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Application of nitroarene dioxygenases in the design of novel strains that degrade chloronitrobenzenes

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Summary

Widespread application of chloronitrobenzenes as feedstocks for the production of industrial chemicals and pharmaceuticals has resulted in extensive environmental contamination with these toxic compounds, where they pose significant risks to the health of humans and wildlife. While biotreatment in general is an attractive solution for remediation, its effectiveness is limited with chloronitrobenzenes due to the small number of strains that can effectively mineralize these compounds and their ability to degrade only select isomers. To address this need, we created engineered strains with a novel degradation pathway that reduces the total number of steps required to convert chloronitrobenzenes into compounds of central metabolism. We examined the ability of 2-nitrotoluene 2,3-dioxygenase from *Acidovorax* sp. strain JS42, nitrobenzene 1,2-dioxygenase (NBDO) from *Comamonas* sp. strain JS765, as well as active-site mutants of NBDO to generate chlorocatechols from chloronitrobenzenes, and identified the most efficient enzymes. Introduction of the wild-type NBDO and the F293Q variant into *Ralstonia* sp. strain JS705, a strain carrying the modified *ortho* pathway for chlorocatechol metabolism, resulted in bacterial strains that were able to sustainably grow on all three chloronitrobenzene isomers without addition of co-substrates or co-inducers. These first-generation engineered strains demonstrate the utility of nitroarene dioxygenases in expanding the metabolic capabilities of bacteria and provide new options for improved biotreatment of chloronitrobenzene-contaminated sites.

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Introduction

Chloronitrobenzenes (CNBs) are industrial chemicals that are used in the production of pesticides, fungicides, dyes and polymers (Hartter, 1985). These compounds are toxic, and there are reports that the 2- and 4-isomers of CNB are both mutagenic and carcinogenic (Shimizu *et al*., 1983; Matsumoto *et al*., 2006a,b). CNBs have been detected in industrial waste and in environmental water samples (Hartter, 1985; Feltes *et al*., 1990), and their presence poses health risks to humans and animals.

The electron-withdrawing nature of the nitro- and chloro-groups makes these compounds resistant to microbial degradation, and consequently, relatively few bacterial strains have been reported to be capable of growth on CNBs. *Pseudomonas acidovorans* CA50 (Hinteregger *et al*., 1994; Kuhlmann and Hegemann, 1997) converts all three CNB isomers to chloroanilines by direct reduction of the nitro-group (Fig. 1). The strain is able to grow on chloroanilines, using an aniline dioxygenase to oxidize the chloroanilines to chlorocatechols (Fig. 1), which in turn are converted to TCA cycle intermediates by a modified *ortho* pathway. However, acetate and nitrate were required as co-substrates for the initial reduction of CNBs, and therefore strain CA50 cannot use CNBs as sole carbon and energy sources. In another study, 3- and 4-chloronitrobenzene (3CNB; 4CNB) degradation was achieved by sequential action of strains *P. putida* HS12 and *Rhodococcus* sp. strain HS51 (Park *et al*., 1999). In this system, the reductive pathway for nitrobenzene degradation in HS12 converted 3CNB and 4CNB into 2-amino-4-chlorophenol and 2-amino-5-chlorophenol, respectively (Fig. 1). These products were then converted to the corresponding chloro-hydroxyacetanilides. *Rhodococcus* sp. strain HS51 was isolated for its ability to grow on chloro-hydroxyacetanilides, which are further metabolized to TCA cycle intermediates using a modified *ortho* cleavage pathway. Together, these strains were able to completely degrade 3- and 4CNB (Fig. 1); however, strain HS12 required the presence of the co-substrate succinate for the initial reduction of CNBs, and nitrobenzene and chlorobenzene were necessary to induce the expression of the upper and lower pathways in strains HS12 and HS51, respectively.

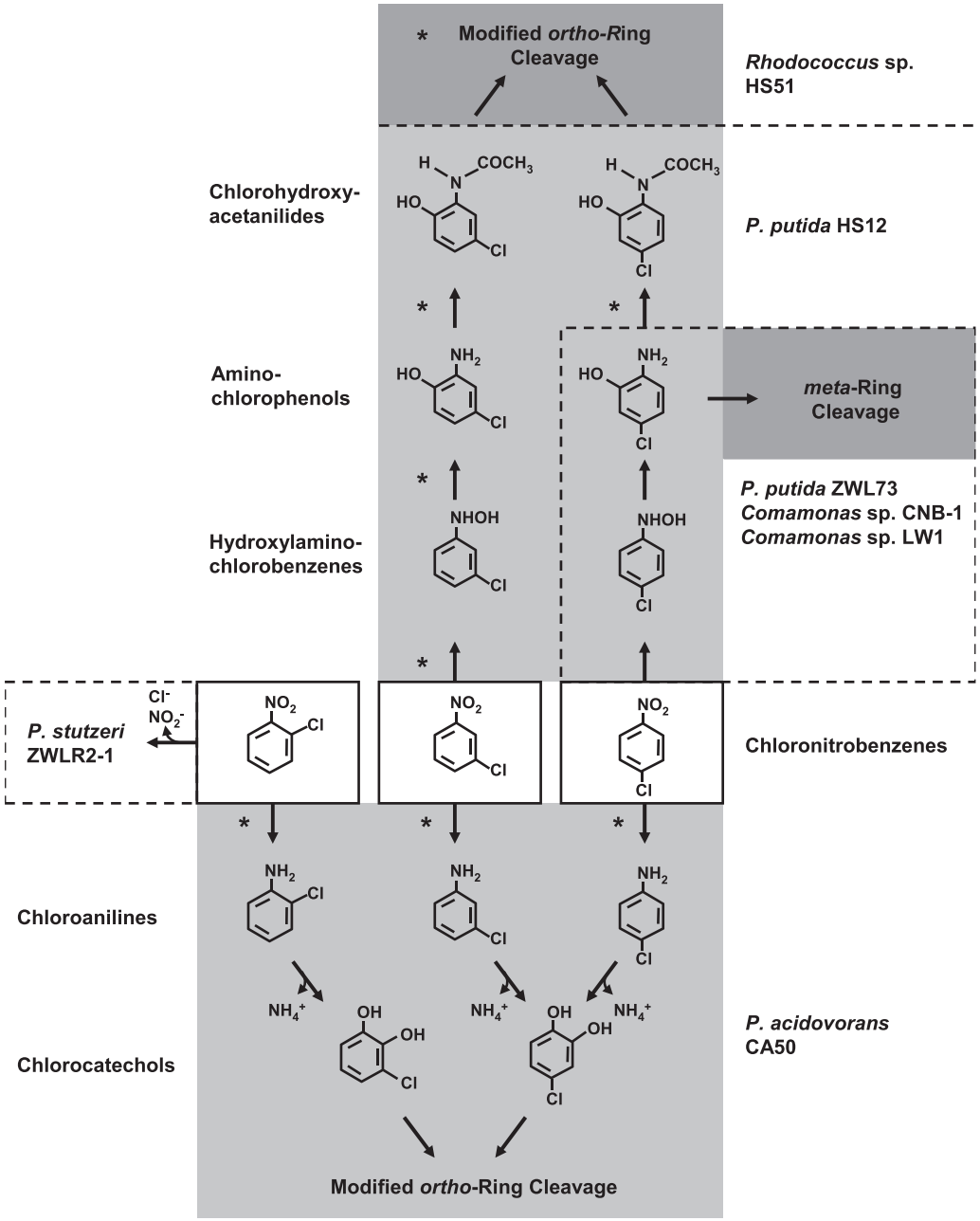

Fig. 1. Chloronitrobenzene degradation pathways previously characterized from bacterial isolates and co-cultures. Asterisks (*) indicate steps that require the addition of a co-substrate or co-inducer in order to facilitate metabolite transformation.

Pseudomonas stutzeri ZWLR2-1 was isolated for its ability to grow on 2-chloronitrobenzene (2CNB), and it was reported to release chloride and nitrite from this substrate (Liu *et al.*, 2005). However, further characterization of its degradation pathway has not been reported. *Comamonas* sp. strain CNB-1 (Wu *et al.*, 2006), *Pseudomonas putida* ZWL73 (Zhen *et al.*, 2006) and *Comamonas* sp. strain LW1 (Katsivela *et al.*, 1999) each contain a reductive pathway for 4CNB degradation (Fig. 1). Metabolism is initiated by a nitroreductase that converts 4CNB into 1-chloro-4-hydroxyl-aminobenzene, which is further transformed into 2-amino-5-chlorophenol by a hydroxylaminobenzene mutase or via Bamberger rearrangement. Ring-cleavage by 2-aminophenol 1,6-dioxygenase produces 2-amino-5-chloromuconic acid, which is converted to TCA cycle intermediates after additional enzymatic steps (Wu *et al.*, 2006).

In this study we examined the ability of wild-type and mutant nitroarene dioxygenases to oxidize CNBs to chlorocatechols, which are easily degraded by strains carrying

the modified *ortho* pathway. Nitroarene dioxygenases act in the first step of nitrobenzene and (di)nitrotoluene degradation pathways, catalysing the insertion of both atoms of oxygen at the nitro-substituted and adjacent carbon atoms of the aromatic ring to produce catechols and release nitrite. Nitroarene dioxygenases are capable of removing nitro-groups from nitrobenzene, nitrotoluenes and aminonitrotoluenes (Suen *et al.*, 1996; Parales *et al.*, 1998a; Johnson *et al.*, 2001; Lessner *et al.*, 2002). They are unique in their ability to overcome the electron-withdrawing nature of the nitro-group, which provides high energetic stability of nitroaromatic compounds and environmental recalcitrance, and we hypothesized that at least some of the isomers of CNB would be similarly oxidized by nitroarene dioxygenases. We characterized the activity of 2-nitrotoluene 2,3-dioxygenase (2NTDO) from *Acidovorax* sp. strain JS42 (Parales *et al.*, 1998a), nitrobenzene 1,2-dioxygenase (NBDO) from *Comamonas* sp. strain JS765 (Lessner *et al.*, 2002), as well as active-site mutants of NBDO (Ju and Parales, 2006) with the substrates 2CNB, 3CNB and 4CNB. Studies on the catalytic and biochemical properties of these enzymes (Parales *et al.*, 1998a; 2005; Lessner *et al.*, 2002; Lee *et al.*, 2005), as well as an available crystal structure (Friemann *et al.*, 2005) have allowed the identification of specific amino acids that control substrate specificity. The availability of this information makes these enzymes good candidates for defining the activity of nitroarene dioxygenases with CNBs, a topic that was unexplored prior to this study.

Genes encoding the enzymes with the best activity were introduced into *Ralstonia* sp. JS705, a robust chlorobenzene-degrading strain isolated from contaminated groundwater. The chlorobenzene degradation pathway in this strain has been well characterized by physiological and genetic analyses (van der Meer *et al.*, 1998; Muller *et al.*, 2003). Chlorobenzene is converted to 3-chlorocatechol in *Ralstonia* sp. JS705, and 3-chlorocatechol is degraded to TCA cycle intermediates using the modified *ortho* cleavage pathway (van der Meer *et al.*, 1998). Introduction of NBDO and one of its variants resulted in new strains that grow on and completely degrade all three isomers of CNBs.

Results

Regiospecificity of CNB oxidation

We tested the activity of 2NTDO, NBDO and active-site mutants of NBDO with 2CNB, 3CNB and 4CNB in *Escherichia coli* strains expressing the cloned dioxygenase genes. These substrates allowed us to characterize the behaviour of these dioxygenases towards molecules with two different electronegative functional groups. With each of these substrates, dioxygenation can occur at: (i) the nitro-substituted carbon, (ii) the chloro-substituted carbon or (iii) at positions distal to the nitro- or chloro-substituted carbons. A variety of different substituted catechols or *cis*-dihydrodiols could be produced depending on the regiospecificity of attack (Fig. 2).

2CNB, 3CNB and 4CNB were substrates for 2NTDO, NBDO and the NBDO variants. The relative product ratios from biotransformation reactions of these dioxygenases with the three CNB isomers are presented in Table 1. The majority of the tested dioxygenases favoured attack at the nitro-substituted region of 2CNB to form 3-chlorocatechol as the sole or major product. Both 2NTDO and NBDO had slight activity ($\leq$ 3%) towards the chloro-substituted region of 2CNB and formed small amounts of 3-nitrocatechol. However, mutations at amino acid positions 258 and 350 of the catalytic subunit of NBDO altered the position of oxidation and increased the relative amount of 3-nitrocatechol produced. The I350F variant showed the greatest change, reversing the regiospecificity of NBDO, to favour dechlorination rather than nitro-group removal. Substitution of the phenylalanine at position 293 with histidine, isoleucine or glutamine increased the specificity of NBDO by eliminating 3-nitrocatechol formation from 2CNB.

All of the tested dioxygenases except the N258V and F293H variants of NBDO preferentially attacked the nitro-substituted positions of 3CNB, yielding 4-chlorcatechol as the sole oxidation product. The substitution of asparagine 258 by valine changed the regiospecificity of NBDO with 3CNB, resulting in dechlorination and the formation of 4-nitrocatechol (16%). Although 4-chlorocatechol remained the major product (83%), the N258V mutant also formed a minor amount of 3-chlorocatechol (1%). The F293H variant of NBDO retained the ability to attack the nitro-substituted carbon but had the opposite regiospecificity, forming 3-chlorocatechol instead of 4-chlorocatechol. 3-Nitrocatechol was not detected in any of the reactions with 3CNB.

NBDO preferentially oxidized the nitro-substituted carbon of 4CNB to yield 4-chlorocatechol as the major product, but this enzyme also had minor activity at the chloro-substituted carbon, forming 4-nitrocatechol (1%). The N258V, I350F and I350T mutants of NBDO produced 4-chlorocatechol as the major product, but were also able to dechlorinate 4CNB to form 4-nitrocatechol (16–34%). In contrast, 2NTDO and the F293H, F293I and F293Q mutants of NBDO had increased specificity, oxidizing at the nitro-substituted carbon and producing only 4-chlorocatechol.

In general, most of the tested dioxygenases preferentially oxidized at the nitro-substituted positions rather than the chloro-substituted positions of the aromatic ring. No catechol or *cis*-dihydrodiols were detected in any biotransformation reactions based on the analysis of

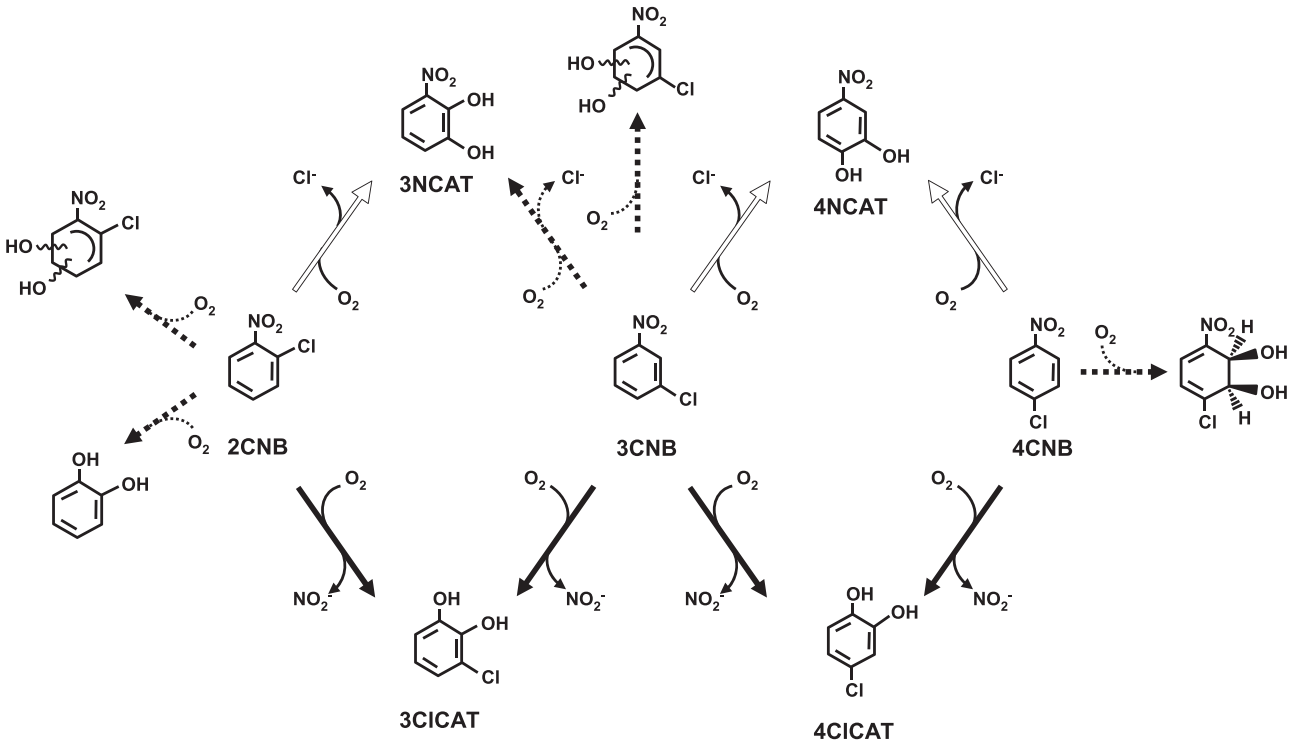

Fig. 2. Possible reactions catalysed by nitroarene dioxygenases with CNBs. Solid black and white arrows indicate reactions detected by some or all of the nitroarene dioxygenases tested in this study. All of the reactions shown require NADH as a cofactor. Black arrows indicate reactions whose products are substrates for further metabolism by the modified *ortho*-ring cleavage pathway (shown in Fig. 5). No evidence for the reactions indicated by dotted lines was obtained. 3ClCAT, 3-chlorocatechol; 4ClCAT, 4-chlorocatechol; 3NCAT, 3-nitrocatechol; 4NCAT, 4-nitrocatechol.

Table 1. Relative ratios of products formed from CNBs by wild-type and mutant nitroarene dioxygenases expressed in *E. coli*.

		Catechols produced (% of total products)			
Substrate	Dioxygenase	3ClCAT	3NCAT	4ClCAT	4NCAT
2CNB	NBDO	97 ± 1	3 ± 1	n/a	n/a
	N258V	88 ± 1	12 ± 1	n/a	n/a
	F293H	100	–	n/a	n/a
	F293I	100	–	n/a	n/a
	F293Q	100	–	n/a	n/a
	I350F	22 ± 3	78 ± 3	n/a	n/a
	I350T	85 ± 4	15 ± 4	n/a	n/a
	2NTDO	98 ± 1	2 ± 1	n/a	n/a
3CNB	NBDO	–	–	100	–
	N258V	1 ± 1	–	83 ± 1	16 ± 1
	F293H	100	–	–	–
	F293I	–	–	100	–
	F293Q	–	–	100	–
	I350F	–	–	100	–
	I350T	–	–	100	–
	2NTDO	–	–	100	–
4CNB	NBDO	n/a	n/a	99 ± 0.4	1 ± 0.4
	N258V	n/a	n/a	79 ± 4	21 ± 4
	F293H	n/a	n/a	100	–
	F293I	n/a	n/a	100	–
	F293Q	n/a	n/a	100	–
	I350F	n/a	n/a	66 ± 5	34 ± 5
	I350T	n/a	n/a	84 ± 0.3	16 ± 0.3
	2NTDO	n/a	n/a	100	–

3ClCAT, 3-chlorocatechol; 4ClCAT, 4-chlorocatechol; 3NCAT, 3-nitrocatechol; 4NCAT, 4-nitrocatechol; $n = 3$; n/a, not applicable, i.e. not a possible product from this substrate; –, none detected.

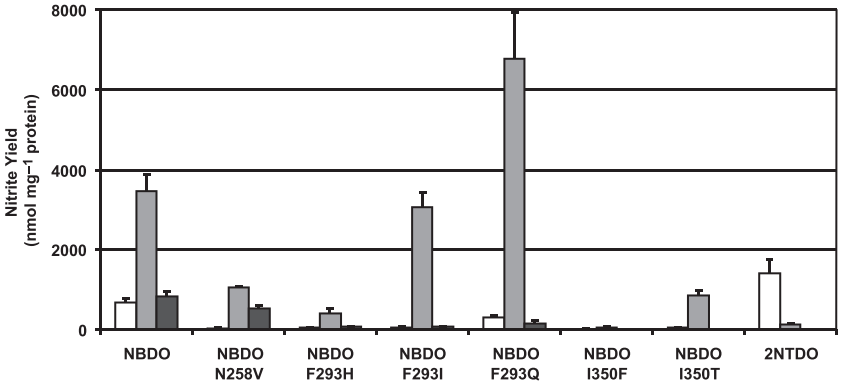

Fig. 3. Nitrite released from CNBs by *E. coli* strains expressing wild-type and mutant nitroarene dioxygenases. 2CNB, white bars; 3CNB, grey bars; 4CNB, black bars. $N = 3$; error bars indicate standard deviations.

extracts by gas chromatography-mass spectrometry (GC-MS). NBDO and 2NTDO exhibited nearly identical regiospecificities, forming 3-chlorocatechol and 4-chlorocatechol as the major or exclusive products from the three CNB isomers. Amino acid substitutions at position 293 in NBDO directed enzyme activity specifically to the nitro-substituted regions of the substrates, eliminating dechlorination reactions and nitrocatechol formation. Mutations at 258 and 350 decreased the regiospecificity of attack by NBDO, resulting in increased production of nitrocatechols.

Oxidation activity

The activity of the nitroarene dioxygenases towards the different CNBs and their ability to form chlorocatechols was determined as a function of the amount of nitrite released at the end of 6 h biotransformation reactions (Fig. 3). NBDO had comparable activities with 2CNB and 4CNB, but had the highest activity with 3CNB. In contrast, 2NTDO produced approximately twice as much product from 2CNB compared with NBDO, but its average activities with 3CNB and 4CNB were only 4% and < 1% of the NBDO activities, respectively.

All of the mutations in NBDO resulted in decreased activity towards 2CNB and 4CNB. With 2CNB as the substrate, the F293Q variant was 45% as active as wild-type NBDO, while the other variants retained only 1–7% of the original activity (Fig. 3). The mutations at 293 and 350 reduced activity with 3CNB to ≤ 8% of NBDO, but the N258V variant was still 65% as active. The F293I mutation had no effect on the activity with 3CNB; in contrast, the F293Q enzyme showed a twofold improvement in nitro-group removal with this substrate. All other mutations resulted in a 70–98% reduction in chlorocatechol production from 3CNB.

Construction and activity of CNB-degrading strains

The results of the biotransformation experiments described above suggested that some of the tested dioxygenase enzymes might be useful biocatalysts in the production of substituted catechols or for improving biodegradation processes. We hypothesized that the successful expression of the appropriate nitroarene dioxygenases in strains containing a chlorocatechol degradation pathway would allow CNBs to be degraded and utilized as a source of carbon and energy for growth. To test the application of these enzymes, broad-host-range expression plasmids carrying selected dioxygenase genes were constructed and introduced into the chlorobenzene-degrading strain *Ralstonia* sp. JS705. NBDO and the NBDO-F293Q variant were chosen based on their high specificity for nitro-group removal and chlorocatechol formation.

To determine if the nitroarene dioxygenases were functional in the JS705 strains, we measured their activity with CNBs. Introduction of the dioxygenase-containing expression plasmids allowed JS705 to remove nitrite from CNBs and form chlorocatechols, while cultures with the control plasmid (pBBR1MCS2) had no activity. JS705 expressing NBDO had comparable activities towards 2CNB (3.4 ± 1.3 nmol nitrite min^{-1} mg^{-1} protein) and 4CNB (3.3 ± 0.6 nmol nitrite min^{-1} mg^{-1} protein), and the highest activity with 3CNB (22.7 ± 1.3 nmol nitrite min^{-1} mg^{-1} protein). JS705 expressing NBDO-F293Q had higher specific activity with 3CNB (29.3 ± 3.4 nmol nitrite min^{-1} mg^{-1} protein), while activity in this strain was reduced 74% with 2CNB and 88% with 3CNB compared with NBDO.

Growth on CNBs

Ralstonia sp. JS705 strains carrying plasmids expressing wild-type NBDO or the NBDO-F293Q variant were able to grow on all three CNB isomers as sole carbon sources (Table 2). Neutral resin (XAD-7) was included to mitigate the toxicity of CNBs. 3CNB was the best substrate for both strains (Table 2, Fig. 4A), and nitrite release correlated with growth (Fig. 4B). JS705(pKSJ114) carrying NBDO-F293Q grew 23% faster on 3CNB than the strain carrying wild-type NBDO (Table 2), which is consistent

Table 2. Doubling times and cell yields of engineered *Ralstonia* sp. JS705 strains.

Substrate	Strain	Dioxygenase present	Doubling time[a] (h)	Maximum cell yield[a] (OD_{660})
2CNB	JS705 (pKSJ99)	NBDO	58.3 ± 3.5	0.18 ± 0.01
	JS705 (pKSJ114)	F293Q	57.8 ± 2.7	0.31 ± 0.02
3CNB	JS705 (pKSJ99)	NBDO	26.0 ± 0.3	0.40 ± 0.02
	JS705 (pKSJ114)	F293Q	20.0 ± 1.3	0.40 ± 0.02
4CNB	JS705 (pKSJ99)	NBDO	42.4 ± 3.7	0.27 ± 0.01
	JS705 (pKSJ114)	F293Q	37.3 ± 0.3	0.24 ± 0.01

a. $n = 2$; ± indicate standard deviations. The control strain JS705 (pBBR1MCS2) did not grow on any of the CNBs.

with the activities of the two enzymes with 3CNB. In comparison, growth on 4-chlorocatechol was inhibited even in the presence of XAD-7, with cultures reaching a maximum cell density of 0.084 ± 0.004 with a 48 h doubling time. This is not a surprising result, as it is well known that catechols are cytotoxic even at low concentrations (Haigler *et al*., 1988; Munoz *et al*., 2007). The two strains were also capable of growth on all three CNBs as sole nitrogen sources in liquid cultures with succinate as the carbon source (data not shown).

Discussion

Although CNBs provide several possible sites for aromatic ring oxidation (Fig. 2), 2NTDO and NBDO were highly specific towards the nitro-substituted positions and produced chlorocatechols as the major products (Table 1). Dechlorination activity was detected, but nitrocatechols were produced only from 2CNB and 4CNB, and resulted in less than 5% of the total products generated by both enzymes. Attack at positions distal to the chloro- and nitro-groups (which would result in the formation of chloronitro-*cis*-dihydrodiols), or the simultaneous removal of both the chloro- and nitro-groups (which would result in the formation of catechol; Fig. 2) was not observed. Analysis of product formation from CNBs by nitroarene dioxygenases allowed us to assess the activity of these enzymes when presented with two electronegative functional groups on a single aromatic ring. Electronegativity calculations (Huheey *et al*., 1993; Garner-O'Neale *et al*., 2003) show that nitro- (3.29 Pauling units) and chloro-groups (3.16 Pauling units) have comparable affinities for electrons. This suggests that differences in size and charge localization may be responsible for differences in regiospecificity towards the two functional groups by these enzymes. Another significant difference between

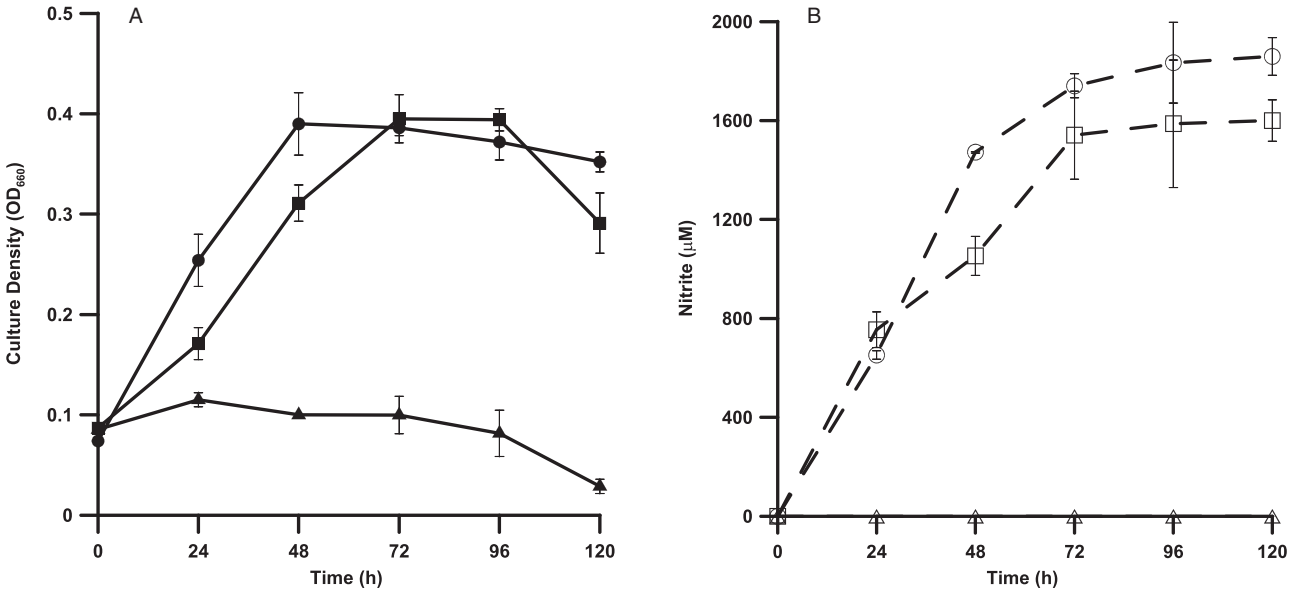

Fig. 4. Growth and nitrite production from 3CNB by *Ralstonia* sp. JS705 derivatives.
A. Growth on 3CNB.
B. Nitrite released in culture supernatants. JS705(pBBR1MCS2), vector control, indicated by triangles; JS705(pKSJ99), expressing wild-type NBDO, indicated by squares; and JS705(pKSJ114), expressing NBDO-F293Q, indicated by circles. $N = 2$; error bars indicate standard deviations.

these functional groups is the ability to participate in hydrogen bonding, and previous work indicated that proper positioning of nitrobenzene and nitrotoluenes for ring oxidation was controlled by hydrogen bonding of the nitro-group of the substrate to Asn 258 in the active site of NBDO (Friemann *et al.*, 2005; Ju and Parales, 2006). The strong preference for nitro-group removal from CNBs is consistent with previous NBDO substrate specificity studies with chloronitrotoluenes, in which chloromethylcatechols were formed as the dominant or exclusive products (Lessner *et al.*, 2002; Johnson and Spain, 2006). Similar to results with CNBs, dechlorination was only a minor activity with select isomers of chloronitrotoluenes and the simultaneous removal of both the chloro- and nitro-groups was not detected.

There are both similarities and differences in the activities of 2NTDO, NBDO, and the active-site mutants of NBDO towards nitrotoluenes and CNBs. Similar to the changes in activity with nitrotoluenes, substitution of amino acids at positions 258, 293 and 350 altered the activity and regiospecificity of NBDO towards CNBs. Substitutions at positions 258 and 350 increased oxidation at the chloro-substituted carbon (Table 1). Biotransformation of nitrotoluenes by these dioxygenases (Ju and Parales, 2006) and 2NTDO mutants with the same amino acid substitutions at positions 258 and 350 (Lee *et al.*, 2005) also led to decreased oxidation on the ring, with nitrobenzyl alcohols formed as the dominant products instead of methylcatechols. Although the asparagine at 258 is critical for positioning nitrobenzene and mononitrotoluenes in the active site of NBDO to allow attack at the nitro-substituted carbon (Friemann *et al.*, 2005; Ju and Parales, 2006), the N258V substitution only altered the product ratio by 10–20% with CNBs (Table 1). Interestingly, while the NBDO variants with substitutions at position 293 showed reduced oxidation at the nitro-group of 4-nitrotoluene (Ju and Parales, 2006), the same changes increased specificity with 2CNB and 4CNB, resulting in the formation of only chlorocatechols. The same changes did not alter the substrate specificity of NBDO towards 3CNB (wild type and 293 variants produced only 4-chlorocatechol), but resulted in production of small amounts of 3-nitrobenzyl alcohol from 3-nitrotoluene (Ju and Parales, 2006). The hydrophobic methyl-group of nitrotoluenes and the electron-withdrawing chloro-group of CNBs appear to be sufficiently different such that their presence results in differences in regiospecificity of the same NBDO enzymes towards the two classes of substrates.

By expressing the genes encoding the nitroarene dioxygenase enzyme systems in the chlorobenzene-degrading *Ralstonia* sp. JS705, we generated strains containing novel pathways for CNB degradation that have not been previously reported (Fig. 5). In these pathways, only a single enzymatic step is required to convert CNBs to chlorocatechols. Complete degradation of all three CNB isomers was achieved by channelling the chlorocatechols into the TCA cycle via the modified *ortho* pathway for chlorobenzene degradation in JS705 (Fig. 5). Of the handful of isolated strains that can grow on CNBs, partially reductive pathways appear to be the main routes for degradation in these bacteria (Fig. 1). The lone exception is a *P. stutzeri* isolate that grows on 2CNB, releasing chloride and nitrite in the process. This strain may utilize a pathway similar to that in our constructed strains, but analysis of the enzymes and pathway intermediates has not been reported (Liu *et al.*, 2005). Although 3CNB degradation has been demonstrated in a reactor system (Livingston, 1993), and complete transformation of 3CNB was achieved by the *P. putida–Rhodococcus* co-culture (Park *et al.*, 1999) and by cultures of *P. acidovorans* CA50 (Hinteregger *et al.*, 1994; Kuhlmann and Hegemann, 1997), no bacterial isolates capable of growth on 3CNB as sole carbon source have been reported to date. In contrast, the JS705 variants generated here are capable of growing directly on 3CNB as the sole carbon and energy source.

Strategies to engineer catabolic pathways have met with varying success. For example, while the individual steps of a constructed 2-chlorotoluene degradation pathway (using the *P. putida* F1 toluene dioxygenase to convert 2-chlorotoluene to 2-chlorobenzyl alcohol, the TOL plasmid upper pathway to convert 2-chlorobenzyl alcohol to 2-chlorobenzoate and either the *ortho* or modified *ortho* pathway for ring cleavage) each were functional, the constructed strains were unable to grow on 2-chlorotoluene (Haro and de Lorenzo, 2001). In contrast, a strain capable of growth on 1,2,3-trichloropropane was generated by introducing a modified haloalkane dehalogenase into a 2,3-dichloropropanol-degrading *Agrobacterium* strain (Bosma *et al.*, 1999). In designing engineered catabolic pathways, the relative activities of individual enzymes in the pathway and the appropriate regulation of the genes encoding each operon need to be considered in order to optimize flux through the pathway and avoid accumulation of toxic intermediates. Although we were successful in developing strains that grow on all three CNB isomers, several aspects of these metabolic pathways could be optimized to improve degradation. Increasing nitroarene dioxygenase activity might increase the net flux of carbon entering central metabolism and potentially allow for faster growth. Additionally, the pathway might be improved by placing the genes encoding the nitroarene dioxygenase and chlorocatechol dioxygenase under the control of a single induction system. Currently, the upper and lower pathways are differentially expressed, which may contribute to the slow growth. A CNB-inducible system would conserve metabolic energy and streamline the pathway such that expression occurs only when substrate is available for degradation. Furthermore, the low

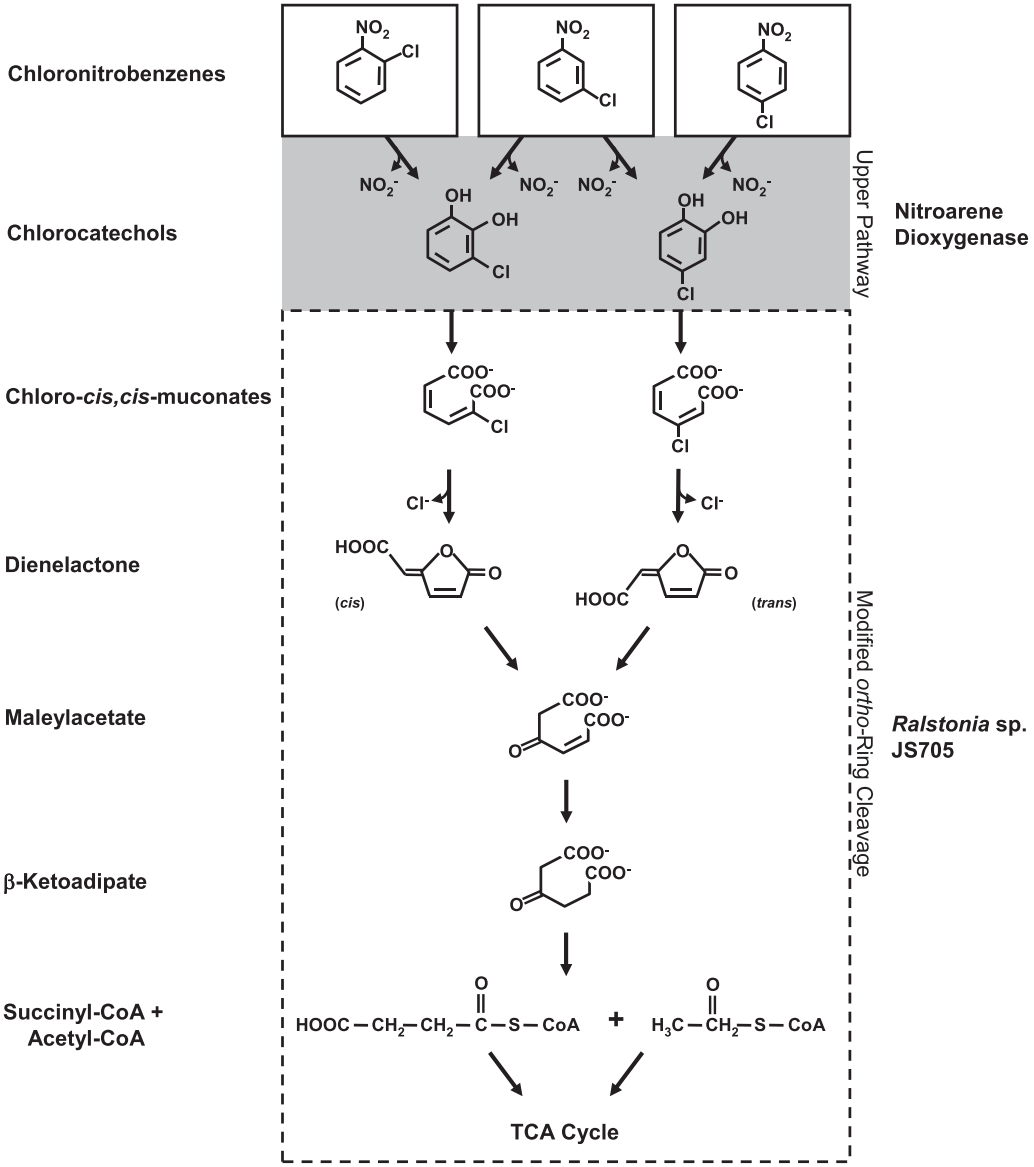

Fig. 5. Constructed pathways for CNB degradation in engineered *Ralstonia* sp. JS705 strains.

n-octanol/water partition coefficients (log P_{ow}) for CNBs [2.21–2.24 (Liu *et al.*, 1996)] indicate that these compounds may accumulate in the cytoplasmic membrane of cells, where they can compromise the proton motive force and other vital metabolic functions mediated (Sikkema *et al.*, 1995). We attempted to minimize the toxicity of CNBs by including XAD-7 resin in the cultures to bind the compounds and allow their slow release into the medium. However, introduction of an appropriate solvent efflux pump (Gallegos *et al.*, 2007) might increase the resistance of these engineered strains to the toxic effects of CNBs, and consequently improve growth and degradation. Despite these considerations, our results demonstrate the modularity of nitroarene dioxygenases and their utility in customizing the metabolic diversity of microbial strains. By taking advantage of the regiospecific oxidation at nitro-substituted carbons, we applied nitroarene dioxygenases to create bacterial strains carrying novel synthetic pathways for the effective degradation of all three isomers of CNBs, thereby expanding the available options for biotreatment of these recalcitrant environmental contaminants.

Experimental procedures

Bacterial strains, plasmids and growth media

The bacterial strains and plasmids used in this study are listed in Table 3. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth (Davis *et al.*, 1980) at 37°C unless otherwise indicated. Minimal-salts broth (MSB) (Stanier *et al.*,

Table 3. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli		
DH5α	Cloning host; *thi*	Invitrogen
S17-1 λ-*pir*	Host for plasmid mobilization; *thi*	de Lorenzo *et al.* (1993)
Pseudomonas putida F1	Wild-type toluene-degrading strain	Gibson *et al.* (1968b); Finette *et al.* (1984)
Ralstonia sp. JS705	Wild-type chlorobenzene-degrading strain	van der Meer *et al.* (1998)
Plasmids		
pBBR1MCS2	Broad-host-range vector; Km^R	Kovach *et al.* (1995)
pDTG800	pUC18 containing *ntdAaAbAcAd* from JS42; Amp^R	Parales *et al.* (1996)
pDTG850	pUC13 containing *ntdAaAbAcAd* from JS42; Amp^R	Parales *et al.* (1998b)
pDTG927	pUC19 containing *nbzAaAbAcAd* from JS765; Amp^R	Lessner *et al.* (2002)
pKSJ4	pUC13 containing *nbzAaAbAcAd* from JS765, NBDO expression plasmid; Amp^R	Ju and Parales (2006)
pKSJ10	Derivative of pKSJ4, NBDO I350T expression plasmid; Amp^R	Ju and Parales (2006)
pKSJ12	Derivative of pKSJ4, NBDO N258V expression plasmid; Amp^R	Ju and Parales (2006)
pKSJ19	Derivative of pKSJ4, NBDO I350F expression plasmid; Amp^R	Ju and Parales (2006)
pKSJ21	Derivative of pKSJ4, NBDO F293Q expression plasmid; Amp^R	Ju and Parales (2006)
pKSJ23	Derivative of pKSJ4, NBDO F293I expression plasmid; Amp^R	Ju and Parales (2006)
pKSJ25	Derivative of pKSJ4, NBDO F293H expression plasmid; Amp^R	Ju and Parales (2006)
pKSJ99	pBBR1MCS2 containing *nbzAaAbAcAd* from pDTG927; Km^R	This study
pKSJ114	Derivative of pKSJ99 containing *nbzAc* AvrII-MfeI gene fragment from pKSJ21; Km^R	This study

Amp^R, ampicillin resistance; Km^R, kanamycin resistance.

1966) containing 1% (vol/vol) modified Balch's vitamins (Gerhardt *et al.*, 1994) solution (without thiamine and *p*-aminobenzoate) was used as the basal medium for growth of other strains as described below. Modified MSB (nitrilotriacetic acid substituted with an equimolar amount of EDTA) was used for growth of *E. coli* cultures expressing recombinant dioxygenases in biotransformation reactions. For plates, LB and MSB were solidified with 1.6% (wt/vol) Difco Agar (Becton, Dickinson and Company, Sparks, MD) and 1.8% (wt/vol) Difco Agar Noble (Becton, Dickinson and Company), respectively. Antibiotics were added at the following concentrations for plasmid selection and maintenance; for *E. coli* strains, ampicillin, 200 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; for *Ralstonia* sp. JS705, kanamycin, 50 μg ml^{-1}.

Chemicals

Catechol (> 99.5%) was purchased from Aldrich (Milwaukee, WI). Chlorobenzene (> 99%) was obtained from Fisher Scientific (Fair Lawn, NJ), nitrobenzene from Acros Organics (Morris Plains, NJ), and 2NT (99%), 2CNB (99%) and 4CNB (98%) were from Avocado (Heysham, Lancashire, UK). 3CNB (98%) was from Lancaster (Pelham, NH). 4-Chlorocatechol and 4-nitrocatechol were generously provided by David T. Gibson (University of Iowa, Iowa City, IA).

3-Chlorocatechol and 3-nitrocatechol were prepared using *P. putida* F1, a toluene-degrading bacterium that is able to produce these compounds from chlorobenzene and nitrobenzene respectively (Gibson *et al.*, 1968a; Haigler and Spain, 1991). F1 was cultured aerobically in 2.8 l Fernbach flasks containing 800 ml of MSB with 40 mM pyruvate and toluene in vapour form, and incubated at 28°C on a rotary shaker at 225 r.p.m. At an OD_{660} of 1.0, cells were harvested by centrifugation, resuspended in 500 ml of 40 mM phosphate buffer (pH 7.3), transferred to a new 2.8 l Fernbach flask with 0.1% chlorobenzene or nitrobenzene, and returned to the shaking incubator. After 12 h, each clarified supernatant was extracted with 2 vols of sodium hydroxide-washed ethyl acetate, dried over anhydrous sodium sulfate and concentrated by rotary evaporation. 3-Chlorocatechol and 3-nitrocatechol were purified from the biotransformation products by flash chromatography over a column (18 mm × 45 mm) of silica gel (60 Å pore diameter). Compounds were eluted with a step gradient of chloroform–acetone, starting with 100% chloroform and transitioning to 100% acetone in 10% gradations of 100 ml of solvent, collected in 5 ml volumes and analysed by thin-layer chromatography (Resnick *et al.*, 1994). Fractions containing purified 3-chlorocatechol or 3-nitrocatechol were combined, concentrated by rotary evaporation and re-crystallized. The final products were > 99% pure as judged by GC-MS analysis.

DNA manipulations

Standard methods were used to manipulate plasmids and DNA fragments (Sambrook *et al.*, 1989). Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Plasmids were purified with a QIAprep Miniprep kit (Qiagen, Valenica, CA). DNA fragments were purified with a QIAquick Gel Extraction kit (Qiagen). Fluorescent automated DNA sequencing was carried out at the University of California, Davis sequencing facility with an Applied Biosystems 3730 automated sequencer. Nucleotide and amino acid sequence analyses were performed using the Vector NTI software suite (Invitrogen, Carlsbad, CA).

E. coli strains were transformed with plasmid DNA by standard procedures (Sambrook *et al.*, 1989). *E. coli* S17-1 λ-*pir* was used to introduce plasmids into *Ralstonia* sp. strain JS705 by conjugative matings. S17-1 λ-*pir* containing plasmids for mobilization were cross-streaked with *Ralstonia* sp. JS705 on LB plates and incubated at 30°C for 24 h. Cells

were resuspended in 10 ml of MSB, homogenized by vortexing and plated on MSB plates containing 10 mM succinate and kanamycin. Exconjugants were purified by repeated single colony isolation on the same medium. The presence of plasmids was confirmed by isolation and diagnostic restriction digests.

Construction of expression clones

To express the nitroarene dioxygenase genes in *Ralstonia sp*. JS705, plasmids pKSJ99 and pKSJ100 were constructed by subcloning the 5 kb SacI-fragments containing gene clusters *ntdAaAbAcAd* from pDTG800 and *nbzAaAbAcAd* from pDTG927 into SacI-digested pBBR1MCS2, which is a broad-host-range cloning vector carrying a kanamycin-resistance gene (Kovach *et al*., 1995). The 2.4 kb AvrII-MfeI fragment from pKSJ21 was ligated to AvrII-MfeI-digested pKSJ99 to generate pKSJ114 (Table 3). Dioxygenase genes were constitutively expressed from the *lac* promoter of the vector.

Biotransformation reactions

E. coli DH5α cultures expressing recombinant dioxygenase enzymes from plasmids pDTG850, pKSJ4, pKSJ10, pKSJ12, pKSJ19, pKSJ21, pKSJ23 or pKSJ25 (Table 3) were cultured aerobically in modified MSB medium containing 10 mM glucose, 1 mM thiamine and 200 μg ml^{-1} ampicillin as previously described (Ju and Parales, 2006). Each culture was harvested by centrifugation during exponential growth (OD_{660} 0.8–1.0), resuspended in 50 ml of 40 mM phosphate buffer (pH 7.3) containing 20 mM glucose and 25 ml volumes were dispensed into 125 ml Erlenmeyer flasks. Reaction mixtures were incubated with 0.1% (wt/vol) of 2-, 3- or 4CNB on a rotary shaker at 30°C and 200 r.p.m. After 6 h, nitrite was analysed (Smibert and Krieg, 1981; Ju and Parales, 2006) from clarified supernatants and then the supernatants were extracted with 3 vols of sodium hydroxide-washed ethyl acetate. Samples were dried over anhydrous sodium sulfate and concentrated by rotary evaporation. Products were transferred into 1-dram glass screw-cap vials, evaporated to dryness, dissolved in 0.5 ml of acetorititrile and analysed by thin-layer chromatography and GC-MS as described previously (Resnick *et al*., 1994; Ju and Parales, 2006). The GC-MS parameters used allowed the separation and detection of catechols, substituted catechols, phenols and single-ring *cis*-dihydrodiols. All products were identified by comparison with chemical standards.

Enzyme assays

Nitroarene dioxygenase activity with 2-, 3- and 4CNB was analysed in *Ralstonia* sp. JS705 carrying cloned NBDO genes. Strains were grown in 125 ml Erlenmeyer flasks containing 25 ml MSB with 10 mM succinate and kanamycin at 30°C and 200 r.p.m. At an OD_{660} between 0.8–0.9, 2 ml volumes of each culture were dispensed into glass culture tubes (20 mm $\times$ 150 mm), supplied with 1 mM substrate, and incubated at 30°C on a rotary shaker at 300 r.p.m. Nitrite released during the initial 30 min was quantified from culture supernatants as previously described (Smibert and Krieg, 1981; Ju and Parales, 2006). Concentrations of total cellular proteins were determined by the method of Bradford (Bradford, 1976) from cell pellets resuspended in 100 mM NaOH and boiled for 10 min, with bovine serum albumin as the standard.

Growth assays

Ralstonia sp. JS705 strains carrying pBBR1MCS2, pKSJ99 or pKSJ114 were tested for growth on 2CNB, 3CNB, 4CNB and 4-chlorocatechol in liquid culture. Dioxygenase genes were expressed from the *lac* promoter of the vector without the need for induction. Strains were grown in 250 ml Fernbach flasks containing 50 ml of MSB, 50 μg ml^{-1} kanamycin and 100 mg of XAD-7 beads (Sigma Chemical Co., St Louis, MO) to provide a gradual release of the CNBs or 4-chlorocatechol and minimize their toxicity as previously described for dinitrotoluenes (Nishino *et al*., 1999; 2000). The CNBs and 4-chlorocatechol were added to a final concentration of 3 mM from methanolic stock solutions to flasks containing sterilized resin and the solvent was evaporated before the addition of sterile culture medium. Growth on CNBs as nitrogen sources was tested in the same fashion except ammonium sulfate was excluded from MSB and 10 mM succinate was added as the carbon source. Flasks were inoculated with overnight cultures of *Ralstonia* strains that had been grown in the same medium, and then incubated on a rotary shaker at 28–30°C and 200 r.p.m. Culture turbidity and nitrite formation were monitored over time (as in enzyme assays).

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