Applied Microbiology and Biotechnology (2019) 103:7741–7750
https://doi.org/10.1007/s00253-019-09994-7

ENVIRONMENTAL BIOTECHNOLOGY

Molecular and biochemical characterization of 2-chloro-4-nitrophenol degradation via the 1,2,4-benzenetriol pathway in a Gram-negative bacterium

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Received: 15 April 2019 / Revised: 17 June 2019 / Accepted: 17 June 2019 / Published online: 1 August 2019

Abstract

2-Chloro-4-nitrophenol (2C4NP) is the most common chlorinated nitrophenol pollutant, and its environmental fate is of great concern. *Cupriavidus* sp. CNP-8, a Gram-negative bacterium, has been reported to degrade 2C4NP via the 1,2,4-benzenetriol (BT) pathway, significantly different from the (chloro)hydroquinone pathways reported in all other Gram-negative 2C4NP-utilizers. Herein, the BT pathway of the catabolism of 2C4NP in this strain was characterized at the molecular, biochemical, and genetic levels. The *hnp* gene cluster was suspected to be involved in the catabolism of 2C4NP because the *hnp* genes are significantly upregulated in the 2C4NP-induced strain CNP-8 compared to the uninduced strain. HnpAB, a two-component FAD-dependent monooxygenase, catalyzes the conversion of 2C4NP to BT via chloro-1,4-benzoquinone, with a K_m of 2.7 ± 1.1 μM and a k_{cat}/K_m of 0.17 ± 0.03 μM^{-1} min^{-1}. *hnpA* is necessary for strain CNP-8 to utilize 2C4NP *in vivo*. HnpC, a BT 1,2-dioxygenase, was proved to catalyze BT ring-cleavage with formation of maleylacetate by HPLC-MS analysis. Phylogenetic analysis indicated that HnpA likely has different evolutionary origin compared to other functionally identified 2C4NP monooxygenases. To our knowledge, this is the first report revealing the catabolic mechanism of 2C4NP via the BT pathway in a Gram-negative bacterium, increasing our knowledge of the catabolic diversity for microbial 2C4NP degradation at the molecular and biochemical level.

Keywords 1,2,4-Benzenetriol pathway · 2-Chloro-4-nitrophenol · Catabolic mechanism · Gram-negative bacterium · *hnp* cluster · Two-component monooxygenase

Introduction

Chlorinated nitrophenols (CNPs) such as 2-chloro-4-nitrophenol (2C4NP), 2-chloro-5-nitrophenol (2C5NP), 4-chloro-2-nitrophenol (4C2NP), and 2,6-dichloro-4-nitrophenol (2,6-DCNP) have been widely used as intermediates in the chemical syntheses of various pesticides, dyes, and pharmaceuticals (Arora et al. 2018). Inevitably, these hazardous pollutants have been extensively introduced into our surrounding environments mainly by anthropogenic activities (Arora et al. 2012). CNPs compounds are resistant to microbial degradation due to the simultaneous existence of electron-withdrawing chlorine and nitro groups on the aromatic ring. Thus, microorganisms with the ability to degrade these xenobiotics have attracted increasing interests as they would bring a feasibility of environment-friendly and in situ bioremediation.

As the most typical representative of CNPs, 2C4NP is mainly used in the synthesis of the pesticide dicapthon and the fungicide nitrofungin (Min et al. 2014). Structurally, 2C4NP is the chlorinated derivative of priority pollutant *para*-nitrophenol (PNP). The microbial catabolism of PNP has been extensively studied, with either the 1,2,4-benzenetriol (BT, hydroxyquinol) pathway initiated by a two-component PNP

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-019-09994-7) contains supplementary material, which is available to authorized users.

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monooxygenase from Gram-positive PNP-utilizers (Kadiyala and Spain 1998; Kitagawa et al. 2004; Perry and Zylstra 2007; Takeo et al. 2008; Liu et al. 2010) or hydroquinone (HQ) pathway initiated by a single-component PNP monooxygenase from Gram-negative PNP-utilizers (Zhang et al. 2009; Shen et al. 2010; Wei et al. 2010; Zhang et al. 2012) having been reported at the biochemical and genetic levels. In contrast to PNP, 2C4NP is more resistant to microbial degradation due to the extra chlorine substituent on the aromatic ring. Therefore, to date, only six 2C4NP-utilizers including *Rhodococcus imtechensis* RKJ300 (Ghosh et al. 2010; Min et al. 2016b), *Burkholderia* sp. RKJ800 (Arora and Jain 2012), *Burkholderia* sp. SJ98 (Pandey et al. 2011; Min et al. 2014), *Cupriavidus* sp. a3 (Tiwari et al. 2017), *Cupriavidus* sp. CNP-8 (Min et al. 2018), and *Arthrobacter* sp. SJCon (Arora and Jain 2011) have been isolated. These bacteria could degrade 2C4NP via either the BT pathway or (chloro)hydroquinone (HQ/CHQ) pathway. Previously, the molecular mechanisms of 2C4NP degradation in strains SJ98 (Min et al. 2014) and RKJ300 (Min et al. 2016b) have been investigated. The catabolism of 2C4NP in these two strains was performed by the enzymes involved in PNP degradation. Similar with the catabolism of PNP, the Gram-positive RKJ300 degrades 2C4NP via the BT pathway initiated by a two-component PNP monooxygenase (PnpA1A2) (Min et al. 2016b), and the Gram-negative SJ98 was reported to degrade 2C4NP via CHQ pathway initiated by a single-component PNP monooxygenase (PnpA) (Min et al. 2014).

Cupriavidus sp. strain CNP-8, previously isolated by us from the pesticide-contaminated soil in Yantai, China (Min et al. 2017), has the ability to degrade several halogenated nitrophenols including 2C4NP, 2C5NP, and 2,6-dibromo-4-nitrophenol (2,6-DBNP). It initiated the catabolism of 2C5NP by an NADPH-dependent nitroreductase (Min et al. 2017), while an FAD-dependent monooxygenase was reported to initiate 2,6-DBNP degradation (Min et al. 2019). Although the biochemical characterization of 2C4NP degradation was not reported, strain CNP-8 was proposed to degrade 2C4NP via the BT pathway (Fig. 1a) (Min et al. 2018), which is significantly different from the HQ/CHQ pathways reported in all other Gram-negative 2C4NP-utilizers (Arora and Jain 2012; Min et al. 2014; Tiwari et al. 2017). Superficially, the catabolic pathway of 2C4NP in Gram-negative CNP-8 was similar with that in the Gram-positive RKJ300. However, strain RKJ300 can utilize both 2C4NP and PNP for growth and degrade these two nitrophenols by the enzymes encoded in the same gene cluster (Min et al. 2016b), whereas strain CNP-8 only utilizes 2C4NP for growth (Min et al. 2018). Therefore, strain CNP-8 likley has the unique evolutionary pattern in acquiring 2C4NP catabolic ability as compared to other 2C4NP-utilizers, motivating us to identify and characterize the enzymes involved in the catabolism of 2C4NP in this strain.

Here, we reveal the molecular mechanism of 2C4NP degradation via the BT pathway in a Gram-negative bacterium. HnpAB, a two-component monooxygenase, catalyzes the conversion of 2C4NP to BT, which was then transformed to maleylacetate by HnpC. Moreover, HnpA was proposed to have different evolutionary origin compared to other functionally identified 2C4NP monooxygenases. This report presents new molecular and biochemical information about microbial 2C4NP degradation, increasing our understanding of the microbial catabolic diversity of this pollutant.

Materials and methods

Strains, primers, chemicals, media, and culture conditions

The strains and primers used here are shown in Table S1. *Cupriavidus* sp. CNP-8 (CCTCC No. M 2017546) and its derivative strains were cultured aerobically at 30 °C in lysogeny broth (LB) or minimal medium (MM) (Liu et al. 2005) with 2C4NP as the sole carbon and nitrogen source. *Escherichia coli* strains were grown at 30 °C in LB medium. When necessary, kanamycin (50 μg/ml) or chloromycetin (34 μg/ml) was added to the medium. The reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Fluka Chemical Co. (Buchs, Switzerland).

Whole cell biotransformation assay

The whole cell biotransformation assays were performed as described (Chen et al. 2014) with little modification. Strain CNP-8 was initially cultured in MM + 20% LB to an OD_{600} of 0.2, followed by induction for 6 h by adding 0.4 mM of substrate (PNP, 2C4NP, or 2,6-DBNP). Cultures performed in the absence of substrate were used as uninduced controls. The cells were harvested by centrifugation at 8000×*g* at 4 °C for 5 min, washed twice, and suspended in phosphate buffer (20 mM, pH 7.2) to OD_{600} of 2 before 0.4 mM of substrate was added. Samples (1 ml) were taken at 10-min intervals and centrifuged (13,000×*g* at 4 °C, 10 min). The concentrations of (halogenated-)nitrophenols in the supernatant were assayed by high-performance liquid chromatography (HPLC).

RNA preparation and transcription analysis

Total RNA from the uninduced and induced cells of strain CNP-8 was extracted using an RNAprep pure kit (TransGen, Beijing, China), followed by reverse transcription into cDNA using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). Real-time quantitative PCR (RT-qPCR) was performed on a 7500 Fast real-time PCR detection system (Applied Biosystems) using the TransStart Tip Green qPCR

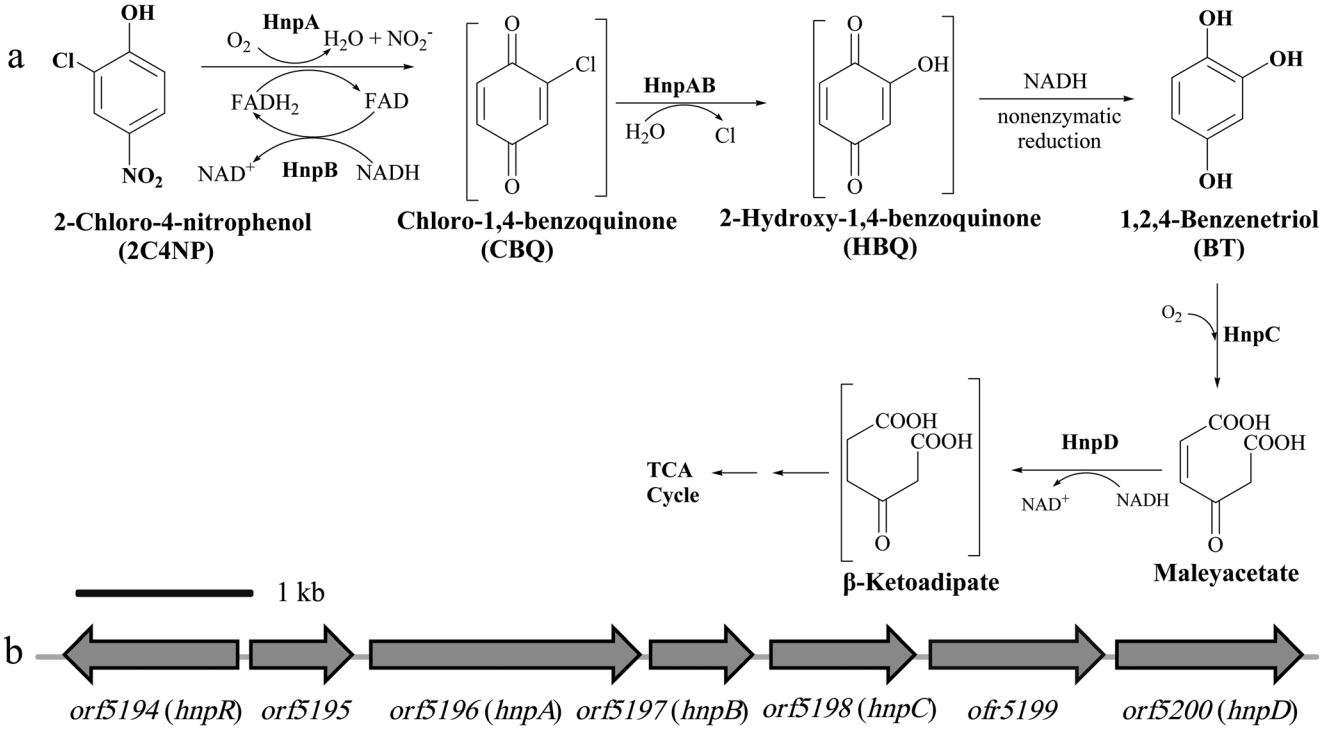

Fig. 1 The proposed pathway of the catabolism of 2C4NP in strain CNP-8 (**a**) and the organization of the *hnp* gene cluster (**b**)

SuperMix (TransGen). The primers used in RT-qPCR assay was listed in Table S1. The transcriptional levels of the target genes were calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) with 16S rRNA gene as control.

Protein purification and enzymatic assays

The *hnpA*, *hnpB*, and *hnpC* genes were respectively cloned into vector pET28a pre-digested with *Nde*I and *Xho*I, and the N-terminal His-tagged HnpA, HnpB, and HnpC were overexpressed in *E. coli* BL21(DE3) and purified (Min et al. 2019). Protein concentrations were determined as reported elsewhere (Bradford 1976). The activity of HnpAB against 2C4NP and PNP was assayed as previously described (Min et al. 2019). In the case of the kinetic assays, the concentrations of substrate range from 0.5 to 30 μM with FAD and NADH fixed concentration (0.02 mM and 0.2 mM, respectively). One unit of enzyme activity was defined as the amount of protein required to catalyze the consumption of 1 μmol of substrate per min. The specific activity was expressed in enzyme activity units per milligram of protein. For the products' identification, the reaction products generated from 2C4NP and PNP after incubations with HnpAB were acetylated, dried over sodium sulfate, and analyzed by gas chromatography-mass spectrometry (GC-MS). For quantitative assay of the 2C4NP derivatives (CHQ and BT), 1 mM ascorbic acid was added to minimize the autooxidative effect. CHQ and BT were quantified by HPLC. The enzymatic assay of HnpC against BT was performed by monitoring the spectral changes from 289 to 243 nm (Min et al. 2019), and the reaction product was identified by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

Analytical methods

HPLC analysis was carried out using an Agilent 1200 system equipped with a DAD detector and an Agilent XDB-C18 column (4.6 × 250 mm, 5 μm). The mobile phase was the same as described (Min et al. 2014). The phenolic compounds (PNP, 2C4NP, 2,6-DBNP, CHQ, and BT) were quantified at 280 nm. GC-MS analysis was carried out on a Thermo Trace GC Ultra gas chromatograph equipped with an Agilent HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm) and coupled with an ITQ 900 ion trap mass spectrometer (Thermo Scientific). The GC-MS conditions were the same reported by Zhang et al. (2009). The acetylated derivatives of CHQ and BT were identified by comparing the retention times and mass spectra with those of acetylated standards. HPLC-MS analysis was performed as previously described (Min et al. 2016a).

Accession number and phylogenetic analysis

The nucleotide sequence of the gene cluster responsible for the catabolism of 2C4NP in strain CNP-8 has been deposited in NCBI (GenBank No. MH271067). The phylogenetic tree for HnpA and its related proteins were constructed by the neighbor-joining method using the programs BioEdit version 7.2 (Hall 1999) and MEGA version 6.06 (Tamura et al. 2013).

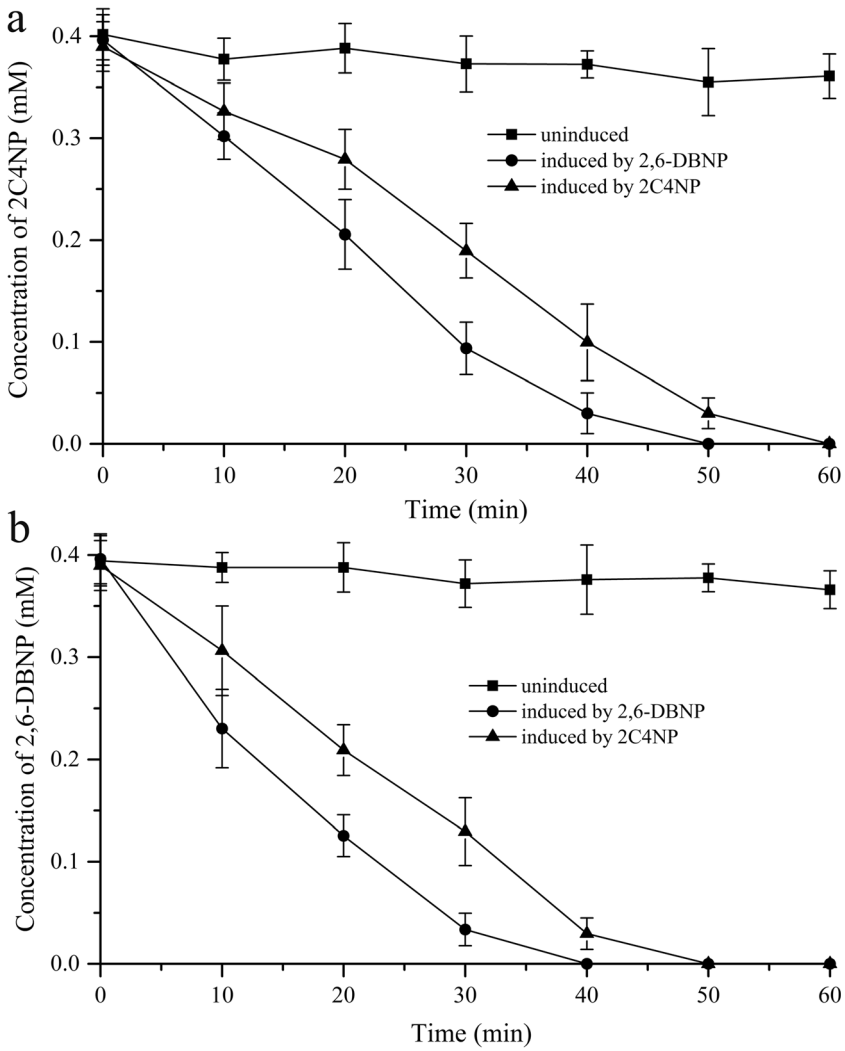

Fig. 2 Biotransformation of 2C4NP (**a**) and 2,6-DBNP (**b**) by the induced and uninduced cells of strain CNP-8. (square) uninduced cells; (circle) 2,6-DBNP-induced cells; (triangle) 2C4NP-induced cells

The topologic accuracy of the tree was evaluated by using 1000 bootstrap replicates.

Results

The catabolism of 2C4NP in strain CNP-8 is likely performed by the enzymes involved in 2,6-DBNP degradation

Considering that the BT (1,2,4-benzenetriol) pathway of 2C4NP degradation is similar with the 6-bromohydroxyquinone (6-bromo-1,2,4-benzenetriol) pathway of the catabolism of 2,6-DBNP in this strain (Min et al. 2019), whole cell biotransformation was initially performed to investigate whether the enzymes catalyzing 2,6-DBNP degradation were also involved in the catabolism of 2C4NP. As shown in Fig. 2, the uninduced cells of strain CNP-8 showed undetectable activity for 2C4NP and 2,6-DBNP, indicating that the enzymes involved in the catabolism of these two halogenated nitrophenols are inducible. However, the 2,6-DBNP-induced bacteria was found to be able to degrade both 2,6-DBNP and 2C4NP rapidly, with a rate of 0.302 ± 0.043 and 0.275 ± 0.034 mM/h/OD_{600}, respectively. Similarly, the 2C4NP-induced cells also degraded both 2C4NP (0.215 ± 0.035 mM/h/OD_{600}) and 2,6-DBNP (0.270 ± 0.024 mM/h/OD_{600}). This indicated that the enzyme initiating 2,6-DBNP degradation is likely also involved in the catabolism of 2C4NP in this strain.

The *hnp* genes are indeed upregulated in the 2C4NP-induced CNP-8

In addition to being involved in the catabolism of 2,6-DBNP (Min et al. 2019), we tentatively proposed that the *hnp* cluster, as outlined in Fig. 1b, was also responsible for the catabolism of 2C4NP in strain CNP-8. Subsequently, the transcriptional analysis of the *hnp* genes was performed to investigate whether these genes were upregulated after 2C4NP induction. Similar to 2,6-DBNP induction, RT-qPCR showed that the transcription

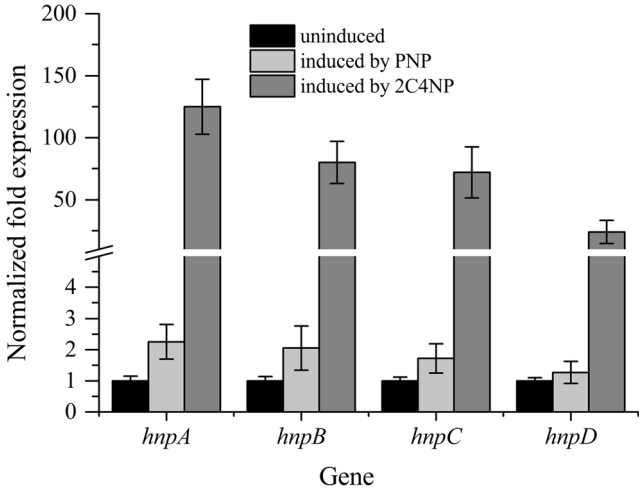

Fig. 3 Transcriptional analyses of *hnpA*, *hnpB*, *hnpC*, and *hnpD* under 2C4NP- and PNP-induced conditions as compared to the uninduced condition

levels of *hnpA*, *hnpB*, *hnpC,* and *hnpD* in the 2C4NP-induced cells increased 125-, 80-, 72-, and 24-fold, respectively, as compared to those in the uninduced cells of strain CNP-8 (Fig. 3). This suggested that the *hnp* genes were likely responsible for the catabolism of 2C4NP in this strain. Conversely, the transcriptional levels of these *hnp* genes were not upregulated when PNP (a structural analogue of 2C4NP) was used as the induced substrate. This is significantly different from the reports about other two 2C4NP-utilizers including *Burkholderia* sp. SJ98 (Min et al. 2014) and *Rhodococcus imtechensis* RKJ300 (Min et al. 2016b), in which the genes involved in 2C4NP catabolism were induced by both 2C4NP and PNP.

HnpAB has the ability to catalyze the transformation of 2C4NP to BT

To further confirm the involvement of the Hnp protein in the catabolism of 2C4NP, HnpA and HnpB were purified to homogeneity as His-tagged proteins (Fig. S1), followed by their catalytic activity against 2C4NP. As shown in Fig. 4a, HnpAB has the ability to degrade 2C4NP and NADH rapidly, together with the production of a new product that was proposed as 2-chloro-1,4-benzoquinone (CBQ, $\lambda_{max} \approx 255$ nm) as described (Min et al. 2014). The maximal activity of HnpAB against 2C4NP was 0.23 ± 0.08 U mg^{-1}. Enzyme kinetic assays showed that the apparent K_m of HnpAB against 2C4NP was 2.7 ± 1.1 μM, and the k_{cat}/K_m was 0.17 ± 0.03 μM^{-1} min^{-1}.

Two compounds with GC retention times of 16.87 and 19.34 min (Fig. 5a), respectively, were detected by the GC-MS analysis of the acetylated products of 2C4NP catalyzed by the purified HnpAB. The mass spectrum of the compound at 16.87 min exhibited a molecular ion peak at *m/z* 228.05, together with the fragments at *m/z* 186.12 (losing one -$COCH_3$) and *m/z* 144.14 (losing two -$COCH_3$) (Fig. 5c), in line with the mass spectrum property of acetylated CHQ (Fig. 5b). The compound at 19.34 min had a molecular ion peak at *m/z* 251.98, with the fragments at *m/z* 209.97 (losing one -$COCH_3$), *m/z* 168.02 (losing two -$COCH_3$), and *m/z* 126.05 (losing three -$COCH_3$) (Fig. 5e), consistent with the mass spectrum property of acetylated BT (Fig. 5d).

In a time course assay of 2C4NP conversion by purified HnpAB, 11.7 μM of CHQ and 23.6 μM of BT were produced when 40.4 μM of 2C4NP was completely consumed (Fig. 4b), indicating that 2C4NP was nearly stoichiometrically transformed to CHQ and BT. In addition, the purified HnpAB is also able to catalyze the conversion of CHQ to BT in the presence of NADH and FAD, although with a slow rate of 0.004 ± 0.002 U mg^{-1}. Although no transcription increase of *hnpA* and *hnpB* was found after PNP induction, purified HnpAB can catalyze the transformation of PNP, together with the formation of HQ and BT (Fig. S2).

hnpA is necessary for strain CNP-8 to utilize 2C4NP

Strains CNP-8Δ*hnpA* and CNP-8Δ*hnpA*[pRK-*hnpA*] (Table S1), the derivative strains of CNP-8 with *hnpA* deletion and complementation, respectively, were constructed to confirm the physiological function of HnpA in 2C4NP catabolism. The *hnpA*-deleted strain (CNP-8Δ*hnpA*) was found to lose the ability to utilize 2C4NP for growth (Fig. 6), while the *hnpA*-complemented strain (CNP-8Δ*hnpA*[pRK-*hnpA*]) recovered the ability to degrade 2C4NP, confirming that *hnpA1* was necessary for strain CNP-8 to utilize 2C4NP.

HnpC catalyzes the 1,2-dioxygenation of BT to maleylacetate

hnpC, clustered with *hnpA*, was highly transcribed after 2C4NP induction. So, HnpC, a BT dioxygenase, was purified to characterize its function in the catabolism of 2C4NP (Fig. S1). As shown in Fig. 4c, purified H_6-HnpC catalyzes the transformation of BT (λ_{max} = 289 nm) rapidly, resulting in the formation of a new product, which was proposed as maleylacetate (MA) (Yamamoto et al. 2011). Subsequently, HPLC-MS analysis confirmed that it was indeed MA, with deprotonated ion peak at *m/z* 157.16 and its fragment at *m/z* 113.05 (lossing a -COOH) (Fig. 4d).

Phylogenetic analysis reveals the origin of HnpA

To reveal the evolutionary origin of HnpA, the phylogenetic relationships among the functionally identified chlorophenol/nitrophenol 4-monooxygenases was investigated. As shown in Fig. 7a, the phylogenetic tree is divided into two larger clades. HnpA is located closely related to the oxygenase components of the two-component monooxygenases involved in the catabolism of chlorophenols such as 4-chlorophenol

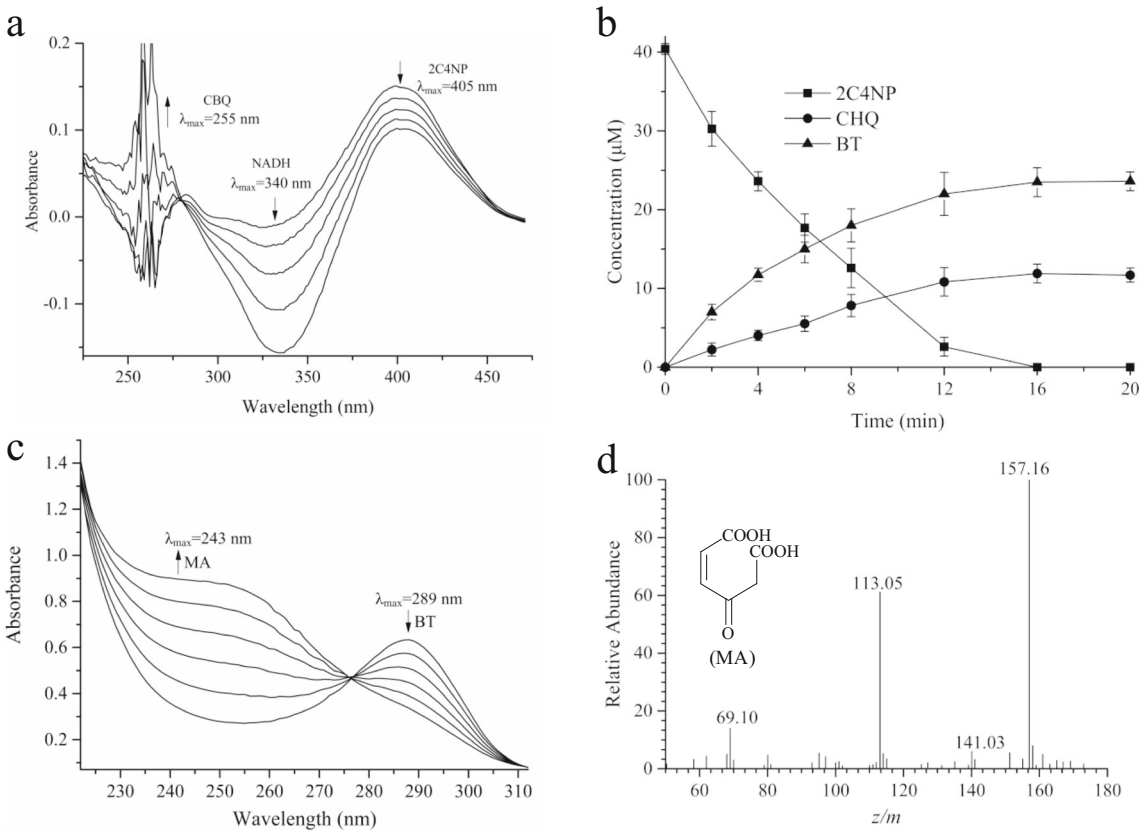

Fig. 4 Enzymatic activity of the purified HnpAB against 2C4NP, as well as HnpC against BT. **a** Spectral changes during 2C4NP transformation by HnpAB. **b** Quantitative assay of CHQ and BT in the time course of 2C4NP degradation by HnpAB. (square) 2C4NP; (circle) CHQ; (triangle) BT. **c** Spectral changes during BT transformation by HnpC. **d** Identification of the product of BT catalyzed by HnpC by HPLC-MS

(Pimviriyakul et al. 2017), 2,4,6-trichlorophenol (Louie et al. 2002), and 2,4,5-trichlorophenol (Hubner et al. 1998). So far, two 2C4NP monooxygenases including the single-component 2C4NP monooxygenases (PnpA) from strain SJ98 (Min et al. 2014) and the two-component 2C4NP monooxygenase (PnpA1A2) from strain RKJ300 (Min et al. 2016b) have been functionally characterized. However, HnpA exhibits no sequence identity with PnpA, and it is located in an unrelated clade with PnpA. Although HnpA exhibited moderate identity (48%) with the oxygenase component (PnpA1) of the 2C4NP monooxygenase from strain RKJ300, HnpA and PnpA1 are distantly related and belong to different sub-clades (Fig. 7a).

Discussion

So far, several 2C4NP-utilizers have been isolated and have been reported to degrade 2C4NP via either BT or HQ (or its derivative CHQ) pathways (Ghosh et al. 2010; Arora and Jain 2011; Pandey et al. 2011; Arora and Jain 2012; Min et al. 2014; Min et al. 2016b; Tiwari et al. 2017; Min et al. 2018). The BT pathway is primarily found in Gram-positive bacteria such as *Rhodococcus imtechensis* RKJ300 (Min et al. 2016b), while the HQ/CHQ pathway is predominantly reported in Gram-negative bacteria such as *Burkholderia* sp. SJ98 (Min et al. 2014), *Cupriavidus* sp. a3 (Tiwari et al. 2017), and *Burkholderia* sp. RKJ800 (Arora and Jain 2012). To date, only the molecular mechanism of 2C4NP degradation in strains RKJ300 (Min et al. 2016b) and SJ98 (Min et al. 2014) have been reported. In current study, by genetic and biochemical characterization, the Gram-negative *Cupriavidus* sp. CNP-8 was proved to degrade 2C4NP via BT pathway, which is significantly different from the HQ/CHQ pathways reported in all other Gram-negative 2C4NP-utilizers. This study will enhance our understanding of the biochemical and genetic diversity of microbial 2C4NP degradation.

In addition to being involved in the catabolism of 2,6-DBNP in strain CNP-8 (Min et al. 2019), the enzymes encoded by the *hnp* genes were proposed to be also responsible for 2C4NP degradation in this study. A similar case has been reported in the biodegradation of 2-chloronitrobenzene and its brominated analogue (2-bromonitrobenzene) by *Pseudomonas stutzeri* ZWLR2-1. In strain ZWLR2-1, the *cnb* gene cluster was reported to be responsible for the catabolism of these two halogenated nitrobenzenes (Liu et al. 2011; Wang et al. 2019). Our study provides another example of a bacterium using the same set of enzymes to utilize different substrates and makes the *hnp* cluster well adapted for

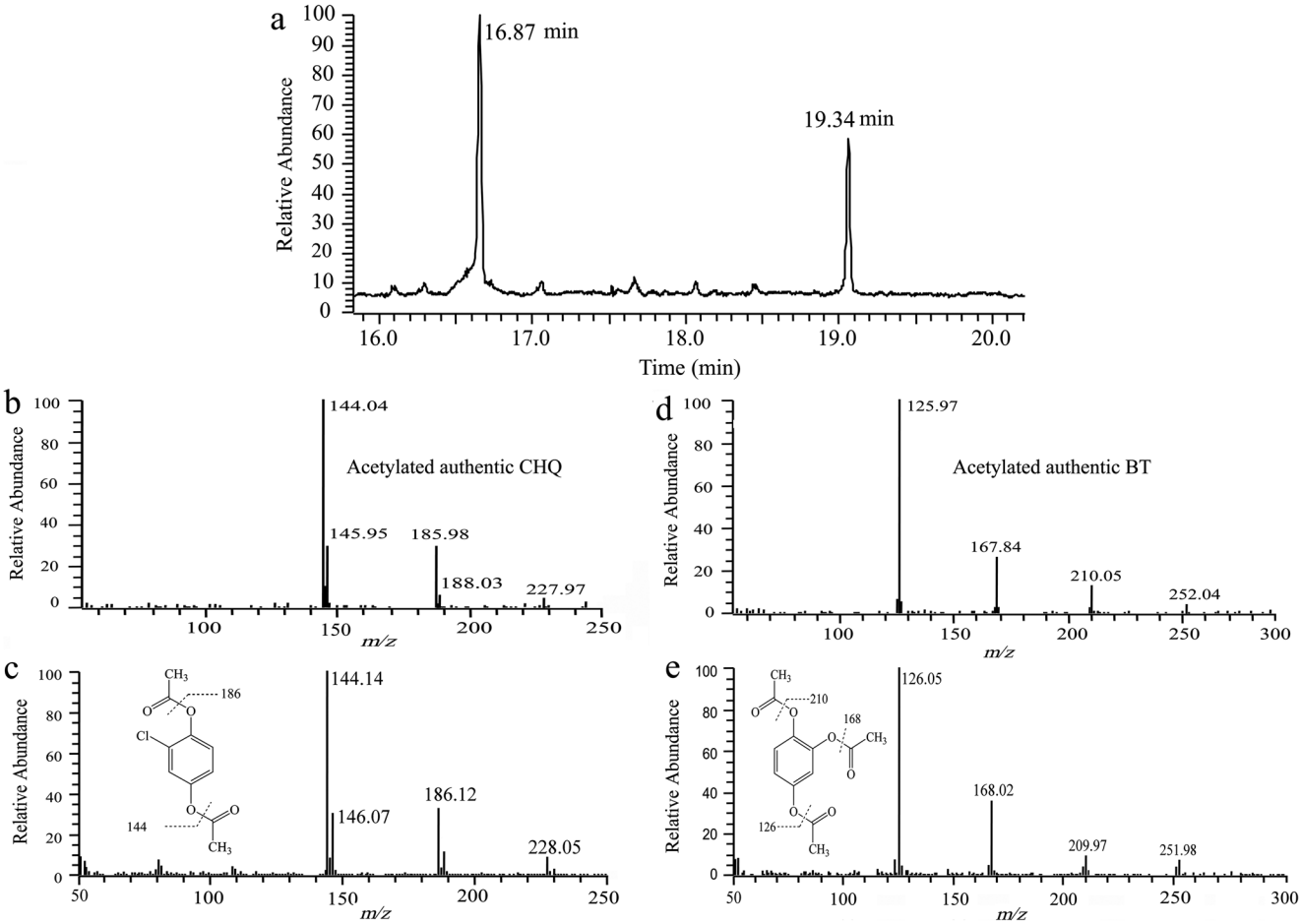

Fig. 5 Identification of the acetylated products of 2C4NP catalyzed by the purified HnpAB by GC-MS. **a** The gas chromatogram. **b** The mass spectra of acetylated authentic CHQ. **c** The mass spectra of acetylated intermediate at 16.87 min. **d** The mass spectra of acetylated authentic BT. **e** The mass spectra of acetylated intermediate at 19.34 min

bioremediation of environments contaminated by multiple halogenated nitrophenols. The identification of CHQ and BT in the conversion of 2C4NP by purified HnpAB suggested that HnpAB is capable of catalyzing 2C4NP's denitration and dechlorination twice in tandem. Generally, monooxygenases attack the phenolic ring substituted with nitro or chlorine resulting in the formation of quinones (Hubner et al. 1998; Xun and Webster 2004; Perry and Zylstra 2007; Zhang et al. 2009; Min et al. 2014; Pimviriyakul et al. 2017; Min et al. 2019). Therefore, the identification of CHQ suggested that 2-chloro-1,4-benzoquinone (CBQ) was likely the authentic product of 2C4NP catalyzed by HnpAB (Fig. 1a), although it was not directly detected in this study. The accumulation of CHQ may be due to CBQ's non-enzymatic reduction by NADH in the reaction system, which is similar with many previous reports (Xun and Webster 2004; Perry and Zylstra 2007; Liu et al. 2010; Min et al. 2014; Min et al. 2016a, b). Although the formation of by-product CHQ may also occur during the catabolism of 2C4NP *in vivo*, its toxicity against the cells could be eliminated by the transformation of CHQ to BT. In addition to CHQ, the identification of BT during 2C4NP conversion by HnpAB indicated that CBQ from the first hydroxylation step would be further hydroxylated to BT, likely via intermediate 2-hydroxy-1,4-benzoquinone (Fig. 1a).

So far, two types of monooxygenases initiating the catabolism of 2C4NP have been functionally characterized. The two-component 2C4NP/PNP monooxygenase (PnpA1A2) from RKJ300 was reported to convert 2C4NP to BT via CBQ (Min et al. 2016b). The single-component 2C4NP/PNP monooxygenase (PnpA) from SJ98 also catalyzes the monooxygenation of 2C4NP to CBQ (Min et al. 2014), but it is unable to catalyze the further transformation of CBO to BT. Both HnpAB and PnpA1A2 can catalyze the oxidation of 2C4NP to BT. However, these two oxygenases was proposed to have different evolutionary origins. In parallel to this work, a two-component monooxygenase (BnpAB) from *Cupriavidus* sp. strain NyZ375 was recently reported to catalyze the oxidation of 2-bromo-4-nitrophenol (2B4NP) to BT, but via an intermediate 4-nitrocatechol (4-NC) (Li et al. 2019). Moreover, although bromohydroquinone (BHQ, a structural analogue of CHQ) was detected during 2B4NP degradation, BnpAB had

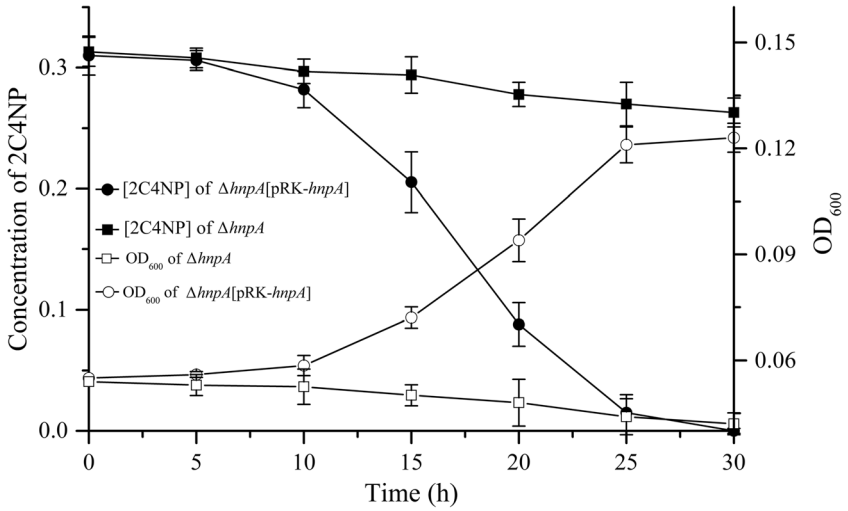

Fig. 6 Time course of 2C4NP degradation by strains CNP-8Δ*hnpA* and CNP-8Δ*hnpA*[pRK-*hnpA*], together with the bacterial growth

no catalytic activity against this by-product. In contrast, HnpAB in this study can catalyze the conversion of CHQ to BT, although with a slow rate. Therefore, the catalytic mechanism of HnpAB against 2C4NP was likely different from that of BnpAB against 2B4NP. BnpAB initiated 2B4NP catabolism with dechlorination resulting in the formation of 4-NC, followed by denitration of 4-NC to BT (Li et al. 2019). However, HnpAB was proposed to initiate 2C4NP catabolism with denitration to form CBQ, followed by dechlorination of CBQ to BT (Fig. 1a).

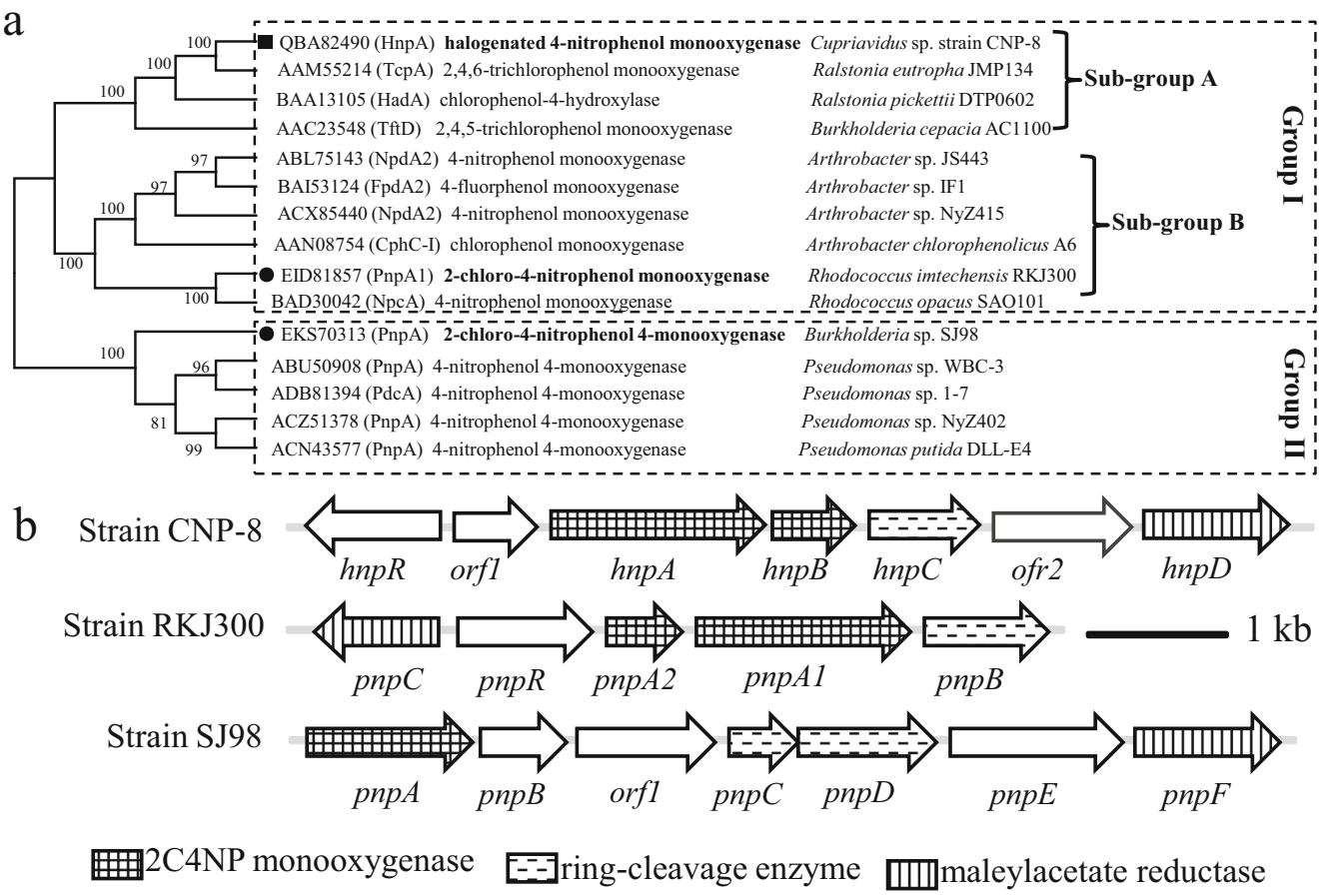

Fig. 7 **a** Phylogenetic relationships of HnpA and other chlorophenol/nitrophenol monooxygenases. HnpA in this study is indicated by a square, and other two functionally characterized 2C4NP monooxygenases including PnpA from *Burkholderia* sp. SJ98 (Min et al. 2014) and PnpA1 from *Rhodococcus imtechensis* RKJ300 (Min et al. 2016b) are indicated by a circle. **b** Comparison of the genetic organization of 2C4NP catabolic clusters from different 2C4NP-utilizers

Previously, the catabolism of 2C4NP and PNP was proved to share the same *pnp* gene cluster in the 2C4NP/PNP utilizers RKJ300 (Min et al. 2016b) and SJ98 (Min et al. 2014). In this study, although the purified HnpAB was found to be able to catalyze PNP transformation *in vitro*, PNP can not induce *hnpAB* transcription. These indicated that the *hnp* gene cluster responsible for 2C4NP degradation in strain CNP-8 likely has different regulatory mechanism from the *pnp* clusters in strains RKJ300 and SJ98. In other words, the *pnp* cluster in strains RKJ300 and SJ98 can be induced by both 2C4NP and PNP, whereas the *hnp* cluster in strain CNP-8 only be induced by 2C4NP. Indeed, in addition to the different evolutionary origins between HnpA and its counterparts for 2C4NP degradation in strains SJ98 and RKJ300, the genetic organization of *hnp* cluster in strain CNP-8 is also significantly different from those of the *pnp* clusters responsible for the catabolism of 2C4NP in other two 2C4NP-utilizers (Fig. 7b). In contrast, *hnp* cluster exhibited similar genetic organization with the *tcp* cluster responsible for the catabolism of 2,4,6-trichlorophenol (2,4,6-TCP) in *Cupriavidus necator* JMP134 (Louie et al. 2002), apart from the closely phylogenetic relationship between HnpA and TcpA (monooxygenase initiating 2,4,6-TCP catabolism in strain JMP134) (Fig. 7). Therefore, we proposed that the *hnp* cluster in strain CNP-8 is evolutionary closer to the *tcp* cluster involved in 2,4,6-TCP degradation in strain JMP134 than the *pnp* clusters responsible for 2C4NP/PNP degradation in strains SJ98 and RKJ300.

Funding information This work was supported by the National Natural Science Foundation of China (No. 31600085), the State's Key Project of Research and Development Plan (2016YFC1402300), the Foreword Key Priority Research Program of Chinese Academy of Sciences (QYZDB-SSW-DQC013), the Yantai Science and Technology Project (2017ZH092), and the State Key Laboratory of Microbial Metabolism, Shanghai JiaoTong University (MMLKF17-04).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by authors.

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