A Functional Phenylacetic Acid Catabolic Pathway Is Required for Full Pathogenicity of *Burkholderia cenocepacia* in the *Caenorhabditis elegans* Host Model[†]

Robyn J. Law, Jason N. R. Hamlin, Aida Sivro,[‡] Stuart J. McCorrister, Georgina A. Cardama,[§] and Silvia T. Cardona*

Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada

Received 8 April 2008/Accepted 26 August 2008

Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex, a group of metabolically versatile bacteria that have emerged as opportunistic pathogens in cystic fibrosis and immunocompromised patients. Previously a screen of transposon mutants in a rat pulmonary infection model identified an attenuated mutant with an insertion in *paaE*, a gene related to the phenylacetic acid (PA) catabolic pathway. In this study, we characterized gene clusters involved in the PA degradation pathway of *B. cenocepacia* K56-2 in relation to its pathogenicity in the *Caenorhabditis elegans* model of infection. We demonstrated that targeted-insertion mutagenesis of *paaA* and *paaE*, which encode part of the putative PA-coenzyme A (CoA) ring hydroxylation system, *paaZ*, coding for a putative ring opening enzyme, and *paaF*, encoding part of the putative beta-oxidation system, severely reduces growth on PA as a sole carbon source. *paaA* and *paaE* insertional mutants were attenuated for virulence, and expression of *paaE* in *trans* restored pathogenicity of the *paaE* mutant to wild-type levels. Interruption of *paaZ* and *paaF* slightly increased virulence. Using gene interference by ingested double-stranded RNA, we showed that the attenuated phenotype of the *paaA* and *paaE* mutants is dependent on a functional p38 mitogen-activated protein kinase pathway in *C. elegans*. Taken together, our results demonstrate that *B. cenocepacia* possesses a functional PA degradation pathway and that the putative PA-CoA ring hydroxylation system is required for full pathogenicity in *C. elegans*.

The *Burkholderia cepacia* complex (Bcc) is a group of closely related bacteria that was originally described by W. H. Burkholder as the plant pathogen *Pseudomonas cepacia* (34). During the past decade, polyphasic-taxonomic studies have demonstrated that Bcc represents a group of at least nine taxonomically related species sharing moderate levels of DNA-DNA hybridization (34). Bcc strains occupy multiple niches from soil to water supplies and can establish beneficial or detrimental associations with plants and fungi. Unfortunately, the Bcc have emerged as opportunistic pathogens in patients with cystic fibrosis, chronic granulomatous disease, and other medical conditions associated with a compromised immune system (34, 53). Representatives of all Bcc species have been isolated from both environmental and human clinical sources. Two species in North America, *Burkholderia cenocepacia* and *Burkholderia multivorans*, account for the majority of cystic fibrosis infections (34, 53). While the molecular basis of Bcc pathogenesis in the human host is far from being understood, recent evidence shows that in contrast to the case with other bacterial pathogens, pathogenicity of Bcc appears to be polygenic and mainly involves genes related to survival under stress

conditions (18, 36, 46). These capacities, in addition to the catabolic versatility of Bcc, may explain the multiple niches where Bcc bacteria thrive. Bcc strains can survive in polluted environments, where they metabolize constituents of crude oils, herbicides, and various man-made recalcitrant aromatic compounds (34). The understanding of nonintermediary aromatic biodegradation processes has benefited from the relevance these processes have for biotechnological applications (12). However, much less is known about microbial catabolism of natural aromatic compounds and the role, if any, of these metabolic pathways in host-pathogen interactions.

The phenylacetic acid (PA) catabolic pathway is the central route where catabolism of many aromatic compounds, such as styrene, *trans*-styrylacetic acid, phenylalanine, 2-phenylethylamine, phenylacetaldehyde, and several *n*-phenylalkanoic acids, converge and are directed to the Krebs cycle (33). Many microbial genomes contain gene clusters encoding putative PA catabolic genes, yet experimental evidence for a functional pathway is available for only a few bacteria: *Escherichia coli* (12, 15, 26), *Azoarcus evansii* (38), *Pseudomonas putida* (27, 30, 42), and *Rhodococcus* sp. (40). In these microorganisms, the PA catabolic gene cluster is organized as a single operon encoding enzymes involved in four steps. The PA-activating enzyme, phenylacetyl-coenzyme A (PA-CoA) ligase, PaaK (15), and the PA-CoA ring hydroxylation system, comprised of PaaA, PaaB, PaaC, PaaD, and PaaE (15, 26), are involved in the first and second steps, respectively. The third step, the opening of the aromatic ring, may be performed by PaaZ (15) or by PaaZ, PaaG, and PaaJ (26), followed by further degradation of the resulting aliphatic compound through a β -oxidation-like pathway complex by PaaF, PaaH (26), and PaaJ (41).

* Corresponding author. Mailing address: Department of Microbiology, Buller Building, Room 418, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2. Phone: (204) 474-8997. Fax: (204) 474-7603. E-mail: cardona@cc.umanitoba.ca.

[‡] Present address: Department of Medical Microbiology and Infectious Diseases, Basic Medical Sciences Building Room 507, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3.

[§] Present address: Laboratory of Molecular Oncology, Quilmes National University, Bernal, Argentina.

[†] Published ahead of print on 5 September 2008.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Features ^a	Reference or source
<i>B. cenocepacia</i> strains		
K56-2 (LMG18863)	ET12 clone related to J2315, CF clinical isolate	35
STC155- <i>paaE</i>	K56-2 <i>paaE</i> ::pSC152 Tp ^r	This study
STC179- <i>paaA</i>	K56-2 <i>paaA</i> ::SC175 Tp ^r	This study
STC181- <i>paaK1</i>	K56-2 <i>paaK1</i> ::SC176 Tp ^r	This study
STC183- <i>paaZ</i>	K56-2 <i>paaZ</i> ::pSC177 Tp ^r	This study
STC199- <i>paaF</i>	K56-2 <i>paaF</i> ::pSC186 Tp ^r	This study
<i>E. coli</i> strains		
DH5α	F ⁻ φ80 <i>lacZ</i> ΔM15 <i>endA1</i> <i>recA1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> <i>thi-1</i> Δ <i>gyrA96</i> (Δ <i>lacZYA-argF</i>)U169 <i>relA1</i>	Invitrogen
SY327	<i>araD</i> Δ(<i>lac pro</i>) <i>argE</i> (Am) <i>recA56</i> Rif ^r <i>nalA</i> λ <i>pir</i>	37
Plasmids		
pGPΩTp	<i>ori</i> _{r6K} ΩTp ^r <i>mob</i> ⁺	18
pRK2013	<i>ori</i> _{colE1} RK2 derivative, Km ^r <i>mob</i> ⁺ <i>tra</i> ⁺	16
pSC152	pGPΩTp, internal fragment from <i>paaE</i>	This study
pSC175	pGPΩTp, internal fragment from <i>paaA</i>	This study
pSC176	pGPΩTp, internal fragment from <i>paaK1</i>	This study
pSC177	pGPΩTp, internal fragment from <i>paaZ</i>	This study
pSC186	pGPΩTp, internal fragment from <i>paaF</i>	This study
pTp-backbone	<i>ori</i> _{PBBR1} Tp	J. Lamothe
pAP1	<i>ori</i> _{PBBR1} Cm ^r	This study
pAP2	<i>ori</i> _{PBBR1} P _{DHFR} Cm ^r	This study
pAP20	<i>ori</i> _{PBBR1} P _{DHFR} Cm ^r Cm duplicated region deleted	This study
pAS1	pAP20, <i>paaE</i>	This study
pRL1	pAP20, <i>paaF</i>	This study

^a Cm, chloramphenicol; Km, kanamycin; Tp, trimethoprim; CF, cystic fibrosis.

In this article, we describe the creation and characterization of *B. cenocepacia* K56-2 insertional mutants that are defective in the PA catabolic pathway and show that the putative PA-CoA ring hydroxylation system is required for full pathogenicity of *B. cenocepacia* in the *Caenorhabditis elegans* model of infection. Using gene interference by ingested double-stranded RNA, we have demonstrated that the observed attenuated pathogenicity is dependent on a functional *C. elegans* p38 mitogen-activated protein (MAP) kinase pathway.

MATERIALS AND METHODS

Bacterial strains, nematode strains, and growth conditions. Bacterial strains and plasmids are listed in Table 1. *B. cenocepacia* K56-2 was grown at 37°C in Luria-Bertani (LB) or M9 medium supplemented, as required, with 100 μg/ml trimethoprim (Tp), 50 μg/ml gentamicin (Gm), and 200 μg/ml chloramphenicol (Cm). *Escherichia coli* strains were grown at 37°C in LB medium supplemented with 50 μg/ml Tp, 40 μg/ml kanamycin, or 20 μg/ml Cm. The nematode *Caenorhabditis 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 medium according to standard practices at the CGC. *E. coli* HT115 carrying the L4440 expression vector for each targeted gene was provided by Geneservice Ltd.

Bioinformatics analysis. BLASTP searches of the genome of *B. cenocepacia* strain J2315 were performed using the *B. cenocepacia* BLAST Server at the

Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_cenocepacia) using the protein sequences of PA catabolic genes from *E. coli* as the query sequence. J2315 belongs to the same clonal lineage as strain K56-2 (10). Bidirectional best hits having an E value of <1e-10 in both directions were considered homologous gene pairs (50). Gene clusters were visualized using the Artemis (45) and VectorNTI (Invitrogen) software programs.

Molecular biology techniques. DNA ligase (New England Biolabs) was used as recommended by the manufacturers. *E. coli* DH5α cells were transformed using the calcium chloride protocol (8), and electroporation was used for transformation of *E. coli* SY327 cells (37). Conjugation into *B. cenocepacia* K56-2, STC155-*paaE*, or STC199-*paaF* was accomplished by triparental mating (9), with *E. coli* DH5α carrying the helper plasmid pRK2013 (16). DNA was amplified using a PTC-221 DNA engine (MJ Research) or an Eppendorf Mastercycler ep gradient S thermal cycler with either *Taq* DNA polymerase or the Phusion high-fidelity PCR kit (New England Biolabs). Amplification conditions were optimized for each primer pair. PCR products and plasmids were purified using the QIAquick purification kit (Qiagen) and the QIAprep Miniprep kit (Qiagen), respectively.

Construction of PA catabolic gene insertional mutants of *B. cenocepacia* K56-2. Several PA catabolic genes were disrupted using single-crossover mutagenesis with pGPΩTp, a derivative of pGP704 that carries the *dhfr* gene flanked by terminator sequences (18). Briefly, internal 300-bp fragments of the target genes were amplified by PCR using appropriate primers (Table 2). The *paaE* PCR-amplified product and the *paaA*, *paaK1*, *paaZ*, and *paaF* PCR-amplified products were digested with XbaI or XbaI and EcoRI, respectively, cloned into the XbaI- or XbaI-EcoRI-digested vector, and maintained in *E. coli* SY327. The resulting plasmids (Table 1) were 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) and Gm (50 μg/ml). Integration of the suicide plasmids was confirmed by colony PCR, using primer SC025, which anneals to the R6K origin of replication of pGPΩTp, and primers upstream of the expected site of insertion (Table 2). All mutant strains were confirmed by sequencing of PCR-amplified DNA fragments containing the insertion site.

Construction of the constitutive expression vector pAP20 and complementation of the *B. cenocepacia* *paaE* and *paaF* mutants. pAP20 was constructed using pTp-backbone, a pMLBAD (32) derivative in which the arabinose system was deleted (J. Lamothe and M. A. Valvano, unpublished), as follows. To construct pAP1, pTp-backbone was amplified by inverse PCR using primers 1548 and 1549 (Table 2). The DNA fragment was digested with ClaI and ligated to a ClaI-restricted DNA fragment obtained from PCR amplification of a Cm resistance cassette from pKD3 (11) using primers 1474 and 1475. To construct pAP2, pAP1 was then amplified by inverse PCR using primers 1550 and 1551, digested with NdeI and XhoI, and ligated to a DNA fragment containing the *dhfr* promoter amplified from pSCrhaB2 (5) with primers 1552 and 1553 and digested with the same restriction enzymes. Finally, a duplicated region was removed from pAP2 by inverse PCR amplification using primers 2167 and 2168, digestion with NsiI, and religation. The resulting plasmid, pAP20, was used to clone *paaE* and *paaF* under the control of the constitutive *dhfr* promoter. DNA fragments carrying the complete coding sequence of the *paaE* or *paaF* gene were PCR amplified with primers SC005 and SC006 or SC036 and SC037 (Table 2). The PCR products were digested with NdeI and XbaI, ligated into NdeI/XbaI-digested pAP20, and transformed into *E. coli* DH5α. The resulting plasmids, pAS1 and pRL1, were introduced into *B. cenocepacia* STC155-*paaE* and STC199-*paaF*, respectively, by triparental mating. pAP20 was also introduced in the mutant strains as a negative control for complementation experiments.

Bacterial growth. Ninety-six-well microplates containing 150 μl of M9 plus different carbon sources were inoculated with 3 μl from overnight culture grown in LB, washed with M9, and adjusted to an optical density at 600 nm (OD₆₀₀) of 2.0 with M9 salts. Microplates were incubated for 48 h at 37°C with shaking at 200 rpm. The OD₆₀₀ was measured using a Biotek Synergy 2 plate reader at various time intervals, and values were converted to a 1-cm-path-length OD₆₀₀ by prior calibration with an Ultraspec 3000 spectrophotometer.

Nematode killing assays. Slow-killing assays were performed as previously described (6, 31). Briefly, 35-mm nematode growth (NG) agar plates were inoculated with 50 μl of overnight cultures grown in LB broth, adjusted to an OD₆₀₀ of 1.7, and incubated overnight at 37°C to allow formation of a bacterial lawn. Twenty to forty hypochlorite-synchronized L4 larvae of *C. elegans* DH26 were added to each plate and incubated at 25°C. Plates were scored for live worms at the time of inoculation and every 24 h subsequently for a total of 5 days using a Fisher Scientific Stereomaster 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

TABLE 2. Primers

Name	Oligonucleotide sequence, 5'–3'	Purpose or location
1474	CGATCGATAAGTATAGGAACTTCGGC	Amplification of Cm resistance cassette
1475	CGATCGATTTCATCGAGTACTGTT	Amplification of Cm resistance cassette
1548	GAGCTCATCGATTTTCGTTCCACTGA	Inverse PCR of pTp-backbone
1549	TCATCGATCTGCACTTGAACGTGTGGCC	Inverse PCR of pTp-backbone
1550	GTTTGACCATATGTCATCGACACCATGGTACCC	Inverse PCR of pAP1
1551	GTGTCTCGAGTAAGCTGTCAAACATGAGCA	Inverse PCR of pAP1
1552	ATCCTCGAGTATGCTAGCGATGAGCTCGC	Amplification of <i>dhfr</i> promoter
1553	GCACGATCATATGTAGAATTCGGAATCCTTCTT	Amplification of <i>dhfr</i> promoter
1711	ACTCTAGACGCGCAGCACGTTACGCTG	Amplification of <i>paaE</i> internal fragment
1712	TGTTAGAGCCTCGTCGATCGCGTCGGCC	Amplification of <i>paaE</i> internal fragment
2045	CTTCTAGATCTTCAACTACCCGACGCCG	Amplification of <i>paaA</i> internal fragment
2046	CCATGAATTCGACTGACTGCTGTGGACTGA	Amplification of <i>paaA</i> internal fragment
2047	AGTCTAGAGTCGTCGGCTATACGGCTGC	Amplification of <i>paaK1</i> internal fragment
2048	AATTGAATTCGCGAGCGAGCTTTGCACGGG	Amplification of <i>paaK1</i> internal fragment
2049	ACTCTAGAAAGCCGCAAGGCAAGAACCC	Amplification of <i>paaZ</i> internal fragment
2050	GGCGGAATTCCTCAGATCGTCGGTCAATC	Amplification of <i>paaZ</i> internal fragment
2063	GATCTAGAGGATGTCTATCGGGGCGACT	Amplification of <i>paaF</i> internal fragment
2064	CACGGAATTCCTTCGACAGCGTCCATGAAGC	Amplification of <i>paaF</i> internal fragment
1436	CCTACTGCATATGGCGACCCGCAATTTCA	5' end of <i>paaE</i>
2069	TAAGCCATGGATTGTGCCGAGAAGATGCC	172 bp upstream of <i>paaA</i>
2067	GGAGACACATATGACTACCCGCTACCGCT	5' end of <i>paaK1</i>
2089	AAGTCGCCATATGACCCATGCCCTGTTAC	5' end of <i>paaZ</i>
2091	GGAGAAACATATGGCTTACGAGAACATCCT	5' end of <i>paaF</i>
2167	ACCATGCATAGCTCCTGAAAATCTCGATA	Inverse PCR of pAP2
2168	CATATGCATTAGCTTTTGCCATTCTCACC	Inverse PCR of pAP2
SC005	AATTCTACATATGGCGACCCGCAATTTCA	Cloning of <i>paaE</i> gene
SC006	TAGCTCTAGATCAACGTTCTGTCGAAGCTC	Cloning of <i>paaE</i> gene
SC036	GGAGGAGCATATGGCTTACGAGAACATCCTG	Cloning of <i>paaF</i> gene
SC037	ACACCTCTAGATCAGCGGTGCTTGAAGACCG	Cloning of <i>paaF</i> gene
SC025	TAACGGTTGTGGACAACAAGCCAGGG	Mutagenesis with pGPΩTp

^a Restriction sites are underlined.

statistical significance using GraphPad Prism, version 4.0. *P* values of <0.05 were considered statistically significant. Worm pictures were taken with a Nikon SMZ 1500 stereomicroscope equipped with a Nikon Coolpix 8400 digital camera.

Quantification of nematode intestinal colonization and pumping rates. Bacterial colonization of the *C. elegans* intestine was quantified as per the method of Moy et al. (39). Briefly, nematodes were allowed to feed on 35-mm NG agar plates seeded with *B. cenocepacia* strain K56-2 or STC155-*paaE* for up to 48 h. At 8, 24, and 48 h postinfection, approximately 10 to 15 nematodes were manually transferred to a 1.5-ml Eppendorf tube of M9 buffer containing 1 mM Na₂SO₄, washed three times, and brought to a final volume of 250 μl. One millimolar NaN₃ was used to prevent expulsion of *B. cenocepacia* from the *C. elegans* intestine. A 50-μl aliquot was removed from each Eppendorf tube, serially diluted, and plated to determine viable external CFU/worm. To the remaining 200 μl, 400 mg of 1.0-mm silicon carbide particles were added. Tubes were then vortexed intermittently at 2,000 rpm for a total of 90 s to disrupt worms. The resulting suspension was serially diluted and plated on LB plus 50 μg/ml gentamicin to determine viable internal CFU/worm. An unpaired *t* test was used to measure the statistical significance of colonization differences. *P* values of <0.05

were considered statistically significant. Pumping rates were quantified by eye using a Fisher Scientific Stereomaster dissecting microscope as described elsewhere (2, 54). Briefly, pharyngeal pumps were counted during five successive 1-min periods, and the average of the five counts was taken as the worm's pumping rate.

RNAi knockdown experiments. RNA interference (RNAi)-immunocompromised worms were obtained by growing the nematodes as previously described (19, 52). Briefly, NG agar plates containing 1 mM isopropyl-β-D-thiogalactopyranoside and 100 μg/ml ampicillin were inoculated with overnight bacterial cultures of *E. coli* HT115 carrying the L4440 expression vector for each targeted gene. *C. elegans* DH26 hypochlorite-obtained eggs were added and allowed to hatch at 25°C. After 48 h, 20 to 40 L4 larvae were transferred to NG plates containing bacterial lawns of the strains to be tested and slow-killing assays were performed.

Gibbs assay. The total phenolic content of supernatants was determined according to the method in reference 51. Briefly, to a 1-ml sample, 0.1 ml buffer (60 g Na₂CO₃ and 40 g NaHCO₃ per liter adjusted to pH 8.5 with HCl) and 0.1 ml Gibbs reagent (0.2% [wt/vol] 2,6-dichloroquinone-4-chloroimide [95%]) in

FIG. 1. Proposed PA catabolic pathway of *B. cenocepacia* strain J2315. (A) Genetic organization of the PA catabolic gene clusters in *B. cenocepacia* strain J2315. (B) PA catabolic enzymes and putative intermediates of the PA catabolic pathway. Genes disrupted by insertional mutagenesis are shown in bold. Disrupted steps are marked with an "X" and the observed pathogenic phenotype summarized as follows: solid lines, attenuated pathogenicity; dashed lines, increased pathogenicity. The gene names are in accordance with those listed in reference 12.

absolute ethanol, made fresh on the day of analysis and stored at 4°C, were mixed in a 1.5-ml microcentrifuge tube by inversion four to six times and then incubated in a water bath at 40°C for 30 min. A standard curve using phenol (0 to 100 μ M; Acros Organics) was prepared similarly. The absorbance of the undiluted or 1/10-dilution sample was measured at 620 nm using an Ultraspec 3000 spectrophotometer.

Nucleotide sequence accession number. The nucleotide sequence of plasmid pAP20 was deposited in GenBank under accession no. EU606014.

RESULTS

Identification of PA catabolic gene clusters in *B. cenocepacia* J2315. Preliminary evidence of a link between the *B. cenocepacia* K56-2 PA catabolic pathway and pathogenesis came out of the isolation of the signature-tagged transposon mutant 4A7 (25). This mutant could not be recovered from intratracheal lung infections in rats and was nonpathogenic in the *C. elegans* host model of infection (6). The transposon insertion site in the 4A7 mutant was identified as having interrupted BCAL0212, a putative *paaE* gene, prompting us to investigate the occurrence of this metabolic pathway in *B. cenocepacia*.

We searched the sequenced genome of *B. cenocepacia* J2315 for genes encoding homologues of the PA catabolic pathway of *E. coli* and found PA genes organized in three separate clusters (Fig. 1A): two gene clusters were located in chromosome one (BCAL0212 to BCAL0216 and BCAL0404 to BCAL0409), while

the third (BCAM1711 and BCAM1712) was located in chromosome two. Our functional assignment based on bidirectional BLAST searches matched the draft annotation of the *B. cenocepacia* J2315 genome. The genes were assigned as follows: BCAL0404 and BCAL1711 are homologues of the *paaK* gene, which encodes a PA-CoA ligase in *E. coli* (15); BCAL0212 to BCAL0216 encode a putative five-component oxygenase that hydroxylates PA-CoA in *E. coli* (14); BCAL0406 and BCAL0408 correspond to the *paaG* and *paaZ* genes, which are proposed to encode enoyl-CoA isomerization/hydration, ring opening, and dehydrogenation activities (26); and BCAL0409 and BCAM1712 code for homologues of *paaF* and *paaH*, respectively, whose gene products are responsible for β -oxidation, the last step of the PA catabolic pathway, (26). The only discrepancy with Sanger annotation is that of BCAL0407, which was annotated as a *pcaF* homolog of the gene coding for a β -ketoadipyl-CoA thiolase involved in the degradation of 4-hydroxybenzoate (23). PaaJ from *E. coli* (CAA66099), however, which is also a β -ketoadipyl-CoA thiolase, matched the BCAM2568 (E value of $<10^{-145}$) and BCAL0407 (E value of $<10^{-143}$) proteins in a BLAST search. Although both putative proteins are highly similar to PaaJ, BCAL0407 clusters together with other PA catabolic genes and therefore most likely corresponds to a *paaJ* gene. Both open reading frames returned a PcaF homolog in a BLAST search

TABLE 3. Growth of PA catabolism-defective mutants^a

Strain	Mean growth [OD ₆₀₀] ± SD ^b (relative growth ^c) with indicated carbon source		
	Glucose (0.2%)	PA (10 mM)	L-Phenylalanine (10 mM)
K56-2	1.41 ± 0.04 (100)	0.84 ± 0.00 (100)	1.21 ± 0.01 (100)
STC181- <i>paaK1</i>	0.96 ± 0.02 (68)	0.71 ± 0.04 (85)	1.06 ± 0.01 (88)
STC179- <i>paaA</i>	0.79 ± 0.00 (56)	0.19 ± 0.01 (23)	0.25 ± 0.06 (20)
STC155- <i>paaE</i>	1.02 ± 0.03 (73)	0.16 ± 0.01 (19)	0.16 ± 0.01 (13)
STC183- <i>paaZ</i>	0.95 ± 0.03 (67)	0.21 ± 0.01 (25)	0.27 ± 0.00 (22)
STC199- <i>paaF</i>	0.75 ± 0.01 (53)	0.18 ± 0.01 (22)	0.36 ± 0.03 (30)
STC155- <i>paaE</i> / pAS1 (<i>paaE'</i>)	1.00 ± 0.01 (71)	0.61 ± 0.02 (73)	1.03 ± 0.02 (85)
STC199- <i>paaF</i> / pRL1 (<i>paaF'</i>)	0.71 ± 0.02 (51)	0.48 ± 0.00 (57)	0.90 ± 0.03 (74)

^a Cells were cultured at 37°C in M9 medium with different carbon sources, and growth was measured by determining the optical density at 24 h (see Material and Methods).

^b Standard deviations of two independent experiments.

^c Percentage of growth relative to wild-type growth under the same conditions.

against the *E. coli* K-12 genomic sequence. In summary, all the genes required for a functional PA catabolic pathway are present in *B. cenocepacia* strain J2315 (Fig. 1B).

Functional characterization of strains carrying insertional mutations in several genes of the PA catabolic pathway. Since *B. cenocepacia* J2315 is difficult to genetically manipulate, we conducted our research on strain K56-2, which has been shown to be clonally related to J2315 (35) but is more amenable to genetic manipulation. Until recently (17), single-crossover mutagenesis was the only available tool to genetically manipulate *B. cenocepacia*. Although selection of double-recombination events is possible with many bacteria using *sacB*-mediated counterselection (21), attempts to select for double-crossover mutants in *B. cenocepacia* K56-2 using this system have been unsuccessful, probably due to the presence of a *sacB* gene, as revealed by genome sequencing. We therefore used site-directed insertional inactivation of genes by integration of the suicide plasmid pGPΩTp (18). Cloning of internal fragments of *paaA*, *paaE*, and *paaK1* in pGPΩTp and introduction of these plasmids into *B. cenocepacia* by conjugation rendered the mutant strains STC179-*paaA*, STC155-*paaE*, and STC181-*paaK1*, respectively (Table 1). We expected that insertion of the suicide plasmid into the *paaA* gene would prevent transcription of the putative *paaABCDE* operon, since pGPΩTp introduces transcriptional terminators downstream of the insertion site (18). Given its location at the end of the cluster, we expected that disruption of *paaE* would affect only the expression of *paaE* itself. The mutants STC183-*paaZ* and STC199-*paaF*, which have insertions in *paaZ* (probably also affecting downstream genes) and *paaF*, respectively, were created in the same manner (Table 1). Glucose, PA, and L-phenylalanine were used as sole carbon sources in growth experiments performed in 96-well plates (Table 3). Microplate growth kinetics were comparable to those with standard cultivation methods (data not shown), as reported elsewhere (24). *B. cenocepacia* K56-2 was able to grow using glucose, PA, or phenylalanine as a sole carbon source. Cultures reached stationary phase at approximately 24 h (data not shown). PA and phenylalanine supported relative growths of 60% and 85% in comparison with glucose, respectively. The mutant strains grew equally on M9 medium with glucose, although not to wild-type levels. All

TABLE 4. Total phenolic content of supernatants as measured by Gibbs assay

Strain	Total phenolic content (μM) with indicated medium ^a	
	LB	LB + 1 mM PA
K56-2	9.77 (1.88)	12.92 (0.99)
STC179- <i>paaA</i>	6.76 (1.48)	7.81 (2.25)
STC155- <i>paaE</i>	3.01 (0.38)	1.89 (2.16)
STC155- <i>paaE</i> /pAS1 (<i>paaE'</i>)	17.5 (4.29)	16.64 (0.74)
STC183- <i>paaZ</i>	158 (2.31)	302 (39.2)
STC199- <i>paaF</i>	214 (36.1)	345 (18.9)
STC199- <i>paaF</i> /pRL1 (<i>paaF'</i>)	23.0 (2.77)	57.8 (4.90)

^a Cells were grown for 18 h at 37°C on different media and spun down, supernatants were collected, and the total phenolic content was determined. Parenthetical numbers represent the standard deviations for three independent experiments.

gene disruptions severely reduced growth in PA or phenylalanine (Table 3). The only exception was STC181-*paaK1*, which has an insertion in one of two putative *paaK* genes. This mutant grew in PA or phenylalanine to levels similar to those of the wild type.

To further demonstrate that PA degradation was impaired in the PA growth-defective mutants, we measured the total phenolic content of supernatants as an indirect measure of PA degradation. It has been proposed that the product of the PaaABCDE enzymatic complex of *E. coli* is 1,2-dihydroxy-1,2-dihydro-PA-CoA and subsequent dehydration releases the product 2-hydroxy-PA to the culture supernatants (14, 26). Using the Gibbs assay (22, 51), we were able to detect phenolic metabolites in culture supernatants of the wild-type and mutant strains grown on LB or LB containing 1 mM PA (Table 4). *B. cenocepacia* K56-2 released approximately 10 μM of phenolic compounds when grown in LB medium. The *paaF* and *paaZ* culture supernatants showed an increase in phenolic content, in contrast with the supernatants of the *paaA* and *paaE* mutants, which showed decreased levels. The *paaA* and *paaE* mutant supernatants contained the lowest levels of phenolic compounds under both conditions, in accordance with the interruption of the ring hydroxylation system. On the contrary, the significant increase of phenolic content in the supernatants of the *paaZ* and *paaF* cultures was consistent with accumulation of a dihydrodiol derivative due to a downstream blockage of the degradation of this compound.

Disruption of *paaA* or *paaE* but not *paaZ* or *paaF* diminishes virulence of *B. cenocepacia* K56-2 in the *C. elegans* model of infection. It has been shown that *B. cenocepacia* causes a persistent intestinal infection in *C. elegans* (31). The 4A7 transposon mutant had shown a nonpathogenic phenotype in *C. elegans* (6), and for this reason, strains with insertional mutations in the PA catabolic pathway were studied using this nematode model. *C. elegans* has emerged as a convenient host model for the study of host-pathogen interactions (1, 13, 49), since it has been shown that there exists some overlap between virulence factors employed by bacterial pathogens upon infection of vertebrate and invertebrate hosts. The abilities of *paaA* and *paaE* mutants to kill *C. elegans* were compared to that of the wild-type strain K56-2. L4 larvae raised on *Escherichia coli* OP50 were transferred onto plates containing lawns of wild-type or mutant strains, and the number of live worms was scored over time. The nematode strain DH26 has a tempera-

FIG. 2. The virulence of *paaA* and *paaE* mutants is diminished in the *C. elegans* infection model. (A) Kaplan-Meier survival plots for DH26 worms fed with mutant strains STC179-*paaA* and STC155-*paaE*. The killing ability of the wild-type *B. cenocepacia* strain K56-2 ($n = 66$) was compared with that of STC179-*paaA* ($n = 113$; $P < 0.0001$) or STC155-*paaE* ($n = 67$; $P < 0.0001$) in slow-killing assays using *C. elegans* strain DH26. solid lines, K56-2; dashed lines, STC155-*paaE* and STC179-*paaA*. (B) Appearance of worms after 2 days of bacterial exposure. Worms exposed to the nonpathogenic *E. coli* OP50 or *B. cenocepacia* strains were randomly chosen and photographed (magnification, $\times 80$). (C) Wild-type *B. cenocepacia* K56-2 and the mutant STC155-*paaE* accumulate to similar levels in the *C. elegans* intestine. Data represent the mean numbers of CFU per worm from five independent experiments, with error bars signifying standard errors of measurement. gray bars, K56-2; lined bars, STC155-*paaE*. P values for 8, 24, and 48 h were 0.8766, 0.1666, and 0.5745, respectively.

ture-sensitive mutation in the spermatogenesis *fer-15* gene rendering worms sterile at 25°C, thus permitting the scoring of original worms for longer periods of time without the interference of progeny worms. As shown in Fig. 2A, STC179-*paaA* and STC155-*paaE* exhibited decreased pathogenicity relative to the wild-type K56-2 strain. The attenuated pathogenicity phenotype was visually evident after 2 days of infection. When fed on wild-type K56-2 bacteria, nematodes did not develop further and became immobile during the second day of infection. However, it should be noted that sick worms are scored as live since they respond to mechanical stimulus. In contrast, worms fed on the *paaA* or *paaE* mutants developed as adults and were more motile (Fig. 2B). Pharyngeal pumping rates were similar in worms fed with wild-type or mutant strains. Next, we hypothesized that intestinal bacterial loads of the PA catabolism-defective mutants would be reduced in comparison with that of the wild-type strain, in accordance with the attenuated phenotype. To determine whether intestinal titers of

K56-2 differed from those of STC155-*paaE*, worms fed on these strains were removed from plates, washed, and disrupted by vortexing with silicon carbide particles to recover intestinal bacteria at 12-h intervals postinfection. As a control, bacterial cell cultures were vortexed in both the presence and absence of silicon carbide particles and plated to assess effects on bacterial viability. This procedure did not affect bacterial survival (data not shown). Bacteria accumulated in the intestinal lumen of *C. elegans*, reaching approximately 10^5 CFU per worm at 48 h. *C. elegans* fed on STC155-*paaE* had approximately the same numbers of CFU in their intestines as worms fed on K56-2 at 8, 24, and 48 h after infection (Fig. 2C). Thus, the attenuated infection phenotype of STC155-*paaE* is due not to a reduced number of bacteria but most likely to either less-virulent bacteria or worms that are capable of mounting a more efficient defense response to STC155-*paaE* or both. To test if lower steps of the PA catabolic pathway were required for full pathogenicity, we conducted killing assays using strains STC183-*paaZ* and

FIG. 3. The virulence of the *paaZ* and *paaF* mutants is enhanced in comparison with that of *B. cenocepacia* K56-2 in the *C. elegans* infection model. Kaplan-Meier survival plots for DH26 worms fed with the STC199-*paaZ* (A) or STC183-*paaF* (B) mutant strain are shown. The killing ability of the wild-type *B. cenocepacia* strain K56-2 ($n = 118$) was compared with that of STC183-*paaZ* ($n = 99$; $P < 0.006$) or STC199-*paaF* ($n = 114$; $P < 0.0001$) in slow-killing assays using *C. elegans* strain DH26. solid lines, K56-2; dashed lines, STC183-*paaZ* or STC199-*paaF*.

STC199-*paaF*. These mutants were defective for growth with PA as a sole carbon source (Table 3) due to interruption of the putative ring opening system and β -oxidation steps, respectively. However, these mutants did not present an attenuated phenotype in *C. elegans* (Fig. 3B). On the contrary, they were slightly but significantly more pathogenic than the wild type. Taken together, these results suggest that the reduced killing ability of STC155-*paaE* and STC179-*paaA* is related not to a reduced growth rate in the presence of PA but to the interruption of the putative PA-CoA hydroxylation system, which results in bacteria that are able to colonize and persist in the intestinal tract to the same levels as wild-type bacteria but are less virulent in *C. elegans*.

Complementation analysis of *paaE* and *paaF* mutants. The observed attenuated phenotype of the *paaE* mutant in *C. elegans* could be due to polar effects of pGPQTP transcriptional terminators on downstream genes of the *paaABCDE* gene cluster. To test this hypothesis, a complementation analysis was performed. The *paaE* gene of *B. cenocepacia* K56-2 was cloned into pAP20, a constitutive expression vector, to obtain pAS1. These plasmids were introduced into STC155-*paaE* by conjugation and the transformants investigated with respect to their in vitro and in vivo phenotypes. When the *paaE* gene was provided in *trans* in strain STC155-*paaE*/pAS1, growth with PA or phenylalanine was restored to 73% and 85% of that of the wild type, respectively (Table 3). Similarly, the presence of

paaE in *trans* restored and even increased the total phenolic content detected in supernatants (Table 4). As shown in Fig. 4, STC155-*paaE*/pAP20 was attenuated for virulence in *C. elegans*, while pathogenicity of STC155-*paaE*/pAS1 was equal to that of the *B. cenocepacia* wild-type strain K56-2. Thus, the observed phenotype of the *paaE* mutant is due to the interruption of *paaE* and not to polar effects on downstream genes or secondary spontaneous mutations. When the *paaF* gene was expressed in *trans* in STC199-*paaF*/pRL1, the ability to grow with PA or phenylalanine as a sole carbon source was restored to 57% and 74%, respectively (Table 3). However, neither the enhanced pathogenicity nor the total phenolic content observed in this mutant strain could be restored to wild-type levels (Table 4; also data not shown).

Interaction of *C. elegans* innate immune system and *B. cenocepacia* PA catabolism. The reasons for the requirement of a functional *paaABCDE* gene cluster for full pathogenicity of *B. cenocepacia* K56-2 are totally unknown. In an effort to elucidate the mechanism of attenuation of the *paaA* and *paaE* mutants, we examined the response of immunocompromised *C. elegans* to *B. cenocepacia*. We decided to target the *pmk-1* and *elt-2* genes using specific interference by ingested double-stranded RNA (19, 52). It has been shown that inhibition of *pmk-1*, the coding gene for the p38 MAP kinase homolog, produces worms with an enhanced susceptibility to pathogens phenotype that is independent of fitness, feeding, or defecation (29). On the other hand, ELT-2 is a specific GATA transcriptional factor identified as a major regulator of epithelial innate immune responses of *C. elegans* to *Pseudomonas aeruginosa* (48) and other pathogens (28). Consistent with previous results showing enhanced bacterially mediated killing of worms in which the p38 MAP kinase pathway or the GATA transcription factor is inhibited (28, 29, 48), *pmk-1* (RNAi) and *elt-2* (RNAi) worms were hypersusceptible to *B. cenocepacia* K56-2 in comparison with DH26 nematodes (Fig. 5; also data not shown). We then reasoned that the diminished virulence of the *paaA* and *paaE* mutants could be explained if *C. elegans* exhibits an enhanced immune response to these strains. If this were the case, interruption of specific innate immune effectors should result in loss of the attenuated pathogenicity phenotype. When we exposed the *pmk-1* (RNAi) worms to the *paaA* mutant, STC179-*paaA*, the worms were highly susceptible to

FIG. 4. Complementation of STC155-*paaE* with the *paaE* gene in *trans* restores full pathogenicity in *C. elegans* DH26. Kaplan-Meier survival plots for DH26 worms fed with K56-2 ($n = 67$), STC155-*paaE*/pAP20 ($n = 91$; $P < 0.0001$), or STC155-*paaE*/pAS1 ($n = 78$; $P = 0.05740$) are shown. Squares and solid lines, K56-2; crosses and solid lines, STC155-*paaE*/pAP20; triangles and dashed lines, STC155-*paaE*/pAS1.

FIG. 5. *pmk-1* (RNAi) worms are hypersusceptible to *B. cenocepacia* K56-2 and *paaA* mutant strains. (A) Kaplan-Meier survival plots for DH26 and *pmk-1* (RNAi) worms, fed with *B. cenocepacia* K56-2 or STC179-*paaA*. The killing abilities of *B. cenocepacia* K56-2 (solid lines; $P < 0.0001$) and STC179-*paaA* (dashed lines; $P < 0.0001$) were assayed in killing assays using *C. elegans* DH26 (triangles) or *pmk-1* (RNAi) worms (circles). (B) Appearance of worms exposed to *B. cenocepacia* K56-2 or STC179-*paaA* for 2 days. Five to ten worms were chosen randomly, and pictures were taken. Representative pictures are shown at magnification $\times 80$.

killing (Fig. 5), contrasting with the DH26 worms, which were more resistant to STC179-*paaA* than to K56-2. The survival median of the *pmk-1* (RNAi) worms was reduced to 1 day in the presence of either of the two strains. On the contrary, STC179-*paaA* was less pathogenic than K56-2 to *elt-2* (RNAi) worms (data not shown), which had a survival median of 3 days compared to 2, respectively. The killing ability of the *paaE* mutant was next compared with that of the complemented strain STC155-*paaE*/pAS1 using *C. elegans* DH26 and *pmk-1* (RNAi) worms (Fig. 6). As shown earlier (Fig. 4), the *paaE* mutant showed a diminished ability to kill *C. elegans* DH26 in comparison with STC155-*paaE*/pAS1. However, the *pmk-1* (RNAi) worms were similarly hypersusceptible to both STC155-*paaE* and STC155-*paaE*/pAS1 (Fig. 6). Taken together, these data show that due to inhibition of the p38 MAP kinase pathway, immunocompromised *C. elegans* worms are equally hypersusceptible to *B. cenocepacia* K56-2 and the *paaA* and *paaE* mutants, which is in contrast to DH26 worms.

DISCUSSION

We provide evidence for a functional PA catabolic pathway in *B. cenocepacia* K56-2. First, interruption of the *paaA*, *paaE*, *paaF*, and *paaZ* genes severely reduces growth with PA and phenylalanine. This is not surprising given that many aromatic compounds, such as phenylalanine, are degraded through the

FIG. 6. Kaplan-Meier survival plots for DH26 and *pmk-1* (RNAi) worms, fed with *B. cenocepacia* STC155-*paaE* or STC155-*paaE*/pAS1. The killing ability of STC155-*paaE* (dashed lines) was compared with that of STC155-*paaE*/pAS1 (solid lines) in slow-killing assays using *C. elegans* DH26 (triangles) ($P < 0.0016$) or *pmk-1* (RNAi) (circles) ($P = 0.4354$) worms.

PA catabolic pathway (40). Second, the *paaF* and *paaZ* mutants release high levels of phenolic compounds, as has been shown for equivalent *E. coli* mutant supernatants (26). The only strain with a mutation in a PA catabolic gene that did not show a PA-reduced growth phenotype is STC181-*paaK1*. However, a second potentially functional *paaK* gene (*paaK2*) (Fig. 1A) most likely explains this phenotype. To test this hypothesis, a double-knockout strain is currently under development using genetic tools that have recently become available for *Burkholderia* species (7, 17).

The PA catabolic pathway and its relationship to pathogenicity in *B. cenocepacia* first captured our interest during a screening of signature-tagged mutagenesis mutants defective for survival in vivo (25). The 4A7 mutant, which failed to survive in a rat model of infection, had a transposon insertion in the *paaE* gene and was not pathogenic to *C. elegans* (6). In this study we demonstrate that interruption of putative PA-CoA ring hydroxylation activity but not the lower steps of PA degradation results in an attenuated pathogenicity phenotype in *C. elegans*. The *paaA* and *paaE* insertional mutants, however, do not present an attenuation phenotype as severe as that of the 4A7 mutant. Recently it was shown that *B. cenocepacia* K56-2 can spontaneously undergo colony morphology transition from a rough phenotype to different shiny colony variants, many of which are associated with decreased virulence (4). Visual examination of the 4A7 mutant evidenced shiny colony morphology (data not shown). Therefore, the nonpathogenic phenotype of the 4A7 mutant in *C. elegans* is most likely a combination of both the defective PA catabolic pathway and a secondary site mutation related to the cell surface modification. Nevertheless, the *paaA* and *paaE* insertional mutants present a rough phenotype (data not shown) and are attenuated for pathogenesis in *C. elegans*. While many bacterial genes have been associated with nematode-killing ability, the reduced virulence of bacteria carrying mutations in these genes is very often associated with reduced colonization or survival in the intestinal tract (3, 20). Surprisingly, the attenuated pathogenicity phenotype of the *paaE* mutant is not due to decreased accumulation of bacteria (Fig. 2C). It should be noted, however, that accumulation of bacteria in the nematode gut does not necessarily cause killing; many clinical isolates of *Enterococcus faecium* accumulate in *C. elegans* but do not result in significant killing (20). This seems to be the case for STC155-*paaE*. Although the intestinal accumulation of the

paaE mutant equals that of the wild type, the killing ability of this strain is reduced.

We first hypothesized that PA catabolism mutants presented reduced growth in *C. elegans*. A number of observations led us to rule out this hypothesis. First, the *paaE* mutant strain accumulates in the *C. elegans* intestine to levels equal to those of the wild type. Second, interruption of lower steps of the PA catabolic pathway does not cause attenuation of pathogenicity. On the contrary, the *paaF* and *paaZ* mutants were slightly but significantly more pathogenic than the wild-type strain. It should be noted that complementation in *trans* with the *paaF* gene did not restore the pathogenic phenotype to the same level as that of the wild type (data not shown). A possible explanation is that the levels of phenolic compounds released by the complemented mutants were reduced but still higher than the ones of K56-2 (Table 4). It is possible, then, that the mutants accumulate or release PA-CoA intermediates or hydrolyzed PA products like those found in supernatants of PA-degrading cells (38), and *C. elegans* may respond to these chemicals.

The effect of PA and its derivatives on eukaryotic cells appears to be pleiotropic and is poorly understood at the molecular level. PA has been described as an inhibitor of inducible nitric oxide synthase (iNOS) and lipopolysaccharide-induced expression of cytokines in rat primary astrocytes, microglia, and macrophages (43). Additionally, PA has been described as a repressor of DNA binding and transcriptional activities of NF- κ B, an important upstream modulator for cytokine and iNOS expression in macrophages (44), and a ligand of PPAR γ (peroxisome proliferator-activated receptor γ), a member of the nuclear hormone receptor superfamily (47). The *C. elegans* genome does not appear to contain homologs of iNOS-, NF- κ B-, or PPAR γ -coding genes, though many *C. elegans* nuclear hormone receptor genes share a high degree of similarity with the PPAR γ ligand binding domain (data not shown). Whether or not nuclear receptor genes are involved in cell signaling by the effect of PA derivatives in *C. elegans* remains to be determined. Hence, the reasons behind the requirement for a functional ring hydroxylation system for full pathogenicity of *B. cenocepacia* in *C. elegans* remain elusive.

Finally, further investigation is needed to determine if PA or its phenolic derivatives may act as interkingdom signal molecules mediating pathogenesis and host response in mammalian host-pathogen interactions. This is a tantalizing hypothesis given the widespread occurrence of natural precursors and metabolites of PA across domains of life and the effect of exogenous PA on mammalian immune responses.

ACKNOWLEDGMENTS

We are grateful to Miguel A. Valvano and Cristina Marolda for facilitating preliminary experiments and providing us with strains and plasmids. We thank Theresa Stiernagle, CGC Center, University of Minnesota, for kindly providing us with *C. elegans* strains; Julian Parkhill and Mathew Holden for allowing us access to the draft annotation of *B. cenocepacia* J2315, and Ivan Oresnik for critically reading the manuscript.

R.J.L. was previously supported by a graduate scholarship from the Faculty of Science, University of Manitoba, and is currently supported by a Canada Graduate Scholarship from the Natural Science and Engineering Research Council of Canada (NSERC). J.N.R.H. is supported by a graduate scholarship from the Manitoba Health Research

Council (MHRC). This study was supported by the NSERC grant no. 327954.

REFERENCES

- Aballay, A., and F. M. Ausubel. 2002. *Caenorhabditis elegans* as a host for the study of host-pathogen interactions. *Curr. Opin. Microbiol.* **5**:97–101.
- Avery, L., and H. R. Horvitz. 1990. Effects of starvation and neuroactive drugs on feeding in *Caenorhabditis elegans*. *J. Exp. Zool.* **253**:263–270.
- Begun, J., J. M. Gaiani, H. Rohde, D. Mack, S. B. Calderwood, F. M. Ausubel, and C. D. Sifri. 2007. Staphylococcal biofilm exopolysaccharide protects against *Caenorhabditis elegans* immune defenses. *PLoS Pathog.* **3**:e57.
- Bernier, S. P., D. T. Nguyen, and P. A. Sokol. 2008. A LysR-type transcriptional regulator in *Burkholderia cenocepacia* influences colony morphology and virulence. *Infect. Immun.* **76**:38–47.
- Cardona, S. T., and M. A. Valvano. 2005. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. *Plasmid* **54**:219–228.
- Cardona, S. T., J. Wopperer, L. Eberl, and M. A. Valvano. 2005. Diverse pathogenicity of *Burkholderia cepacia* complex strains in the *Caenorhabditis elegans* host model. *FEMS Microbiol. Lett.* **250**:97–104.
- Choi, K. H., T. Mima, Y. Casart, D. Rhol, A. Kumar, I. R. Beacham, and H. P. Schweizer. 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. *Appl. Environ. Microbiol.* **74**:1064–1075.
- Cohen, S. N., A. C. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* **69**:2110–2114.
- Craig, F. F., J. G. Coote, R. Parton, J. H. Freer, and N. J. Gilmour. 1989. A plasmid which can be transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and conjugation. *J. Gen. Microbiol.* **135**:2885–2890.
- Darling, P., M. Chan, A. D. Cox, and P. A. Sokol. 1998. Siderophore production by cystic fibrosis isolates of *Burkholderia cepacia*. *Infect. Immun.* **66**:874–877.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Diaz, E., A. Ferrandez, M. A. Prieto, and J. L. Garcia. 2001. Biodegradation of aromatic compounds by *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* **65**:523–569.
- Ewbank, J. 2003. The nematode *Caenorhabditis elegans* as a model for the study of host-pathogen interactions. *J. Soc. Biol.* **197**:375–378.
- Fernandez, C., A. Ferrandez, B. Minambres, E. Diaz, and J. L. Garcia. 2006. Genetic characterization of the phenylacetyl-coenzyme A oxygenase from the aerobic phenylacetic acid degradation pathway of *Escherichia coli*. *Appl. Environ. Microbiol.* **72**:7422–7426.
- Ferrandez, A., B. Minambres, B. Garcia, E. R. Olivera, J. M. Luengo, J. L. Garcia, and E. Diaz. 1998. Catabolism of phenylacetic acid in *Escherichia coli*. Characterization of a new aerobic hybrid pathway. *J. Biol. Chem.* **273**:25974–25986.
- Figurski, D. H., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. USA* **76**:1648–1652.
- Flannagan, R. S., T. Linn, and M. A. Valvano. 2008. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. *Environ. Microbiol.* **10**:1652–1660.
- Flannagan, R. S., D. Aubert, C. Kooi, P. A. Sokol, and M. A. Valvano. 2007. *Burkholderia cenocepacia* requires a periplasmic HtrA protease for growth under thermal and osmotic stress and for survival in vivo. *Infect. Immun.* **75**:1679–1689.
- Fraser, A. G., R. S. Kamath, P. Zipperlen, M. Martinez-Campos, M. Sohrmann, and J. Ahringer. 2000. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**:325–330.
- Garsin, D. A., C. D. Sifri, E. Mylonakis, X. Qin, K. V. Singh, B. E. Murray, S. B. Calderwood, and F. M. Ausubel. 2001. A simple model host for identifying Gram-positive virulence factors. *Proc. Natl. Acad. Sci. USA* **98**:10892–10897.
- Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado. 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J. Bacteriol.* **164**:918–921.
- Gibbs, H. D. 1927. The indophenol test. *J. Biol. Chem.* **72**:649–664.
- Harwood, C. S., N. N. Nichols, M. K. Kim, J. L. Ditty, and R. E. Parales. 1994. Identification of the *pcrRKF* gene cluster from *Pseudomonas putida*: involvement in chemotaxis, biodegradation, and transport of 4-hydroxybenzoate. *J. Bacteriol.* **176**:6479–6488.
- Horakova, K., M. Greifova, Z. Seemannova, B. Gondova, and G. M. Wyatt. 2004. A comparison of the traditional method of counting viable cells and a quick microplate method for monitoring the growth characteristics of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **38**:181–184.
- Hunt, T. A., C. Kooi, P. A. Sokol, and M. A. Valvano. 2004. Identification of *Burkholderia cenocepacia* genes required for bacterial survival in vivo. *Infect. Immun.* **72**:4010–4022.
- Ismail, W., M. El-Said Mohamed, B. L. Wanner, K. A. Datsenko, W. Eisen-

- reich, F. Rohdich, A. Bacher, and G. Fuchs. 2003. Functional genomics by NMR spectroscopy. Phenylacetate catabolism in *Escherichia coli*. *Eur. J. Biochem.* **270**:3047–3054.
27. Jimenez, J. I., B. Minambres, J. L. Garcia, and E. Diaz. 2002. Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**:824–841.
28. Kerry, S., M. TeKippe, N. C. Gaddis, and A. Aballay. 2006. GATA transcription factor required for immunity to bacterial and fungal pathogens. *PLoS ONE* **1**:e77.
29. Kim, D. H., R. Feinbaum, G. Alloing, F. E. Emerson, D. A. Garsin, H. Inoue, M. Tanaka-Hino, N. Hisamoto, K. Matsumoto, M. W. Tan, and F. M. Ausubel. 2002. A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. *Science* **297**:623–626.
30. Kim, Y. H., K. Cho, S. H. Yun, J. Y. Kim, K. H. Kwon, J. S. Yoo, and S. I. Kim. 2006. Analysis of aromatic catabolic pathways in *Pseudomonas putida* KT 2440 using a combined proteomic approach: 2-DE/MS and cleavable isotope-coded affinity tag analysis. *Proteomics* **6**:1301–1318.
31. Kothe, M., M. Antl, B. Huber, K. Stoecker, D. Ebrecht, I. Steinmetz, and L. Eberl. 2003. Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the cep quorum-sensing system. *Cell Microbiol.* **5**:343–351.
32. Lefebvre, M. D., and M. A. Valvano. 2002. Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. *Appl. Environ. Microbiol.* **68**:5956–5964.
33. Luengo, J. M., J. L. Garcia, and E. R. Olivera. 2001. The phenylacetyl-CoA catabolon: a complex catabolic unit with broad biotechnological applications. *Mol. Microbiol.* **39**:1434–1442.
34. Mahenthalingam, E., T. A. Urban, and J. B. Goldberg. 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat. Rev. Microbiol.* **3**:144–156.
35. Mahenthalingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. Govan, P. Taylor, and P. Vandamme. 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **38**:910–913.
36. Maloney, K. E., and M. A. Valvano. 2006. The *mgtC* gene of *Burkholderia cenocepacia* is required for growth under magnesium limitation conditions and intracellular survival in macrophages. *Infect. Immun.* **74**:5477–5486.
37. Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
38. Mohamed, M., W. Ismail, J. Heider, and G. Fuchs. 2002. Aerobic metabolism of phenylacetic acids in *Azoarcus evansii*. *Arch. Microbiol.* **178**:180–192.
39. Moy, T. I., A. R. Ball, Z. Anklesaria, G. Casadei, K. Lewis, and F. M. Ausubel. 2006. Identification of novel antimicrobials using a live-animal infection model. *Proc. Natl. Acad. Sci. USA* **103**:10414–10419.
40. Navarro-Llorens, J. M., M. A. Patrauchan, G. R. Stewart, J. E. Davies, L. D. Eltis, and W. W. Mohn. 2005. Phenylacetate catabolism in *Rhodococcus* sp. strain RHA1: a central pathway for degradation of aromatic compounds. *J. Bacteriol.* **187**:4497–4504.
41. Nogales, J., R. Macchi, F. Franchi, D. Barzaghi, C. Fernandez, J. L. Garcia, G. Bertoni, and E. Diaz. 2007. Characterization of the last step of the aerobic phenylacetic acid degradation pathway. *Microbiology* **153**:357–365.
42. Olivera, E. R., B. Minambres, B. Garcia, C. Muniz, M. A. Moreno, A. Ferrandez, E. Diaz, J. L. Garcia, and J. M. Luengo. 1998. Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the phenylacetyl-CoA catabolon. *Proc. Natl. Acad. Sci. USA* **95**:6419–6424.
43. Pahan, K., F. G. Sheikh, A. M. Namboodiri, and I. Singh. 1997. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. *J. Clin. Investig.* **100**:2671–2679.
44. Park, J. S., E. J. Lee, J. C. Lee, W. K. Kim, and H. S. Kim. 2007. Anti-inflammatory effects of short chain fatty acids in IFN-gamma-stimulated RAW 264.7 murine macrophage cells: involvement of NF-kappaB and ERK signaling pathways. *Int. Immunopharmacol.* **7**:70–77.
45. Rutherford, K., J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajan-dream, and B. Barrell. 2000. Artemis: sequence visualization and annotation. *Bioinformatics* **16**:944–945.
46. Saldias, M. S., J. Lamothe, R. Wu, and M. A. Valvano. 2008. *Burkholderia cenocepacia* requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. *Infect. Immun.* **154**:440–453.
47. Samid, D., M. Wells, M. E. Greene, W. Shen, C. N. Palmer, and A. Thibault. 2000. Peroxisome proliferator-activated receptor gamma as a novel target in cancer therapy: binding and activation by an aromatic fatty acid with clinical antitumor activity. *Clin. Cancer Res.* **6**:933–941.
48. Shapira, M., B. J. Hamlin, J. Rong, K. Chen, M. Ronen, and M. W. Tan. 2006. A conserved role for a GATA transcription factor in regulating epithelial innate immune responses. *Proc. Natl. Acad. Sci. USA* **103**:14086–14091.
49. Sifri, C. D., J. Begun, and F. M. Ausubel. 2005. The worm has turned—microbial virulence modeled in *Caenorhabditis elegans*. *Trends Microbiol.* **13**:119–127.
50. Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin. 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* **28**:33–36.
51. Thoss, V., M. S. Baird, M. A. Lock, and P. V. Courty. 2002. Quantifying the phenolic content of freshwaters using simple assays with different underlying reaction mechanisms. *J. Environ. Monit.* **4**:270–275.
52. Timmons, L., and A. Fire. 1998. Specific interference by ingested dsRNA. *Nature* **395**:854.
53. Valvano, M. A., K. E. Keith, and S. T. Cardona. 2005. Survival and persistence of opportunistic *Burkholderia* species in host cells. *Curr. Opin. Microbiol.* **8**:99–105.
54. Woombs, M., and J. Laybourn-Parry. 1984. Feeding biology of *Diplogasterius nudicapitus* and *Rhabditis curvicaudata* (Nematoda) related to food concentration and temperature, in sewage treatment plants. *Oecologia* **64**:164–167.