhamnose to produce PAA-CoA. White 96-well microplates with clear
622 bottoms were used and the lids were treated with a TritonX-100/Ethanol solution. Plates were
623 incubated at 30°C with agitation in a Biotek Synergy 2 plate reader. OD_{600} and relative
624 luminescence were measured at 30 min intervals for 4 h. Inhibition was measured at the
625 timepoint with peak signal intensity (2 h for PAA and 3 h for PAA-CoA).
626
627 *Reverse Transcription quantitative PCR (RT-qPCR)*
628 *B. cenocepacia* cells were harvested from stationary cultures (OD_{600} ~7). RNA was
629 purified and DNase treated using the Ribopure bacteria kit (Ambion) with the DNase treatment
630 extended to 2 h. RNA quality was confirmed by running on a 2% agarose gel. cDNA was
631 synthesized using iScript Reverse Transcriptase kit (Bio-Rad) and quantified using iQ SYBR
632 Green mastermix (Bio-Rad) performed on a CFX96 Touch Real-Time PCR Detection System
633 (Bio-Rad). Primer efficiency was calculated for each primer set and efficiencies between 95%
634 and 105% were deemed acceptable. Data were analyzed using the comparative C_T method (43,

635 44). Genes were normalized to the RNA polymerase sigma factor *sigE* (BCAM0918) as a
636 reference gene (primers 750 and 751) (7, 45, 46).
637
638 *LC-MS/MS quantification of AHLs*
639 For AHL extraction, five milliliters of *B. cenocepacia* strains were harvested at the same
640 time as the samples used for RT-qPCR (see above). Samples were extracted and analyzed as
641 described previously (47) and 5,6,7,8-Tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d4) was
642 used as an internal standard at a concentration of 0.2 ppm.
643
644 **Acknowledgements**
645 This study was supported by a discovery grant from the Natural Sciences and Engineering
646 Research Council of Canada (NSERC) awarded to S.T. Cardona. The authors would like to
647 acknowledge the preliminary work of Samuel J. Wolfram, Vincent Henega and Stacey Line. The
648 authors thank Dr. Miguel Valvano for generously sharing plasmids pDelCepR and pDelCepI.
649 pBBRlux was a gift from Dr. Aleksandra Sikora. TJL and STC were responsible for the
650 experimental design and writing the manuscript. TJL and KLF performed the experimental work.
651 CR detected PAA and PAA-CoA using IP-RP-UHPLC-MS/MS. SB and LRC performed the
652 CepI enzyme activity assay. MCG and ED performed the LC-MS/MS and data analysis for AHL
653 quantifiaction. JLS provided knowledge and equipment for the initial detection of PAA by HPLC
654 and edited the manuscript.
655

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786
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788

789 **Supplemental Material Figure Legends**

790 (see Supplemental Material document for Tables and Figures)

791

792 **Table S1. Bacterial strains used in this study**

793 **Table S2. Plasmids used in this study.**

794 **Table S3. Primers used in this study.**

795 **Table S4. Gene fragment sequences flanking the regions of target genes.**

796 **Fig. S1: 0.02 µM (20 nM) is the effective concentration (EC_{50}) of C8-HSL with the CepR reporter**

797 **system.** The CepR reporter system was tested with varying concentrations of *N*-octanoyl homoserine

798 lactone (C8-HSL) to determine the concentration required for half maximal signal. The signal was

799 measured over 4 hours and the time point where the signal was highest (2 hours) was used. Data was

800 graphed and the EC_{50} was calculated using GraphPad Prism. Error bars are standard deviations of two

801 replicates. For the conditions of this reporter system the EC_{50} of C8-HSL is 20 nM.

802

803 **Fig. S2: Growth of *paaK* ligase mutants on M9 with 5 mM PAA was restored by pKF2, a rhamnose-**

804 **inducible *paaK* ligase expression vector.** *B. cenocepacia* strains were grown on M9 with 25 mM of

805 glucose or 5 mM of PAA as a sole carbon source. There was no difference of growth of *B. cenocepacia*

806 K56-2 containing the empty vector (pSCrhaB2) in the presence of 0.01% (v/v) rhamnose on glucose or

807 PAA as a sole carbon source. When the *paaK* ligase expression vector (pKF2) was induced with

808 rhamnose in the K56-2*ΔpaaK1ΔpaaK2* and the K56-2*ΔpaaK2ΔpaaK1* strains growth was restored on

809 PAA. This was not the case for the empty vector controls, indicating that the PAA pathway interruption in

810 this mutant can be complemented with the introduction of BCAM1711 (*paaK2*) in trans. Error bars

811 represent standard deviations of three biological replicates.

812

813 **Fig. S3: PAA does not accumulate intracellularly and PAA-CoA is barely detectable extracellularly.**
814 Cellulare levels of PAA and PAA-CoA were measured with ion pair reversed phase ultra high
815 performance liquid chromatography tandem mass spectrometry (IP-RP-UHPLC-MS/MS)**.A)**
816 PAA levels are very low compared to those measured extracellularly. **B)** PAA-CoA was barely detectable
817 in extracellular samples. Two biological replicates with three technical replicates were performed.
818
819 **Fig. S4: *C. elegans* slow killing assays show that the *paaK* single deletion mutants have wild type**
820 **levels of virulence but the double *paaK* knockout mutants have increased virulence compared to**
821 **wild type in *C. elegans* and exoprotease assays. A)** *ΔpaaK2ΔpaaK1* had slightly increased virulence
822 compared to wild type and was determined as significant according to the log-rank test. **B)** The *ΔpaaK1*
823 and *ΔpaaK2* single deletion mutants have wild type levels virulence in a *C. elegans* model of infection.
824 This figure is one assay representative of at least three independent experiments. **C)** Proteolytic activity
825 was measured as the area of the zone of clearance (excluding colonies) on agar containing 2% skim milk
826 with or without the addition of 2 mM of phenylalanine. The error bars represent the SD of three
827 independent experiments.
828
829 **Fig. S5: Transcription of *cepI* and *cepR* and C8-HSL production is not decreased in any of the PAA**
830 **pathway mutants. A)** RT-qPCR shows that *cepI* and *cepR* gene expression is not decreased compared to
831 wild type in the PAA pathway mutants. Error bars are standard deviations of three biological replicates.
832 **B)** C8-HSL levels were not decreased in any of the mutants, but C8-HSL levels were higher in the
833 Δ*paaABCDE* and Δ*paaK2*Δ*paaK1* mutants. The bar is the mean of three biological replicates. Virulence
834 is for *C. elegans* slow killing assays and exoprotease assays as seen in Fig. 3.
835

836 **Fig. S6: Growth of QS mutants in the PAA pathway mutants show no growth defects in LB.**
837 Mutants were grown in LB from a starting OD_{600} of 0.04 over 24 hours and showed no significant growth
838 defects compared to wild type. Error bars are standard deviation of three biological replicates.
839

840 **Fig. S7: The virulence of Δ*paaK2*Δ*paaK1*Δ*cepR* is independent of the CepIR QS system. A)**
841 *cepI* and *cepR* deletions in wild type and Δ*paaABCDE* mutant backgrounds are attenuated for virulence in
842 *C. elegans*. The Δ*paaABCDE* mutant is attenuated compared to wild type but is less attenuated than *cepI*
843 and *cepR* mutants. **B)** As seen with the Δ*paaK1*Δ*paaK2*Δ*cepR* mutant, the *paaK2*Δ*paaK1*Δ*cepR* mutant
844 displays CepR-independent virulence in *C. elegans*. A *cepI* deletion in the Δ*paaK2*Δ*paaK1* mutant is
845 attenuated similar to the *cepI* and *cepR* mutants in wild type backgrounds. **C)** The Δ*paaK2*Δ*paaK1*Δ*cepR*
846 mutant has wild type levels of protease activity with or without phenylalanine whereas the Δ*cepR* mutant
847 has decreased protease activity. Protease activity was measured as the area of the zone of clearance
848 (excluding colonies) on agar containing 2% skim milk with or without the addition of 2 mM of
849 phenylalanine. **D)** *cepR* transcription is restored to wild type levels in Δ*paaABCDE*Δ*cepI* and
850 Δ*paaK2*Δ*paaK1*Δ*cepI* indicating that PAA increases *cepI* transcription. **E)** *cepI* is downregulated in all of
851 the Δ*cepR* mutants. **F)** C8-HSL levels were measured and none of the QS mutants had detectable levels of
852 C8-HSL. Experiments were performed in triplicate. The error bars represent the SD of three independent
853 experiments.