



3-Hydroxyphenylacetic acid induces the *Burkholderia cenocepacia* phenylacetic acid degradation pathway – toward understanding the contribution of aromatic catabolism to pathogenesis

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The phenylacetic acid (PA) degradative pathway is the central pathway by which various aromatic compounds (e.g., styrene) are degraded. Upper pathways for different aromatic compounds converge at common intermediate phenylacetyl-CoA (PA-CoA), which is then metabolized to succinyl-CoA and acetyl-CoA. We previously made a link in *Burkholderia cenocepacia* between PA degradation and virulence by showing that insertional mutagenesis of *paaA* and *paaE* genes, that encode part of a multicomponent oxidase of PA-CoA, results in PA-conditional growth and an attenuated killing phenotype in the *Caenorhabditis elegans* model of infection. However, insertional mutagenesis of *paaK1*, which encodes a phenylacetate-CoA ligase, did not result in a PA-conditional growth probably due to the presence of a putative paralog gene *paaK2*. Recently published crystallographic and enzyme kinetics data comparing the two PaaK ligases showed that PaaK1 is more active than PaaK2 and that the larger binding pocket of PaaK1 can accommodate hydroxylated PA derived molecules such as 3-hydroxyphenylacetic (3-OHPA) acid and 4-hydroxyphenylacetic acid (4-OHPA). The higher activity and broader substrate specificity suggested a more active role in pathogenesis. In this work, we aimed to determine the relevance of PaaK1 activity to the killing ability of *B. cenocepacia* to *C. elegans*. Using reporter activity assays, we demonstrate that 3-OHPA activated PA degradation gene promoters of *Burkholderia cenocepacia* K56-2 in a *paaK1*-dependent manner, while 4-OHPA had no effect. We compared the pathogenicity of a *paaK1* deletion mutant with that of the wild type in *C. elegans* and observed no differences in the killing ability of the strains. Taken together, these studies suggest that 3-OHPA, but not 4-OHPA, can induce the PA pathway and that this induction is dependent on the *paaK1* gene. However, the more active PaaK1 does not play a distinct role in pathogenesis of *B. cenocepacia* as previously suggested.

Keywords: *Burkholderia cepacia*, 3-hydroxyphenylacetic, phenylacetic acid, PA-CoA, CoA ligase, gene reporter

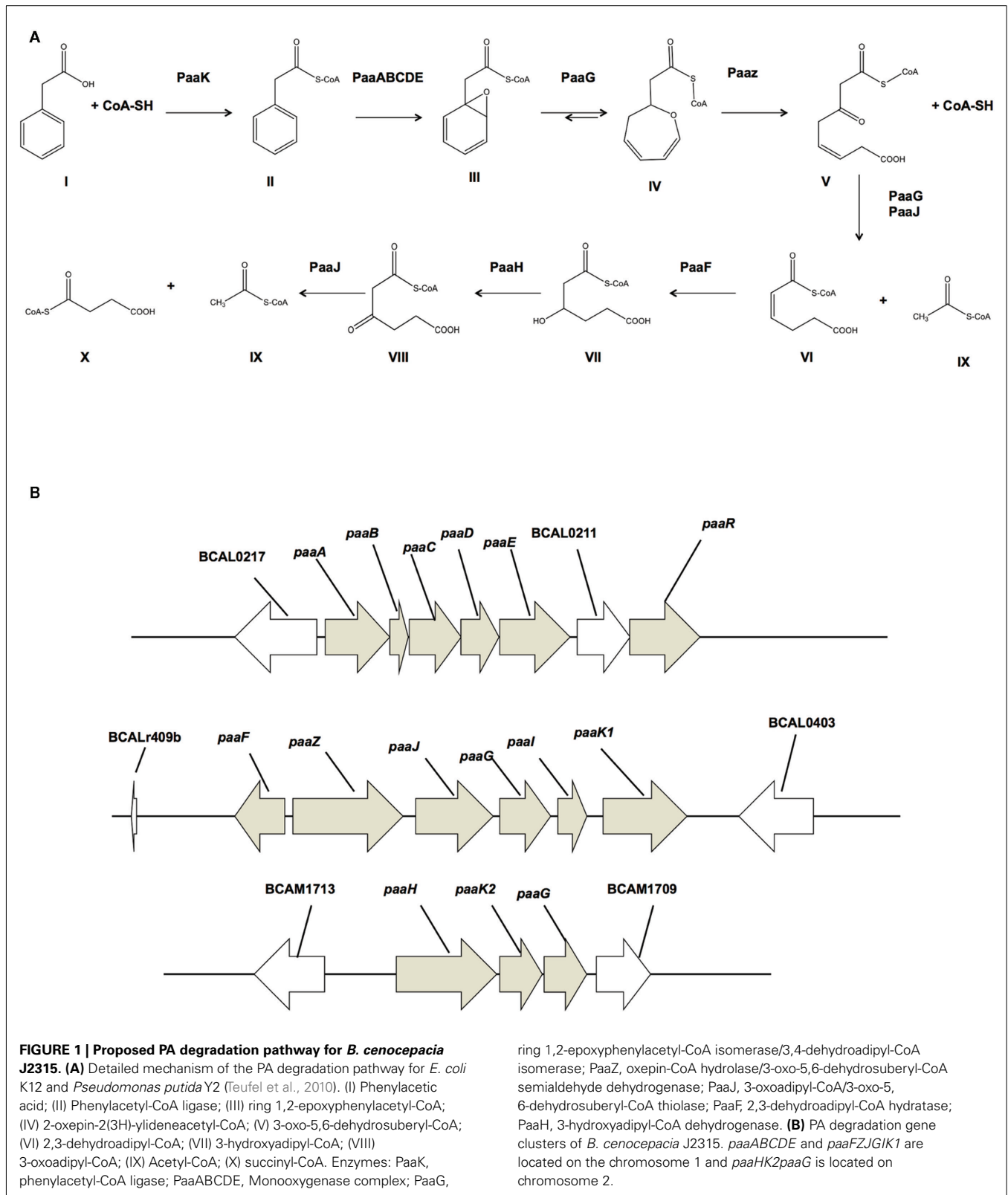
INTRODUCTION

The phenylacetic acid (PA) degradative pathway is a well-conserved, central pathway in bacteria utilized in the breakdown of many aromatic compounds such as styrene and phenylalanine (Luengo et al., 2001). A unique feature of the PA degradation pathway is that it exploits CoA activation of PA in aerobic conditions. Thus, intermediates are processed as CoA thioesters that are finally broken down into the TCA intermediates, acetyl-CoA, and succinyl-CoA.

The detailed mechanism of the PA degradation pathway was recently elucidated for *E. coli* K12 and *Pseudomonas putida* Y2 (Teufel et al., 2010; Figure 1A). The initial step is crucial as it consists of the activation of PA by CoA mediated by the phenylacetate-CoA ligase PaaK (Martinez-Blanco et al., 1990; El-Said Mohamed, 2000). In *Burkholderia cenocepacia*, the PA degradation genes are organized in three clusters across two of three chromosomes (Figure 1B). There are two similar yet non-identical genes that

encode phenylacetate-CoA ligases (Law et al., 2008). Each gene (BCAL0404 or *paaK1* and BCAM1711 or *paaK2*) is located on chromosome 1 and chromosome 2 respectively. Recently, Law and Boulanger (2011) provided a rationale for the presence of the two-paralog PA-CoA ligases of *B. cenocepacia* by *in vitro* studies, showing that PaaK1 has a much higher affinity for PA than PaaK2 with K_m values of 62 and 150 μ M, respectively. A striking difference was that PaaK1 was able to accommodate 3' and 4' substituted PA due to a larger binding pocket. Although both enzymes were able to accept 3-OHPA, PaaK1 showed 6.5-fold greater affinity for 3-OHPA than PaaK2. PaaK1 could also use 4-OHPA as substrate, while the activity of PaaK2 with 4-OHPA was undetectable (Law and Boulanger, 2011).

We previously demonstrated that the *B. cenocepacia* K56-2 catabolic pathway for phenylacetic acid (PA) degradation is a requirement for full pathogenesis of *B. cenocepacia* (Law et al., 2008) in the *Caenorhabditis elegans* model of infection (Aballay and Ausubel,



2002) but the role of the PaaK enzymes in pathogenicity and the biological relevance of 3-OHPA and 4-OHPA as substrates was not addressed. With the benefit of reporter activity assays and *C.*

elegans as a host model organism, we aimed to address the relevance of the more active and substrate-promiscuous PaaK1 for pathogenesis of *B. cenocepacia* against *C. elegans*. We demonstrate

that a hydroxylated PA derived molecule such as 3-OHPA can induce the PA degradative pathway and that this induction is *paaK1*-dependent. A clean deletion of *paaK1* resulted however, in wild type-like pathogenicity of *B. cenocepacia* in *C. elegans*.

MATERIALS AND METHODS

BACTERIAL STRAINS, NEMATODES, AND GROWTH CONDITIONS

Bacterial strains and plasmids are listed in **Table 1**. *B. cenocepacia* strains were grown at 37°C in Luria Bertani (LB), M9 media, or modified nematode growth media (mNGM; 3 g L⁻¹ NaCl, 17 g L⁻¹ bacto-agar, 3.5 g L⁻¹ peptone, 1 mL 5 mg mL⁻¹ cholesterol, 1 mL 1 M CaCl₂, 1 mL 1 M MgSO₄, 25 mL phosphate buffer) supplemented, as required, with 100 μg/mL trimethoprim (Tp), and 200 μg/mL chloramphenicol (Cm). Unless indicated otherwise, glycerol and phenylacetic acid were added at 0.1% w/v and 5 mM, respectively. The nematode *C. elegans*, strain DH26 and *E. coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota, Minneapolis. *C. elegans* strains were maintained on nematode growth media (NGM) according to standard practices at the CGC.

MOLECULAR BIOLOGY TECHNIQUES

E. coli DH5α cells were transformed using the calcium chloride protocol (Cohen et al., 1972) and electroporation was used for transformation of *E. coli* SY327 cells (Miller and Mekalanos, 1988). Conjugation into *B. cenocepacia* K56-2, and IAI1 was accomplished by tri-parental mating (Craig et al., 1989) with *E. coli* DH5α carrying the helper plasmid pRK2013 (Figurski and Helinski, 1979). DNA was amplified using an Eppendorf Mastercycler ep gradient S thermal cycler with Taq DNA polymerase PCR Kit (Qiagen). Amplification conditions were optimized for each primer

pair. PCR products and plasmids were purified with QIAquick purification kit (Qiagen) and QIAprep Miniprep kit (Qiagen), respectively.

CONSTRUCTION OF *paaK1* DELETION MUTANT IAI1

IAI1 (*paaK1*) was created using the techniques outlined in Flanagan et al. (2008) using *B. cenocepacia* wild type K56-2. Briefly, introduction of plasmid pRL5 (**Table 1**), which contains the flanking regions of *paaK1* as well as a yeast I-SceI restriction site (TAGGGATAACAGGGTAAT) was performed by tri-parental mating. The first homologous recombination event and the recombination site were confirmed by colony PCR with primer pairs 154/165 or 153/164 (**Table 2**), which anneal to the plasmid and the genome, respectively. Following homologous recombination a second plasmid pDAI-SceI, which constitutively expresses the yeast homing endonuclease I-SceI, was introduced by tri-parental mating. Resulting clones were screened for trimethoprim sensitivity and tetracycline resistance by replica plating on LB containing 100 μg/mL of tetracycline with and without 100 μg/mL of trimethoprim. Subsequently, trimethoprim sensitive clones were screened for the deletion of *paaK1* with primers 166 and 167 (**Table 2**). Following confirmation by colony PCR that the gene had been deleted, pDAI-SceI was cured by serial dilution in LB broth. Loss of the plasmid was confirmed by colony PCR using primers 3 and 4, which anneal external to the multi-cloning site of pDAI-SceI. Deletion of the *paaK1* in *B. cenocepacia* IAI1 gene was further confirmed by sequencing.

BACTERIAL GROWTH

One-milliliter aliquots of M9 plus different carbon sources were inoculated with 100 μL from overnight culture grown in LB,

Table 1 | Bacterial strains and plasmids.

Strain or plasmid	Features	Reference or source
E. COLI STRAINS		
DH5α	F_φ80 <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> (r _K -m _K +) <i>supE44 thi-1Δ gyrA96</i> (Δ <i>lacZYAarg-F</i>) <i>U169 relA1</i>	Invitrogen
SY327	<i>A(lac pro) argE(Am) rif 20 nalA recA56</i>	Miller and Mekalanos (1988)
MM290	Host strain for pRK2013	Izard et al. (1992)
B. CENOCEPACIA STRAINS		
K56-2	ET12 clone related to J2315, CF clinical isolate	Mahenthalingam et al. (2000)
IAI1	K56-2Δ <i>paaK1</i>	This study
STC179 (<i>paaA</i>)	K56-2::pGPomegaTp <i>paaA</i> , Tp ^r	Law et al. (2008)
STC155 (<i>paaE</i>)	K56-2::pGPomegaTp <i>paaE</i> , Tp ^r	Law et al. (2008)
PLASMIDS		
pRL5	pGPISceI, <i>paaK1</i> flanking regions	Law (2009)
pJH1	<i>DHFR</i> promoter controlling <i>e-gfp</i>	Hamlin et al. (2009)
pJH2	Promoterless <i>e-gfp</i>	Hamlin et al. (2009)
pJH6	<i>paaZ</i> promoter controlling <i>e-gfp</i>	Hamlin et al. (2009)
pJH7	<i>paaA</i> promoter controlling <i>e-gfp</i>	Hamlin et al. (2009)
pJH8	<i>paaH</i> promoter controlling <i>e-gfp</i>	Hamlin et al. (2009)
pDAI-SceI	Constitutive expression vector, <i>ori</i> _{colE1} , <i>DHFR</i> promoter, Tc ^r , I-SceI	Aubert et al. (2008)
pRK2013	<i>ori</i> _{colE1} , RK2 derivative, Km ^r <i>mob</i> ⁺ <i>tra</i> ⁺	Figurski and Helinski (1979)

Cm, chloramphenicol; Tp, trimethoprim; CF, cystic fibrosis.

washed with PBS, and adjusted to an optical density at 600 nm (OD₆₀₀) of 2.0. One hundred fifty microliters aliquots were added in triplicate to 96-well format plates and the microplates were incubated 24 h at 37°C with shaking at 210 rpm. Growth was measured as the OD₆₀₀ was measured with a BioTek Synergy 2 plate reader at hourly intervals.

NEMATODE INFECTION ASSAYS

Caenorhabditis elegans slow killing assays were performed as previously described (Law et al., 2008). Thirty 5 mm plates filled with mNGM were seeded with 50 µL of stationary phase culture adjusted to an OD₆₀₀ of 1.7. In these growth conditions, the PA degradation pathway is activated. Plates were incubated overnight at 37°C to allow growth of a bacterial lawn before 30–40 hypochlorite-synchronized L4 larvae of *C. elegans* DH26 were inoculated to each plate and plates were then incubated at 25°C for the duration of the experiment. Plates were scored for live worms at the time of inoculation and every 24 h subsequently for 7 days using a stereo-master dissecting microscope. Worms were considered dead when unresponsive to touch with a sterile wire pick. Assays were performed in triplicate and analyzed using survival curves generated by the Kaplan–Meier statistical method. The log rank test was used to compare survival differences for statistical significance using GraphPad Prism, version 5.02. A *P* value <0.05 was considered to be significant.

REPORTER ACTIVITY ASSAYS

Green Fluorescent Protein fluorescence was measured according to its excitation/emission wavelengths of 488/509 (Cormack et al., 1996). Relative fluorescence, defined as the ratio between arbitrary fluorescence and OD₆₀₀ was measured with a BioTek Synergy 2 plate reader, using excitation 485/20 and emission 528/20 filter sets.

Table 2 | Primers.

Name	Oligonucleotide sequence	Purpose
SC003	GGCGTAGAGGATCTGCTCATGTTTG	External to MCS in pDAI- <i>Scel</i> , forward
SC004	GCTACTGCCGCCAGGCCAAATTCTGT	External to MCS in pDAI- <i>Scel</i> , reverse
SC137	CCAGT <u>TCTAGAC</u> GTTCGAGCAGTTTCATCCA	<i>paaK1</i> upstream forward
SC140	CATTGATATCGAGGCGTTCTTCTCTGCA	<i>paaK1</i> downstream reverse
SC153	GTGGATGACCTTTTGAATGACCTTT	External to MCS in pRL5 forward
SC154	ACAGGAACACTTAACGGCTGACATG	External to MCS in pRL5, reverse
SC166	GCGTGCAACTCGTACAACCTGAGTG	<i>paaK1</i> upstream forward
SC167	CGGATGTGGCTGCTGTTCCCGAATT	<i>paaK1</i> downstream reverse
SC172	GAGACA <u>CATATGACT</u> ACCCCGCTACCGCT	<i>paaK1</i> 5'
SC173	AACGTCTAGAT <u>CAGCC</u> CTTGCGCTTGTCGA	<i>paaK1</i> 3'

Restriction sites are underlined.

RESULTS

BURKHOLDERIA CENOCEPACIA UTILIZES 3-OHPA AND 4-OHPA AS SOLE CARBON SOURCES, BUT NOT EXCLUSIVELY THROUGH PA DEGRADATION

To address the biological relevance of 3-OHPA and 4-OHPA as substrates of the PaaK enzymes, we started out by asking if *B. cenocepacia* could use 3-OHPA and 4-OHPA as sole carbon sources. Given the evidence that 3-OHPA and 4-OHPA can be substrates of the PaaK enzymes (Law and Boulanger, 2011) we also considered that these compounds could be funneled exclusively through the PA catabolic pathway. *B. cenocepacia* K56-2 was grown in 3-OHPA or 4-OHPA as sole carbon sources and growth was recorded as final optical density. The hydroxylated phenolic acids supported 80 and 54% growth related to growth in glucose, respectively while 3-OHPA and 4-OHPA supported growth to 94 and 75% respectively, in comparison to growth in PA (Figure 2). Similarly, the *paaK1* deletion mutant IA11 did not show any growth-defective phenotype in PA according to the presence of a second *paaK* gene (*paaK2*). Moreover, deletion of the *paaK1* gene did not affect the ability of this strain to use 3-OHPA or 4-OHPA (87% and 80% growth related to glucose, respectively). Concomitantly, when the PA degradation defective mutants *PaaA* and *PaaE* were grown with 3-OHPA, 4-OHPA, or PA the growth-defective phenotype was observed only in the presence of PA (3.4 and 4% growth, respectively) while both mutants exhibited wild type-like growth in the presence of the hydroxylated phenolic acids. This suggests that 3-OHPA and 4-OHPA are degraded through other routes, possibly the homogentisate and homoprotocatechuate pathways (Méndez et al., 2011). Taken together, these results suggest that 3-OHPA and 4-OHPA can serve as carbon sources for *B. cenocepacia* and that these compounds do not seem to be degraded exclusively through the PA degradative pathway.

3-OHPA BUT NOT 4-OHPA INDUCES THE PA DEGRADATIVE PATHWAY

We reasoned that 3-OHPA and 4-OHPA, as substrates for PaaK1, could induce the PA degradative pathway in *Burkholderia*

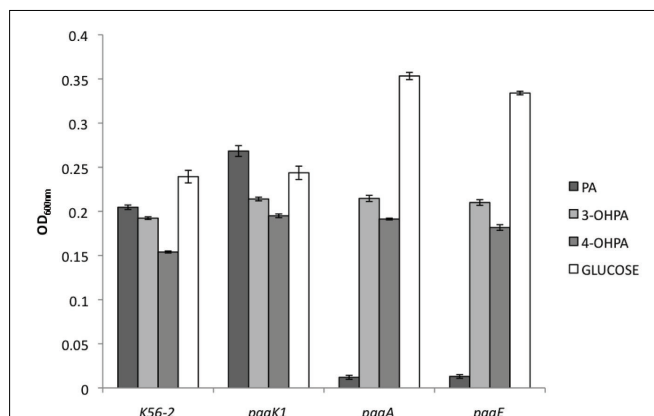


FIGURE 2 | Growth of *B. cenocepacia* strains with 3-OHPA, 4-OHPA as sole carbon sources. *B. cenocepacia* K56-2 (wild type), deletion mutant IA11 (*paaK1*), and insertional mutants STC179, (*paaA*) and STC155 (*paaE*) were grown for 24 h in M9 media supplemented with 5 mM PA, 3-OHPA, 4-OHPA, or 0.2% glucose as sole carbon sources. Error bars represent the SD of three replicates.

cenocepacia. In order to test this, we utilized a PA pathway induction reporter assay previously developed in our laboratory (Hamlin et al., 2009). The translational reporter consists of a plasmid with the enhanced green fluorescent protein gene (*e-gfp*) controlled by the *paa* promoter for each of the three *paa* gene clusters of the *B. cenocepacia* *paa* operons that start with the *paaZ*, *paaA*, and *paaH* genes. Growth of a strain carrying the reporter plasmid in minimal media with PA results in expression of eGFP, which can be measured by fluorescence as an indication of the induction of the PA pathway. Plasmids pJH1 and pJH2 carry *e-gfp* under control of the constitutive expression promoter *DHFR* and a promoterless *e-gfp*, respectively.

Burkholderia cenocepacia K56-2 reporter strains were grown in M9 minimal media with glycerol supplemented with 5 mM PA, 3-OHPA, or 4-OHPA (Figure 3). After 24 h of incubation at 37°C the relative fluorescence of wild type and reporter strains was analyzed. Low levels of background fluorescence were observed for *B. cenocepacia* K56-2 wild type and the reporter strain carrying a promoterless *e-gfp* gene. On the contrary, when *B. cenocepacia*/pJH1 was grown with glycerol or glycerol plus phenolic acids, increased fluorescence was observed. The levels of relative fluorescence due to PA, 3-OHPA, or 4-OHPA were comparable suggesting that there are no metabolic effects of these compounds on plasmid copy number. When PA pathway reporter strains were grown in the presence of PA, an increase in relative fluorescence was observed as previously described. However, only 3-OHPA (but not 4-OHPA) was capable of inducing the *paaZ*, *paaA*, and *paaH* promoters (Figure 2). Across the board, we noted an average of five times less relative fluorescence in media containing 4-OHPA than in media containing PA. Interestingly, *B. cenocepacia* K56-2/pJH6 registered a twofold increase in relative fluorescence for 3-OHPA in comparison with PA (7389 and 4281, respectively). However, this was not the case for *B. cenocepacia* K56-2 harboring pJH7 or pJH8. These strains showed slightly higher relative fluorescence in response to PA in comparison with 3-OHPA (11206 and 9966 units, respectively for pJH7 and 4454 and 3502 units, respectively for pJH8). For all carbon sources tested, pJH7 registered a phenotype characterized with strongest levels of relative fluorescence compared to the other reporter strains, as described previously (Hamlin et al., 2009).

INDUCTION OF PA DEGRADATION BY 3-OHPA IS DEPENDENT ON THE *paaK1* GENE

To shed light on the role of *paaK1* gene in the induction of PA degradation by 3-OHPA, we constructed a *B. cenocepacia* K56-2 *paaK1* deletion mutant, IAI1 and introduced the reporter plasmid pJH7 in this strain. Next, we compared the relative fluorescence of the reporter system in the *paaK1* genetic background to the one in the wild type upon induction by 3-OHPA. Figure 4 shows an increase in relative fluorescence in the presence of PA in both wild type and IAI1 reporter strains. This is expected due to the presence of at least one functioning *paaK* gene. It is probable then, that the PaaK2 enzyme is able to synthesize PA-CoA, which is necessary to activate the *paa* promoters (Yudistira et al., 2011). Nevertheless, compared to K56-2/pJH7, IAI1/pJH7 exhibited 10% less fluorescence possibly due to the presence of two functioning ligases in the wild type variant compared to one in the mutant. K56-2/pJH7

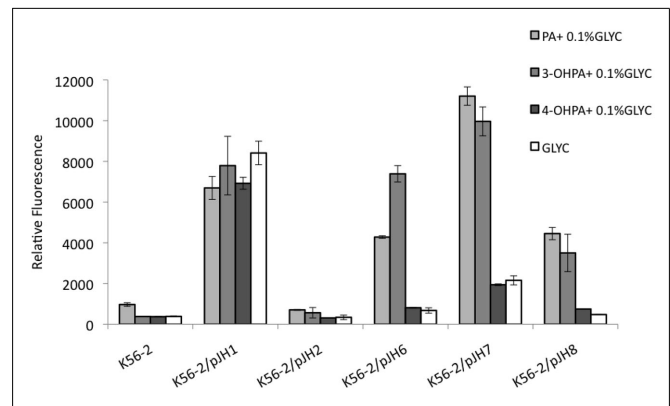


FIGURE 3 | *B. cenocepacia* K56-2 reporter activity assays. PA pathway induction in *B. cenocepacia* K56-2 (wild type) measured as relative fluorescence at 12 h. Strains were grown in M9 minimal media with PA, 3-OHPA, or 4-OHPA (plus 0.1% glycerol) or 0.2% glycerol. pJH7, *paaA* promoter reporter; pJH8, *paaH* promoter reporter; pJH6, *paaZ* promoter reporter; pJH1, positive control; pJH2, negative control. Error bars represent the SD of three replicates.

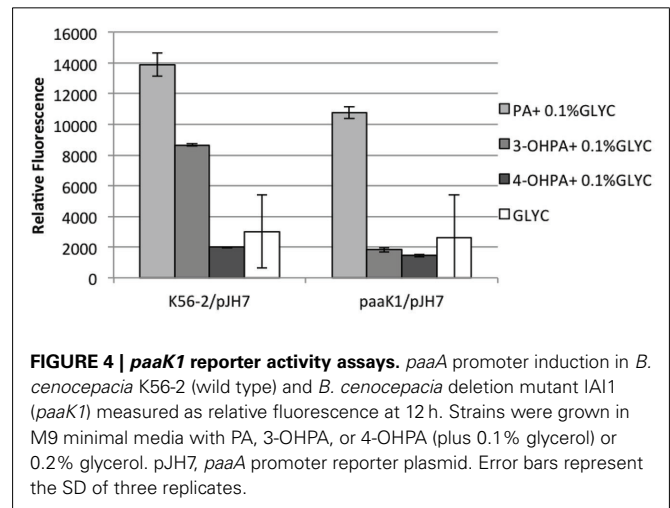


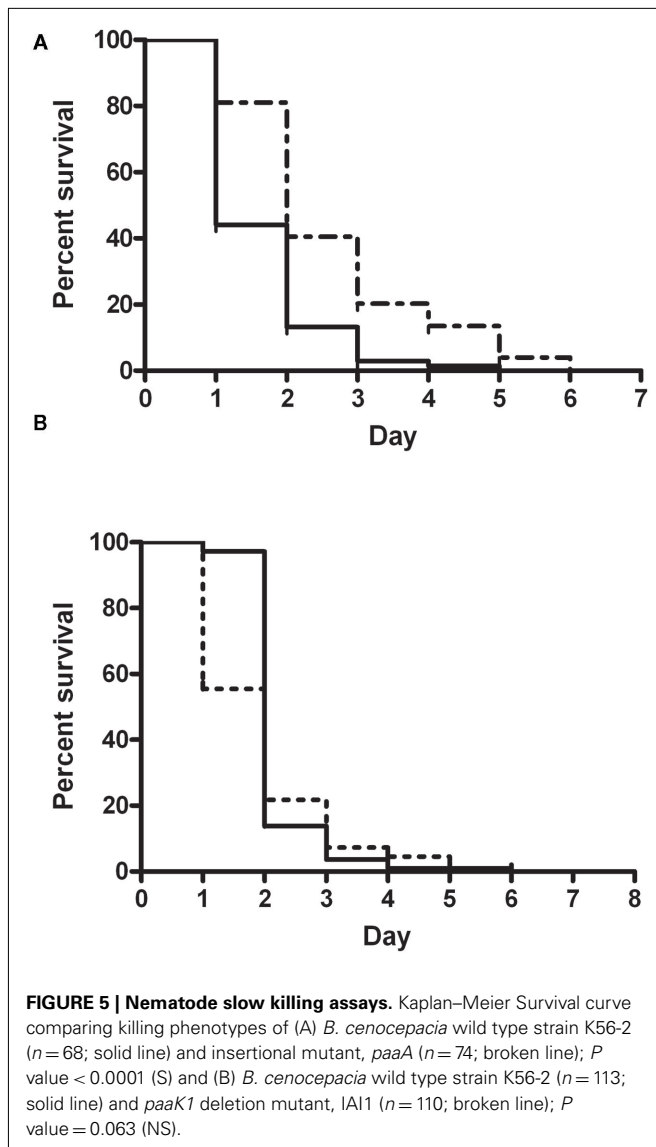
FIGURE 4 | *paaK1* reporter activity assays. *paaA* promoter induction in *B. cenocepacia* K56-2 (wild type) and *B. cenocepacia* deletion mutant IAI1 (*paaK1*) measured as relative fluorescence at 12 h. Strains were grown in M9 minimal media with PA, 3-OHPA, or 4-OHPA (plus 0.1% glycerol) or 0.2% glycerol. pJH7, *paaA* promoter reporter plasmid. Error bars represent the SD of three replicates.

registered induction of the PA degradative pathway in 3-OHPA, (but not 4-OHPA) converse to IAI1/pJH7, which did not for either carbon source. We can therefore deduce that induction of the PA degradative pathway by 3-OHPA is dependent on the presence of PaaK1 and not PaaK2.

PaaK1 DOES NOT PLAY A ROLE IN *B. CENOCEPACIA* PATHOGENICITY

Previous preliminary work done by Law (2009) in our laboratory had suggested that a *paaK1* deletion mutant in *B. cenocepacia* K56-2 (RJL1) resulted in an attenuated phenotype in *C. elegans*. However, introduction of a *paaK1* gene *in trans* in this strain did not restore the phenotype to wild type levels (data not shown) suggesting that the attenuated phenotype was not related to *paaK1* but to a secondary site mutation.

In order to finally elucidate the role of PaaK1 in pathogenicity, we conducted slow killing assays with the *paaA* strain as a control of attenuated pathogenicity (Figure 5A) and with the newly



constructed deletion mutant IA11 (Figure 5B). Survival of the worms exposed to the wild type strain, K56-2, registered a sharp drop by more than 75% on day 2 of the assay with complete loss of survival at day 4. For worms infected with IA11, a 50% drop in survival occurred on day 1 followed by a wild type-like decline in survival in subsequent days. Overall, we found no significant

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difference (P value = 0.063) in pathogenicity between wild type and *paaK1* mutant. This finding supports a strong likelihood that PaaK1 may not play a role in PA pathway related killing.

DISCUSSION

The premise that PaaK1, the activating enzyme of the PA pathway, can also bind 3-OHPA and 4-OHPA (Law and Boulanger, 2011) suggests that these phenolic acids may also be involved in inducing the pathway particularly 3-OHPA which binds PaaK1 with a lower K_m than 4-OHPA. We supported this assertion with the results from our reporter activity assays showing PA pathway induction in minimal media containing 3-OHPA. Of particularly note is the observation that the strain harboring reporter plasmid (pJH6), featuring the *paaZ* promoter, displays double the strength of induction in 3-OHPA than in PA unlike the other two reporter plasmids (pJH7 and pJH8), which register only slightly stronger induction in PA. This is noteworthy because *paaK1* is downstream of and under the control of the *paaZ* promoter. More interestingly, the gene upstream of *paaK1* is the *paaI* gene, which encodes a hotdog thioesterase PaaI that preferentially binds 3-OHPA-CoA and 4-OHPA-CoA as substrates compared to PA-CoA (Song and Ko, 2007).

In toto, our results show that 3-OHPA induces the PA degradative pathway whereas 4-OHPA does not and that this induction is dependent on the presence of a functional *paaK1* gene. Thus, we provide *in vivo* evidence for the role of 3-OHPA as a substrate of PaaK1 in *Burkholderia cenocepacia*, while 4-OHPA is likely not physiologically relevant as a substrate of PaaK1.

The basis of this study was preliminary data showing that a deletion of the *paaK1* gene in *B. cenocepacia* RJL1 resulted in an attenuated killing phenotype in the nematode host model (Law, 2009). According to this finding, Law and Boulanger (2011) suggested that the more active, and substrate-versatile PaaK1 might be more beneficial during infection. However, our results show that PaaK1 (and possibly 3-OHPA as a PA degradation inducer) does not play a significant role in pathogenicity.

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