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Functional genomic analysis of phthalate acid ester (PAE) catabolism genes in the versatile PAE-mineralising bacterium *Rhodococcus* sp. 2G

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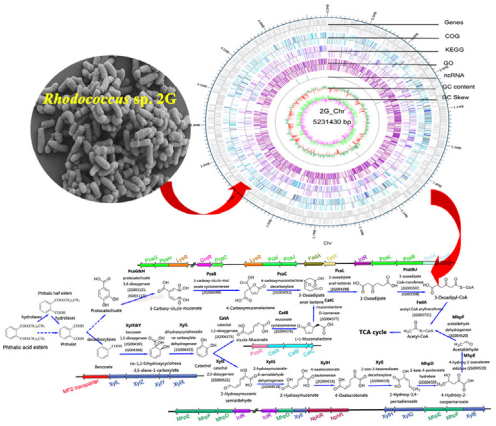
HIGHLIGHTS

- The strain 2G could efficiently degrade a wide range of PAEs.
- A repertoire of the whole PAEs-catabolism process was obtained.
- An array of genes involved in PAEs catabolic pathway in the 2G genome were excavated.
- Strain 2G has two gene clusters of ring cleavage pathways in catechol degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial degradation is considered the most promising method for removing phthalate acid esters (PAEs) from polluted environments; however, a comprehensive genomic understanding of the entire PAE catabolic process is still lacking. In this study, the repertoire of PAE catabolism genes in the metabolically versatile bacterium *Rhodococcus* sp. 2G was examined using genomic, metabolic, and bioinformatic analyses. A total of 4930 coding genes were identified from the 5.6 Mb genome of the 2G strain, including 337 esterase/hydrolase genes and 48 transferase and decarboxylase genes that were involved in hydrolysing PAEs into phthalate acid (PA) and decarboxylating PA into benzoic acid (BA). One gene cluster (*xyl*) responsible for transforming BA into catechol and two catechol-catabolism gene clusters controlling the *ortho* (*cat*) and *meta* (*xyl* & *mhp*) cleavage pathways were also identified. The proposed PAE catabolism pathway and some key degradation genes were validated by intermediate-utilising tests and real-time quantitative polymerase chain reaction. Our results provide novel insight into the mechanisms of PAE biodegradation at the molecular level and useful information on gene resources for future studies.

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1. Introduction

Phthalate acid esters (PAEs) are a class of refractory organic compounds that are widely used as additives or plasticisers in the manufacture of plastics (Ahuactzin-Pérez et al., 2016). Due to the absence of chemical binding between polymer mesh and the plasticiser, PAEs are readily released from plastic products and enter various environments such as soil, air, water, and sediments (Sálamo et al., 2018; Wang et al., 2015; Zhang et al., 2014; Zhao et al., 2015). PAEs have been widely detected in aquatic and terrestrial environments (Net et al., 2015; Zhao et al., 2016a, 2016b). Studies have shown that PAE exposure results in human health concerns, including dysfunctions of the endocrine, reproductive, and nervous systems, as well as acquired diseases such as cancers, allergies, and birth defects (Bui et al., 2016; Gao et al., 2017; Meng et al., 2015; Wójtowicz et al., 2017). Due to their risks to human health and the environment, the United States Environmental Protection Agency (US EPA) and China National Environmental Monitoring Center have listed PAEs as environmental priority pollutants (Zhang et al., 2016). Therefore, it is necessary to develop remediation strategies to eliminate PAEs from the environment.

Microbial degradation is considered the most promising method for removing PAEs from polluted environments. To date, a large number of PAE-degrading bacteria have been isolated from various environments; these include strains from genera such as *Achromobacter* (Pradeep et al., 2015), *Agromyces* (Zhao et al., 2016a), *Agrobacterium* (Wu et al., 2011), *Bacillus* (Surhio et al., 2017), *Curvibacter* (Ma et al., 2016), *Enterobacter* (Fang et al., 2014), *Gordonia* (Jin et al., 2012), *Microbacterium* (Zhao et al., 2017) *Providencia* (Zhao et al., 2016b), *Pseudoxanthomonas* (Meng et al., 2015), and *Rhodococcus* (He et al., 2014). Biodegradation pathways for PAEs have been proposed in several studies by detecting generated metabolites using mass spectrometry (Liang et al., 2008; Gao and Wen, 2016). Generally, PAEs are first hydrolysed into phthalate monoesters and then into phthalic acid (PA) by esterases. PA is further metabolised into protocatechuate (PCA) via 4,5-dihydroxyphthalate in Gram-negative bacteria, or into PCA via 3,4-dihydroxyphthalate in Gram-positive bacteria. PCA is finally metabolised through either the *ortho* or *meta* cleavage pathway (Liang et al., 2008). However, the molecular mechanisms of PAE biodegradation are not yet known, and a comprehensive genomic understanding of the steps of PAE degradation is still lacking. Whole-genome sequencing and functional annotation of PAE-degrading isolates are promising approaches for identifying functional genes and understanding their roles in the degradation pathways (Pan et al., 2017). To the best of our knowledge, the degradation genes encoding the enzymes involved in PAE biodegradation have not been explored by integrated genomic and experimental validation.

In this study, we explored the molecular mechanisms of PAE biodegradation using genome-sequence analyses of the bacterium *Rhodococcus* sp. 2G, which has versatile PAE-degrading capacity. Our findings may provide unique insights into the catabolic pathways of PAEs and related degrading genes; this knowledge can be used as a resource for constructing genetically engineered microbes.

2. Materials and methods

2.1. Chemicals and bacterial strain

The chemicals used in this study were of high purity (>98%) and were purchased from commercial sources (Aladdin, China; Sigma-Aldrich, Germany). These included dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), di-(2-ethylhexyl) phthalate (DEHP), di-*n*-octyl phthalate (DnOP), diisononyl phthalate (DINP), mono-(2-ethylhexyl) phthalate (MEHP), phthalate acid (PA), benzoic acid (BA), protocatechuate (PCA), and catechol. All other chemical reagents were of analytical grade, and all solvents were of high-performance liquid chromatography (HPLC) grade. The bacterial strain was previously isolated from activated sludge and identified as *Rhodococcus* sp. 2G, which can utilise PAEs as its sole carbon and energy source.

2.2. Biodegradation of PAEs by the 2G strain

The 2G strain was inoculated in sterile mineral salt medium (MSM) supplemented with one of the following PAEs as the sole source of carbon and energy (200 mg L^{-1}): DMP, DEP, DBP, BBP, DEHP, DnOP, and DINP. The microorganisms were cultured in triplicate in 250 mL Erlenmeyer flasks under optimum conditions (pH 7.1 and 29 °C) for 5 days. A non-inoculated medium was used as control. Subsamples were collected periodically from the cultures to analyse the PAEs using gas chromatography–mass spectrometry (GC–MS) (QP2010 Plus, Shimadzu). The GC–MS program and the quality assurance/quality control (QA/QC) used in this study are described in a previous study (Zhao et al., 2016a, 2016b). Briefly, an HP-5 column (0.25 μm × 0.25 mm × 30 m) with helium as carrier gas at a flow rate of 1.0 mL min^{-1} was employed; an injection temperature of 250 °C and an ion source temperature of 220 °C was applied. The GC oven temperature was programmed as follows: 100 °C for 2 min, raised to 129 °C at 15 °C min^{-1}, and then raised to 280 °C at 40 °C min^{-1} (held for 5 min). Quantitative analyses were performed using external calibration based on a five-point calibration curve (0–4.0 mg L^{-1}). The instrument was calibrated daily using calibration standards. The detection limits of PAEs in samples, based on three times the signal-to-noise ratio, ranged from 1.3 to 8.6 μg L^{-1}. The average concentration of PAEs ranged from 4.7 to 7.2 μg L^{-1} in all method blanks ($n = 12$); this concentration was subtracted from the sample values. The recovery of spiked PAE compounds was in the range of 85.3–109.7%.

2.3. Whole-genome sequencing and annotation

The genomic DNA of strain 2G was extracted using genome kits (Omega, USA) and the concentration and purity of DNA were detected using a NanoDrop-2000 spectrophotometer. The complete genome was sequenced by HengChuang Gene, Limited (Shenzhen, China) using a hybrid of the HiSeq 2500 (Illumina, USA) and PacBio RS II (Pacific Biosciences, USA) platforms. Annotations for the unique genes were performed using BLAST to align them to the National Center for Biotechnology Information (NCBI) non-redundant protein (NR), gene ontology (GO), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups (COG) databases. The complete genome of strain *Rhodococcus* sp. 2G was deposited to GenBank under accession numbers CP018063 and CP018064 (submission ID SUB2094340, BioProject ID PRJNA353162, and BioSample ID SAMN06011250).

2.4. Intermediate utilisation tests and reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

The 2G strain was incubated in MSM supplemented with 100 mg L^{-1} DEHP. Potential intermediates in the DEHP-degradation product were mono-(2-ethylhexyl) phthalate (MEHP), PA, PCA, benzoate, and catechol. MSM without inoculation was used as a negative control and MSM containing 0.1% glucose with inoculation was used as a positive control. Substrate utilisation was assessed in triplicate by measuring the increase in biomass (OD_{600}) using a UV-2450 spectrophotometer at 6 h intervals over 5 days.

The expression levels of six key genes selected from the genome sequence of strain 2G were determined via RT-qPCR; these are likely involved in the DEHP-catabolism pathway. The primer sequences of these genes are provided in Table S1. Total RNA was extracted from the above DEHP-induced and non-induced (glucose growth) bacteria with three biological replicates using RNAiso Plus (Takara, China) at 24 h intervals over 5 days. The first strand of cDNA was generated by purifying the total RNA using a PrimeScript RT reagent kit (Takara, China).