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Phenylalanine induces *Burkholderia cenocepacia* phenylacetic acid catabolism through degradation to phenylacetyl-CoA in synthetic cystic fibrosis sputum medium

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ABSTRACT

Synthetic cystic fibrosis sputum medium (SCFM) is rich in amino acids and supports robust growth of *Burkholderia cenocepacia*, a member of the *Burkholderia cepacia* complex (Bcc). Previous work demonstrated that *B. cenocepacia* phenylacetic acid (PA) catabolic genes are up-regulated during growth in SCFM and are required for full virulence in a *Caenorhabditis elegans* host model. In this work, we investigated the role of phenylalanine, one of the aromatic amino acids present in SCFM, as an inducer of the PA catabolic pathway. Phenylalanine degradation intermediates were used as sole carbon sources for growth and gene reporter experiments. In addition to phenylalanine and PA, phenylethylamine, phenylpyruvate, and 2-phenylacetamide were usable as sole carbon sources by wild type *B. cenocepacia* K56-2, but not by a PA catabolism-defective mutant. EMSA analysis showed that the binding of PaaR, the negative regulator protein of *B. cenocepacia* PA catabolism, to PA regulatory DNA could only be relieved by phenylacetyl-Coenzyme A (PA-CoA), but not by any of the putative phenylalanine degradation intermediates. Taken together, our results show that in *B. cenocepacia*, phenylalanine is catabolized to PA and induces PA catabolism through PA activation to PA-CoA. Thus, PaaR shares the same inducer with PaaX, the regulator of PA catabolism in *Escherichia coli*, despite belonging to a different protein family.

1. Introduction

The *Burkholderia cepacia* complex (Bcc) comprises a group of at least fifteen taxonomically related species of extremely versatile Gram-negative bacteria [1,2]. Initially exploited for biocontrol and bioremediation, Bcc strains are now well known for their capacity to cause infections in patients with the genetic disease cystic fibrosis (CF) [3–5]. Bcc has evolved large genomes that allow them to deal with a variety of nutrient sources, predation, and competition. The three chromosomes of *B. cenocepacia*, one of the most common Bcc species found in CF patients [6], encode a broad array of catabolic functions, many of them seemingly redundant. Yet, the contribution of these metabolic capacities to *B. cenocepacia*'s ability to colonize and grow in its host is unclear.

The particular environment of the CF lung may influence the first events leading to bacterial colonization and growth. The CF airways are characterized by impaired ion transport across the lung epithelia, poor hydration [7], and a significant increase in sputum amino acid levels [8]. Recent progress in mimicking the nutritional components of CF sputum in the laboratory, through the use of a synthetic CF sputum medium (SCFM) [9], has shown that the nutrient composition of CF sputum induces a transcriptional response in *Pseudomonas aeruginosa* [9] and *B. cenocepacia* [10]. In particular, the global gene expression profile of *B. cenocepacia* during growth in SCFM showed that several genes encoding phenylacetic acid (PA) degradation enzymes [11–13] were highly induced [10]. Although the physiological significance of such activation remains elusive, a link between PA degradation and pathogenicity is emerging. Our laboratory recently demonstrated that interruption of different steps of the *B. cenocepacia* PA catabolic pathway produces diverse pathogenic phenotypes [14], and that the promoter of *B. cenocepacia paaA* gene is activated during growth in SCFM and in the presence of phenylalanine, one of the aromatic amino acids of SCFM [15]. In this work, we investigated the role of phenylalanine, as an inducer of the PA catabolic pathway. We show that phenylalanine, and putative intermediates of

Abbreviations: SCFM, synthetic cystic fibrosis sputum medium; PA, phenylacetic acid; PA-CoA, phenylacetyl-Coenzyme A; Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; EMSA, electrophoretic mobility shift assay.

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phenylalanine catabolism, mediate activation of PA catabolism through degradation to PA-CoA, which, in turn, binds to the TetR-like negative regulator, PaaR [15], releasing the interaction between the regulatory protein and promoters.

2. Results

2.1. Phenylalanine activates PA catabolism reporter systems during growth in SCFM

The PA catabolic pathway is a central route through which the catabolism of many aromatic compounds, such as styrene, phenylethylamine and poly-hydroxyphenyl alkanoates converge, and are directed to the TCA cycle [12,16]. In *Pseudomonas* sp. strain Y2, styrene degradation and PA degradation are co-regulated [17,18] through the action of PaaX, a negative regulator of the GntR family [19,20]. Because the only aromatic compounds of the SCFM are the amino acids phenylalanine, tyrosine, and tryptophan, we sought to investigate the possible co-regulation of aromatic amino acid degradation and PA catabolism. To find out if the aromatic amino acids present in SCFM activated the expression of the PA catabolic pathway, we conducted gene reporter analyses with the translational reporter plasmids pJH6, pJH7 and pJH8 [15], that contain the enhanced green fluorescent protein gene (*eGFP*) [21] under control of P_{paaA}, P_{paaH} and P_{paaZ}, the promoters of *paaA*, *paaH* and *paaZ*, respectively. When bacteria were grown in MOPS minimal medium containing glycerol plus each of the aromatic amino acids at the same concentration found in SCFM (Fig. 1) phenylalanine was capable of activating the *paaA*, *paaH* and *paaZ* reporter systems. As previously noted [15], the *paaA* reporter strain showed the strongest levels of relative fluorescence (Fig. 1 top right panel), while eGFP expression driven by P_{paaH} and P_{paaZ} was lower (Fig. 1 bottom left and right panels, respectively). Relative fluorescence of the reporter strains grown in the presence of tyrosine or tryptophan was comparable to the one of strains grown with glycerol only, and to background fluorescence of *B. cenocepacia* K56-2 cells (Fig. 3 top left panel). Only the *paaA* reporter strain showed an increase in relative fluorescence in the presence of tyrosine (Fig. 1 top right panel) although this activation was negligible in comparison with the strongest signal due to phenylalanine. Relative fluorescence of the reporter strains grown in the presence of tryptophan was comparable to that of the strains grown with glycerol only, and to background fluorescence of *B. cenocepacia* K56-2 cells (Fig. 3 top left panel).

To further confirm that phenylalanine was the major inducer of PA catabolism during growth in SCFM, the reporter strains were grown in phenylalanine-depleted-SCFM and their fluorescence compared with

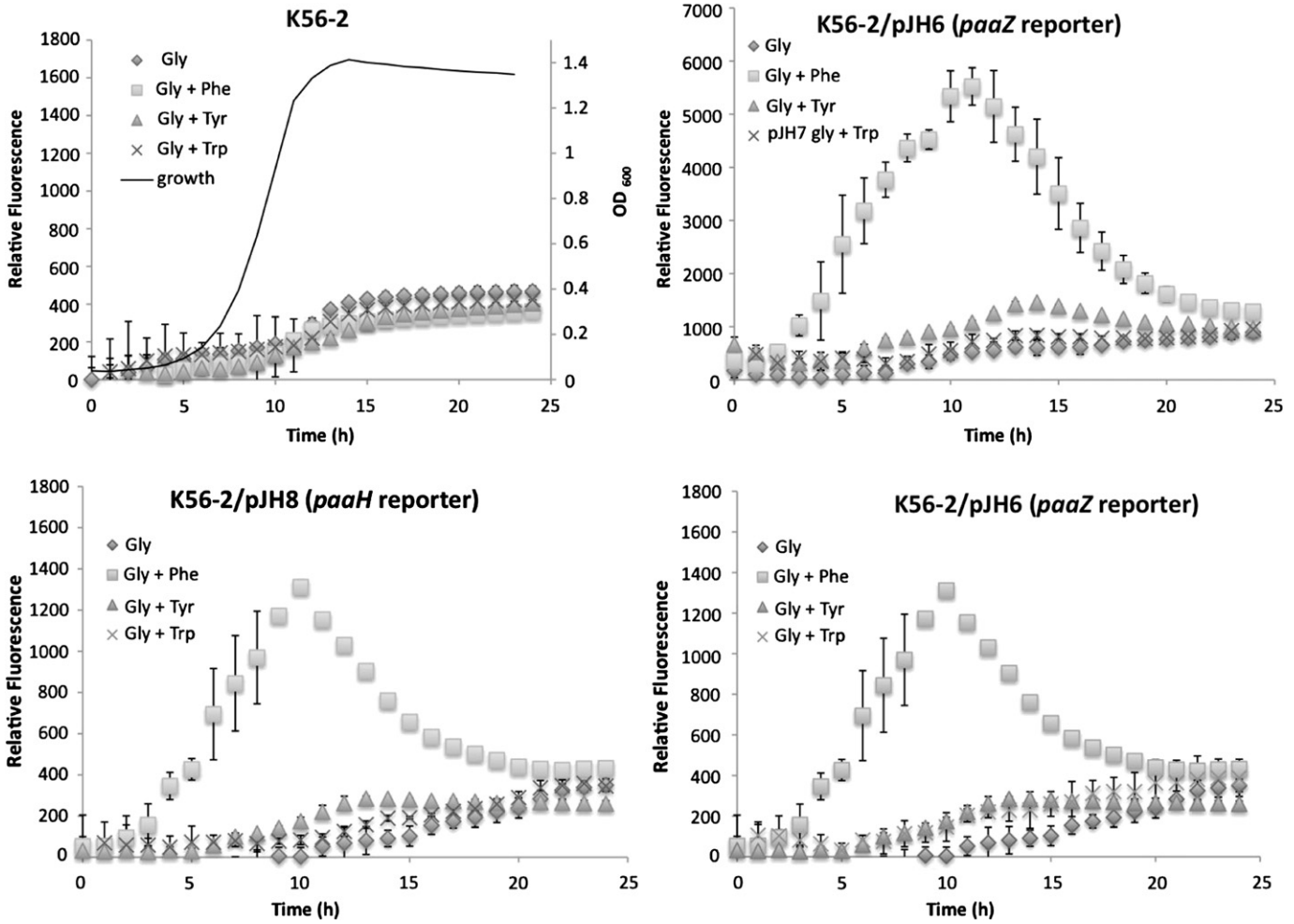

Fig. 1. Phenylalanine activates PA catabolic gene promoters. *B. cenocepacia* K56-2 wild type P_{paaA}, P_{paaZ} and P_{paaH} reporter strains were inoculated in 96-well plates in MOPS minimal medium containing 10 mM glycerol and each of the aromatic amino acids phenylalanine, tyrosine or tryptophan at the same concentrations present in SCFM. Plates were incubated in a Biotek Synergy 2 plate reader at 37 °C with shaking and optical density (OD_{600}) and fluorescence were automatically recorded every hour. Top left panel, a representative growth curve of *B. cenocepacia* K56-2 in MOPS 10 mM glycerol is shown. Top right panel, note the different scale of the *y*-axis. Error bars represent the standard deviation of three independent experiments.

the one of reporter strains grown in SCFM (Fig. 2). P_{paaA}, P_{paaH} and P_{paaZ}, reporter strains showed increased fluorescence in SCFM in comparison with the ones grown in phenylalanine-depleted SCFM.

2.2. Phenylalanine degradation proceeds through PA catabolism in *B. cenocepacia*

Although experimental evidence is limited, the catabolism of phenylalanine in bacteria is thought to proceed through the homogentisic acid pathway [22–24] or via the PA catabolic pathway [25]. In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database predicts the occurrence of both pathways in many microorganisms [26–28]. The *B. cenocepacia* J2315 genome [6], a clonal strain of *B. cenocepacia* K56-2 [29], contains multiple open reading frames, which putatively encode phenylalanine degradation enzymes through both the homogentisic acid pathway and the PA catabolic pathway (Fig. 3a). To investigate if phenylalanine degradation proceeds exclusively through the PA catabolic pathway in *B. cenocepacia*, we used phenylalanine to PA intermediates as sole carbon sources for growth experiments using the previously developed *B. cenocepacia paaE* mutant [14], which is unable to catabolize PA and an isogenic strain with a *paaE* gene expressed *in trans*. When phenylalanine, phenylacetamide, phenylethylamine, phenylpyruvate, and phenylacetic acid were used as sole carbon sources, wild type *B. cenocepacia* K56-2 was able to grow although not to the levels supported by glucose (Fig. 3b). However, none of these carbon sources were able to support the growth of the *B. cenocepacia* K56-2 *paaE* mutant. Complementation with the *paaE* gene in trans restored wild type-like growth with the aforementioned sole carbon sources. The only phenylalanine putative intermediate that did not support growth as a sole carbon source for any of the strains tested was phenylacetaldehyde. The lack of growth could be probably due to poor solubility or defective transport of this compound when exogenously added. Finally, 2-hydroxyphenylacetic acid supported limited growth of all strains regardless of the PA catabolism defect, in accordance with this compound not being a true intermediate of PA degradation [16]. In contrast to *paaE* mutant, insertional mutagenesis of *phhA* and *hpd* genes, putatively encoding enzymes of the homogentisic acid pathway did not cause a defective growth phenotype in phenylalanine as a sole carbon source (data not shown).

2.3. Phenylacetyl-CoA disrupts the interaction between PaaR and promoter DNA of paaA, paaZ, and paaH

Insertional mutagenesis of the *BCAL0210* gene, which encodes PaaR, a TetR type transcriptional regulator located downstream of

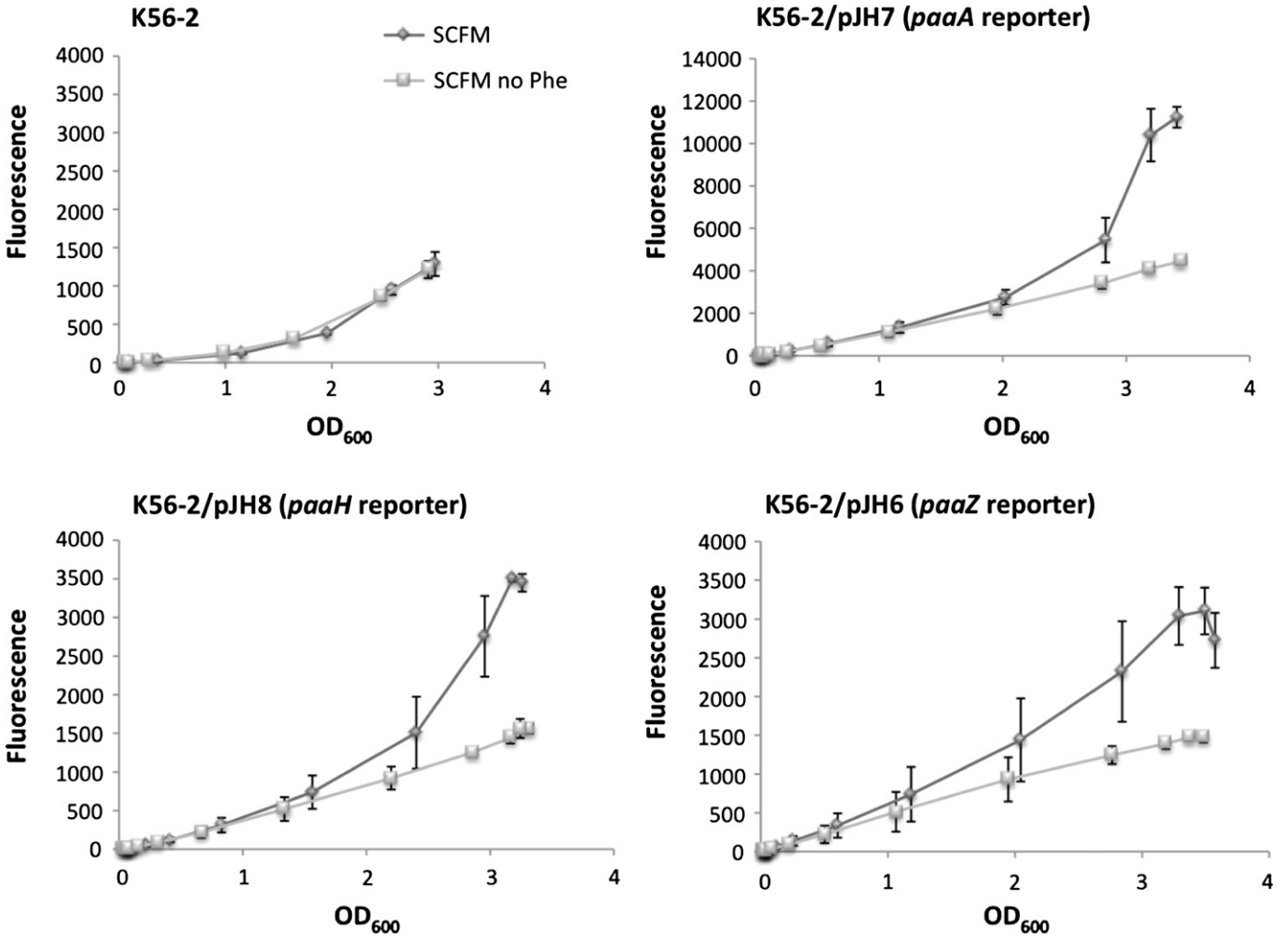

Fig. 2. Phenylalanine is the major inducer of the PA catabolic pathway during growth in SCFM. *B. cenocepacia* K56-2 wild type and P_{paaA}, P_{paaZ} and P_{paaH} reporter strains were inoculated in 96-well plates containing SCFM or phenylalanine-depleted SCFM (SCFM no Phe) and incubated in a Biotek Synergy 2 plate reader at 37 °C with shaking. OD_{600} and fluorescence were automatically recorded every hour. Error bars represent the standard deviation of three independent experiments.

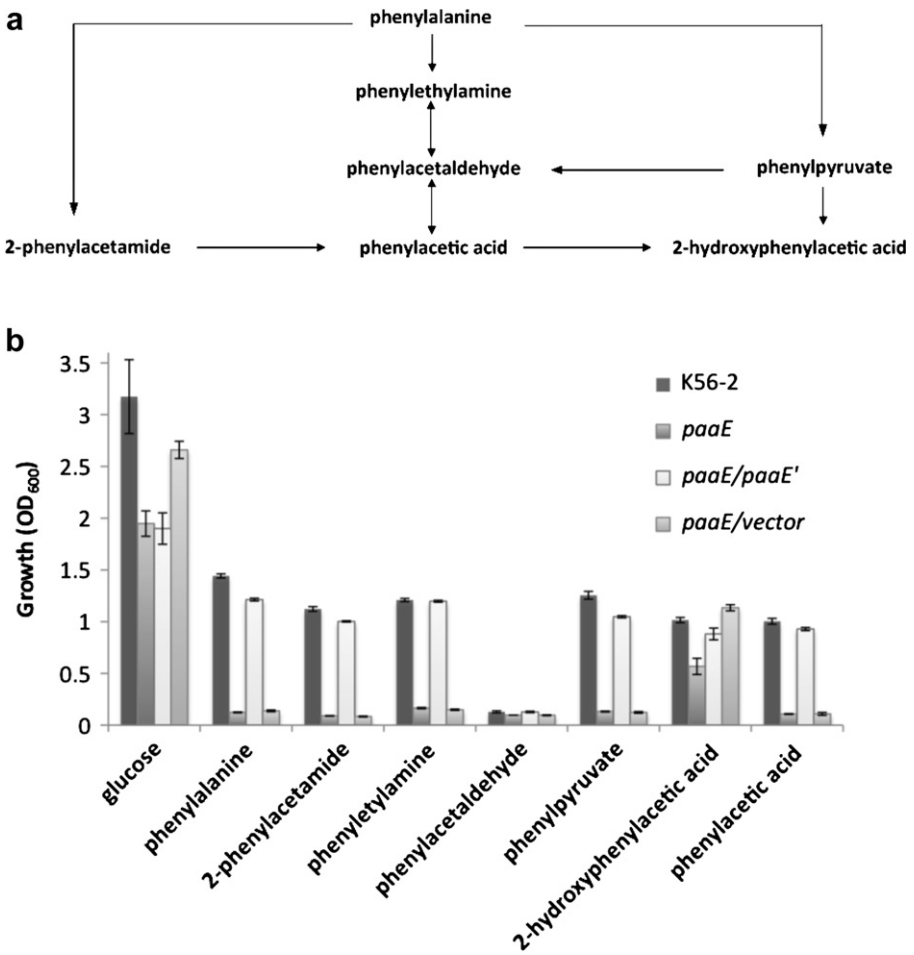

Fig. 3. Phenylalanine degradation proceeds through the PA degradation pathway in *B. cenocepacia* K56-2. a) Putative pathway for phenylalanine degradation to PA. Pathway was derived from map00360 of the KEGG database [26] and references therein. b). A phenylacetic acid degradation-defective mutant is unable to grow in phenylalanine or putative phenylalanine degradation intermediates as sole carbon sources. *B. cenocepacia* K56-2 (wild type), *B. cenocepacia* STC1155-*paaE* (*paaE*), STC1155-*paaE*/pAS1, (*paaE*/*paaE'*) and STC1155-*paaE*/pAP20 (*paaE*/vector) were inoculated in MOPS minimal medium containing 20 mM glucose or 5 mM of the indicated carbon sources and incubated in a Biotek Synergy 2 plate reader at 37 °C with shaking for 24 h. Error bars represent the standard deviation of three independent experiments.

the *paaABCDE* cluster, yielded PA-independent activity of the promoters of *paaA*, *paaZ*, and *paaH*, P_{paaA}, P_{paaZ}, and P_{paaH}, respectively [15]. Thus, PaaR is likely the negative transcriptional regulator for the catabolic PA pathway in *B. cenocepacia*. TetR type regulators are characterized by a high degree of sequence similarity in the DNA binding domain [30], although the ligand binding domains are not conserved. Remarkably, many TetR type regulators can accommodate structurally diverse molecules due to the presence of large ligand binding pockets [31–35]. To address whether PaaR is directly involved in the co-regulation of phenylalanine and PA degradation by binding degradative intermediates, we investigated the binding capacities of PaaR. First, we performed electrophoretic mobility shift assays (EMSA) in which each of the previously described PA catabolic gene promoter regions (P_{paaA}, P_{paaZ}, and P_{paaH}) were incubated with PaaR-His_6, then the DNA, and the DNA-protein mixes, were resolved on a polyacrylamide gel and visualized with SYBR® Green fluorescent dye. The addition of PaaR-His_6 retarded the mobility of P_{paaA}, P_{paaZ}, and P_{paaH} through the gel (Fig. 4), but not the mobility of unspecific DNA (data not shown), thereby indicating the formation of PA catabolic gene promoter DNA-PaaR complexes. Two different retardation complexes were observed in the presence of 181.5 nM, and 363 nM of PaaR while 543 nM of PaaR seemed to favor the formation of the higher molecular weight complex. Next, phenylalanine and its degradative intermediates were each separately incubated with the DNA-protein complexes, and the samples were analyzed by EMSA. Neither the aromatic amino acid phenylalanine, nor any of the phenylalanine intermediates were able to interfere with the DNA-protein complexes, suggesting that they do not act as ligands of PaaR (data not shown). To investigate the possibility that PA or its intermediate PA-CoA is an effector ligand for PaaR, increasing amounts of PA or PA-CoA where added to the DNA-protein complexes and analyzed by EMSA (Fig. 5). In contrast with previous observations, the lower molecular weight DNA-protein complex was not observed when PaaR was incubated with the

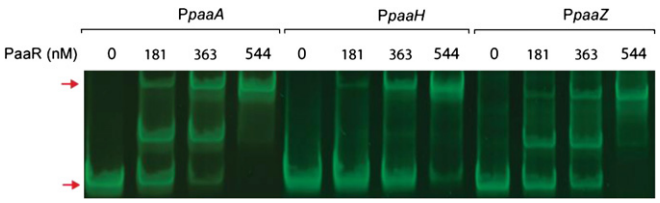

Fig. 4. PaaR binds the promoter regions P_{paaA}, P_{paaH}, and P_{paaZ}. EMSA of PaaR-His_6 binding to P_{paaA}, P_{paaH}, and P_{paaZ} with 13.5 nM of DNA and increasing concentrations of PaaR-His_6. The lower and upper arrows correspond to the 0.5 kb DNA band and the shifted DNA-protein complex, respectively.

DNA. An increasing concentration of PA-CoA dissociated PaaR from the P_{paaA}, P_{paaH} and P_{paaZ} promoter regions, but similar amounts of PA had no effect on PaaR binding activity. In these conditions, the addition of PA-CoA dissociated the higher molecular weight DNA-protein complexes and induced the formation of the lower molecular weight complex in the presence of P_{paaA} and P_{paaZ} but not in the presence of P_{paaH} promoter regions (Fig. 5).

3. Discussion and conclusion

Research on the catabolic pathways utilized by pathogenic bacteria during growth in their hosts is emerging as a relevant field in bacterial pathogenesis [36]. Until recently, the importance of aromatic amino acid catabolic pathways in bacterial pathogenesis was largely overlooked. These carbon utilization routes may be especially relevant in CF infection, given the high concentration of aromatic amino acids found in the CF sputum. In accordance with this hypothesis, the evidence that tryptophan acts as a quorum sensing signal precursor in *Pseudomonas aeruginosa* [37] provides a link between the nutritional content of the CF lung and the production of signal molecules with involvement in pathogenesis. During growth in CF like conditions, activation of the *B. cenocepacia* PA catabolic pathway was observed, but the reasons underlying this induction were unknown [10,15]. In this work, we show that during growth of *B. cenocepacia* K56-2 in SCFM, phenylalanine mediates activation of PA catabolism through its degradation to PA-CoA, which binds the TetR-like negative regulator, PaaR [15], releasing the interaction of the regulatory protein with the PA catabolic gene promoters. The increase in the relative fluorescence due to phenylalanine degradation observed between 4 h and 12 h of growth suggests that the PA catabolic genes are activated during the exponential and early stationary phase. This is consistent with PA catabolism being part of the central metabolism of *B. cenocepacia*. Given that the presence of phenylalanine at levels similar to those found in SCFM was enough to induce the PA catabolism reporter systems (Fig. 1) and the relative fluorescence of the P_{paaA}, P_{paaH} and P_{paaZ} reporter strains was reduced in phenylalanine-depleted SCFM, PA catabolism in SCFM may be active due to phenylalanine degradation. In *Pseudomonas putida*, the degradation of phenylalanine via tyrosine and homogentisate and via the PA catabolic pathway has recently been described [25]. However, in contrast to *P. putida*, a mutant strain defective in PA degradation could not grow with phenylalanine or its degradative intermediates as sole carbon sources (Fig. 3b) raising the question of the role of the predicted homogentisic acid pathway present in the genome of *B. cenocepacia* J2315. If phenylalanine is degraded exclusively through its PA catabolic pathway, then the homogentisic acid route should have another role for example as an intermediate pathway for the synthesis of secondary metabolites [38].

We also provide additional evidence to support that PaaR is a negative transcriptional regulator of the phenylacetic acid catabolic genes in *Burkholderia cenocepacia* [15]. PaaR specifically binds the promoter regions of the first genes in each of *B. cenocepacia*'s three PA catabolic gene clusters – *paaA*, *paaZ*, and *paaH* (Fig. 4) – and these specific interactions are disrupted only by PA-CoA but not by other phenylalanine metabolism intermediates (Fig. 5 and data not shown). Thus, co-regulation between phenylalanine degradation and PA degradation does not seem to occur by phenylalanine degradation intermediate binding to PaaR. The PaaR-DNA interaction could be observed by the formation of two complexes (Figs. 4 and 5). However, only the high molecular weight complex was consistently observed in the same conditions in successive experiments (Fig. 5). The presence of unstable different complexes during incubation of DNA promoter with their cognate DNA binding protein is not uncommon [39]. Yet, the reasons of the differences observed in the formation of the lower molecular weight DNA-protein complex are unknown to us. It is possible that the concentrations of DNA were slightly different, and this difference is not observed in the figures due to the auto exposure settings used. Nevertheless, the DNA and DNA-protein mixes with or without effectors shown in each figure were processed and ran in parallel in the same polyacrylamide gel and show that the presence of PaaR causes a shift in the migration of the DNA corresponding to the promoter region of *paaA*, *paaH* and *paaZ* genes, and that this retardation is abolished by PA-CoA but not by any of the other phenylalanine intermediates or PA.

The mechanism of regulation of the PA catabolic pathway found in *B. cenocepacia*, differs from methods observed in both *E. coli* and strains of *Pseudomonas* sp. [16,17,19,40,41]. Unlike the PaaX binding sites of *Pseudomonas* sp. strain Y2, which are found in PA and styrene catabolic gene promoters [17], the conserved inverted repeat sequence necessary for the binding of PaaR to P_{paaA}, P_{paaZ}, and P_{paaH}, identified by Hamlin et al. (2009) was absent from other promoters in *B. cenocepacia* J2315 genome [15]. The PA catabolon of *P. putida* CA-3 has also been examined and a homolog of PaaX was

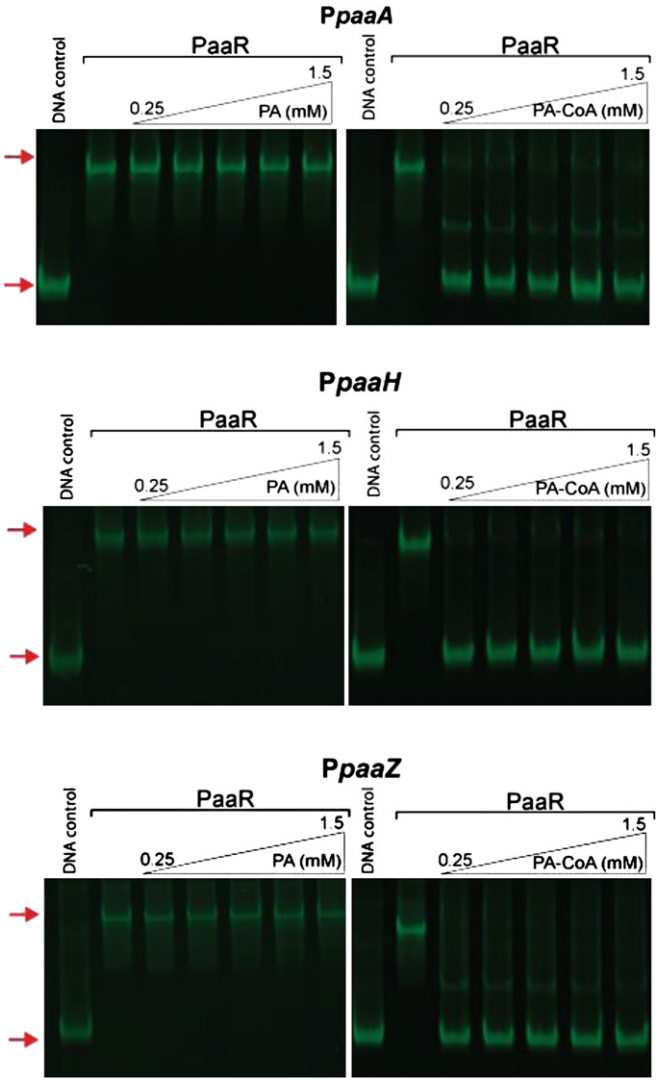

Fig. 5. PA-CoA, but not PA, disrupts PaaR binding to the promoter regions P_{paaA}, P_{paaH}, and P_{paaZ}. EMSA of PaaR-His_6 binding to the promoter regions containing 13.5 nM of DNA, 181.5 nM of PaaR-His_6, and the increasing concentrations of PA or PA-CoA 0.25 mM, 0.5 mM, 0.75 mM, 1 mM and 1.5 mM. The lower and upper arrows correspond to the 0.5 kb DNA band and the shifted DNA-protein complex, respectively.

found [42], which may play a similar role as in strain Y2, given that evidence of coordinated regulation of the styrene and phenylacetic acid catabolons was found [43,44]. Despite their differences, PaaR and the PaaX proteins involved in the regulation of the PA catabolons in other microorganisms are similar in that they bind PA-CoA. This congruency may imply a particular importance of tight regulation of PA-CoA levels. The fact that these proteins share their ligand specificity for PA-CoA without belonging to the same family merits further investigation into their evolutionary relationship. Particularly, comparisons of the crystal structures of both PaaR and PaaX would be beneficial in helping to determine the degree of relatedness between the proteins, if any.

Finally, a key question that remains unanswered is how the *B. cenocepacia* phenylalanine degradation pathway is related to pathogenesis and, moreover, to CF infection. The high levels of phenylalanine present in the CF sputum and its involvement in the activation of PA catabolic pathway, together with our previous finding that the PA catabolic pathway is related to *B. cenocepacia* pathogenesis to *C. elegans*, raises the possibility of this pathway being utilized to generate precursors of signal molecules that trigger pathogenic responses. This hypothesis is currently under investigation in our laboratory.

4. Material and methods

4.1. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this work are shown in Table 1. *E. coli* BL21-GOLD (DE3) carrying plasmid pHB1 was grown in Luria-Bertani (LB) medium supplemented with 35 μg ml^{-1} kanamycin at 37 °C with aeration on a shaking incubator at 190 rpm. *E. coli* DH5 α containing plasmids pJH5, pJH6, pJH7, pJH8, or pJH10 were grown at 37 °C with aeration at 200 rpm in LB medium plus 20 μg ml^{-1} chloramphenicol. *B. cenocepacia* strains used were grown at 37 °C in synthetic cystic fibrosis sputum medium (SCFM) [9] or MOPS-buffered minimal medium [45] containing the indicated carbon sources.

4.2. Growth of B. cenocepacia in 96-well format

B. cenocepacia strains were grown overnight, with shaking, at 37 °C in 5 mL tubes containing LB medium. The cells were then collected, washed in MOPS-buffered medium, diluted to an initial optical density of 600 nm (OD_{600}) of 0.04 with 150 μL of MOPS-buffered minimal medium containing the indicated carbon sources, in 96-well plates. For continuous monitoring by automated reading of OD_{600}, plates were incubated in a Biotek Synergy 2 plate reader for 24 h at 37 °C, with continuous, fast shaking. For endpoint readings, plates were incubated in a Biotek Synergy 2 plate reader at 37 °C with shaking and the endpoint growth was monitored at 24 h. OD_{600} values were converted to 1 cm-path length OD_{600} using a standard curve.

4.3. Reporter activity assays

B. cenocepacia K56-2 wild type and P_{paaA}, P_{paaZ} and P_{paaH} reporter strains [15] were grown overnight with shaking at 37 °C in 5 mL SCFM or LB medium. The cells were then washed in PBS, diluted to an OD_{600} of 0.04 in 150 μL-aliquots of SCFM or MOPS containing the indicated carbon sources, and arranged in 96-well format. Plates were incubated with continuous, fast shaking at 37 °C in a Biotek Synergy 2 plate reader for 24 h, until stationary phase was reached. Fluorescence was quantified using excitation 485/20, emission 528/20 filter sets as previously described [15]. Relative fluorescence was defined as the ratio between the arbitrary fluorescence and the OD_{600} of *B. cenocepacia* reporter strain-containing wells.

4.4. Cloning, expression and purification of PaaR-His_6

The *BCAL0210* gene, encoding the PaaR protein, was PCR-amplified using primers SC131 (CCTTCGCATATGGCCCGAACCCGAGCGCC) and SC134 (TGTGCGACTAGTGGCCGCTGTCGACAGCCCGC), containing restriction sites *Nde*I and *Spe*I, respectively. The resulting DNA amplicon was cloned into the C-terminal 6X histidine T7 expression vector p1-86-8BM, a pET-28 derivative with a modified multiple cloning site. The resulting recombinant plasmid, pHB1, was introduced in *E. coli* BL21-GOLD (DE3) and the cultures were grown in 500 mL LB with 35 μg ml^{-1} kanamycin at 37 °C with aeration until OD_{600} reached 0.5. Cultures were then induced with 1 mM IPTG and incubated at 28 °C with aeration for 4 h. Cells were harvested by centrifugation at 7000 rpm (1096 × *g*) for 15 min, resuspended in 50 mM Tris pH 8.0, 1 M NaCl, and 1 mM phenylmethylsulphonyl fluoride (PMSF) with 10 μg ml^{-1} Protease Inhibitor Cocktail (Sigma, Oakville, ON). Cells were then passed through a French Press for lysis. Lysate was centrifuged at 10,000 rpm (2236 × *g*) for 60 min and

Table 1
Bacterial strains and plasmids.

Strain or plasmid	Features[a]	Reference or source
B. cenocepacia strains		
K56-2 (LMG18863)	ET12 clone related to J2315, CF clinical isolate	[29]
STC155-*paaE*	K56-2 *paaE*:: SC152, Tp^r	[14]
LEK47	C5424 *hppD*::pLK2, Tp^r	[38]
RB6	K56-2 *phhA*::pRB2, Tp^r	This study
E. coli strains		
DH5α	F^-, φ 80 *lacZ*ΔM15 *endA*1 *hsdR*17 ($r_K^-m_K^+$) *supE*44 *thi*−1Δ*gyrA*96 (Δ*lacZYA-argF*)U169 *relA*1	
BL21-GOLD	*E. coli* B F-*ompT hsdS*(rB-mB-) *dcm*+ Tet^r *gal* λ (DE3) *endA* Hte	Invitrogen, Burlington, ON
Plasmids		Stratagene, La Jolla, CA
pAP20	$ori_{PBBR1}P_{DHFR}$ Cm^r Cm duplicated region deleted	[14]
pAS1	pAP20, paaE	[14]
p1-86-8BM	pET-28 derivative, ori_{pBR322}, T7 promoter, C-terminal 6X His tag, Km^r, modified MCS	B. Mark, unpublished
pHB1	P1-86-8BM, *BCAL0210* cloned in-frame with C-terminal 6x His tag, Km^r	This study
pGPΩTp	ori_{R6K} Tp^R	[46]
pRB2	pGPΩTp; 300-bp *phhA* mutagenesis fragment	This study
pJH6	pJH2, *paaZ* promoter region (P_{paaZ}), Cm^r	[15]
pJH7	pJH2, *paaA* promoter region (P_{paaA}), Cm^r	[15]
pJH8	pJH2, *paaH* promoter region (P_{paaH}), Cm^r	[15]

Cm, chloramphenicol; Km, kanamycin; MCS, multiple cloning site.

supernatant was applied to a nickel-nitriloacetic acid (NTA) column (Qiagen), and then eluted with buffer containing 1 M NaCl, 250 mM imidazole, and 50 mM Tris pH 8.0. Fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE Novex Bis-Tris Gels with MOPS SDS Running Buffer (Invitrogen, Burlington, ON) and SimplyBlue SafeStain (Invitrogen, Burlington, ON). BCAL0210-containing fractions were pooled and dialyzed overnight in binding buffer (BB1) composed of 15 mM Tris pH 7.5, 500 mM KCl, 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol (DTT). Binding buffer was changed, and then fractions were dialyzed further for an additional 4 h. Protein concentrations were determined using Pierce's Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific, Rockford, IL). All SDS-PAGE assays were performed using Invitrogen's NuPAGE® system (Burlington, ON). Sample preparation utilized NuPAGE® LDS Sample Buffer and NuPAGE® Reducing Agent containing 500 mM DTT. Polyacrylamide gels used were Invitrogen's Novex® Bis-Tris pre-cast 4–12% polyacrylamide mini-gels and MOPS SDS Running Buffer with Antioxidant. Gels were run at 200 V for 50–60 min and subsequently stained using SimplyBlue SafeStain containing Coomassie G-250 (Invitrogen, Burlington, ON). Protein concentrations were determined using Pierce's Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific, Rockford, IL).

4.5. Construction of a phhA insertional mutant in B. cenocepacia K56-2

BCAL0010 was disrupted using single crossover mutagenesis with plasmid pGPΩTp, a derivative of pGP704 that carries the *dhfr* gene flanked by terminator sequences [46]. Briefly, an internal 300-bp fragment of BCAL0010 was PCR–amplified using primers 5′-ATTATGAATTCTGAATCGCCAGCTGAAG and 5′-ATTATTCTAGAAC CGTATACCAATAGAGGC, containing *Eco*RI and *Bgl*II restriction sites respectively (underlined). The PCR-amplified was digested with *Eco*RI and *Bgl*II respectively, cloned into the EcoRI-BglII digested vector and maintained in *E. coli* SY327. The resulting plasmid, RB2 (Table 1) was conjugated into *B. cenocepacia* strain K56-2 by triparental mating. Conjugants that had the plasmid integrated into the K56-2 genome were selected on LB agar plates supplemented with Tp 100 μg ml^{-1} and Gm 50 μg ml^{-1}. Integration of the suicide plasmids was confirmed by colony PCR.

4.6. Amplification of PA degradation gene promoter regions

The PA degradation gene promoter regions, P_{paaA}, P_{paaZ}, and P_{paaH} were PCR-amplified using plasmids pJH6, pJH7, and pJH8 (Table 1) as templates with an Eppendorf Mastercycler ep gradient S thermocycler and ProofStart PCR Kit (Qiagen,) with primers SC43-SC44, SC38-SC39, and SC45-SC46, respectively [15]. Amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen).

4.7. Electrophoretic mobility shift assays (EMSA)

EMSA analyses were performed using 13.5 nM purified P_{paaZ}, P_{paaA}, or P_{paaH} DNA, and increasing concentrations of PaaR-His_6 diluted in binding buffer (BB2) containing 15 mM Tris pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, and 10% glycerol, v/v. Promoter DNA and PaaR-His_6 were incubated together for 20 min at room temperature in BB2. After incubation, 9 μL aliquots of binding reaction were mixed with 1 μL 5X Hi-Density TBE Sample Buffer (Invitrogen), and loaded into Novex 6% DNA Retardation Gels (Invitrogen) in XCell SureLock MiniCell System (Invitrogen). Electrophoresis was run using 0.5X TBE Running Buffer, containing 4.5 mM Tris pH 8, 4.5 mM boric acid, and 0.05 mM EDTA for 100 min at 100 V. Gels were stained with SYBR® Green EMSA nucleic acid dye (Invitrogen) following procedures recommended by manufacturer.

4.8. Ligand binding assays

Ligand binding assays were performed using 13.5 nM purified P_{paaA}, P_{paaZ}, or P_{paaH}, 181.5 nM purified PaaR-His_6, and increasing concentrations of phenylalanine, phenylpyruvate, phenylacetaldehyde, phenylethylamine, phenylacetic acid, or phenylacetyl-CoA. For each compound, a 5 mM stock solution was prepared in BB2. Promoter DNA and PaaR-His_6 were incubated together for 20 min at room temperature, followed by the addition of the appropriate compound and further incubation for an additional 15 min at room temperature. DNA-protein complexes were analyzed by EMSA as described previously.

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