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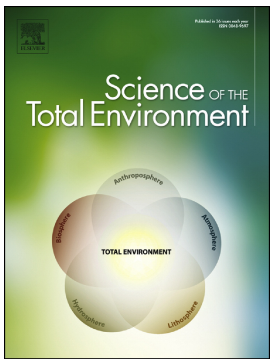

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An angular dioxygenase gene cluster responsible for the initial phenazine-1-carboxylic acid degradation step in *Rhodococcus* sp. WH99 can protect sensitive organisms from toxicity

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Abstract

A bacterial strain, *Rhodococcus* sp. WH99, capable of degrading phenazine-1-carboxylic acid (PCA) was isolated and characterized. Genome comparison revealed that a 21499-bp DNA fragment containing a putative angular dioxygenase gene cluster consisting of the dioxygenase-, ferredoxin reductase- and ferredoxin-encoding genes (*pzcA1A2*, *pzcC* and *pzcD*) is missed in the PCA degradation-deficient mutant WH99M. The *pzcA1A2CD* genes were expressed in *Escherichia coli* respectively and hydroxylation of PCA to 1,2-dihydroxyphenazine occurred *in vitro* only when all components were present. However, *in vivo* analyses showed that *pzcA1A2* and *pzcD* were indispensable for PCA degradation, while PzcC can be partially replaced by other ferredoxin reductases. Hydroxylation of PCA not only initiates degradation of PCA in strain WH99 but also provides protection to sensitive organisms that would otherwise be inhibited by PCA toxicity. This study illustrates a new initial PCA degradation step in Gram-positive bacteria and enhances our understanding of the genes responsible for PCA hydroxylation, thus enabling targeted studies on protection by PCA degradation in diverse environments.

Keywords: Phenazine-1-carboxylic acid; *Rhodococcus* sp. WH99; Degradation; Dioxygenase；Protection

1. Introduction

Phenazine-1-carboxylic acid (PCA), the main active component of benzimidazole-derived biocide shenqinmycin, is the precursor of all phenazines mainly produced by *Pseudomonas* species (Mavrodi et al., 2012; Voggu et al., 2016). Owing to its high fungicidal efficiency, low toxicity and good environmental compatibility, PCA is widely used to suppress diseases and protect plants from colonization and infection by pathogenic fungi in the rhizosphere (Puopolo et al., 2013; Renée et al., 2015; Xu et al., 2015).

Despite being a promising biocide, PCA can be toxic to even non-target microorganisms by producing reactive oxygen species (ROS) and interfering with the respiratory electron transport chain (Voggu et al., 2006; Cezairliyan et al., 2013), thus reducing species diversity (Costa et al., 2015). Therefore, it is necessary to study the degradation characteristics of PCA to prevent the potential hazards of PCA and its intermediates on the environment.

Several PCA-degrading bacteria have been reported to date, including *Sphingomonas* sp. DP58 (Yang et al., 2007; Zhao et al., 2017), *Mycobacterium* sp. CT6, *Mycobacterium* sp. DNK1213, *Mycobacterium* sp. ATCC6841, *Rhodococcus* sp. JVH1 (Costa et al., 2015) and *Nocardia* sp. LAM0056 (Costa et al., 2018). Although the degradation pathway of PCA has been investigated in different strains, only the two initial degradation steps have been reported so far. In the Gram-negative bacterium *Sphingomonas* sp. DP58, PCA is initially hydroxylated to 1,2-dihydroxyphenazine by a dioxygenase system PcaA1A2A3A4 under aerobic

conditions (Zhao et al., 2017). In the Gram-positive bacterium *Mycobacterium* sp. CT6, PCA is converted to phenazine by a decarboxylase system PhdAB under anoxic conditions, a mechanism also seen in other *Actinobacteria* strains capable of degrading PCA, such as *Nocardia* sp. LAM0056 and *Rhodococcus* sp. JVH1 (Costa et al., 2018). As *Actinobacteria* strains can also grow aerobically, the degradation mechanisms they employ under aerobic conditions may differ from those under anoxic conditions (Costa et al., 2015). Thus, more PCA-degrading bacteria, especially *Actinobacteria* strains, need to be isolated to investigate the degradation mechanism of PCA under different oxygen conditions.

In this study, we isolated and characterized a PCA-degrading strain, *Rhodococcus* sp., designated as WH99. The genes involved in the catabolic pathway in this organism were identified by genome comparison, heterologous expression, gene knockout and complementation experiments. Also, the protective effect of PCA degradation on some PCA sensitive organisms was investigated. These findings enhance our understanding of the microbial degradation mechanism of PCA as well as the protective effects of its degradation.

2. Materials and methods

2.1. Chemicals and media

PCA (98%) was purchased from Shanghai Qinba Chemical Co. Ltd. (Shanghai, China). Phenazine (98%) was purchased from J&K Scientific Ltd. (Shanghai, China). All enzymes used in DNA manipulations were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China). All chemicals used in this study were of highest analytical

grade. Luria-Bertani (LB) medium contained (g L^{-1}): tryptone 10.0, yeast extract 5.0, NaCl 10.0. LB medium was diluted 1:5 by distilled water to obtain 1/5 LB medium. The Mineral salts medium (MSM) contained (g L^{-1}): NaCl 1.0, K_2HPO_4 1.5, KH_2PO_4 0.5, NH_4Cl 1.0, $MgSO_4$ 0.2. MMP was generated by supplementing MSM with 0.10 mM PCA as the sole carbon source unless otherwise stated. Solid media were obtained by adding 15 g L^{-1} agar into respective liquid media. The initial pH of the media was adjusted to 7.0 and autoclaved at 121°C for 30 min.

2.2. Strains, plasmids, primers and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The primers used in this study are listed in Table 2. All strains were grown at 30°C in LB broth except *E. coli* (37°C). If necessary, the antibiotics kanamycin or ampicillin was added to media at final concentrations of 50 and 100 mg mL^{-1} respectively unless otherwise stated.

2.3. Isolation and identification of a PCA-degrading strain

Strain isolation was performed as previously described by Sun et al (2019) with some modifications (Mallick et al., 2014; Lepuschitz et al., 2019; Liu et al., 2019). Briefly, soil samples collected from the wheat field (Anhui, China) were used as initial inoculants to enrich PCA-degrading bacteria. 5 g of soil was added into a 250 mL flask containing 100 mL MMP and incubated at 30°C, 180 rpm for three days. From this, 5 mL of the culture was transferred into fresh 100 mL MMP and incubated under the same conditions for another three days. After three successive rounds of such enrichment, ten-fold serial dilutions of the resulting bacterial culture were made;

10^{-5}, 10^{-6} and 10^{-7} dilutions were spread onto MMP agar plates and incubated at 30°C for 3 days. One isolate capable of utilizing PCA as the sole carbon source was obtained and designated as WH99.

Strain WH99 was identified according to *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994) and by 16S rRNA sequence analysis. Genomic DNA was extracted by the high-salt-concentration precipitation method (Miller et al., 1988). 16S rRNA gene was amplified by PCR using standard procedures (Lane et al., 1991). The 16S rRNA gene sequence was compared for homology with the available sequences using EzTaxon-e server (https://www.ezbiocloud.net/) (Kim et al., 2012). Phylogenetic analysis was performed using MEGA software (version 6.0) after multiple alignments of data by Clustal X (version 2.1) (Thompson et al., 1997; Tamura et al, 2013). Phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).

2.4. Relationship between growth of strain WH99 and PCA degradation

The cells of strain WH99 were collected by centrifugation at 6000 × *g* for 5 min at room temperature after precultured in LB broth at 30°C, 180 rpm till the later-exponential phase. The cell pellets were then washed and resuspended in 100 ml MMP to a final OD_{600} of 0.10. The cell suspensions were incubated at 30°C with shaking at 180 rpm. The samples were then used to estimate the growth of strain WH99 by the colony counting method and analyze the concentration of PCA by HPLC.

2.5. Screening of the PCA degradation-deficient mutant

As previously described by Li et al (2018) with some modifications, briefly, a lawn of strain WH99 grown on 1/5 LB agar plates was streaked onto fresh 1/5 LB agar plates and incubated at 30°C for 3-4 days. After approximately 50 continuous transfers, the lawn was suspended in MSM. Serial dilutions of this suspension were spread onto 1/5 LB agar plates and incubated until colonies developed. Each colony was then simultaneously streaked using a sterilized toothpick onto a 1/5 LB agar plate and an MMP agar plate. Colonies that grew on 1/5 LB agar plate but failed to grow on MMP agar plate were selected and their ability to degrade PCA in liquid MMP was determined; one mutant incapable of degrading PCA was selected and designated as WH99M.

2.6. Sequencing, assembly, annotation and genome comparison

Total genomic DNA from the wild-type strain WH99 and the mutant strain WH99M were extracted by high-salt-concentration precipitation method (Miller et al., 1988). Genome sequencing was performed by Shanghai Biozeron Technology Co. Ltd. (Shanghai, China). Complete genome sequences were assembled using SOAP *de novo* (version 1.05) method (http://soap.genomics.org.cn/soapdenovo.html), on the Pacific Biosciences platform and validated by PCR. The coverage depth of the genome was 380x and the coverage extent was 100% with a BUSCO assembly rate of 99.7%, which indicated that the assembly result was very good. Functional annotation was accomplished by BLAST analysis (www.blast.ncbi.nlm.nih.gov/) using nonredundant protein (NR), Swiss-Prot, KEGG and COG databases. An all-versus-all genome alignment between strain WH99 and strain WH99M was performed to

identify the missing DNA fragment in strain WH99M using Mauve (version 1.2.3) software package (Darling et al., 2004).

For phylogenetic analysis, all protein sequences were first aligned by Clustal X (version 2.1) (Thompson et al., 1997; Larkin et al., 2007) and then imported into MEGA software (version 6.0) (Tamura et al., 2013) to construct the phylogenetic tree by neighbor-joining method. Distances were calculated using the Kimura two-parameter distance model. Confidence values for the branches of the phylogenetic tree were determined using bootstrap analysis based on 1000 resampling iterations.

2.7. Co-expression of predicted dioxygenase-encoding genes in *E. coli*

pzcA1A2, *pzcB1B2*, *pzcCD* and *pzcB1B2CD* were amplified from strain WH99 using primer pairs pMDA12-F/R, pMDCD-F/R, pMDB12-F/R and pMDB12CD-F/R, respectively. *pzcA1A2* and *pzcCD* were combined by overlap PCR, yielding *pzcCD-pzcA1A2*. *pzcA1A2*, *pzcB1B2*, *pzcCD-pzcA1A2*, and *pzcCDB1B2* were individually ligated into *Hind*Ⅲ-*Eco*R Ⅰ sites of plasmid pMD19-T using ClonExpress II one-step cloning kit (Vazyme Biotech, Nanjing, China), yielding pMD*pzcA1A2*, pMD*pzcB1B2*, pMD*pzcA1A2CD* and *pzcB1B2CD*, respectively. The recombinant plasmids were transformed into *E. coli* DH5α and verified by sequencing. The transformants were grown to late log phase in LB broth containing ampicillin (100 mg L^{-1}) at 37°C, 180 rpm, harvested by centrifugation, washed and resuspended in 20 mL MMP to a final OD_{600} of 1.0. The suspensions were incubated at 37°C, 180 rpm for 12 hours. The samples were collected to determine the concentration of PCA and its

intermediates.

2.8. Expression and purification of each component of PzcA1A2CD

pzcA1, *pzcA2*, *pzcC* and *pzcD* genes were amplified from strain WH99 using primer pairs pETA1-F/R, pETA2-F/R, pETC-F/R and pETD-F/R, respectively. The fragments were ligated into *Nde*I-*Xho*I sites of plasmid pET28a(+) using ClonExpress II one-step cloning kit, yielding pET*pzcA1*, pET*pzcA2*, pET*pzcC* and pET*pzcD*, respectively. The coding sequences of genes in the recombinant plasmids were verified by sequencing to be in frame with the N terminus His6 tag of the vector. His6-tagged PzcA1, PzcA2, PzcC and PzcD were overexpressed in *E. coli* BL21 (DE3) carrying pET*pzcA1*, pET*pzcA2*, pET*pzcC* and pET*pzcD*, respectively.

E. coli BL21 (DE3) harboring the appropriate expression vector was grown in LB broth containing kanamycin (50 mg L^{-1}) at 37°C to OD_{600} of 0.6-0.8. Gene expression was induced by addition of Isopropyl β-α-1-Thiogalactopyranoside (IPTG) (Lutz et al., 1997) to a final concentration of 0.2 mM and the culture was incubated at 16°C for another 10 h, following which cells harvested by centrifugation and disrupted by ultrasonication (Auto Science, UH-650B ultrasonic processor, 40% intensity) for 10 min. Intact cells were removed by centrifugation at 12,000 × *g* for 30 min (4°C).

A nickel-nitrilotriacetic acid (Ni^{2+}-NTA) resin was used to purify the enzyme from the supernatant on a gradient of imidazole. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of the protein and the protein concentration was estimated using

Bradford assay (Bradford, 1976).

The standard enzyme reaction was performed at 30°C for 1 h in 1 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.4 μg PzcA1, 0.4 μg PzcA2, 0.1 μg PzcC, 0.1 μg PzcD, 1 mM NADH/NADPH and 0.5 mM Fe^{2+}. The assay was initiated by addition of PCA to a final concentration of 0.10 mM. The reaction was performed at 30°C for 1 h and terminated by addition of 10 μL of 6 M HCl.

2.9. Knockout and complementation of *pzcA1, pzcA2, pzcC* and *pzcD* genes

The knockouts of *pzcA1*, *pzcA2*, *pzcC* and *pzcD* genes in strain WH99 were performed using the suicide plasmid pK18*mobsacB* (Schäfer et al., 1994). The knockout plasmid pK18*mobsacB*-Δ*pzcA1* was constructed by cloning the DNA upstream and downstream of *pzcA1* in tandem into *Pst* Ⅰ-digested pK18*mobsacB* using ClonExpress II One Step Cloning Kit. Knockout plasmids for *pzcA2*, *pzcC* and *pzcD* genes were generated similarly. The plasmid carrying the region flanking the gene of interest was introduced into strain WH99 by electrotransformation as previously described by Chen et al (2017). Single-crossover clones were selected on LB agar plates containing kanamycin (200 mg L^{-1}), while double-crossover strains were screened on LB agar plates containing 10% (m/v) sucrose. Mutants with gene disruptions were confirmed by colony PCR and DNA sequencing.

For gene complementation, plasmid pRE*pzcA1* was constructed by cloning PCR amplified *pzcA1* into *Spe*I-digested pRESQ. The plasmid was transformed into WH99Δ*pzcA1* by electrotransformation to generate the *pzcA1*-complemented strain WH99Δ*pzcA1*(pRE*pzcA1*). Strains WH99Δ*pzcA2*(pRE*pzcA2*),

WH99Δ*pzcC*(pRE*pzcC*) and WH99Δ*pzcD*(pRE*pzcD*) were generated similarly.

The wild-type strain, the mutant strains and the corresponding complemented strains were streaked onto MMP agar plates and incubated at 30°C for 3 days. PCA-degrading ability of these strains was performed as described in section 2.5 above.

2.10. Protective effects assay of PCA degradation to sensitive organisms

As described above, cultures of WH99 and *E. coli* DH5α/pMD*pzcA1A2CD* in MMP were incubated under their respective growth conditions until PCA in the medium was completely degraded or transformed as indicated by HPLC analysis. The supernatants from the cultures were then collected by centrifugation and sterilized by filtration through a 0.2-μm-pore-size filter. The two supernatants recovered were called RW (spent MMP from the WH99 culture) and RD (spent MMP from the DH5α/pMDpzcA1A2CD culture). To test the protective effects of PCA degradation, PCA-sensitive strains, *E. coli* DH5α, *Bacillus subtilis* 168 (Wu et al., 2019), *Rhodococcus* sp. YL-1 (Chen et al., 2017) and *Pichia pastoris* GS115 (Chan et al., 2017), which could not degrade PCA (data not shown), were individually suspended in 20 mL MSM, MMP, RW and RD to a final OD_{600} of 0.1, cultured for 24 h, and 5 μL of 10-fold serial dilutions of each culture were spotted on LB agar plates to determine the CFU of the cultures.

2.11. Analytical methods

To analyze PCA and its metabolites, the cultures or enzyme assay samples were centrifuged at 12,000 × *g* for 5 min. Supernatants were filtered through

0.2-μm-pore-size filters and subjected to high-performance liquid chromatography (HPLC) analysis using a system (Dionex UltiMate 3000, USA) equipped with a C_{18} reverse phase column (4.6 × 250 mm, 5 μm). The mobile phase was a mixture of acetonitrile (A) and water (B) at a 1.0 mL min^{-1} flow rate, the column temperature was 30°C and the injection volume was 20 μL. A stepwise solvent gradient was used as follows: 0 to 4 min, 20% A; 4.01 to 20 min, 40% A; and 20.01 to 30 min, 20% A. Column elution was monitored by measuring absorbance at 367 nm. For mass spectrometry analysis, an AB SCIEX Triple TOF 5600 plus High-Resolution Mass Spectrometry System equipped with a Turbo V^{TM} probe was used. Data acquisition was performed in positive ionization mode.

2.12. Nucleotide sequence accession numbers

Sequences of the 16S rRNA gene and the DNA fragment (21499 bp) containing the dioxygenase gene cluster *pzcA1A2CD* from strain WH99 were deposited in the GenBank database under the accession numbers MN029024 and MN038158 respectively.

3. Results and discussion

3.1. Isolation and identification of a PCA-degrading strain

A PCA-degrading bacterial strain WH99 was isolated that could utilize PCA as the sole carbon source. Colonies grown on MMP agar plate were smooth, opaque, dirty white or slightly pink (Fig. S1A). It is a rod-shaped bacterium about 0.4 - 0.6 μm wide and 0.9 - 1.2 μm in length. (Fig. S1B). The optimal pH and temperature for the growth of strain WH99 were 7.0 and 30°C, respectively. It was negative for oxidase, starch

hydrolysis, methyl red and nitrate reductase tests, and positive for phenylalanine aryl aminase, tyrosine aryl aminase and L-lactic acid salinization tests. These characteristics were consistent with the general properties of *Rhodococcus* species. The 16S rRNA gene sequence of WH99 was highly similar to those of known *Rhodococcus* strains such as *Rhodococcus equi* NBRC 101255^T (100%), *Rhodococcus soli* DSD51W^T (99.3%) and *Rhodococcus agglutinans* CFH S0262^T (99.0%). A phylogenetic tree constructed on the basis of 16S rRNA gene sequences of WH99 and its close relatives is presented in Fig. S2. Based on these preliminary observations, WH99 was identified as *Rhodococcus* species.

Among the reported PCA-degrading strains, apart from the only Gram-negative *Sphingomonas* sp. DP58 (Yang et al., 2007; Zhao et al., 2017), all others are Gram-positive *Actinobacteria* including *Mycobacterium* sp. CT6, *Mycobacterium* sp. DNK1213, *Mycobacterium* sp. ATCC6841, *Rhodococcus* sp. JVH1 (Costa et al., 2015) and *Nocardia* sp. LAM0056 (Costa et al., 2018). Strain WH99 is also Gram-positive and is the second PCA-degrading strain belonging to the genus *Rhodococcus. Rhodococcus sps* have an excellent ability to degrade a wide range of pesticides including buprofezin (Chen et al., 2017), carbendazim (Zhang et al., 2017) and carbaryl (Zhu et al., 2018). They can also degrade organosulfur compounds (Van Hamme et al., 2004) and nitroaromatic compounds (Subashchandrabose et al., 2018). This is owing to strong environmental adaptability and presence of diverse cellular oxygenases in the genus *Rhodococcus* (Janssen et al., 2010; Chen et al., 2017).

3.2. Utilization of PCA for growth by strain WH99

The degradation of PCA and growth of WH99 were investigated simultaneously **(**Fig. 1**)**. The strain displayed a growth lag for the first 6 h in conjunction with modest decrease in PCA, suggesting that the PCA-degrading enzyme might be inducible, following which it degraded PCA rapidly. After 12 h of incubation, 0.10 mM PCA was almost completely depleted (99.4%) and the cell density had increased from 2.01×10^7 CFU mL^{-1} to about 3.88×10^7 CFU mL^{-1}, indicating that strain WH99 can degrade PCA and use it as the sole carbon source.

Although five PCA-degrading Gram-positive bacteria have been reported, only *Rhodococcus* sp. JVH1 has been examined for its degradation rate. This strain can only degrade half of 0.2 mM PCA in 48 h upon inoculation at an OD_{600} of ~2 to 3 (Costa et al., 2015). In our study, strain WH99 was capable of degrading 99.4% of 0.10 mM PCA in 12 h with a small amount as inoculum (OD_{600} of 0.10), indicating that the degradation efficiency of strain WH99 is higher than that of the previously characterized strain JVH1. Considering that the concentration of PCA is low (~0.1 μg g^{-1} dry soil) in the rhizosphere (Letourneau et al., 2018), strain WH99 may have more potential for degradation of PCA in the rhizosphere.

3.3. Genome comparison and prediction of candidate dioxygenase genes

Occasionally, we found that after successive streaking on 1/5 LB agar plates, few WH99 descendants grew on LB agar plates but not on MMP agar plates. One such isolate, designated WH99M could not degrade PCA in liquid MMP, indicating loss of function of the gene responsible for PCA degradation.

To identify the genes mutated in WH99M, the complete genomes of WH99 and

WH99M were sequenced. Both genomes consisted of one chromosome and two circular plasmids, named plasmid1 (P1) and plasmid2 (P2), respectively. Pairwise comparison of both sequences revealed that a 21.5-kb DNA fragment from P1 (329611 to 351109 bp) was deleted in WH99M (Fig. 2A, Table 3); this was further confirmed by PCR.

The physical map of this fragment is shown in Fig. 2. Computational analysis of this fragment by online ORF Finder and BLASTx program (www.ncbi.nlm.nih.gov/) identified 21 complete ORFs. Of these, *pzcA1*, *pzcA2*, *pzcB1*, *pzcB2*, *pzcC* and *pzcD* encode putative 45-, 19-, 49-, 23-, 45-, 12-kDa proteins consisting of 400, 160, 442, 190, 413, 110 amino acids, respectively. The protein sequences encoded by these ORFs were used as queries in a BLASTP search (NR and Swiss-Port database), and the functions for each ORF were predicted (Table 3). PzcA1 and PzcA2 share 33% and 29% identities with the anthranilate 1,2-dioxygenase α subunit from *Burkholderia cepacia* and salicylate 5-hydroxylase small oxygenase component from *Ralstonia* species, respectively. PzcB1 and PzcB2 share 44% and 49% identities with the 3-phenylpropionate/cinnamic acid dioxygenase α subunit from *Photorhabdus laumondii* TTO and biphenyl dioxygenase β subunit from *Comamonas testosterone*, respectively. PzcC and PzcD share 37% and 38% identities with the ferredoxin reductase component from *Sphingomonas wittichii* DC-6 and biphenyl dioxygenase ferredoxin subunit from *Pseudomonas* sp. KKS102, respectively.

Two different steps for the initial degradation of PCA are known. One is the oxidative decarboxylation reaction occurring in Gram-positive bacteria, represented

by *Mycobacterium* sp. CT6, catalyzed by the decarboxylase system PhdAB to yield phenazine (Costa et al., 2018); the other is the hydroxylation reaction occurring in the Gram-negative strain DP58, catalyzed by a dioxygenase system PcaA1A2A3A4 to form 1,2-dihydroxyphenazine (Zhao et al., 2017). Interestingly, accumulation of phenazine was not observed during the degradation of PCA by strain WH99, and this strain could not degrade phenazine in either aerobic or anaerobic conditions (data not shown). This suggested that the initial degradation step of PCA in strain WH99 may be not a decarboxylation reaction, but a hydroxylation reaction catalyzed by a dioxygenase under aerobic conditions. Thus, of the genes identified, two pairs of dioxygenases (PzcA1A2 and PzcB1B2), reductase (PzcC) and ferredoxin (PzcD), may degrade PCA (Fig. 2A, Table 3).

3.4. Confirmation the function of PzcA1A2CD

To determine the function of the putative PCA-degradation dioxygenase, *E. coli* DH5α harboring the corresponding vectors with predicted genes were constructed. After 12 h of culturing, PCA was converted to a brown complex in MMP only with DH5α carrying pMD*pzcA1A2CD* (Fig. S3). Culture media with of DH5α carrying pMD*pzcA1A2*, pMD*pzcB1B2* or pMD*pzcB1B2CD* did not show any changes, which was confirmed by absence of any signs of degradation upon HPLC analysis.

In the culture that formed the brown complex, two compounds (compounds Ⅰ and Ⅱ) were detected by the HPLC with retention times of 16.61 min and 8.07 min, respectively (Fig. 3A). The prominent protonated molecular ion mass of compound I was *m/z* 225.0661 $[M+H]^+$, which is consistent with the protonated derivative of PCA

($C_{13}H_9N_2O_2^+$, *m/z* 225.0659), with a 0.9 ppm error. The molecular ion mass of compound II was *m/z* 213.0665 $[M+H]^+$, which was identified as 1,2-dihydroxyphenazine ($C_{12}H_9N_2O_2^+$, *m/z* 213.0659), with a 2.8 ppm error (Fig. 3B). Generally, a mass error between - 5 ppm and 5 ppm is acceptable for the identification of compounds (Blake et al., 2011). Therefore, the initial step of degradation in this organism is hydroxylation of PCA to 1,2-dihydroxyphenazine by PzcA1A2CD (Fig. 2B), an unstable compound that is spontaneously transformed to a brown complex at room temperature (Fig. S3). This observation is consistent with previous observations (Zhao et al. 2017). Interestingly, HPLC analysis of degradation of PCA by WH99 only showed the transient peak of 1,2-dihydroxyphenazine without any brown complex (data not shown) possibly owing to a series of quick downstream reactions responsible for ring cleavage of 1,2-dihydroxyphenazine, yielding the carbon source required for the growth of strain WH99.

3.5. Expression and purification of each component of the PzcA1A2CD

To test the PCA dioxygenase activity of enzymes *in vitro*, purified recombinant proteins His6-PzcA1 (45kDa), His6-PzcA2 (19kDa), His6-PzcC (45kDA) and His6-PzcD (12kDa) were analyzed by SDS-PAGE (Fig. 4), and their molecular weights were found to be in agreement with the theoretically calculated molecular weights. The proteins did not display any PCA conversion capabilities when tested individually or in pairs, but did so when all four proteins were present (Table 4). These observations indicated that the dioxygenase complex contains two subunits of a nonheme iron dioxygenase, a [2Fe-2S] ferredoxin and a GR-type reductase. Thus,

PCA 1,2-dioxygenase is a type IV dioxygenase.

While PzcA1A2CD can utilize both NADH and NADPH as cofactors for reduction, NADH is preferred (data not shown). We performed Phylogenetic analysis on the basis of the amino acid sequences alignment of PzcA1 with the α-subunits of characterized RHOs (Fig. S4). In the phylogenetic tree, PzcA1 was found to be clustered with the oxygenase components of RHOs responsible for the degradation of benzoates and substituted benzoates, and forms a subclade with PcaA1, BphA1, AhdA1, NagG, HybB, AhdA1, AhdA1 and PhnA1. However, PzcA1 shares low amino acid sequence identities with these oxygenases (27%-45%, NR databases) except the *Sphingomonas* sp. DP58 PcaA1 (68%) (Zhao et al., 2017). However, it is strange that the amino acid sequence of PcaA1 is not available in the NR or Swiss-Prot databases.

3.6. Confirmation of the roles of each gene in *pzcA1A2CD*

To further verify the roles of dioxygenase (PzcA1A2)- ferredoxin reductase (PzcC)- and ferredoxin (PzcD)- encoding genes, unmarked individual knockout strains of these genes were constructed. WH99Δ*pzcA1*, WH99Δ*pzcA2* and WH99Δ*pzcD* were incapable of growing on MMP agar plates and degrading PCA (Fig. 5) while the complemented strains WH99Δ*pzcA1*(pRE*pzcA1*), WH99Δ*pzcA2*(pRE*pzcA2*) and WH99Δ*pzcD*(pRE*pzcD*) regained the ability to grow on MMP agar plates. Although WH99Δ*pzcC* could still grow on MMP agar plates, the growth was poor when compared to the wild strain (Fig. 5A). The degrading ability assay of the wild-type, mutant and the corresponding complemented strains showed similar results (Fig. 5B). These observations suggested that PzcA1A2CD is the only

dioxygenase system catalyzing the first degradation step of PCA in strain WH99. The dioxygenase component (PzcA1A2) of this system has a high specificity for the ferredoxin component (PzcD), while ferredoxin reductase PzcC can be partially replaced by other ferredoxin reductases *in vivo*.

3.7. PCA degradation is protective to sensitive organisms

Since PCA can function as an antimicrobial (Voggu et al., 2006, 2016), it is ecologically important to study whether degradation might influence other members of the microbial community. We hypothesized that PCA degradation would be protective to some organisms that are otherwise susceptible, such as *E. coli* DH5α, *Bacillus subtilis* 168 (Wu et al., 2019), *Pichia pastoris* GS115 (Chan et al., 2017) and *Rhodococcus* sp. YL-1 (Chen et al., 2017), none of which can degrade PCA (data not shown). After 24 h of incubation in MMP containing 0.1 mM PCA, all strains except *E. coli* DH5α were inhibited by PCA to various degrees (Fig. 6). In comparison with treatment in MMP, incubation in RW or RD, *Bacillus subtilis* 168 (Wu et al., 2019), *Rhodococcus* sp. YL-1 (Chen et al., 2017) and *Pichia pastoris* GS115 (Chan et al., 2017) rescued growth to varying degrees, though not always to the same extent as in MSM. Therefore, the initial step of transformation of PCA could decrease its toxicity against some sensitive members of the microbial community and protect them.

Phenazines are a class of polycyclic, aromatic, nitrogen-containing heterocycles, including PCA, pyocyanin (PYO) and phenazine-1-carboxamide (PCN). Their toxicity varies depending on the phenazine type and environmental conditions. The presence of phenazines is negatively correlated with microbial species richness in

laboratory enrichment cultures (Costa et al., 2015). Our study showed that the degradation or transformation of PCA decreased toxicity to some organisms such as Gram-positive bacteria *Bacillus subtilis* 168 (Wu et al., 2019), *Rhodococcus* sp. YL-1 (Chen et al., 2017) and eukaryote *Pichia pastoris* GS115 (Chan et al., 2017), while the PCA toxicity on *E. coli* DH5α was not obvious. Interestingly, PYO was also found to be less toxic to *E. coli* DH5α than *Staphylococcus aureus* MN8 and *Shewanella oneidensis* MR1 in a previous report (Costa et al., 2015). Thus, further study is needed to reveal the mechanisms of resistance or sensitivity to PCA of different microorganisms. It is tempting to speculate that future work will involve modulating PCA levels or transforming it to impact the fitness of organisms by promoting growth and minimizing toxicity in other contexts or natural environments.

4. Conclusions

A bacterial strain WH99, capable of degrading PCA and using it as the sole carbon source for growth, was isolated and identified as *Rhodococcus* species. An angular dioxygenase encoding gene cluster *PzcA1A2CD* responsible for the initial degradation step of PCA was identified, cloned and expressed in *E. coli*. The dioxygenase (PzcA1A2) has high specificity for the ferredoxin component (PzcD), but low specificity for reductase *in vivo*. PCA degradation by WH99 or DH5α/pMD*pzcA1A2CD* could provide varying degrees of protection to some sensitive microbes. We thus identified a new initial PCA degradation step in a Gram-positive bacterium, which provides more insight into the mechanisms of microbial PCA degradation.

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Declaration of competing interest

The authors declare no competing financial interests.

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Table captions

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Table 2 Primers used in this study

Table 3 Deduced function of each ORF within the missing 21499-bp fragment of mutant WH99M

Table 4 Activities of different combinations of oxygenase, ferredoxin and reductase in PCA degradation

Figure legends

Fig. 1. Growth of *Rhodococcus* sp. WH99 with PCA as the sole carbon source. ○, PCA control; ●, PCA with strain WH99; ■, cell density of strain WH99 with PCA. Error bars represent the standard errors from three replicates. These experiments were performed in triplicate. The results are the averages of three independent experiments, and the error bars show standard deviations.

Fig. 2. The initial PCA degradation steps of Gram-positive strains and the involved genes in strain WH99. (A) Organization of the genes involved in PCA catabolism in strain WH99. (B) Pattern of PCA conversion by dioxygenase system PzcA1A2CD in strain WH99 (green arrows) and decarboxylase system PhdAB in strain CT6 (yellow arrows).

Fig. 3. Identification of the hydroxylation product of PCA by *E. coli* DH5α/pMD*pzcA1A2CD*. (A) HPLC analysis of the hydroxylation product of PCA by *E. coli* DH5α/pMD*pzcA1A2CD*. (B) MS/MS analysis of compound I (*m/z* 225.0659 $[M+H]^+$), which was identified as PCA. (C) MS/MS analysis of compound II (*m/z* 213.0659 $[M+H]^+$), which was identified as 1,2-dihydroxyphenazine.

Fig. 4. SDS-PAGE analysis of dioxygenase, reductase and ferredoxin. M: Protein marker from up to down (kDa) 97.2, 66.4, 44.3, 29.0, 20.1, 14.0; 1: Purified His6-PzcA1 protein; 2: Purified His6-PzcA2 protein; 3: Purified His6-PzcC protein; 4: Purified His6-PzcD protein.

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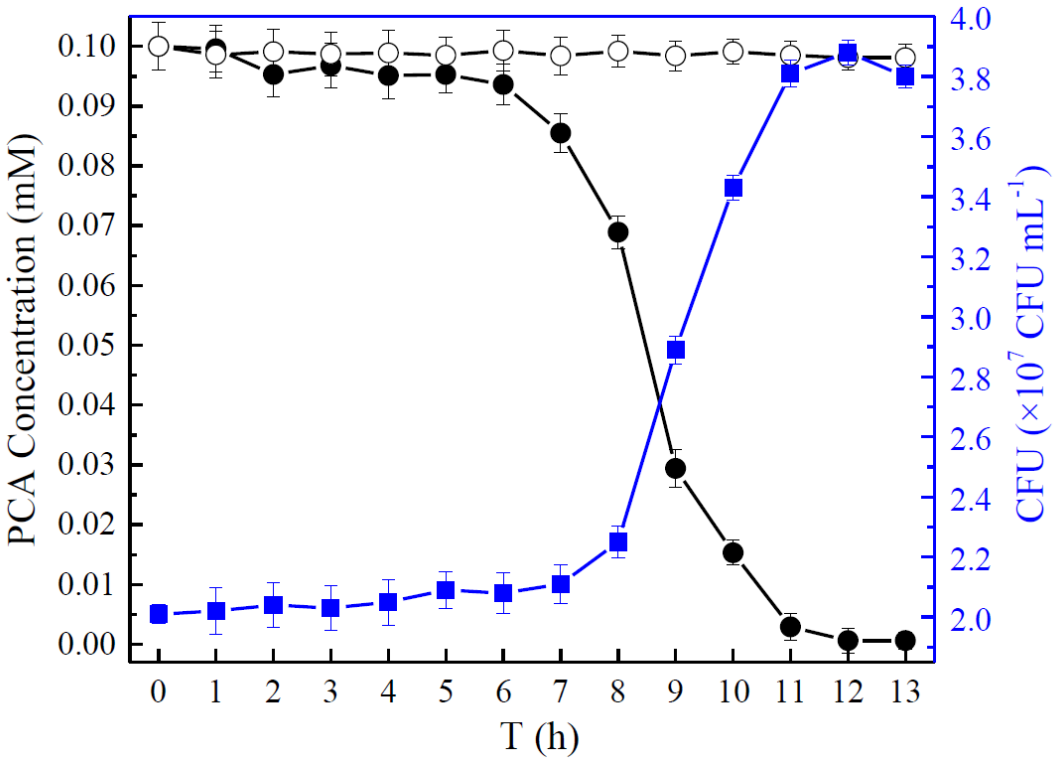
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



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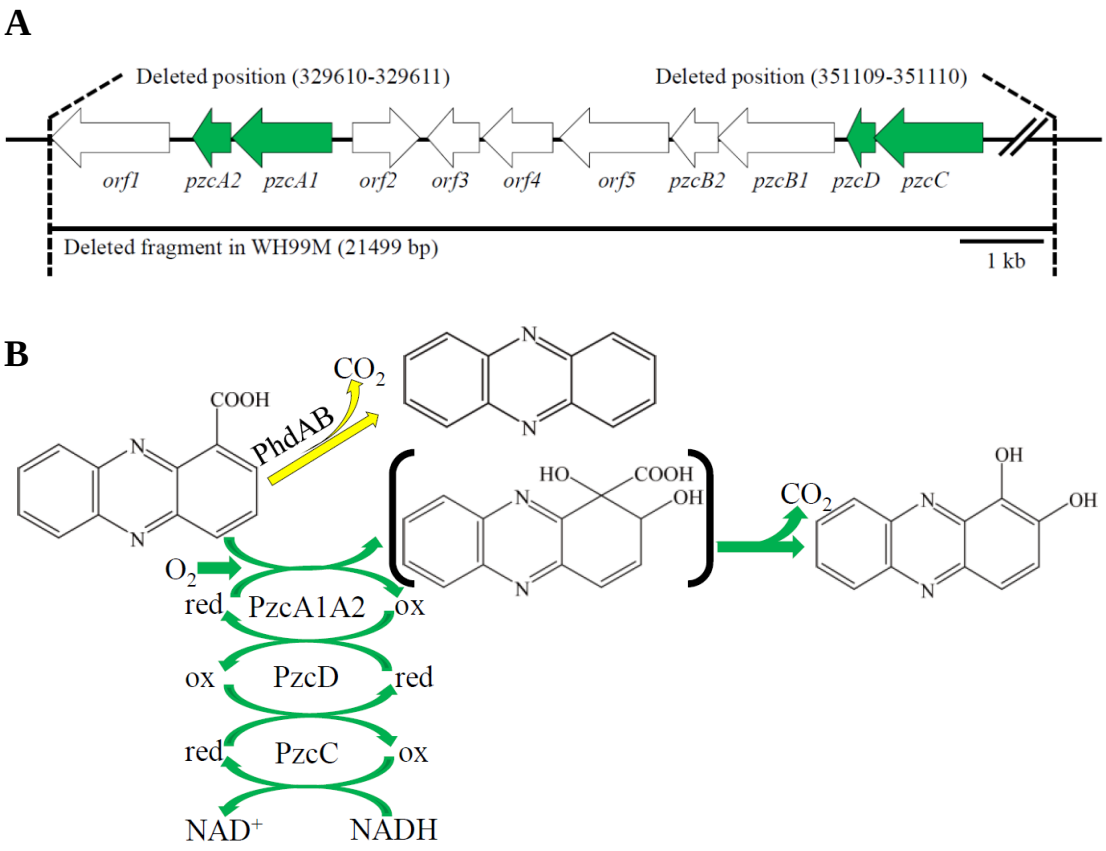

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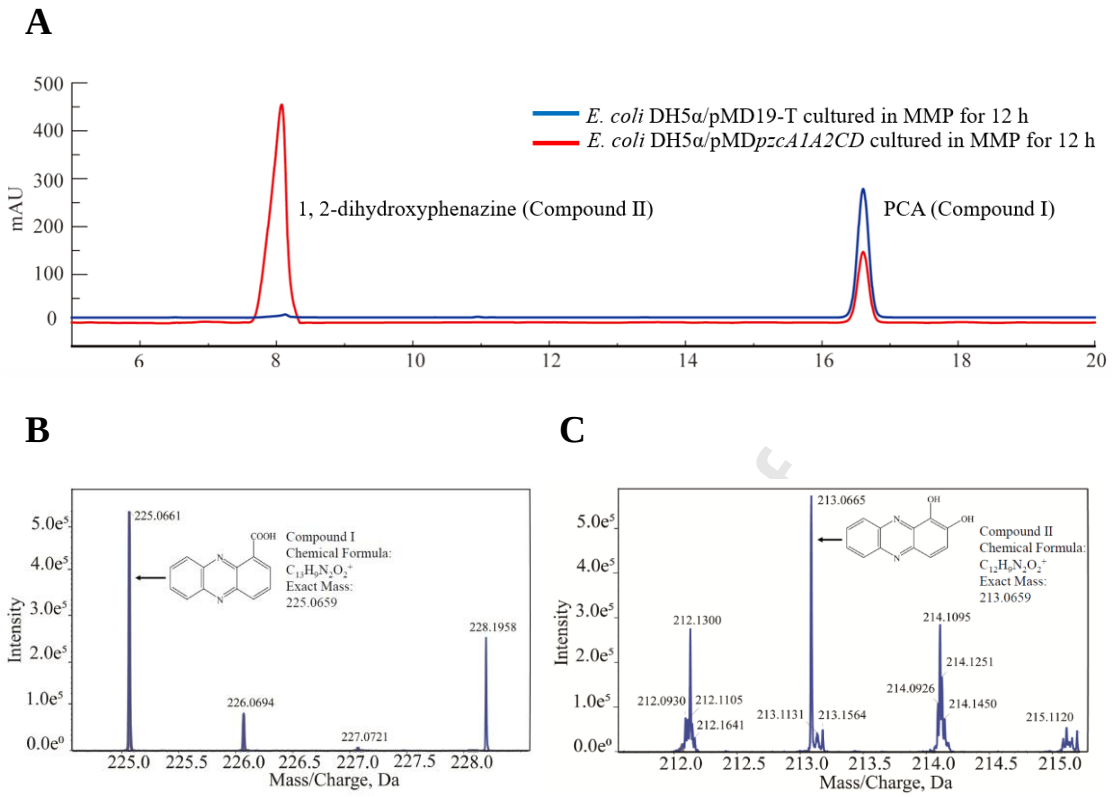

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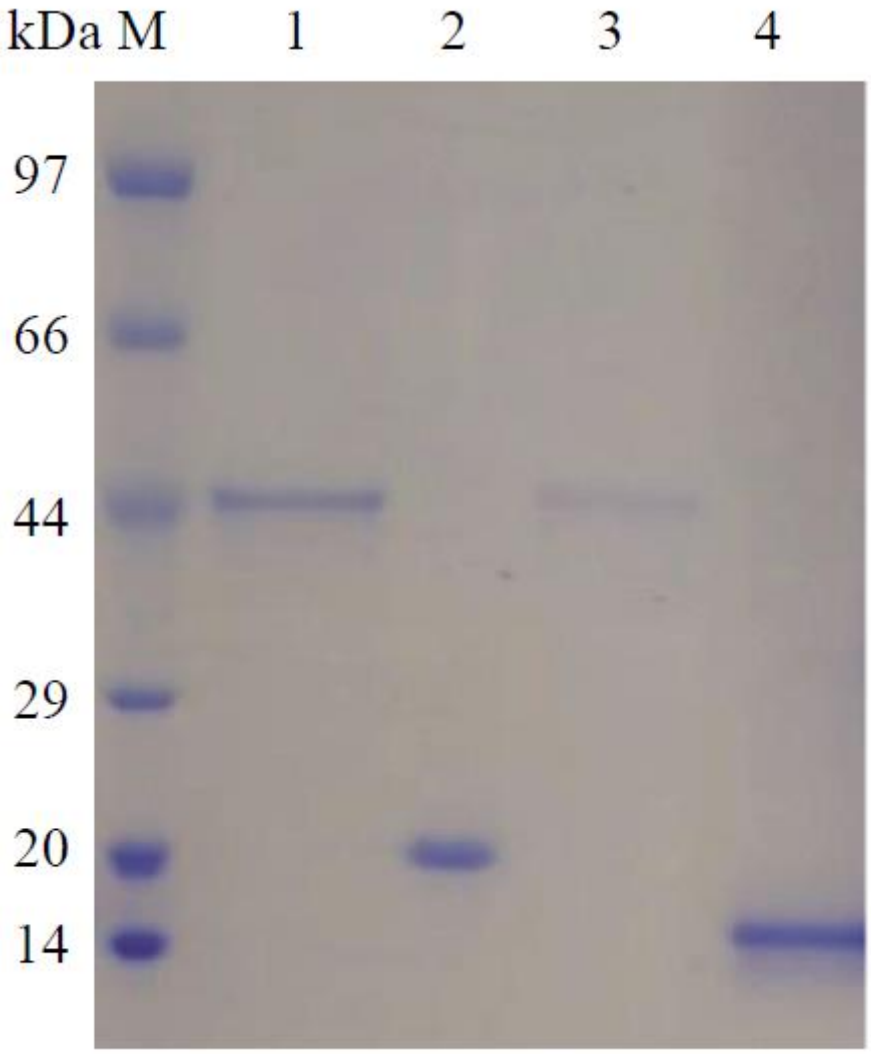

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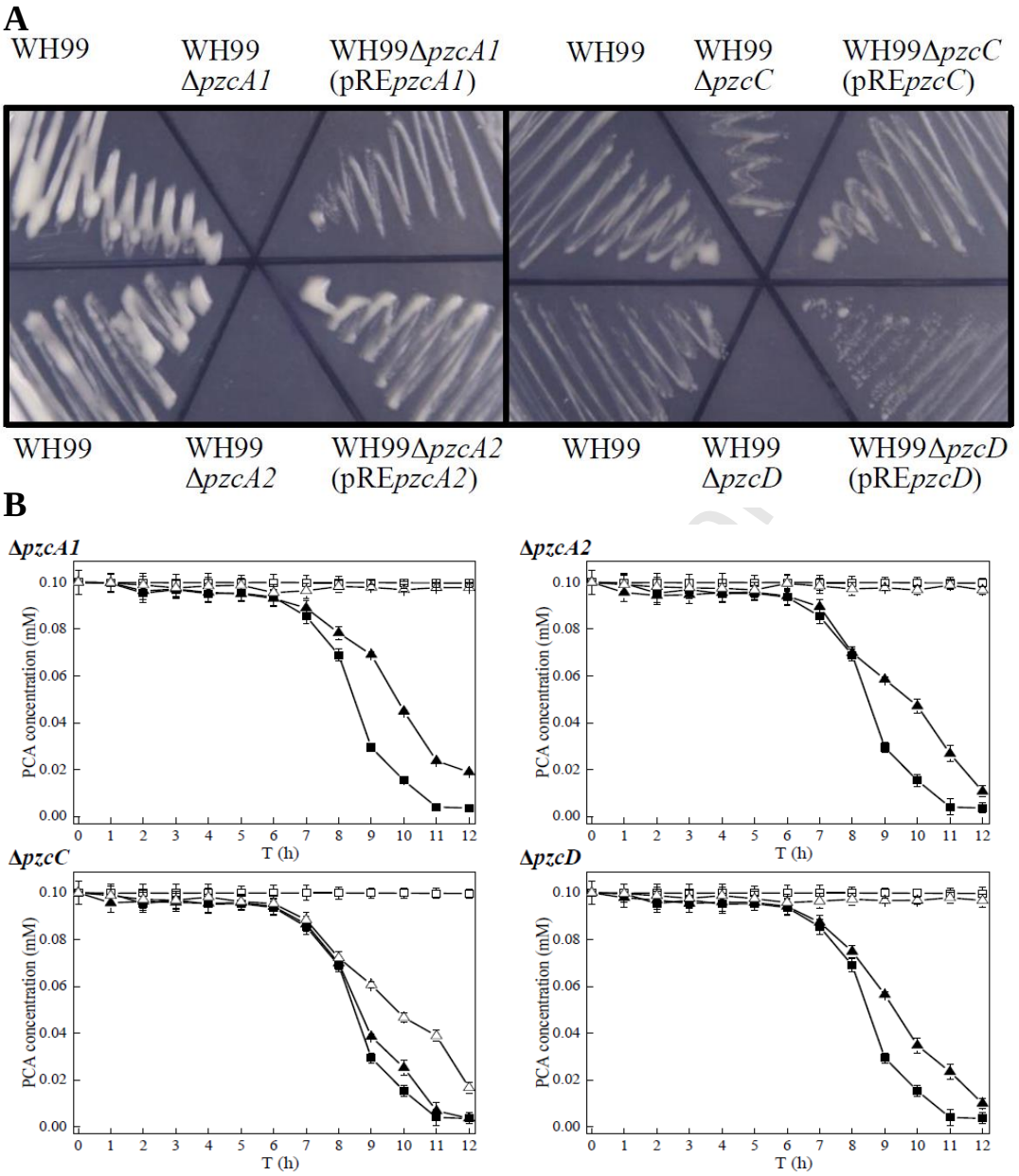

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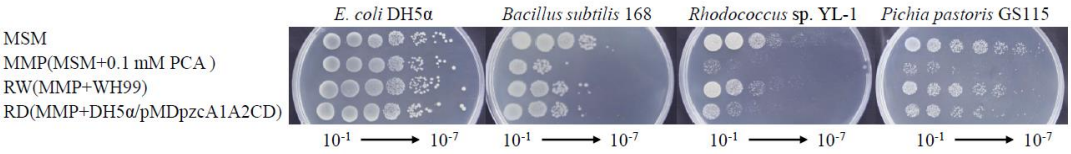

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Table 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
Rhodococcus sp. WH99	Degrades phenazine-1-carboxylic acid; Km^r	This study
E. coli DH5α	Host strain for pMD19-T and pRESQ; Amp^r	TaKaRa
E. coil BL21(DE3)	Host strain for pET28a(+); Km^r	TaKaRa
Bacillus cereus 168	Model strain	Novagen
Rhodococcus sp. YL-1	Degrades buprofezin; Km^r	our laboratory
Pichia pastoris GS115	Model strain	Novagen
Plasmids		
pMD19-T	Cloning vector; Amp^r	TaKaRa
pET28a(+)	Expression vector; Km^r	Novagen
pK18*mobsacB*	Gene knockout vector derived from plasmid pK18; $sacB^+$ Km^r	Novagen
pRESQ	Cloning vector; Km^r	Novagen
pMD*pzcA1A2*	pMD19-T derivative carrying *pzcA1A2*; Amp^r	This study
PMD*pzcB1B2*	pMD19-T derivative carrying *pzcB1B2*; Amp^r	This study
pMD*pzcA1A2CD*	pMD19-T derivative carrying *pzcA1A2CD*; Amp^r	This study
pMD*pzcB1B2CD*	pMD19-T derivative carrying *pzcB1B2CD*; Amp^r	This study
pET*pzcA1*	pET28a(+) derivative carrying *pzcA1*; Km^r	This study
pET*pzcA2*	pET28a(+) derivative carrying *pzcA2*; Km^r	This study
pET*pzcC*	pET28a(+) derivative carrying *pzcC*; Km^r	This study
pET*pzcD*	pET28a(+) derivative carrying *pzcD*; Km^r	This study
pK18*mobsacB*-Δ*pzcA1*	pK18*mobsacB* derivative carrying two DNA fragments homologous to upstream and downstream regions of *pzcA1*; $sacB^+$ Km^r	This study
pK18*mobsacB*-Δ*pzcA2*	pK18*mobsacB* derivative carrying two DNA fragments homologous to upstream and downstream regions of *pzcA2*; $sacB^+$ Km^r	This study
pK18*mobsacB*-Δ*pzcC*	pK18*mobsacB* derivative carrying two DNA fragments homologous to upstream and downstream regions of *pzcC*; $sacB^+$ Km^r	This study
pK18*mobsacB*-Δ*pzcD*	pK18*mobsacB* derivative carrying two DNA fragments homologous to upstream and downstream regions of *pzcD*; $sacB^+$ Km^r	This study
pRE*pzcA1*	pRESQ derivative carrying *pzcA1*; Km^r	This study
pRE*pzcA2*	pRESQ derivative carrying *pzcA2*; Km^r	This study
pRE*pzcA3*	pRESQ derivative carrying *pzcA3*; Km^r	This study
pRE*pzcA4*	pRESQ derivative carrying *pzcA4*; Km^r	This study

[a] Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

Table 2 Primers used in this study

Primer	Sequence (5'-3')[a]	Purpose
pMDA12-F	TACGTCGTCGTCAGCGGTTGAATGGTGAACACCACCATTT	Amplification a fragmen
pMDA12-R	AAAACGACGGCCAGT**GAATTC**TTAGAGCGGGTAGACCAGGT	
pMDCD-F	GACCATGATTACGCC**AAGCTT**ATGACTGAAACCTACGCCGT	Amplification a fragmen
pMDCD-R	AAATGGTGGTGTTCACCATTCAACCGCTGACGACGACGTA	
pMDB12-F	GACCATGATTACGCC**AAGCTT**ACGATATCGAGAGAACTCA	Amplification a fragmen
pMDB12-R	AAAACGACGGCCAGT**GAATTC**TCAACCGCTGACGACGACG	
pMDB12CD-F	GACCATGATTACGCC**AAGCTT**ATGACTGAAACCTACGCCG	Amplification a fragmen
pMDB12CD-R	AAAACGACGGCCAGT**GAATTC**CTAGAACAGGACGCTGAGG	
pETA1-F	GTGCCGCGCGGCAG**CCATATG**ATGGTGAACACCACCATTT	Amplification a fragmen
pETA1-R	GTGGTGGTGGTGGTG**CTCGAG**TCAGTGATCAAAACCCATCACCT	
pETA2-F	GTGCCGCGCGGCAGC**CATATG**ATGATCACTGAAACAGCCA	Amplification a fragmen
pETA2-R	GTGGTGGTGGTGGTG**CTCGAG**TTAGAGCGGGTAGACCA	
pETC-F	GTGCCGCGCGGCAGC**CATATG**ATGACTGAAACCTACGCCGTAG	Amplification a fragmen
pETC-R	GTGGTGGTGGTGGTG**CTCGAG**TCACGCGATACCGCTCGA	
pETD-F	GTGCCGCGCGGCAGC**CATATG**ATGACAACGACCGAGAAGTCC	Amplification a fragmen
pETD-R	GTGGTGGTGGTGGTG**CTCGAG**TCAACCGCTGACGACGACG	
pKA1u-F	TCCTCTAGAGTCGAC**CTGCAG**GGGAGTTACCTCCGCATCGA	Amplification of upstrea
pKA1u-R	CTCGAATGTCGTTGGCTGTTCCCTGCGCGGACCATTCGAG	
pKA1d-F	CTCGAATGGTCCGCGCAGGGAACAGCCAACGACATTCGAG	Amplification of downst
pKA1d-R	GCCAAGCTTGCATGC**CTGCAG**GACCATTTCGTCTCGTCGAT	
pKA2u-F	TCCTCTAGAGTCGAC**CTGCAG**TGCGGTTCGCCCGCCC	Amplification of upstrea
pKA2u-R	GCGTCGTGTTGCCTCGCTCGAACCCATCACCTTCCGGTAG	
pKA2d-F	CTACCGGAAGGTGATGGGTTCGAGCGAGGCAACACGACGC	Amplification of downst
pKA2d-R	GCCAAGCTTGCATGC**CTGCAG**TACTGGAAGCGGCTCATCGG	
pKCu-F	TCCTCTAGAGTCGAC**CTGCAG**CTTCATGCTCGAGGTCACCA	Amplification of upstrea
pKCu-R	AACTTTCCTCCTCGTCACCGCTGAGTTCTCTCGATATCGT	
pKCd-F	ACGATATCGAGAGAACTCAGCGGTGACGAGGAGGAAAGTT	Amplification of downst
pKCd-R	GCCAAGCTTGCATGC**CTGCAG**TGGCCAGTGGTTCCGTTG	
pKDu-F	TCCTCTAGAGTCGAC**CTGCAG**CTGGTCCGACCAGGGCG	Amplification of upstrea
pKDu-R	TCTAGTTTCTGGAACAAAGCAACTTTCCTCCTCGTCACCG	
pKDd-F	CGGTGACGAGGAGGAAAGTTGCTTTGTTCCAGAAACTAGA	Amplification of downst
pKDd-R	GCCAAGCTTGCATGC**CTGCAG**ATTGGGGATCATGCTGTCGT	
pREA1-F	ACCGAGCTCAGATCT**ACTAGT**CCCTGCGCGGACCATTCGAG	Amplification of *pzcA1*
pREA1-R	ACACTGGCGGCCGTT**ACTAGT**AACAGCCAACGACATTCGAG	
pREA2-F	ACCGAGCTCAGATCT**ACTAGT**CCCTGCGCGGACCATTCGAG	Amplification of *pzcA2*
pREA2-R	ACACTGGCGGCCGTT**ACTAGT**TTAGAGCGGGTAGACCAGGT	
pREC-F	ACCGAGCTCAGATCT**ACTAGT**CTTCATGCTCGAGGTCACCAA	Amplification of *pzcC* f
pREC-R	ACACTGGCGGCCGTT**ACTAGT**CGGTGACGAGGAGGAAAGTT	
pRED-F	ACCGAGCTCAGATCT**ACTAGT**CTTCATGCTCGAGGTCACCAA	Amplification of *pzcD* f
pRED-R	ACACTGGCGGCCGTT**ACTAGT**CAACCGCTGACGACGACGT	

[a] Underlined bases indicate that they overlapped and were used to construct plasmids by homologous recombination. The bold bases indicate restriction enzyme sequences.

Table 3 Deduced function of each ORF within the missing 21499-bp fragment of mutant WH99M

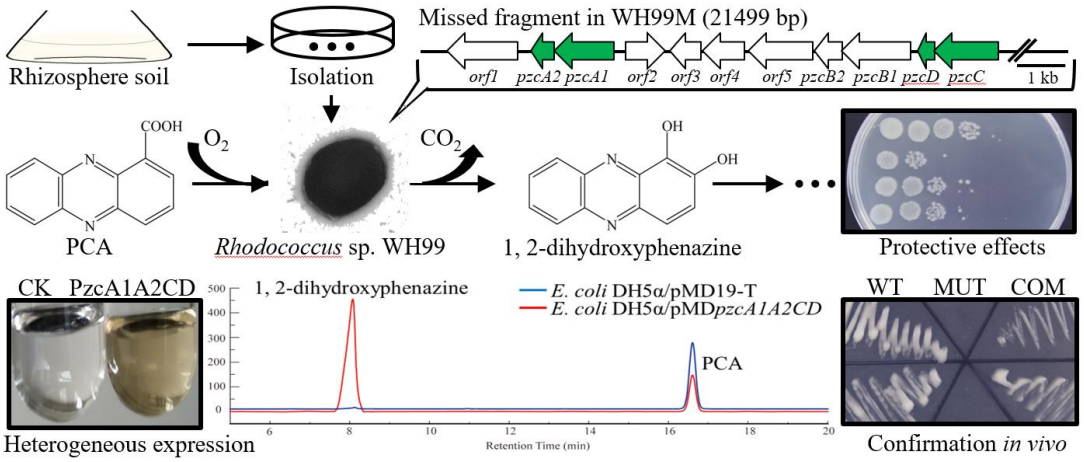
Gene name	Position in P1	Size (amino acids)	Proposed product	Database	Homologous protein (GenBank accession no.), source	Identity%
orf1	331002-329611	463	Hypothetical protein	NR	MFS transporter (WP_072751183.1), *Rhodococcus*	78
				Swiss-P	4-hydroxybenzoate transporter PcaK (Q51955.1), *Pseudomonas*	30
pzcA2	331735-331253	160	Dioxygenase βsubunit	NR	Hypothetical protein (WP_0094786021), *Rhodococcus* sp.	84
				Swiss-P	Salicylate 5-hydroxylase, small oxygenase component	29
pzcA1	332927-331725	400	Dioxygenase αsubunit	NR	Aromatic ring-hydroxylating dioxygenase α subunit	93
				Swiss-P	Anthranilate 1,2-dioxygenase α subunit (Q84BZ3.1),	33
ort2	333121-333921	266	Reductase	NR	Glucose 1-dehydrogenase (WP_009478600.1), *Rhodococcus*	81
				Swiss-P	Dihydroanticapsin 7-dehydrogenase (P39640.2), *Bacillus*	38
orf3	334611-334009	200	Hypothetical protein	NR	FMN reductase (SEK56253.1), *Rhodococcus maanshanensis*	81
				Swiss-P	FMN-dependent NADPH-azoreductase (Q2FJ80.1),	30
orf4	335507-334617	296	Glyoxalase	NR	Glyoxalase (WP_063017100.1), *Nocardia nova*	90
				Swiss-P	Biphenyl-2,3-diol 1,2-dioxygenase (P47228.2),	38
orf5	336740-335571	389	Putative pigment protein	NR	Acyl-CoA dehydrogenase (WP_009478598.1), *Rhodococcus*	86
				Swiss-P	Flavin-dependent monooxygenase, oxygenase subunit HsaA	34
pzcB2	337389-336817	190	Dioxygenase βsubunit	NR	3-Phenylpropionate/cinnamic acid dioxygenase β subunit	77
				Swiss-P	Biphenyl dioxygenase β subunit (Q46373.1), *Comamonas*	49
pzcB1	338714-337386	442	Dioxygenase αsubunit	NR	Aromatic ring-hydroxylating dioxygenase α subunit	89
				Swiss-P	3-phenylpropionate/cinnamic acid dioxygenase α subunit	44
pzcD	339188-338856	110	[2Fe-2S] ferredoxin	NR	Non-heme iron oxygenase ferredoxin subunit	89
				Swiss-P	Biphenyl dioxygenase ferredoxin subunit (Q52440.1),	38
pzcC	340450-339209	413	Ferredoxin reductase	NR	Pyridine nucleotide-disulfide oxidoreductase	72
				Swiss-P	Ferredoxin reductase component (X5CY81.1), *Sphingomonas*	37

Table 4 Activities of different combinations of oxygenase, ferredoxin and reductase in PCA degradation

Enzyme	Activity (nmol · min^{-1} · mg^{-1}) [a]
PzcA1A2	ND
PzcA1A2C	ND
PzcA1A2D	ND
PzcA1A2CD	195.12±8.03

[a] ND, no PCA conversion was detected.

Graphical abstract



Highlights

- A PCA-degrading strain *Rhodococcus* sp. WH99 was isolated.
- A new initial PCA degradation step in Gram-positive bacteria was confirmed.
- A PCA dioxygenase system PzcA1A2CD was cloned and expressed in *E. coli*.
- The roles of each gene in *pzcA1A2CD* were defined *in vivo*.
- The protection by PCA degradation to some sensitive organisms was studied.