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Special Issue Article

A Nag-like dioxygenase initiates 3,4-dichloronitrobenzene degradation via 4,5-dichlorocatechol in *Diaphorobacter* sp. strain JS3050

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Summary

The chemical synthesis intermediate 3,4-dichloronitrobenzene (3,4-DCNB) is an environmental pollutant. *Diaphorobacter* sp. strain JS3050 utilizes 3,4-DCNB as a sole source of carbon, nitrogen and energy. However, the molecular determinants of its catabolism are poorly understood. Here, the complete genome of strain JS3050 was sequenced and key genes were expressed heterologously to establish the details of its degradation pathway. A chromosome-encoded three-component nitroarene dioxygenase (DcnAaAbAcAd) converted 3,4-DCNB stoichiometrically to 4,5-dichlorocatechol, which was transformed to 3,4-dichloromuconate by a plasmid-borne ring-cleavage chlorocatechol 1,2-dioxygenase (DcnC). On the chromosome, there are also genes encoding enzymes (DcnDEF) responsible for the subsequent transformation of 3,4-dichloromuconate to β-ketoadipic acid. The fact that the genes responsible for the catabolic pathway are separately located on plasmid and chromosome indicates that recent assembly and ongoing evolution of the genes encoding the pathway is likely. The regiospecificity of 4,5-dichlorocatechol formation from 3,4-DCNB by DcnAaAbAcAd represents a sophisticated evolution of the nitroarene dioxygenase that avoids misrouting of toxic intermediates. The findings enhance the understanding of microbial catabolic diversity during adaptive evolution in response to xenobiotics released into the environment.

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Introduction

Nitroaromatic compounds such as chloronitrobenzenes (CNBs) and dichloronitrobenzenes (DCNBs) are widely used as industrial intermediates in the synthesis of various pesticides, pharmaceuticals, herbicides and dyes (Booth, 2012). Such compounds can be resistant to biodegradation due to the high stability of the benzene ring along with the electronic effects of chlorine and nitro groups (Ju and Parales, 2010). Halogenated nitroaromatics are acutely biotoxic (Wang *et al*., 2019) and genotoxic (Zeiger *et al*., 1987). Their relatively recent release into the environment has selected for microorganisms that have evolved metabolic pathways to use CNBs (Katsivela *et al*., 1999; Wu *et al*., 2006; Zhen *et al*., 2006), dichlorobenzenes (Spain and Nishino, 1987; Haigler *et al*., 1988) and nitrobenzene (Lessner *et al*., 2003) as sole carbon and nitrogen sources.

Aerobic bacteria initially attack the nitro group via either reductive or oxidative pathways to metabolize nitroaromatic compounds such as 4-chloronitrobenzene (Katsivela *et al*., 1999; Wu *et al*., 2006) and 2-chloronitrobenzene (Liu *et al*., 2011). It was also reported that 2,4,5-trichloronitrobenzene was converted to 3,4,6-trichlorocatechol by *Pseudomonas* strains PS12 and PS14 (Sander *et al*., 1991). Engineered strains that can utilize 2-chloronitrobenzene, 3-chloronitrobenzene and 4-chloronitrobenzene with an oxidative pathway were also reported (Ju and Parales, 2009). Nitroarene dioxygenases that recently diverged from naphthalene dioxygenase (NagAcAd) (Zhou *et al*., 2001) typically

catalyse initial aromatic ring oxygenation and the elimination of nitrite in the catabolism of nitroaromatic compounds. These enzymes are classified in group III of Rieske non-heme iron dioxygenases (Parales and Resnick, 2006) and are collectively called Nag-like nitroarene dioxygenases. A recently described exception, 3-nitrotoluene dioxygenase (BndA1A2A3A4) is more closely related to biphenyl dioxygenase (Gao *et al*., 2020).

The chlorocatechols generated from the initial dioxygenation reactions are metabolized via the modified *ortho* pathways. The *ortho* pathway involves sequential reactions catalysed by chlorocatechol dioxygenase (Dorn and Knackmuss, 1978a; Dorn and Knackmuss, 1978b), chloromuconate cycloisomerase, diene lactone hydrolase and maleyacetate reductase (van der Meer *et al*., 1991; Field and Sierra-Alvarez, 2008). For example, 2-chloronitrobenzene is converted to 3-chlorocatechol by 2CNB dioxygenase (CnbAaAbAcAd). Chlorocatechol 1,2-dioxygenase (CnbC) opens the ring between the hydroxyl groups to form 2-chloro-*cis*, *cis*-muconate, which is further converted into β-ketoadipic acid by CnbDEF. The gene cluster encoding this catabolic pathway is a patchwork assembly of *nag*-like nitroarene dioxygenase genes and the 3-chlorocatechol degradation cluster (Liu *et al*., 2011). Although chloronitroarenes have only recently appeared in the biosphere, the enzymes that catalyse the initial steps in degradation seem to have evolved readily from those involved in catabolic pathways of natural substrates such as naphthalene (Suen and Spain, 1993; Nishino and Spain, 1995). Genes encoding downstream pathways are often recruited from pathways for other natural compounds to assemble complete degradation pathways (Wackett, 2004).

Recently we reported isolation of bacteria able to degrade 3,4-dichloronitrobenzene (3,4-DCNB) and 2,3-dichloronitrobenzene (2,3-DCNB) (Palatucci *et al*., 2019). Growth of *Diaphorobacter* sp. strain JS3050 on 3,4-DCNB resulted in stoichiometric release of nitrite, suggesting an initial attack by a nitroarene dioxygenase to remove the nitrogen substituent of 3,4-DCNB. Although the draft genome sequence of this strain included a putative nitroarene dioxygenase (Palatucci *et al*., 2019), it was not biochemically characterized and no evidence was presented regarding the regiospecificity of the initial ring-hydroxylation reaction. The initial oxidative attack on 3,4-DCNB could occur at the 1,2 position, the 1,6 position, or both. Preliminary evidence from enzyme assays also indicated the presence of an inducible dichlorocatechol 1,2-dioxygenase activity in 3,4-DCNB-grown cells, but the ring-fission substrate and the genes encoding the downstream pathway were not determined (Palatucci *et al*., 2019). In addition, the draft genome provided no insight about the organization of the genes involved. The goal of the present study was to elucidate the molecular basis of the pathway through identification of the genes and corresponding enzymes involved. The catabolic pathway of 3,4-DCNB was established based on the closed genome and by heterologous expression of the key genes.

Results

The complete genome sequence of strain JS3050

Sequencing revealed a 3 983 118 bp circular genome and a 49 405 bp plasmid (pDCNB) with an overall G + C content of 66.48% and 3923 predicted genes. The average length of these genes is 919.3 bp. The genes putatively encoding the previously suspected nitroarene dioxygenase system (Palatucci *et al*., 2019) are located from 2 750 712 to 2 755 732 on the chromosome of strain JS3050 (Fig. 1). The cluster consists of a divergently transcribed LysR-type regulator, highly conserved α and β subunit coding regions of the putative nitroarene dioxygenase genes (*dcnAcAd*), ferredoxin reductase (*dcnAa*) and ferredoxin (*dcnAb*). The *dcnAa* and *dcnAb* are separated by *orf7* encoding a partial ORF, which is similar to the salicylate-5-hydroxylase large subunit NagG from *Ralstonia* sp. strain U2 (Zhou *et al*., 2002). It has a gene arrangement and biochemical motifs ([2Fe-2S] cluster binding site) similar to the reported group III of Rieske nonheme iron dioxygenases (Parales and Resnick, 2006) that catalyses dioxygenation of 2,4-dinitrotoluene (Suen and Spain, 1993), nitrobenzene (Nishino and Spain, 1995), 2-nitrotoluene (An *et al*., 1994), 3-nitrotoluene (Kumari *et al*., 2017), 2-chloronitrobenzene (Liu *et al*., 2011) and naphthalene (Zhou *et al*., 2001). Thus the cluster of genes would be expected to encode synthesis of a functional nitroarene dioxygenase system that would catalyse conversion of 3,4-DCNB to a dichlorocatechol. Also on the chromosome were genes annotated as encoding nitrite assimilation including nitrate- and nitrite sensing domain-containing protein (WP_172208979.1), formate/nitrite transporter family protein (WP_172206882.1) and nitrite reductase (WP_172206998.1, WP_088886406.1). The products encoded by these genes may be related to the utilization of nitrite released from 3,4-DCNB by dioxygenation catalysed by DcnAcAd.

The predicted genes encoding enzymes involved in degradation of dichlorocatechol(s) are non-contiguous in strain JS3050, which suggests relatively recent recruitment and assembly of the functional pathway (Table S1 and Fig. 1). BLAST comparison of the genes to those of the closest biochemically characterized relatives indicates the predicted dichlorocatechol dioxygenase and its regulator are located at plasmid positions 47 213 to 42 237. ORF 2 (tentatively designated *dcnC*) encodes a protein sharing 95% identity with chlorocatechol 1,2-dioxygenase (TetC) from *Pseudomonas chlororaphis*

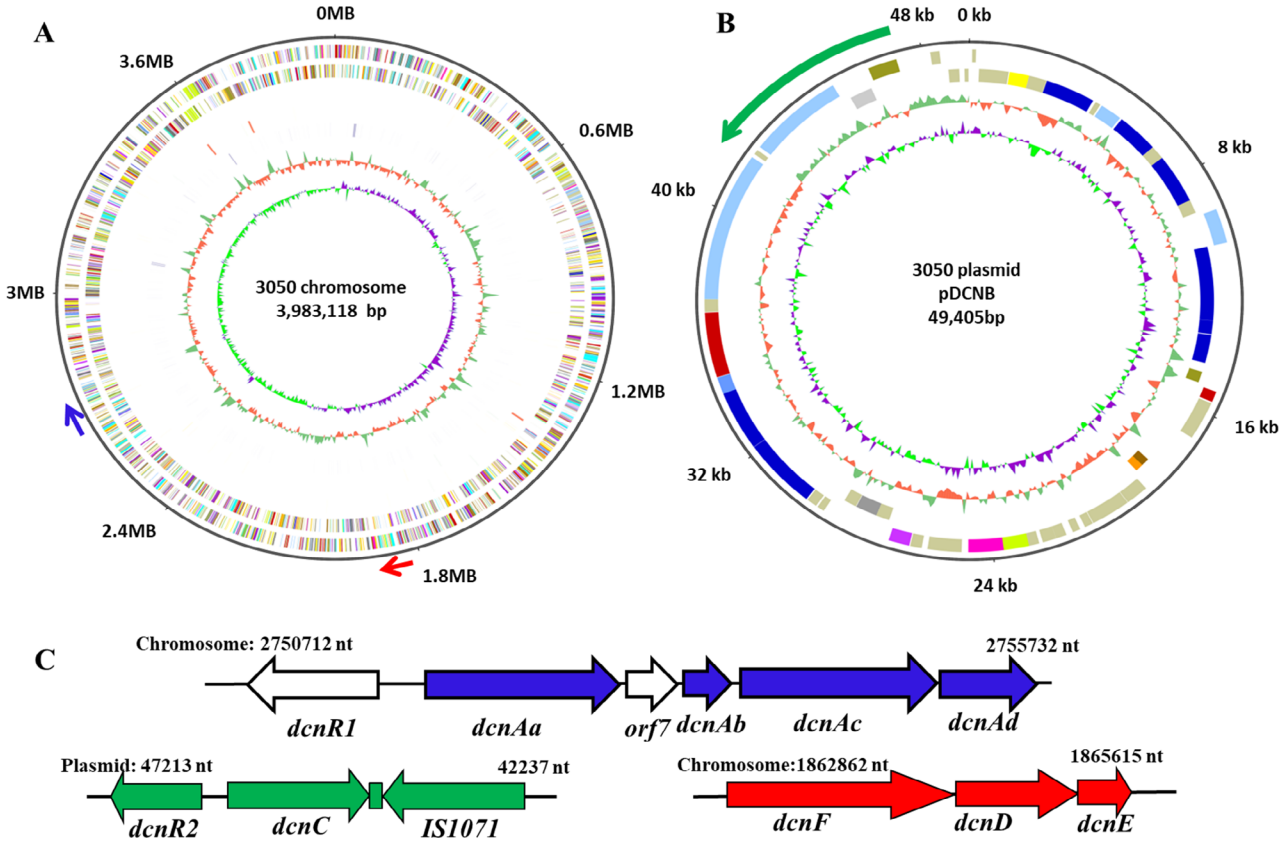


Fig. 1. Circular representation of genome (A) and plasmid (B) of *Diaphorobacter* sp. strain JS3050 (C). Organization of the *dcn* gene cluster. Arrows indicate the size and transcription direction of each gene or ORF. The numbers show the location of the genes. The gene clusters related to the 3,4-DCNB metabolic pathway are marked by arrows of corresponding colour. [Color figure can be viewed at wileyonlinelibrary.com]

RW71, which has a broad substrate range including 3,4- and 4,5-dichlorocatechols (Potrawfke *et al*., 2001). The genes *dcnDEF*, predicted to encode enzymes that catalyse metabolism of the dichlorocatechol ring-cleavage product, are located at chromosome positions 1 862 862 to 1 865 615. ORFs 10 and 12 are predicted to encode chloromaleylacetate reductase and chlorodienelactone hydrolase. ORF 11 is most closely related to genes encoding chloromuconate cycloisomerase. To further investigate functions of the key genes mentioned above, biochemical characterizations were done to establish the roles of the putative genes in 3,4-DCNB degradation.

DcnAaAbAcAd encodes a dioxygenase converting 3,4-DCNB to 4,5-dichlorocatechol

The position of initial attack on the 3,4-DCNB molecule determines the subsequent downstream catabolic pathway. Attack at the 1,2 position would yield 3,4-dichlorocatechol, whereas attack at the 1,6 position would yield 4,5-dichlorocatechol. In order to establish the catalytic function and the regiospecificity of 3,4-DCNB dioxygenase, we constructed plasmid [pUC18-*dcnAaAbAcAd*] and transformed it into *E. coli* strain DH5α to construct *E. coli* [pUC18-*dcnAaAbAcAd*] for evaluation of 3,4-DCNB dioxygenase activity via heterologous expression and whole cell biotransformations.

The heterologously expressed nitroarene dioxygenase transformed 3,4-DCNB stoichiometrically to 4,5-dichlorocatechol and nitrite (Fig. 2, Figs S1, S2). The identity of the product was confirmed by HPLC and GC–MS, using commercial 4,5-dichlorocatechol as a standard, and commercial 3,4-dichlorocatechol as a control. The results established that *dcnAaAbAcAd* encodes a 3,4-DCNB dioxygenase catalysing the conversion of 3,4-DCNB into 4,5-dichlorocatechol with a high degree of regiospecificity.

In a separate experiment the substrate specificity of DcnAaAbAcAd was also analysed by biotransformation assays with a variety of nitroaromatic compounds (Table S2). The enzyme system exhibits a strong preference for substrates substituted in the *meta* and *para* positions with respect to the nitro group. The high activity with 2,4-dinitrotoluene suggested the possibility that the 3,4-DCNB dioxygenase is closely related to 2,4-dinitrotoluene dioxygenase.

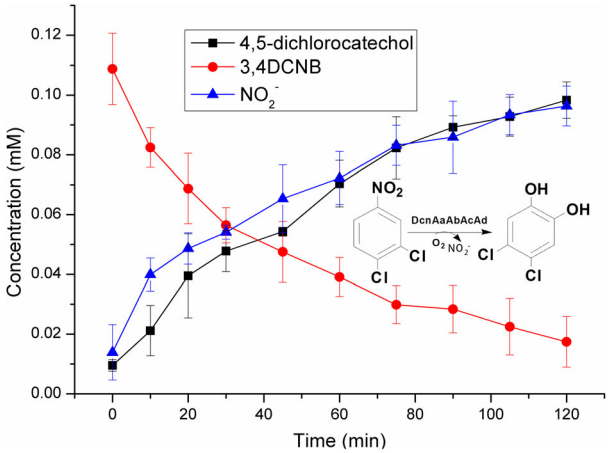


Fig. 2. 3,4-DCNB biotransformation by *E.coli* DH5α [pUC18-*dcnAaAbAcAd*]. The disappearance of 3,4-DCNB and the appearance of the products 4,5-dichlorocatechol were quantified by HPLC. The experiments were performed in triplicate, the results shown are the average values from three independent assays, and error bars indicate standard deviations. [Color figure can be viewed at wileyonlinelibrary.com]

Structure modelling of 3,4-DCNB dioxygenase

It is known that the substrate specificity of nitroarene dioxygenase is determined by the large subunit of the terminal oxygenase (Ac subunit) (Parales *et al*., 1998). The fact that the product of 3,4-DCNB dioxygenation by DcnAcAd is 4,5-dichlorocatechol instead of 3,4-dichlorocatechol indicates that there must be strict control of the orientation of the substrate in the active site. In order to identify the residues that determine the specificity of the oxidation attack, the overall protein structure model of the DcnAc subunit of 3,4-DCNB dioxygenase was built using the 3NT dioxygenase α subunit as template (Fig. 3A). 3,4-DCNB was docked into the substrate pocket of the DcnAc model by Autodock Vina. The mononuclear iron is coordinated by a non-heme iron-binding triad consisting of His206, His211 and Asp360. The substrate 3,4-DCNB binds close to the mononuclear iron, and its aromatic plane faces the ferrous ion with a distance of about 3.9 Å. For substrate binding, some residues, such as Phe200, Val305, Ile207 and Val258, interact with the aromatic ring of 3,4-DCNB through hydrophobic interactions. Tyr222, Gln293 and Asn295 form hydrogen bonds to the nitro group of 3,4-DCNB. A positively charged residue, Lys312, attracts a chlorine atom (4-positon) of 3,4-DCNB with a distance of 4.5 Å (Fig. 3B and C), while the other chlorine atom (3-position) is engaged in halogen–π interaction with the side chain of Trp356 with a distance of 3.1 Å. The key residues mentioned above, together with mononuclear iron atom and water molecules in the active center, place the substrate at a suitable location and orientation. Through analysing this model it can be deduced that C2, remote from catalytic residue Asn295 and close to Val258, cannot be hydroxylated because of the limited space between C2 and Val258 (3.4 Å). Thus this orientation of the substrate makes C1 and C6 the sites of oxidation, rather than C1 and C2. This probably explains why 4,5-dichlorocatechol is the product rather than 3,4-dichlorocatechol. Thus, structure information provides an explanation for the regiospecificity of the 3,4-DCNB dioxygenase catalysed hydroxylation.

dcnC encodes a 4,5-dichlorocatechol 1,2-dioxygenase

In the genome of strain JS3050, *dcnC* is the only gene similar to catechol (or chlorocatechol) 1,2-dioxygenase. To determine the function of DcnC, the expression vector pET28a-*dcnC* was constructed to express *dcnC*. The plasmid was

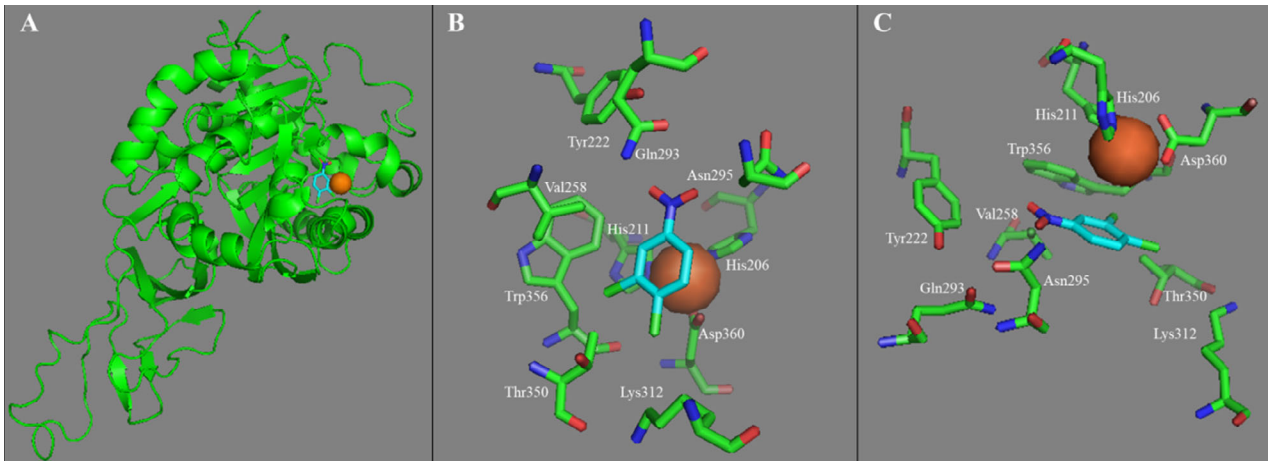
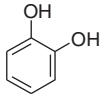
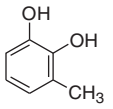
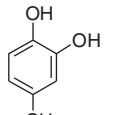
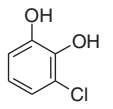
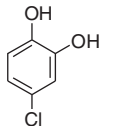
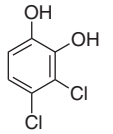
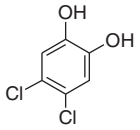


Fig. 3. The structure prediction of the active site of the 3,4-DCNB dioxygenase. A. Overall structure of 3,4-DCNB dioxygenase model. B, C. Model of the active site of 3,4-DCNB dioxygenase. The active site residues are shown in stick and 3,4-DCNB is in cyan. The mononuclear iron center (large orange sphere) is coordinated with two histidines and one aspartic residue. The picture was made using PyMOL (http://www.pymol.org). [Color figure can be viewed at wileyonlinelibrary.com]

Table 1. Substrate specificity of DcnC.

Substrate	Activity of cell extract of strain JS3050 (U mg^{-1})[b]	Activity of cell extract of *E. coli* pET28a-*dcnC* (U mg^{-1})[b]	Relative activity of cell extract of strain JS3050 (%)	Relative activity of cell extract of *E. coli* pET28a-*dcnC* (%)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/Km ($min^{-1} \times \mu M^{-1}$)
 Catechol	1.66 ± 0.04	0.41 ± 0.01	100.0	100.0	8.0 ± 0.68	163.9	20.5
 3-Methylcatechol	1.45 ± 0.04	0.42 ± 0.02	88.0	102.0	9.31 ± 0.69	144.4	15.5
 4-Methylcatechol	2.33 ± 0.11	0.66 ± 0.12	140.8	160.3	9.29 ± 1.46	310.1	33.4
 3-Chlorocatechol	0.17 ± 0.01	0.05 ± 0.00	10.0	12.0	0.44 ± 0.07	17.4	39.5
 4-Chlorocatechol	1.30 ± 0.06	0.43 ± 0.02	78.5	103.8	2.44 ± 0.16	174.1	71.3
 3,4-Dichlorocatechol	0.050 ± 0.003	0.021 ± 0.001	3.0	5.1	ND[a]	ND[a]	ND[a]
 4,5-Dichlorocatechol	0.042 ± 0.004	0.018 ± 0.000	2.5	4.4	ND[a]	ND[a]	ND[a]

[a]ND: not detected.
[b]The substrate concentration used was 500 μM.

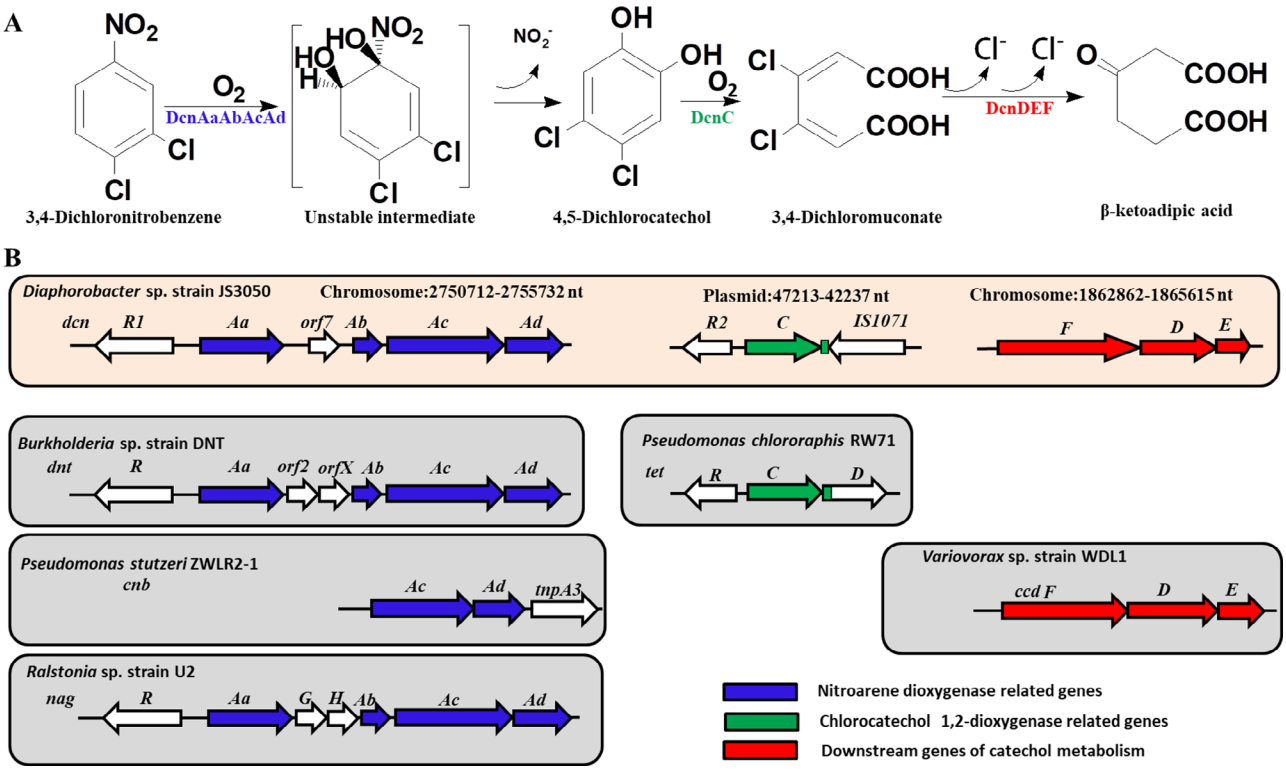


Fig. 4. Catabolic pathway of 3,4-DCNB degradation and the corresponding genes in strain JS3050. (A) Proposed 3,4-DCNB catabolic pathway. (B) Comparison of the three 3,4-DCNB gene clusters with 2,4-dinitrotoluene utilizer *Burkholderia* sp. strain DNT (Suen*et al*., 1996), 2-chloronitrobenzene utilizer *Pseudomonas stutzeri* ZWLR2-1 (Liu*et al*., 2011), naphthalene utilizer *Ralstonia* sp strain U2 (Fuenmayor*et al*., 1998), 1,2,3,4-tetrachlorobenzene utilizer *Pseudomonas chlororaphis* RW71 (Potrawfke *et al*., 1998; Potrawfke *et al*., 2001) and linuron utilizer *Variovorax* sp. strain WDL1 (Bers*et al*., 2013). Related genes are marked by corresponding colours. [Color figure can be viewed at wileyonlinelibrary.com]

transformed into *E. coli* BL21 (DE3), resulting in recombinant strain *E. coli* BL21 (DE3) [pET28a-*dcnC*]. Upon IPTG induction, the cell extract exhibited 1,2-dioxygenase activity toward various substituted catechols (Table 1). Although 4,5-dichlorocatechol is clearly the product of the initial reaction catalysed by 3,4-DCNB dioxygenase, the relative activity of DcnC against 4,5-dichlorocatechol was lower than expected. The results were similar to those seen with the 1,2,3,4-tetrachlorobenzene utilizer *Pseudomonas chlororaphis* RW71, where a chlorocatechol 1,2-dioxygenase TetC exhibited a lower activity toward its natural substrate 3,4,5-trichlorocatechol and 4,5-dichlorocatechol than toward other substituted catechols (Potrawfke *et al*., 2001).

To determine whether DcnC is the native catechol 1,2-dioxygenase in strain JS3050, the catechol 1,2-dioxygenase activity of cell extract of 3,4-DCNB-grown strain JS3050 was also measured. The substrate range was similar to that of the heterologous expressed DcnC (Table 1). Given the fact that no other catechol 1,2-dioxygenase gene (above 30% amino acid identity with DcnC) was found by bioinformatics analysis, the similar substrate ranges and the relative substrate preference of enzymes in cell extracts of strain JS3050 and *E. coli* BL21 (DE3) [pET28a-*dcnC*] provide strong evidence for the functional role of DcnC as the 4,5-dichlorocatechol 1,2-dioxygenase in the 3,4-DCNB pathway. In order to verify the affinity for different substrates to DcnC, the protein was purified (Fig. S3) and the K_m values for different substrates were determined (Table 1, Fig. S4). The K_m values of DcnC for 3,4-dichlorocatechol and 4,5-dichlorocatechol were below the limit of detection.

Discussion

The results presented here establish the proposed catabolic pathway of 3,4-DCNB in strain JS3050 at the molecular level (Fig. 4). In addition to analogy with previously described systems (Johnson *et al*., 2002; Lessner *et al*., 2003; Liu *et al*., 2011), two lines of evidence support the identification of the physiological roles of DcnAaAbAcAd and DcnC in 3,4-DCNB degradation. (i) Heterologous expression studies establish that the candidate genes encode enzymes that catalyse the key reactions. (ii) No other enzymes similar to nitroarene dioxygenase and dichlorocatechol dioxygenase (with protein identity higher than 30%) are encoded in the genome of JS3050. Nevertheless, gene deletions would be required to rigorously establish the roles of the proposed genes for 3,4 DCNB degradation in the wild type strain.

Theoretically, a ring-hydroxylating dioxygenase could attack 3,4-DCNB at either the 1,2- or 1,6- positions (Palatucci *et al*., 2019). Heterologous expression of the functional nitroarene dioxygenase from strain JS3050 established the high regiospecificity of the initial attack on 3,4-DCNB that would avoid misrouting of the resultant dichlorocatechol and ensure proper operation of the downstream pathway.

Based on the sequence information, the putative DcnR is similar to NtdR (with three amino acid differences) from *Acidovorax* sp. JS42 (Lessner *et al*., 2003) and DntR (with four amino acid differences) from *Burkholderia* sp. DNT (de las Heras *et al*., 2011). It is noteworthy that the five residue difference in NtdR (*Acidovorax* sp. JS42), in contrast to NagR (*Ralstonia* sp. strain U2), endowed NtdR with the ability to accept a number of nitroarenes as inducers. Exchange of these five residues revealed that residues at positions 227, 232 and 169, which are located near the predicted inducer-binding pocket, were key for the recognition of new compounds (Ju *et al*., 2009). Preliminary experiments with real-time RT-PCR indicate substantial upregulation of *dcnAc* by 3,4-DCNB (data not shown), which suggests inducibility of the nitroarene dioxygenase in strain JS3050 consistent with findings for 2-nitrotoluene dioxygenase NtdAcAd in JS42 (Lessner *et al*., 2003). This contrasts with the case in *Pseudomonas stutzeri* ZWLR2-1, where constitutive expression of *cnbAcAd* for 2-chloronitrotoluene dioxygenase was observed (Gao *et al*., 2016). Details of the regulation in strain JS3050 are currently under investigation.

DcnAc was aligned with α subunits of other naphthalene/nitroarene dioxygenases to compare the conserved and variable regions of the different enzymes as shown in Fig. S5. The [2Fe-2S] center binding sites are conserved in all of the enzymes. A phylogenetic tree of aromatic ring hydroxylating dioxygenases (Fig. S6) reveals that naphthalene/nitroarene dioxygenases from Gram-negative strains belong to group III aromatic ring hydroxylating dioxygenases (Parales and Resnick, 2006), which include the naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 98164 (Ensley *et al*., 1982; Suen and Gibson, 1993). The results from the amino acid sequence alignment (Fig. S6) as well as Swiss-Model structural model indicate residues in the active sites of 3,4-DCNB dioxygenase and 2,4-dinitrotoluene dioxygenase (Suen and Spain, 1993) are highly similar, particularly 3,4-DCNB dioxygenase/2,4-dinitrotoluene dioxygenase residues Gln293/Gln298, Val258/Val263, Ser350/Thr355, Ile204/ Ile209 and Ile207/Ile212. These two enzymes likely share common substrate binding orientation and substrate preference. The relationship between the amino acid residues and the substrate specify has been studied in nitrobenzene dioxygenase (Ju and Parales, 2006) and 2-nitrotoluene dioxygenase (Lee *et al*., 2005; Ju and Parales, 2011; Mahan *et al*., 2015). Future studies on DcnAc of strain JS3050 would provide additional insight about structure–function relationships.

Some of the metabolic clusters responsible for degradation of chlorinated aromatic compounds are globally dispersed and inherited through horizontal transfer of the catabolic plasmids (Wu *et al*., 2015). There are a number of evolutionary clues to the assembly of the functional 3,4-DCNB catabolic pathway in strain JS3050. ORFs 1, 2, 4 and 6 in the *dcnDEF* cluster contain genes encoding transposases and integrases found on catabolic plasmids in other isolates. They include a transposase gene similar to the one from the linuron and 3,4-dichloroaniline degrading *Variovorax* sp. WDL1 (Bers *et al*., 2013). Thus, the recruitment of *dcnC* might have been a key step in the assembly of the catabolic pathway for 3,4-DCNB.

The α subunit (Ac) is the most important subunit in the three component-nitroarene dioxygenase because it catalyses the dihydroxylation reaction and determines the substrate specificity (Parales *et al*., 1998). The large subunit DcnAc of 3,4-DCNB dioxygenase in this study is most similar (92% identity) to DntAc of 2,4-dinitrotoluene dioxygenase (Suen *et al*., 1996) rather than CnbAc of 2-chloronitrobenzene dioxygenase (88% identity) (Liu *et al*., 2011), although the latter is involved in chloronitroarene degradation (Fig. S6). Interestingly, the chemical structure of 2,4-dinitrotoluene is also similar to the 3,4-DCNB structure. Compared with the reactive nitro group, the two chlorine atoms in 3,4-DCNB are in the same position as the methyl and nitro groups in 2,4-dinitrotoluene.

The catechol and chlorocatechol 1,2-dioxygenases are historically distinguished as type I and type II enzymes, based on their substrate ranges (Dorn and Knackmuss, 1978a). Type I enzymes primarily catalyse conversion of catechol, whereas type II enzymes have a wider substrate range and transform chlorinated catechols more rapidly than catechol. However, type II chlorocatechol 1,2-dioxygenases are strongly inhibited by halocatechols simultaneously substituted in positions 4 and 5 (Haigler *et al*., 1988; Potrawfke *et al*., 2001). In the chlorobenzoate-degrading strain *Pseudomonas putida* AC27, 4,5-dichlorocatechol was a competitive inhibitor for the chlorocatechol 1,2-dioxygenase (Broderick and O'Halloran, 1991). DcnC, the plasmid-encoded 4,5-dichlorocatechol dioxygenase in this study and TetC (type III catechol 1,2-dioxygenase) from *Pseudomonas chlororaphis* RW71 (Potrawfke *et al*., 2001) are 95% identical, and are among the few chlorocatechol 1,2-dioxygenases reported to be active against 4,5-dichlorocatechol. Thus, DcnC in this study is a new example of type III catechol 1,2-dioxygenase.

Within the proposed chloromuconate catabolic cluster (Fig. 4B), DcnD, DcnE and DcnF exhibit high similarity (more than 60% in amino acid sequences) to the chloromuconate cycloisomerase from *Variovorax* sp. WDL1

Table 2. Strains and plasmids used in this study.

	Characteristics	Source or reference
Strains		
Diaphorobacter sp. strain JS3050	3,4-dichloronitrobenzene utilizer	Palatucci *et al*. (2019)
E. coli strains DH5α	*supE44 lacU169* (ϕ80d*lacZ* ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Woodcock *et al*. (1989)
E. coli BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lacY1* (DE3)	Novagen
Plasmids		
pUC18	Ap^r; cloning and expression vector	TaKaRa Bio
pET28a	Expression vector, Kan^r	Novagen
pUC18*cnbA*	pUC18 carrying *cnbAaAbAcAd* which encodes a 2CNB dioxygenase	Liu *et al*. (2011)
pUC18*DcnAaAb(cnbAcAd)*	Intermediate plasmid: *cnbAaAb* of pUC18*cnbA* was replaced by *dcnAaAb* fragment	This study
pUC18-*dcnAaAbAcAd*	*CnbAcAd* of the intermediate plasmid pUC18*dcnAaAb(cnbAcAd)* was replaced by *DcnAcAd fragment*	This study
pET28a-*dcnC*	PCR amplicon of *dcnC* cloned into pET28a	This study
pET28a-His*dcnC*	PCR fragment of *dcnC* cloned into pET28a	This study

(Bers *et al*., 2013), chlorodienelactone hydrolase from *Delftia acidovorans* LME1 (Krol *et al*., 2012) and chloromaleylacetate reductase from *Burkholderia* sp. M701 (Sakai *et al*., 2014) respectively. These three enzymes DcnDEF are proposed to catalyse the sequential reactions in 3,4-dichloromuconate catabolism (Fig. 4 and Table S1), but they remain to be expressed and characterized.

pDCNB in strain JS3050 has high similarity (>90% both in query coverage and percent identity) to several previously reported catabolic plasmids including the 60.7 kb plasmid in the chlorpyrifos degrader *Cupriavidus nantongensis* strain X1 (Fang *et al*., 2019), the 77.2 kb plasmid pENH91 in the 3-chlorobenzoate degrader *Cupriavidus necator* strain NH9 (Ogawa and Miyashita, 1999), the 98.2 kb plasmid pA81 in the 2-chlorobenzoate degrader *Achromobacter xylosoxidans* A8 (Strnad *et al*., 2011), the 91.2 kb plasmid pCNB from the 4-chloronitrobenzene degrader *Comamonas* strain CNB-1 (Wu *et al*., 2005) and the 74.3 kb plasmid pC1-1 in the 3-chloroaniline degrader *Delftia acidovorans* strain CA28 (Boon *et al*., 2001). Notably, as shown in Fig. S7, *dcnC*-related genes are present in some of the plasmids (boxed). These plasmids are all larger than that of strain JS3050 but share the main plasmid backbone and some putative chlorocatechol degradation clusters with the JS3050 plasmid. This suggests all these plasmids may have a common evolutionary history. The IncP-type conjugative transfer protein Trb clusters are conserved on all these plasmids. They are broad host range and self-transmissible and could play an important role in horizontal gene transfer (Dennis, 2005).

There is an incomplete chloromuconate cycloisomerase gene that is truncated by an *IS1070* adjacent to *dcnC* in strain JS3050. This 134-bp fragment and the *tetD* gene next to *tetC* in strain *Pseudomonas chlororaphis* RW71 are identical which suggests a recent common origin and that strain JS3050 may have acquired *dcnC* via horizontal gene transfer. During 3,4-DCNB degradation by strain JS3050, the low specific activity of DcnC toward 4,5-dichlorocatechol might be a bottleneck for the pathway and would account for the relatively slow growth on 3,4-DCNB. In addition, non-contiguous distribution of the genes responsible for 3,4-DCNB catabolism (Fig. 4B) suggests that the catabolic pathway is in the early stages of evolution.

Materials and methods

Bacterial strains, plasmids, culture conditions and chemicals

The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strains were grown in lysogeny broth (LB) at 37°C. *Diaphorobacter* sp. strain JS3050 was grown in 1/5 trypticase soy broth or mineral salts broth (Cohen-Bazire *et al*., 1957) medium supplied with 3,4-DCNB (1–2 mM) and Amberlite XAD-7 resin (3.5 g L^{-1}) (from Sigma-Aldrich) at 30°C. For solid media, Bacto-Agar was added to a final concentration of 12 g L^{-1}. When required, antibiotics were used at the following concentrations: ampicillin (100 mg L^{-1}), kanamycin (50 mg L^{-1}) and tetracycline hydrochloride (20 mg L^{-1}). 3,4-DCNB was purchased from Alfa Aesar (Shanghai, China); 3-chloronitrobenzene, 4-chloronitrobenzene, nitrobenzene, 2,4-dinitrotoluene, 2-nitrobenzyl alcohol, 3-nitrobenzyl alcohol, 2-nitrotoluene, 3-nitrotoluene and 4-nitrotoluene were purchased from Sinopharm Chemical Reagent (Beijing; China) with purity of higher than 98%; catechol, IPTG, 3-methylcatechol, 4,5-dichlorocatechol and 4-chlorocatechol were purchased from Sigma (St. Louis,

Table 3. Primers used in study.

Name	Sequence	Purpose
pUC18 Aa ins	CAGCCATTCGGAGACAACTG	To amplify *dcnAaAb* from strain JS3050 to construct pUC18*dcnAaAb(cnbAcAd)*
pUC18 Ab ina	TTAGTCCAGCTTGAGCATCAC	To amplify *dcnAaAb* from strain JS3050 to construct pUC18*dcnAaAb(cnbAcAd)*
pUC18 Ab vector ins	TGATGCTCAAGCTGGAC TAAgcgcagaggccgcacttctg	To amplify the vector from pUC18*cnbA* to construct pUC18*dcnAaAb(cnbAcAd)*
pUC18 Aavector ina	CAGTTGTCTCCGAATGGCT Ggagctcgaattcgtaatcat	To amplify the vector from pUC18*cnbA* to construct pUC18*dcnAaAb(cnbAcAd)*
pUC18 DcnAc ins	ATGAGTTACCAAAACTTAG	To amplify *dcnAcAd* from strain JS3050 to construct pUC18-*dcnAaAbAcAd*
pUC18 DcnAd ina	TCACAGGAAGATCATCAGGT	To amplify *dcnAcAd* from strain JS3050 to construct pUC18-*dcnAaAbAcAd*
pUC18 Ac invectora	CTAAGTTTTGGTAACTCAT	To amplify the vector from pUC18*DcnAaAb (cnbAcAd)* to construct pUC18-*dcnAaAbAcAd*
pUC18 Ad invectors	ACCTGATGATCTTCCTGTGAACCC	To amplify the vector from pUC18*DcnAaAb (cnbAcAd)* to construct pUC18-*dcnAaAbAcAd*
28nohisvectors	TGAGATCCGGCTGCTAACAAA	To amplify the vector to construct pET28a-*dcnC*
28nohisvectora	GGTATATCTCCTTCTTA AAGTTAAACAAAAT	To amplify the vector to construct pET28a-*dcnC*
dcnC 28nohiss	ctttaagaaggagatatacc ATGAACGAACGAGT GAAGCAGG	To amplify *dcnC* to construct pET28a-*dcnC*
dcnC 28nohisa	ttgttagcagccggatctcaTCATGC GTGCTCCCGGGG	To amplify *dcnC* to construct pET28a-*dcnC*
DcnbC ndei ins	GTGCCGCGCGGCAGCCA TATGATGAACGAACGAGTGAAGC	To amplify *dcnC* to construct pET28a-His*dcnC*
DcnbC ndei ina	ACCAGTCATGCTAGCCA TATGTCATGCGTGCTCCCGGGGTC	To amplify *dcnC* to construct pET28a-His*dcnC*

MO, USA); 2-chloronitrobenzene and 4-methylcatechol were from Fluka Chemical (Buchs, Switzerland); 3-chlorocatechol was from TCI (Tokyo, Japan); 3,4-dichlorocatechol was from CFW Laboratories (Walnut, CA). All the primers were synthesized by Tsingke, Shanghai, China and are listed in Table 3.

Genome sequencing and bioinformatics

DNA was extracted from cells of *Diaphorobacter* sp. strain JS3050 using a Mo Bio Ultra Clean microbial DNA purification kit, and sent for Illumina and PacBio sequencing by BGI Genomics (Shenzhen, China). Assembled sequence results were annotated in RAST Artemis online, and analysed for genes involved in nitroarene and chlorocatechol metabolism. Candidate genes were compared with the top biochemically characterized hits in the NCBI database using BLASTp. The sequences of the genomic DNA and pDCNB are available under accession numbers CP053553 and CP053554 respectively.

General DNA manipulations

Plasmid pUC18-*dcnAaAbAcAd* was constructed by replacing the *cnbAaAb* of pCNBA (Liu *et al*., 2011) with *dcnAaAb* and replacing the *cnbAcAd* with *dcnAcAd*. The primers used were listed in Table 3. pCNBA is a pUC18-based construct containing 2-chloronitrobenzene dioxygenase encoding genes *cnbAaAbAcAd*. Primer pair pUC18 Ab vector ins/pUC18 Aa vector ina was used to amplify the 5.1 kb vector fragment. Primer pair pUC18 Aa ins/pUC18 Ab ina was used to amplify the 2.0 kb fragment *dcnAaAb* from strain JS3050. A 7.1 kb intermediate plasmid pUC18*DcnAaAb(cnbAcAd)* was constructed by the linkage of these two fragments using a cloning kit (see below). Primer pair pUC18 Ac invectora/pUC18 Ad invectors was used to amplify the 5.3 kb vector fragment from the intermediate plasmid pUC18*DcnAaAb (cnbAcAd)*. Primer pair pUC18 DcnAc ins/pUC18 DcnAd ina was used to amplify the 1.9 kb fragment *dcnAcAd* from strain JS3050. And then the plasmid pUC18-*DcnAaAbAcAd* was constructed. A 0.7 kb *dcnC* gene was amplified from strain JS3050 using primer pair dcnC 28nohiss/dcnC 28nohisa. A 5.2 kb vector fragment was amplified from plasmid pET28a using primer pair 28nohisvectors/28nohisvectora. These two fragments were linked to construct plasmid pET28a-*dcnC*. pET28a-His*dcnC* was constructed with Nde I digested plasmid pET28a and *dcnC* fragment amplified by primer pair DcnbC ndei ins/DcnbC ndei ina. pET28a-His*dcnC* was

constructed with Nde I digested plasmid pET28a and *dcnC* fragment amplified by primer pair DcnbC ndei ins/DcnbC ndei ina. The word 'in' means that the primers were used for the 'infusion clone' strategy; the characters 's' and 'a' mean 'sense' or 'antisense' of the primers respectively, 'vector' means that the primers were used to amplify the plasmid. The plasmids and inserted fragments were linked by NovoRec PCR One Step Directed Cloning Kit (Novoprotein, China) or ClonExpress II One Step Cloning Kit (Vazyme China). The molecular constructs were further confirmed by sequencing (General Biosystems, China). Other molecular operations followed standard protocols (Sambrook *et al*., 1989).

Biotransformation of 3,4-DCNB by heterologously expressed 3,4-DCNB dioxygenase

Plasmid pUC18-*dcnAaAbAcAd* was transformed into *E. coli* strain DH5α to construct *E. coli* strain DH5α [pUC18-*dcnAaAbAcAd*]. The cells were incubated overnight at 37°C in 5 ml of LB medium supplied with ampicillin, then transferred (1% inoculum) into 100 ml of the same medium and incubated at 30°C for 3 h. When the OD_{600} of the culture was approximately 0.6, IPTG was added (final concentration of 1 mM) the culture was incubated for another 3 h to induce the expression of *dcnAaAbAcAd*. Cells were collected by centrifugation, washed with sodium phosphate buffer (PB) (pH = 7.4, 50 mM) and suspended in 5 ml PB, 3,4-DCNB was added (0.15 mM final), the suspension was incubated at 30°C, and samples were removed for nitrite analysis at appropriate intervals. *Escherichia coli* strain DH5α [pUC18] was used as the negative control. To determine the substrate range of 3,4DCNB dioxygenase, a series of substrates (3,4-dichloronitrobenzene, nitrobenzene, 3-nitrotoluene, 2-nitrotoluene, 4-nitrotoluene, 2-chloronitrobenzene, 3-chloronitrobenzene, 4-chloronitrobenzene, 2,4-dinitrotoluene, 2,6-dinitrotoluene) were also added from 100 mM methanol solution individually for analysis. The final concentrations were 1 mM. Biotransformations were performed in a shaker (180 rpm) at 30°C with cell densities of 8–10 at OD_{600}. The activities were calculated by the nitrite concentrations at 15 min for reactions against each substrate. The data are shown in Table S2.

Enzyme assays

To determine the 4,5-dichlorocatechol 1,2-dioxygenase activity of DcnC, plasmid pET28a-*dcnC* was transformed into *E. coli* BL21 (DE3) to construct strain *E. coli* BL21(DE3) [pET28a-*dcnC*]. The strain was grown in LB medium supplied with kanamycin at 30°C. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 0.2 mM and the culture was incubated with shaking for another 3 h to induce the expression of *dcnC*. Then the cells were collected by centrifugation, washed, suspended in PB buffer and sonicated in an ice-water bath at 3.0 s on and 7.0 s off for 20 min at 30% of full power. Cell debris was removed by centrifugation at 17 000*g* for 60 min at 4°C. A Lambda 25 spectrophotometer (PerkinElmer/Cetus, Norwalk, CT) was used to determine the 4,5-dichlorocatechol 1,2-dioxygenase activity by a spectrophotometric assay as described previously except that EDTA was omitted (Spain and Nishino, 1987). The initial activity of DcnC against the substituted catechols was assayed by measuring the increase of OD_{260} due to the formation of corresponding muconate. The initial concentration of catechols was 500 or 50 μM. The molar extinction coefficients of muconate and its derivatives 2-methylmuconate, 3-methylmuconate, 2-chloromuconate, 3-chloromuconate, 2,3-dichloromuconate are 16 800,18 000, 13 900, 17 100, 12 400 and 12 400 M^{-1} cm^{-1} respectively (Dorn and Knackmuss, 1978b). To measure the molar extinction coefficient of 3,4-dichloromuconate, 50 μM 4,5-dichlorocatechol was incubated with excess crude cell extract until the reaction was complete to measure the increase of OD_{260}. The molar extinction coefficient of 3,4-dichloromuconate was calculated as 11 000 M^{-1} cm^{-1}. One unit of enzyme activity (U) is defined as the amount of the enzyme required for the production of 1 μmol of product in 1 min at 30°C (Wang *et al*., 2019). Specific activities are expressed as units per milligram of protein. Protein concentrations were measured according to the Bradford method (Bradford, 1976). For further determination of kinetic parameters the DcnC expressed by *E. coli* BL21(λDE3) [pET28a-His*dcnC*] was purified as described previously (Zhao *et al*., 2018). A single colony of *E. coli* BL21(λDE3) [pET28a-His*dcnC*] was cultured in 5 ml of fresh LB medium containing kanamycin, and was incubated in a shaker at 37°C for 14 h. Then 500 μl of bacterial culture was transferred to 500 ml of fresh LB medium and cultured at 37°C to 0.8 (OD_{600}). IPTG with a final concentration of 0.3 mM was added and cells were inculcated at 30°C for 5 h. The culture was centrifuged8000*g*) to collect the cells, which were resuspended with lysate buffer: pH 8.0 Tris–HCl (20 mM), 100 mM NaCl, 5% (vol./vol.) glycerol, 1 mM PMSF (phenylethanesulfonyl fluoride, protease inhibitors). The suspended cells were sonicated in an ice bath for 3.0-s on and 5.0-s off intervals for 150 cycles at 300 W and then centrifuged at 15 000*g* for 30 min to remove the cell debris. The supernatant was filtered through a 0.45 μM filter membrane and then loaded onto a 5-ml HisTrap HP column (GE Healthcare) and ÄKTA start system (GE Healthcare) according to the manufacturer's instructions. Two buffers were used for the protein purification. Buffer A: 20 mM Tris–HCl 8.0, 100 mM NaCl, 5% glycerol. Buffer B: 20 mM Tris–HCl 8.0, 100 mM NaCl, 500 mM imidazole, 5% glycerol. HisTrap HP column was eluted to equilibrium state with buffer A, and then 30 ml of the sample was loaded at the flow rate of 1 ml min^{-1}. Then the HisTrap HP column was

eluted with 5% and 10% buffer B. Finally, the target protein was eluted with 100% buffer B and collected. The protein purified was assessed by SDS-PAGE.

Analytical methods

GC–MS analyses were performed on a TRACE 1310 gas chromatograph (Thermo Fisher Scientific, MA, USA) using a capillary column HP-5MS (0.25 mm × 30 m, Agilent Technologies, CA, USA). The column temperature gradient was 0–2 min, 50°C; 2–11 min, 50–140°C; 11–14 min, 140°C; 14–17.5 min, 140–250°C; 17.5–20 min, 250°C. The detector was a TSQ8000 Triple Quadrupole MS (Thermo Fisher Scientific). The biotransformation samples were extracted with diethyl ether. The extracts were dried over anhydrous Na_2SO_4 before GC–MS analysis.

HPLC analysis of nitroaromatic substrates and their oxidation products was performed on a Waters e2695 Separation Module equipped with a C18 reversed-phase column (5 μm, 4.6 × 250 mm) maintained at 30°C. The monitoring wavelength was 280 nm. The mobile phase consisted of water containing 0.1% (vol./vol.) acetic acid (A) and methanol (B) at a 1.0 ml min^{-1} flow rate. The solvent gradient was 20% to 90% B over 15 min. Under these conditions, authentic 3,4-dichlorocatechol and 4,5-dichlorocatechol had retention times of 13.4 and 14.0 min respectively. The concentration of nitrite was assayed as described previously (Lessner *et al*., 2002), using a BioTek Synergy 2 microplate reader and sodium nitrite as a standard.

Homology modelling

The amino acid sequence of the DcnAc subunit of 3,4-DCNB dioxygenase was submitted to Swiss-model (Waterhouse *et al*., 2018), for homology modelling. The α subunit of 3-NT dioxygenase from *Diaphorobacter* sp. strain DS2 (pdb number: 5xbp), with an 87% amino acid sequence identity with the alpha subunit of 3,4-DCNB dioxygenase, was used as the modelling template (Kumari *et al*., 2017). The GMQE and QMEAN scores of 0.98 and −1.17 respectively showed the reliability of this model. The molecular docking process was performed with Autodock vina (Trott and Olson, 2010). The best pose with a binding affinity of −6.0 kcal mol^{-1} was used for our analysis. Based on the analysis above, PyMOL v1.3 was used to view and analyse the binding of the enzyme and 3,4-DCNB.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: supporting Information