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RESEARCH ARTICLE

Enzymatic Degradation of Phenazines Can Generate Energy and Protect Sensitive Organisms from Toxicity

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ABSTRACT **Diverse bacteria, including several *Pseudomonas* species, produce a class of redox-active metabolites called phenazines that impact different cell types in nature and disease. Phenazines can affect microbial communities in both positive and negative ways, where their presence is correlated with decreased species richness and diversity. However, little is known about how the concentration of phenazines is modulated *in situ* and what this may mean for the fitness of members of the community. Through culturing of phenazine-degrading mycobacteria, genome sequencing, comparative genomics, and molecular analysis, we identified several conserved genes that are important for the degradation of three *Pseudomonas*-derived phenazines: phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), and pyocyanin (PYO). PCA can be used as the sole carbon source for growth by these organisms. Deletion of several genes in *Mycobacterium fortuitum* abolishes the degradation phenotype, and expression of two genes in a heterologous host confers the ability to degrade PCN and PYO. In cocultures with phenazine producers, phenazine degraders alter the abundance of different phenazine types. Not only does degradation support mycobacterial catabolism, but also it provides protection to bacteria that would otherwise be inhibited by the toxicity of PYO. Collectively, these results serve as a reminder that microbial metabolites can be actively modified and degraded and that these turnover processes must be considered when the fate and impact of such compounds in any environment are being assessed.**

IMPORTANCE **Phenazine production by *Pseudomonas* spp. can shape microbial communities in a variety of environments ranging from the cystic fibrosis lung to the rhizosphere of dryland crops. For example, in the rhizosphere, phenazines can protect plants from infection by pathogenic fungi. The redox activity of phenazines underpins their antibiotic activity, as well as providing pseudomonads with important physiological benefits. Our discovery that soil mycobacteria can catabolize phenazines and thereby protect other organisms against phenazine toxicity suggests that phenazine degradation may influence turnover *in situ*. The identification of genes involved in the degradation of phenazines opens the door to monitoring turnover in diverse environments, an essential process to consider when one is attempting to understand or control communities influenced by phenazines.**

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Pseudomonas spp. are important biocontrol agents that produce a variety of secreted metabolites that suppress disease in the rhizosphere (1–4). Of these metabolites, phenazines are an important subclass. Phenazines have long been studied due to their antibiotic activities against diverse cell types as well as their beneficial physiological roles for their producers (5). In agricultural settings, the production of phenazines—particularly phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN)—is thought to protect plants from colonization and infection by pathogenic fungi (1–3). Phenazines accumulate in the rhizosphere of dryland cereals, where they have a half-life of 3.4 days (2). In addition to the rhizosphere, phenazines are present and active in other environmental and clinical contexts, such as crude oil and the lungs of patients with the genetic disorder cystic fibrosis (CF) (6–8); however, their turnover has not been measured in any of these systems. While the relatively short half-life of phenazines in the rhizosphere suggests that there are active mechanisms of removal, it is unclear what they are.

Many natural phenazine compounds with a common nitrogen-heterocyclic core have been described (Fig. 1A). *Pseudomonas* spp. and other bacteria produce several phenazine derivatives with diverse properties (9). The most abundant phenazines in laboratory-grown *Pseudomonas aeruginosa* culture are PCA, PCN, and pyocyanin (PYO). PCA is the precursor from which all other phenazines are derived, PYO is produced by the action of two enzymes (PhzM and PhzS) that modify PCA, and PCN is generated from PCA by the action of PhzH (10). *P. aeruginosa* can also produce 1-hydroxyphenazine through the action of PhzS. Phenazines benefit producing organisms in a variety of ways. In *P. aeruginosa*, phenazines are involved in anaerobic survival, iron acquisition, signaling, and biofilm development (11–14). However, the redox properties of phenazines are harmful to other bac-

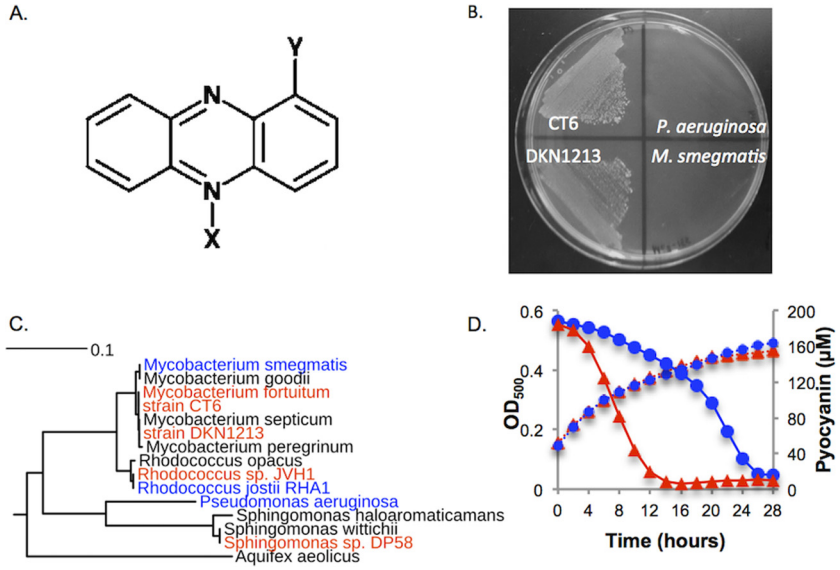

FIG 1 Isolation, identification, and degradation phenotype of phenazine-degrading bacteria. (A) Structure of phenazines. For PCA, Y = COOH; for PCN, Y = $CONH_2$; for PYO, X = CH_3 and Y = OH. (B) Growth of representative isolates CT6 and DKN1213 with PCA as a sole source of carbon and energy. *P. aeruginosa* and *M. smegmatis* were both incapable of growth under this condition. (C) 16S rRNA gene dendrogram of various phenazine-degrading bacteria (red) and close relatives. Organisms in blue were tested for phenazine degradation but showed no activity. (D) Growth and degradation of PYO by strains CT6 (red) and DKN1213 (blue) supplied with 10% LB-Tw as a carbon source. Data are averages and standard deviations (SD) for three cultures. Solid lines, PYO concentration; dashed lines, OD.

teria and eukaryotic organisms that are often found in association with *Pseudomonas* spp. (15–17).

Phenazine toxicity differs depending on the phenazine type and can change under various environmental conditions. For example, *Caenorhabditis elegans* is more sensitive to PCA than PYO at acidic pH, but the opposite is true at alkaline pH (15). Phenazines can cause toxicity by producing reactive oxygen species (ROS) and interfering with the respiratory electron transport chain (16, 17). While defense against the toxic effects of phenazines is generally thought to involve the induction of ROS defense systems, the capacity to degrade or transform phenazines, including PCA and PCN, has also been demonstrated (18–20). A recent study showed changes to phenazines in mixed communities, where diffusion of phenazines between colonies of *P. aeruginosa* and *Aspergillus fumigatus* results in several metabolic transformations (but not removal) of the phenazines (21). Yet, in analogy to what has been shown for acyl-homoserine lactone quorum-sensing signal degradation (22, 23), it is important to consider turnover processes in addition to chemical modifications when one is seeking to understand the fate of phenazines in the environment.

While the capacity to alter or degrade phenazines has been demonstrated by microbes associated with *Pseudomonas* spp. in natural communities (18–21), the genes responsible for this activity have been unknown. Here, we isolated phenazine-degrading organisms and identified genes involved in the degradation of three *Pseudomonas*-derived phenazines. We used these findings to explore the effects of phenazine degradation on phenazine producers (pseudomonads) and degraders (mycobacteria) and to determine whether phenazine degradation can play a protective role for other, phenazine-sensitive organisms. Our findings suggest that the interactions between phenazines and phenazine degraders have the potential to tune the concentrations of different phenazine types, and if phenazine degradation is active *in situ*, it would be expected to impact microbial community structure.

RESULTS

Isolation of PCA-degrading organisms. PCA is the precursor of all phenazines produced by *Pseudomonas* spp. (9, 10). Therefore, we reasoned that the ability to degrade PCA would be common among organisms capable of degrading *Pseudomonas*-derived phenazines. Soil was collected from 16 locations around the California Institute of Technology campus and the nearby San Gabriel mountains. Samples from six sites yielded isolates capable of growing in medium with 2.5 mM PCA as the sole source of carbon and energy (Fig. 1B). Among the isolates that were verified to degrade PCA, all had similar colony morphology; thus, one isolate from each site was randomly selected for follow-up studies. Partial 16S rRNA gene sequences were determined for each isolate and phylogenetic trees were constructed. PCA-degrading organisms grouped with two members of the genus *Mycobacterium*, *Mycobacterium fortuitum* (GenBank accession number NR_118883) and *Mycobacterium septicum* (accession number NR_042916), with >99% sequence identity (Fig. 1C). Strains CT6 (*M. fortuitum*-like) and DKN1213 (*M. septicum*-like) were chosen as representative strains for further study.

Strains CT6 and DKN1213 can additionally degrade PYO. While PCA is produced by all pseudomonads capable of phenazine production, PYO is produced only by *P. aeruginosa* and is the best-studied phenazine due to its clinical relevance. We therefore sought to determine if our isolates were capable of degrading PYO. Because PYO is poorly soluble in aqueous solutions at circumneutral pH, we monitored PYO degradation using micromolar—but physiologically relevant (24)—concentrations with cultures grown in 10% LB-Tw (lysogeny broth with 0.05% Tween 80) medium. Both CT6 and DKN1213 were capable of

TABLE 1 Genes important to this study[a]

CT6		*M. fortuitum* ATCC 6841		*Rhodococcus* JVH1		*Sphingomonas* DP58	
XA26 gene no.	RAST annotation	MFORT gene no.	% ID	Gene GI no.	% ID	MSEDRAFT gene no.	% ID
16600	3-Hydroxy-4-oxoquinaldine 2,4-dioxygenase	16204	100			00241	32
16610	Salicylate hydroxylase	16209	99	497117731	28	05232	33
16620	Hypothetical protein	16214	99	760099504	38	02403	35
16640	4-Hydroxyphenylacetate 3-monooxygenase	16224	100	396930211	32		
16730	Ortho-halobenzoate 1,2-dioxygenase	16269	99	497121742	79	05237	41
16830	2,3-Dihydroxybiphenyl 1,2-dioxygenase	16319	95	497121763	82	04471	52
16860	Large subunit naph/bph dioxygenase[b]	16334	100	497121769	89	04467	73
16890	2,3-Dihydroxybiphenyl 1,2-dioxygenase	16349	99	497121775	80	04486	62
16960	Amidase	30529	99	497116141	32	05146	34
16980	Probable oxidoreductase	14347	99	497109126	27	04185	27
16990	Hypothetical protein	14352	99				

[a] An expanded version of this table is presented as Table S1 in the supplemental material. ID, identity.
[b] naph/bph, naphthalene/biphenyl.

degrading PCA under these conditions. Both strains were additionally capable of degrading PYO from a starting concentration of 200 μM to a final concentration of less than 20 μM (the detection limit of our assay) (Fig. 1D) with a concomitant change of the medium from blue to colorless. No change in medium color or measured PYO was observed for the close relative *Mycobacterium smegmatis* (see Fig. S1 in the supplemental material).

Genome sequence of strain CT6 and identification of candidate dioxygenases involved in PCA degradation. Phenazines are polycyclic, aromatic, nitrogen-containing heterocycles (Fig. 1A); therefore, we hypothesized that the genes responsible for their degradation likely encode dioxygenases, a family of enzymes known to break down aromatics. Several observations lend credence to this notion. First, because the only source of carbon in PCA that is sufficiently reduced to support growth is bound within the ring, it must be cleaved; the carboxylate moiety alone cannot support growth (Fig. 1A). Second, previous studies of the PCA degradation pathway in *Sphingomonas wittichii* strain DP58, the only other known PCA degrader, demonstrated that ring cleavage is essential for PCA catabolism (25). Furthermore, in our cultures, we never observed the accumulation of the unsubstituted core phenazine molecule, despite the fact that our mycobacteria can degrade this molecule (see Fig. S2 in the supplemental material); its absence strongly suggests that the core phenazine ring is cleaved. Finally, the fact that our mycobacteria can also use PCA as a sole nitrogen source (see Fig. S3 in the supplemental material) further indicates that ring cleavage must occur in order for nitrogen to be liberated from PCA (Fig. 1A). Accordingly, we focused on identifying putative dioxygenase enzymes that might catalyze PCA degradation.

The genome of *S. wittichii* strain DP58 has been sequenced and contains 91 predicted dioxygenase genes, though none have been identified as important for PCA degradation (19, 26). We sought to identify candidate dioxygenase genes for PCA degradation by comparative genomic analysis of *S. wittichii* DP58 and one of the phenazine-degrading mycobacteria described here. The genome of strain CT6 was sequenced to completion using the PacBio single-molecule, real-time (SMRT) genome sequencing technology. The strain CT6 genome is 6.25 Mbp and contains no plasmids. There are a predicted 6,051 protein-encoding genes according to the RAST (Rapid Annotation using Subsystem Technology) server, and the genome contains 35 to 47 dioxygenase genes as annotated by RAST or the Bacterial Annotation System (BASys) pipelines, respectively (27–29). Reciprocal best BLAST analysis of annotated dioxygenase genes was performed between *S. wittichii* strain DP58 and strain CT6 (PCA degraders) or *M. smegmatis* mc^2-155 and strain CT6 to identify genes present in both PCA-degrading organisms but absent from *M. smegmatis*. This identified three predicted dioxygenase genes—XA26_16830, XA26_16860, and XA26_16890—that are co-oriented in an ~10-kbp chromosomal locus in strain CT6 (Table 1; also, see Table S1 in the supplemental material). Because the same set of genes is present in both sequenced PCA-degrading organisms, we hypothesized that these genes would be regulated specifically by phenazines. Quantitatve reverse transcription-PCR (qRT-PCR) analysis of the expression of putative dioxygenase genes in strain CT6 demonstrated that these three genes, as well as a nearby fourth gene predicted to encode a dioxygenase (XA26_16730), are highly induced in the presence of phenazines (Fig. 2A). mRNA abundance for these genes was increased >1,000-fold in the presence of both PCA and PYO after a 3-h exposure but not in the presence of 9,10-anthraquinone-2,6-disulfonate (AQDS) or methylene blue (MB), two other 3-ringed, aromatic, redox-active molecules (13). AQDS and MB have midpoint redox potentials that are lower and higher, respectively, than those of phenazines (13) suggesting that a redox switch is not responsible for increased expression. The mRNA abundance of two predicted dioxygenase genes located distantly on the chromosome was unchanged in the presence of any compounds tested.

***Rhodococcus* strain JVH1 and *M. fortuitum* ATCC 6841 are both capable of degrading phenazines.** A comparison of the genome of CT6 to that of the type strain *M. fortuitum* ATCC 6841 revealed the presence of the predicted PCA-degrading genes, and this organism was verified to degrade both PCA and PYO (see Fig. S1 in the supplemental material). The putative dioxygenases predicted to be involved in PCA degradation were analyzed in the Integrated Microbial Genomes/Expert Review (IMG/ER) web server (30, 31), and *Rhodococcus* sp. strain JVH1 was also found to contain these genes (32, 33). Based on their presence, we predicted that *Rhodococcus* sp. strain JVH1 would be capable of PCA degradation. When suspended at high density (optical density at 600 nm [OD_{600}] of ~2 to 3), JVH1 degraded 200 μM PCA to ~50% of the starting concentration in 48 h—a rate of degradation significantly lower than that observed for mycobacteria. No activity was

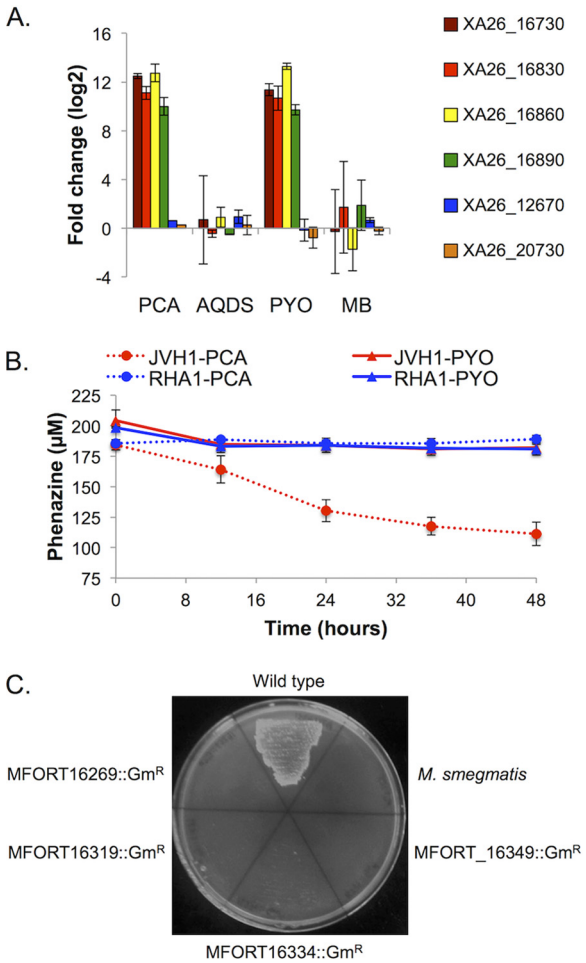

FIG 2 Evidence for the involvement of four putative dioxygenase genes in the breakdown of PCA. (A) qRT-PCR of genes from cultures exposed to various redox-active small molecules. Data are normalized internally to *rpoA* and to a control with no added small molecule. Data represent at least duplicates, and error bars show one SD around the mean for triplicate cultures. (B) Phenazine degradation by *Rhodococcus* strains JVH1 and RHA1. Data are averages and SD for three independent cultures. (C) Growth of individual gene mutants with PCA as a sole carbon source.

seen in *Rhodococcus* sp. strain RHA1, the closest sequenced relative that lacks the genes of interest (Fig. 2B). No degradation activity was observed with PYO for either strain.

Allelic replacement of candidate dioxygenase homologs from *M. fortuitum* ATCC 6841 abolishes the ability to grow with PCA as the sole carbon source. The presence of the same putative dioxygenase genes in all PCA degraders and their phenazine-specific regulation in strain CT6 strongly suggest an involvement in PCA degradation. We used mutagenesis to test this hypothesis. Attempts to genetically manipulate strain CT6 were ineffective, but a similar approach succeeded in the *M. fortuitum* type strain. A recombineering approach was employed to create mutations in the four putative dioxygenase genes (MFORT_16269, MFORT_16319, MFORT_16334, and MFORT_16349) (Table 1) (34), and each gene was replaced with a gentamicin resistance cassette. Gentamicin-resistant colonies with disruptions in each gene were streaked onto agar medium with 2.5 mM PCA as the sole carbon source. None of the mutants grew under these conditions, indicating that the mutated genes are essential for growth with PCA as a carbon source (Fig. 2C). High-performance liquid chromatography (HPLC) analysis of supernatants from cultures grown on LB medium with 100 μM PCA revealed the loss of PCA from Δ16319::Gm^r, Δ16334::Gm^r, and Δ16349::Gm^r strains but not from the Δ16269::Gm^r strain (see Fig. S4 in the supplemental material). This suggests that the product of MFORT_16269 is necessary to catalyze PCA degradation, whereas the other genes are involved in a downstream reaction (these mutants cannot use PCA as a carbon source [Fig. 2C] but still remove it from culture supernatants [see Fig. S4 in the supplemental material]). None of these mutants had a defect in PYO removal.

Notably, only the putative single-subunit, ring-cleavage dioxygenase genes, MFORT_16319 and MFORT_16349, could be complemented in *trans* (see Fig. S5 in the supplemental material). It is unclear why the putative multisubunit, ring-hydroxylating dioxygenase genes MFORT_16269 and MFORT_16334 could not be complemented, but perhaps these enzymes are nonfunctional when overexpressed. The position of the gentamicin resistance cassette on the chromosome was verified by sequencing for each mutation; however, we cannot rule out the possibility that a secondary site mutation resulted in the phenotype for the MFORT_16269 and MFORT_16334 mutants.

RNA-Seq and mutagenesis identifies a small hypothetical protein that is necessary for PYO degradation. Because the four predicted dioxygenase genes were expressed specifically in the presence of phenazines, we took a transcriptome sequencing (RNA-Seq) approach to identify candidate genes important to PYO degradation. Strain CT6 was used for RNA-Seq because the genome of this organism has been closed, whereas the genome of ATCC 6841 exists as 82 contigs (35). As expected, the genes important for PCA-dependent growth showed increased mRNA abundance after a 20-min exposure to either PCA or PYO (Fig. 3A). In fact, the entire region of the CT6 genome that shares homology with *Rhodococcus* sp. strain JVH1 (an organism that can degrade only PCA) was highly expressed in the presence of either PCA or PYO. Additionally, genes flanking this region had increased mRNA abundance and were induced to a greater extent by PYO.

A mutant lacking the entire ~40-kb region (Δ40kb::Gm^r) of the genome induced by phenazines was constructed in *M. fortuitum* and found to completely lack the ability to degrade phenazines, including PYO (Fig. 3B). This locus contains genes for an additional predicted dioxygenase (MFORT_16204 [XA26_16600]) and a monooxygenase (MFORT_16224 [XA26_16640]) in one of the flanking regions; however, mutants with mutations in both genes were still capable of PYO degradation (Fig. 3B). Of note is that Δ16224::Gm^r degrades PYO more slowly than the wild type (WT), suggesting a possible role in a downstream reaction in the PYO degradation pathway. A mutant missing a three-gene operon in the other flanking region (MFORT_14352 to MFORT_30529 [XA26_16990 to XA26_16960]) lacked the ability to degrade PYO. A single gene mutant lacking MFORT_14352, annotated as a hypothetical protein, was deficient in PYO degradation; additionally, expression of this gene in *Rhodococcus* sp. strain JVH1 allowed PYO degradation by this strain (see Fig. S6 in the supplemental material). Therefore, MFORT_14352 is necessary for the first step of PYO degradation and may be sufficient for the removal of this phenazine from the medium.

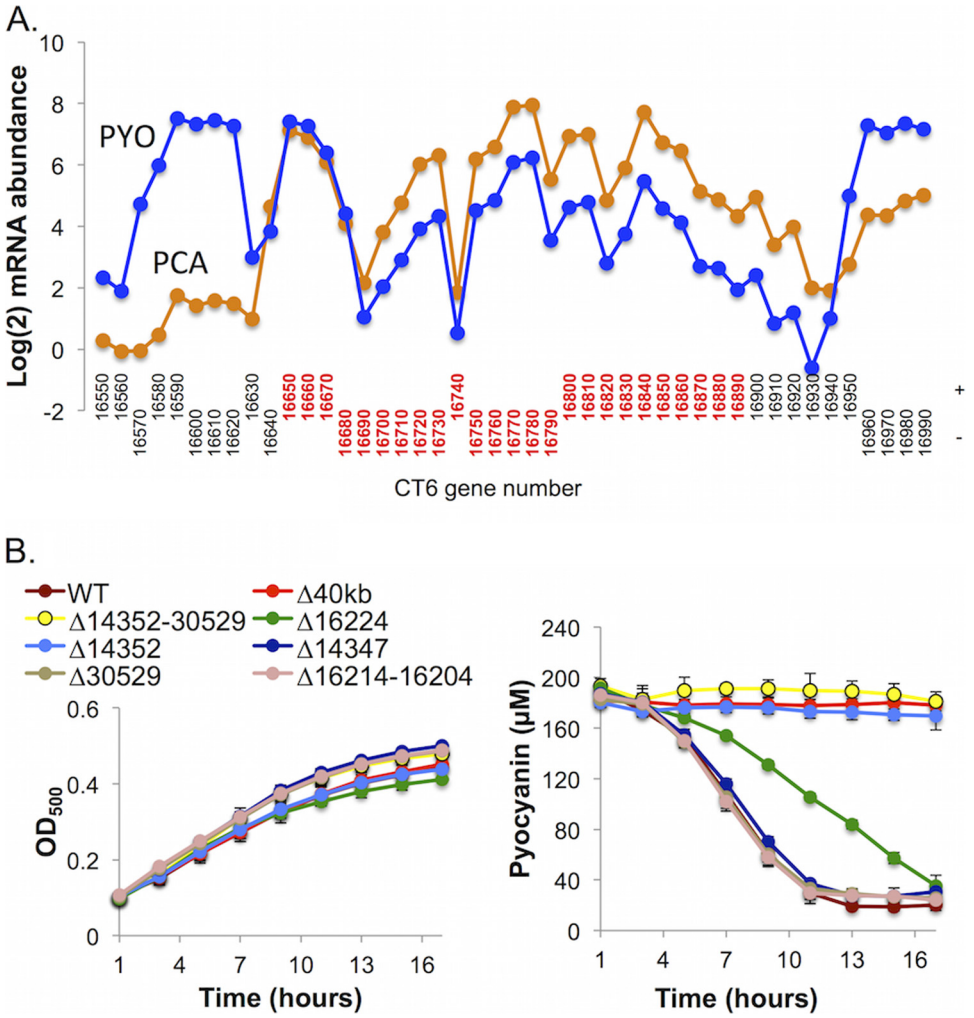

FIG 3 Gene expression and mutational analysis of PYO degradation. (A) mRNA abundance of strain CT6 genes after 20-min exposure to PCA or PYO. Values are given as $\log_2$ fold change versus a mock treatment. An ~40-kb region of the genome shows a large change in gene expression in response to phenazines. Strain CT6 gene numbers are listed on the *x* axis, with genes on the plus strand listed on the top row and genes on the minus strand listed on the bottom row. Genes highlighted in red are present in the genome of *Rhodococcus* strain JVH1 (Table 1; also, see Table S1 in the supplemental material). Blue points represent relative mRNA abundance after PYO exposure, and orange points correspond to PCA exposure. (B) Growth and PYO degradation characteristics of *M. fortuitum* mutants grown in 10% LB-Tw medium supplemented with 200 μM PYO. All mutants could grow with 10% LB as the carbon source, but only a subset displayed a defect in PYO degradation. Data are averages and SD for three cultures. RNA-Seq data for the entire chromosome can be found in Dataset S1 in the supplemental material.

***M. fortuitum* alters the phenazine pool in coculture with *Pseudomonas* spp.** *M. fortuitum* is ubiquitous and found in environments where phenazine-producing pseudomonads are also present (36, 37). Using a subset of the mutants described above, we sought to determine whether a mixed culture of *M. fortuitum* with a pseudomonad would lead to alterations in the phenazine pool. *M. fortuitum* and several mutants were inoculated into LB medium with *P. aeruginosa* PA14, and culture supernatants were analyzed by HPLC after 24 h. The Δ40kb::Gm^r mutant was used as a control to determine the amount of phenazines produced by PA14 under coculture conditions. The WT *M. fortuitum* strain decreased the abundance of both PYO (~50% decreased) and PCN (~90% decreased) but not PCA in coculture with PA14 (Fig. 4A). This level of phenazine degradation impacted neither the rate of *P. aeruginosa* growth nor its transcription of the phenazine biosynthesis genes. As expected, the PYO degradation mutant, Δ14352::Gm^r, decreased the abundance of PCN but not PYO. The mutant Δ30529::Gm^r decreased PYO but not PCN, suggesting that MFORT_30529—annotated as an amidase—may convert PCN to PCA. In fact, cocultures that are proficient for PCN degradation seem to have higher PCA concentrations in their supernatants, supporting this hypothesis (Fig. 4A; also, see Fig. S6 in the supplemental material). The PCN degradation phenotype of Δ30529::Gm^r was assayed in monoculture, and this strain was confirmed to have a specific defect in the removal of PCN from culture supernatants (see Fig. S7 in the supplemental material); additionally, expression of MFORT_30529 in *Rhodococcus* sp. strain JVH1 conferred the ability to degrade PCN to this organism (see Fig. S6).

Because *M. fortuitum* in coculture with PA14 prioritizes PYO and PCN removal from the medium, to determine whether PCA could also be degraded in coculture, we mixed *M. fortuitum* and

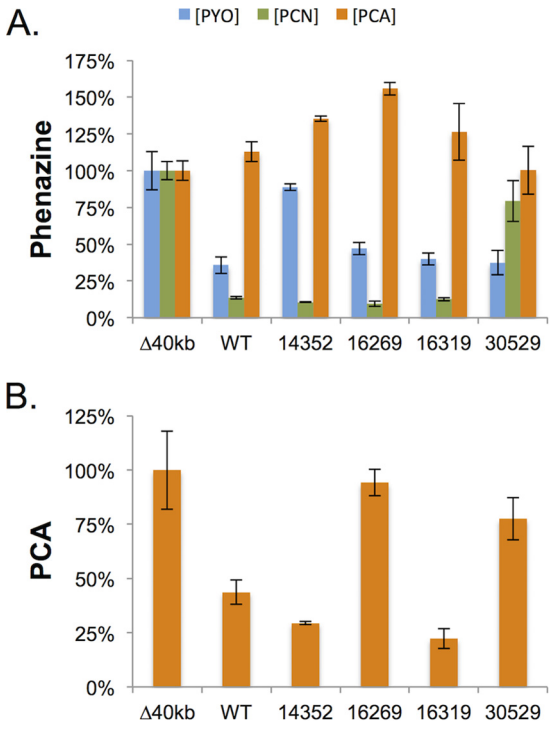

FIG 4 Phenazine degradation by *M. fortuitum* strains grown in coculture with *Pseudomonas* spp. Cocultures were grown for 24 h, and phenazine concentrations were measured by HPLC. Δ40 kb::Gm^r is defective in phenazine degradation, so PYO, PCN, and PCA levels in a coculture with this strain were arbitrarily set to 100%. (A) Phenazine levels in a coculture between the indicated mutant and *P. aeruginosa* PA14. (B) PCA levels in a coculture between the indicated mutant and *P. fluorescens* 2-79. Data are averages and SD from three experiments.

Pseudomonas fluorescens 2-79, an organism that produces PCA as its only phenazine. WT *M. fortuitum* was capable of removing ~50% of the PCA from culture supernatants compared to the Δ40kb::Gm^r control (Fig. 4B). As expected, Δ16269::Gm^r was defective for PCA removal. For unknown reasons, Δ30529::Gm^r was also defective for PCA removal in the coculture condition.

PYO degradation is protective to other organisms in coculture with *M. fortuitum*. Alterations in the abundance of phenazines in cocultures of *M. fortuitum* and *Pseudomonas* spp. demonstrate that phenazine degradation can influence the concentration of structurally diverse phenazines in the coculture environment. Because phenazines can function as antimicrobials (16, 17), it is ecologically important to consider whether degradation might impact other members of a microbial community. We hypothesized that PYO degradation would be protective to organisms that are otherwise susceptible. PYO survival was assayed in cocultures of *M. fortuitum* mixed with diverse bacteria that have a range of sensitivity to different phenazines: *Staphylococcus aureus*, *Agrobacterium tumefaciens*, *Shewanella oneidensis*, and *Escherichia coli* (16, 17, 38, 39). After 24 h of incubation in monoculture in the presence of 100 μM PYO, *S. aureus*, *E. coli*, and *S. oneidensis* were all inhibited by PYO to various degrees (Fig. 5). In all three cases, coculture with WT *M. fortuitum* rescued growth of these organisms, though not always to the same extent as growth in monoculture in the absence of PYO. A PYO degradation mutant provided no protection. *A. tumefaciens* was resistant to PYO, consistent with previous reports (38), and coculture had little effect on this organism.

DISCUSSION

Phenazines can shape both microbial community composition and the chemistry of the environment; however, while much is known about their biosynthesis, regulation and physiological functions (11–14), little is known about their degradation. Previously, *S. wittichii* DP58 was shown to be capable of degrading PCA, yet the genes catalyzing this process were not identified (25). Here, we identified genes involved in the degradation of multiple phenazines in members of the *Mycobacterium fortuitum* complex. The identification of conserved degradation genes in mycobacteria not only broadens the phylogenetic diversity of this activity to include members of both the *Proteobacteria* and *Actinobacteria* phyla it also suggests that the enzymes catalyzing this activity may be widespread.

Mycobacteria are ubiquitous, and *Mycobacterium* spp. and *Pseudomonas* spp. are commonly reported to be present in the same types of environments, including soil, crude oil, and the lungs of patients with CF (6–8). It may thus not be surprising that one organism has evolved the capacity to utilize an excreted product of the other. A common gene cluster that appears to be essential for PCA degradation is shared between the genomes of *M. fortuitum* ATCC 6841, *Rhodococcus* sp. strain JVH1, and strain CT6. Notably, the same four putative dioxygenases display increased mRNA abundance in strain CT6 in the presence of both PCA and PYO. *Rhodococcus* strain JVH1 is incapable of PYO degradation yet possesses close homologs of each of these genes. This suggests that PCA and PYO may share a degradation intermediate. In *P. aeruginosa*, PYO is produced from PCA via the action of PhzS and PhzM (10). One possibility is that strains CT6 and DKN1213 first degrade PYO to PCA and that this product leads to the increased mRNA abundance for PCA-specific genes. A second possibility is that PCA and PYO may be converted to an intermediate

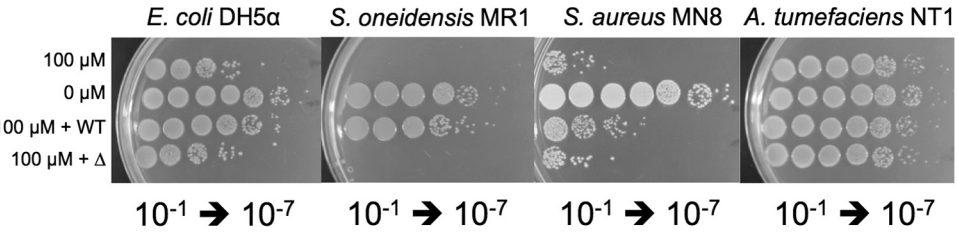

FIG 5 PYO degradation protects sensitive organisms. *E. coli*, *S. oneidensis*, *S. aureus*, and *A. tumefaciens* were plated after coculture with WT *M. fortuitum* or Δ14352::Gm^r (Δ) in the presence of 100 μM PYO. Survival of these organisms in monoculture with 0 or 100 μM PYO is included to verify their sensitivity to PYO. Sensitive organisms had increased cell density after incubation with the WT, suggesting a protective effect for PYO degradation.

that is further degraded through the action of MFORT_16269, MFORT_16319, MFORT_16334, and MFORT_16349 or a subset of these. A small (162-amino-acid) protein is sufficient to catalyze PYO degradation, and a predicted amidase is likely required for the first step in PCN breakdown. An amidase activity suggests that PCN is first converted to PCA before further breakdown takes place. Elucidation of the complete pathway(s) of PCA, PCN, and PYO degradation awaits future research, yet this study provides an important step in that direction.

What consequences might the degradation of different phenazines have on *Pseudomonas* spp.? Though we did not observe significant phenotypic consequences of phenazine degradation on *P. aeruginosa* under standard laboratory growth conditions, it is possible that phenazine degradation may decrease the fitness of phenazine-producing organisms in natural or engineered environments. Each *Pseudomonas*-derived phenazine has unique redox and chemical properties (13, 40) and a distinct impact on the physiology of its producer (9, 41). For example, PYO is important for biofilm maturation in *P. aeruginosa* (42), and PCA rapidly reduces ferric iron, facilitating iron acquisition (12). Phenazine toxicity to other organisms can benefit *Pseudomonas* spp. by inhibiting competitors. Finally, phenazines are terminal signaling factors in the quorum-sensing network of pseudomonads (14). In many cases, the impact of molecular signals is concentration dependent, but how degradation influences the accumulation and fate of bacterial signals is generally poorly characterized. However, as shown by work on acyl-homoserine lactone degradation, the ability to control signal accumulation can significantly affect the behavior of microbes responsive to that signal if it is consumed below a certain threshold (22, 23). We thus speculate that controlling the distribution of phenazines via degradation might similarly attenuate the ability of *Pseudomonas* species to dominate environments where phenazine cycling is beneficial (e.g., hypoxic or anoxic habitats) or provides a competitive advantage.

Just as the degradation of phenazine metabolites would be predicted to impact the fitness of phenazine producers, we might also anticipate a more general effect on ecosystem diversity. *P. aeruginosa* interactions with plants highlight the potential implications of this interaction on the outcome of two different fungal infections. PYO enhances susceptibility of rice to *Rhizoctonia solani* but leads to increased resistance to *Magnaporthe grisea* (43); the presence of a PYO-degrading organism could significantly alter the outcome of infection in each of these cases. While there is evidence of phenazine turnover and degradation in the rhizosphere (2, 19), phenazines also play important roles in other environments where these processes have not been measured or observed. The presence of phenazines is negatively correlated with microbial species richness in both laboratory enrichment cultures and the lungs of patients with CF (6–8, 44). What role might natural or stimulated degradation play in modulating phenazine levels and microbial community development in these and other contexts? Given that phenazine degradation can impact the fitness of organisms by promoting growth and minimizing toxicity, it is temting to speculate that these activities may be important in natural environments. Future work will determine whether this is, in fact, the case.

MATERIALS AND METHODS

Strains, media, and growth conditions. Strains used in this study are listed in Table S2 in the supplemental material. PCA-degrading organisms were isolated by inoculating 5 ml of PCA medium with 100 mg of soil collected on and around the California Institute of Technology campus and growing at 30°C with shaking. PCA medium was composed of minimal medium containing (per liter) KH_2PO_4 (1.93 g), K_2HPO_4 (6.24 g), NaCl (2.5 g), $MgSO_4$ (0.12 g), NH_4Cl (0.5 g) and supplemented with PCA (0.56 g). Trace minerals were also included (45). Cultures were allowed to grow for 5 to 8 days before transfer to fresh PCA medium. After 3 rounds of growth in PCA liquid medium, cultures were streaked on plates containing PCA medium amended with 1.5% Bacto agar. After isolation, strains were routinely grown in lysogeny broth with 0.05% Tween 80 (LB-Tw). PCA and PCN were purchased from Princeton Biomolecular Research Inc., and PYO was purified from cultures of *P. aeruginosa* strain PA14 grown with 40 mM succinate as the sole carbon source using organic extraction into dichloromethane as described previously (14).

Growth curves were performed in 10% LB-Tw medium supplemented with 200 μM PCA, PYO, or PCN. Cultures were grown to mid-exponential phase and diluted to an OD of ~0.1 to 0.2 for growth analysis. Growth curves were carried out in 96-well plate format on a BioTek Synergy 4 microplate reader (BioTek, Inc.) set to 30°C and medium agitation. Ninety-six-well plates were sealed with sterile adhesive film to prevent medium evaporation. OD was monitored at 500 nm; PCA and PCN were monitored at 370 nm, and PYO was monitored at 310 nm. Standards were included to calculate the concentrations of each phenazine. PCA and PYO degradation by *Rhodococcus* was performed at an OD_{600} of ~2 to 3. *Rhodococcus* spp. were suspended in 10% LB medium with 200 μM PCA or PYO in a volume of 5 ml. A 500-μl portion of culture supernatant was collected by centrifugation at various time points and analyzed on a plate reader.

Coculture experiments were carried out in LB with various *M. fortuitum* strains at a starting OD of 0.15. Pseudomonads were inoculated to an OD of 0.05 and other organisms to 0.001. Cocultures were grown for 24 h in all cases except for *A. tumefaciens* (48 h). To test the protective effects of PYO degradation, 100 μM PYO was included, and cultures were plated on LB at the end of growth to enumerate CFU (all organisms formed colonies in 1 to 2 days versus 3 to 5 days for *M. fortuitum*, so selective medium was not necessary). To analyze coculture supernatants, samples were diluted 1:10, sterilized by filtration on a 0.2-μm filter and analyzed by HPLC as described previously (12) using the same method but a flow rate of 950 μl min^{-1}.

Phylogenetic analysis. 16S rRNA genes were amplified using primers 9bf and 1512uR and sequenced with 9bf and 519uF (46, 47). Relatives were found using BLAST (http://blast.ncbi.nlm.nih.gov) and collected for analysis. All phylogenetic analyses were performed using the phylogeny.fr web server and further refined using Interactive Tree Of Life (iTOL) (48–51).

DNA extraction, genome sequencing, gene annotation and analysis. DNA extraction of strain CT6 was performed using a modified protocol from (52). Fifty milliliters of mid-exponential-phase culture was harvested and stored at −80°C before DNA extraction. Pellets were thawed at 80°C for 30 min and suspended in an equal volume of chloroform-methanol (2:1) for 1 h with periodic shaking at room temperature. This mix was centrifuged for 20 min at 2,500 × *g*, and the organic and aqueous phases were discarded without disturbing the cell material. The cell pellet was dried for 10 min at 55°C, suspended in 550 μl of 100 mM Tris (pH 9.5), and incubated overnight at 37°C with lysozyme (final concentration, 0.5 mg ml^{-1}). After lysozyme treatment, SDS (final concentration, 1%) and proteinase K (final concentration, 0.75 mg ml^{-1}) were added, and the tube was left at 60°C for 1 h with periodic mixing. During the last 15 min of incubation, 100 μl each of 5 M NaCl and cetyltrimethylammonium bromide (CTAB) solution (10% CTAB solution in 0.7 M NaCl) was added. DNA was extracted with 1 vol of chloroform-isoamyl alcohol (24:1), and the aqueous phase was treated with RNase A. RNA-free DNA was ethanol precipitated and stored at −80°C before sequencing.

SMRT sequencing was performed using the Pacific Biosciences RS II

platform with 10-kb libraries and P5/C3 chemistry using the standard protocols. The *de novo* genome assembly, using HGAP v2 (53), resulted in a single circular contig. The genome of strain CT6 was annotated using RAST and BASys (27–29). Genomes were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov) and the Integrated Microbial Genomes/Expert Review (IMG/ER) web server (https://img.jgi.doe.gov/er/) (26, 30, 31, 35).

RNA extraction, qRT-PCR, and RNA-Seq. To generate material for qRT-PCR analysis, overnight cultures were diluted 1/50 in LB-Tw and grown overnight to an OD_{600} of ~0.3. At this point, PCA, PYO, AQDS, or MB was added to a final concentration of 200 μM. An equivalent volume of water was added to a set of cultures as a control. After 3 h, cell material was collected, flash frozen, and stored at −80°C until RNA extraction. Frozen cell pellets were suspended in 350 μl AES buffer (50 mM sodium acetate [pH 5.3], 10 mM EDTA, 1% SDS) and 350 μl acid phenol-chloroform (5:1; pH 4.5), and 200 μl glass beads (≤106 μm) were added. Samples were homogenized in an analog Disruptor Genie (Scientific Industries, Inc.) four times for 30-s each with a 30-s interval on ice between disruptions. Glass beads were removed by centrifugation, and liquid was transferred to a heavy phase lock tube containing 300 μl chloroform. The phase lock tube was centrifuged for 5 min at 12,000 × *g*. The aqueous phase was extracted with an additional 300 μl chloroform and transferred to a microcentrifuge tube containing 40 μl 3M sodium acetate (pH 5.2), and RNA was alcohol precipitated and suspended in 100 μl H_2O. This crude RNA extract was cleaned using an RNeasy kit (Qiagen, Inc.) with a modified protocol with optional on-column DNase treatment, as described previously (54). RNA was additionally treated with Turbo DNA-free using the manufacturer's directions (Life Technologies, Inc.).

Purified RNA was converted to cDNA using an iScript cDNA synthesis kit and following the manufacturer's directions (Bio-Rad, Inc.). cDNA was used as a template for qPCR using iTaq universal SYBR green Supermix (Bio-Rad, Inc.) on a 7500 fast real-time PCR system (Applied Biosystems, Inc.). Samples were analyzed in at least duplicate, and the signal from each treatment (PCA, PYO, AQDS, or MB) was first normalized to a water-only control using the following equation: relative expression = $P_e^{-[CT(\text{sample}) - CT(\text{control})]}$ where P_e is the calculated efficiency for a given primer pair. Data were then standardized to *rpoA*. qRT-PCR primers are listed in Table S2 in the supplemental material.

For RNA-Seq, RNA was extracted after a 20-min exposure to 200 μM PCA, PYO, or water. rRNA was depleted with the magnetic RiboZero kit for Gram-negative bacteria (Epicentre). A library was prepared using the NEBNext mRNA library prep master mix set for Illumina (NEB). Sequencing was performed at the Millard and Muriel Jacobs Genetics and Genomics Laboratory at the California Institute of Technology to a depth of 12 to 15 million reads on an Illumina HiSeq2500 and processed using the Illumina HiSeq control software (HCS version 2.0). Low-quality bases were removed using Trimmomatic (LEADING:27 TRAILING:27 SLIDINGWINDOW:4:20 MINLEN:35) (55), mapped using Bowtie (56), and sorted with SAMtools (57). The number of reads per locus was calculated with easyRNAseq (58). The .gff file was generated using the RAST annotations (27, 28). Reads were normalized by number and compared to the water control. RNA-Seq results can be found in Dataset S1 in the supplemental material.

Generation of mutants. *M. fortuitum* ATCC 6841 was used for mutagenesis using a recombineering protocol (34, 59, 60). Plasmids and primers are listed in Table S2 in the supplemental material. *M. fortuitum* ATCC 6841 was electroporated with pJV53 using a protocol established for *M. smegmatis* to make a recombineering strain (34, 59). Cultures were plated on LB plates with 100 μg ml^{-1} kanamycin and allowed to grow for 3 to 5 days. *M. fortuitum* ATCC 6841 pJV53 was grown overnight in LB-Tw to an OD_{600} of 0.5 to 1, induced for 3 h with 0.2% acetamide, washed and suspended in 10% glycerol as described previously, and frozen at −80°C (34).

Linear DNA constructs for generating allele replacement mutants were generated by PCR amplifying genomic regions flanking the target gene and the gentamicin resistance cassette from pMQ30 (61). PCR products were treated with XbaI and NotI (New England Biolabs) when necessary (see Table S2 in the supplemental material) and T4 ligase (Invitrogen). Constructs were PCR amplified and electroporated into acetamide induced *M. fortuitum* ATCC 6841 pJV53 and outgrown overnight in LB-Tw medium at 30°C. Allele replacement mutants were selected on LB plates with 100 μg ml^{-1} gentamicin at 30°C. The position of the gentamicin cassette on the chromosome of single-gene-deletion mutants was confirmed by PCR and sequencing.

To complement mutations in *trans*, cultures were cured of pJV53 by daily passage at 37°C for 5 days and streak purified. Colonies that had lost pJV53 and by extension kanamycin resistance were identified by patch plating on LB and on LB supplemented with kanamycin. Mutants cured of pJV53 were subsequently transformed with plasmid pSD5 (60) bearing the appropriate gene to complement the mutation.

Nucleotide sequence accession number. The complete genome sequence of strain CT6 has been deposited in GenBank (accession number CP011269).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01520-15/-/DCSupplemental.

Dataset S1, XLSX file, 0.4 MB.
Figure S1, TIF file, 2.8 MB.
Figure S2, TIF file, 2.8 MB.
Figure S3, TIF file, 2.8 MB.
Figure S4, TIF file, 2.7 MB.
Figure S5, TIF file, 2.7 MB.
Figure S6, TIF file, 2.7 MB.
Figure S7, TIF file, 2.8 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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