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ORIGINAL ARTICLE

3-Nitrotoluene dioxygenase from *Diaphorobacter* sp. strains: cloning, sequencing and evolutionary studies

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Abstract The first step in the degradation of 3-nitrotoluene by *Diaphorobacter* sp. strain DS2 is the dihydroxylation of the benzene ring with the concomitant removal of nitro group. This is catalyzed by a dioxygenase enzyme system. We report here the cloning and sequencing of the complete dioxygenase gene with its putative regulatory sequence from the genomic DNA of *Diaphorobacter* sp. strains DS1, DS2 and DS3. Analysis of the 5 kb DNA stretch that was cloned, revealed five complete open reading frames (ORFs) encoding for a reductase, a ferredoxin and two dioxygenase subunits with predicted molecular weights (MW) of 35, 12, 50 and 23 kDa respectively. A regulatory protein was also divergently transcribed from the reductase subunit and has a predicated MW of 34 kDa. Presence of parts of two functional ORFs in between the reductase and the ferredoxin subunits reveals an evolutionary route from a naphthalene dioxygenase like system of *Ralstonia* sp. strain U2. Further a 100 % identity of its ferredoxin subunit reveals its evolution via dinitrotoluene dioxygenase like system present in *Burkholderia cepacia* strain R34. A modeled structure of oxygenase$_{3NT}$ from strain DS2 was generated using nitrobenzene dioxygenase as a template. The modeled structure only showed minor changes at its active site. Comparison of growth patterns of strains DS1, DS2 and DS3 revealed that *Diaphorobacter* sp. strain DS1 has been evolved to degrade 4-nitrotoluene better by an oxidative route amongst all three strains.

Keywords Dioxygenase · Biodegradation · Transcriptional regulator · 3-Nitrotoluene · 4-Nitrotoluene · Gene evolution

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Introduction

Nitrotoluene compounds are synthetic molecules mainly used in the production of dyes, drugs, pesticides and explosives (Booth 2007). These compounds pollute the environment due to anthropogenic activities. Some of these compounds are broken down products of large nitroaromatic compounds (Ju and Parales 2010). Nitroaromatic compounds are toxic to humans and animals alike (Watanabe et al. 2010). Microbial degradation of these compounds has gained worldwide attention because of rapid adoptability of bacterial strains for degradation of such recalcitrant compounds (Ye et al. 2004; Kulkarni and Chaudhari 2007; Arora et al. 2012). Isolation of bacterial strains which can utilize 2-nitrotoluene (2-NT) (Haigler et al. 1994; Mulla et al. 2011), 3-nitrotoluene (3-NT) (Singh

and Ramanathan 2013; Tian et al. 2012), 4-nitrotoluene (4-NT) (Haigler and Spain 1993; Rhys-Williams et al. 1993; Spiess et al. 1998) nitrobenzene (NB) (Nishino and Spain 1995) and dinitrotoluenes (Nishino et al. 2000; Spanggord et al. 1991) have been reported from contaminated soil, groundwater and waste water treatment plants. Both oxidative and reductive degradation pathways for different nitroaromatic compounds are known and have been reviewed recently by Ju and Parales (2010). In the oxidative degradation pathways, the degradation usually begins by the action of a ring hydroxylating dioxygenase enzyme system with the release of nitrite.

Dioxygenase enzyme systems are multicomponent enzymes that add two oxygen atoms to the benzene ring simultaneously to form catechol intermediates. Bacteria possess a three component dioxygenase system mostly comprising of a flavoprotein reductase, an iron-sulfur ferredoxin and a terminal iron-sulfur oxygenase. The oxygenase component is the main catalytic subunit whereas reductase and ferredoxin are involved in the transfer of electrons to the oxygenase (Gibson and Parales 2000). During recent years, quite a few bacterial nitroarene dioxygenase systems have been reported. Their genes have been cloned, sequenced and studied. These include genes encoding NB dioxygenase (NBDO) from *Comamonas* sp. strain JS765 (Lessner et al. 2002), 2-chloronitrobenzene dioxygenase from *Pseudomonas stutzeri* strain ZWLR2-1 (Liu et al. 2011), 2-nitrotoluene dioxygenase (2NTDO) from *Acidovorax* sp. JS42 (Parales et al. 1996), dinitrotoluene dioxygenase (DNTDO) from *Burkholderia cepacia* R34 (Johnson et al. 2002) and *Burkholderia* sp. strain DNT (Suen et al. 1996). Recently Ju and Parales (2011) reported a laboratory-evolved pathway for oxidative degradation of 4-NT that resulted in the formation of 4-methylcatechol as an intermediate because of accumulation of few mutations in 2NTDO of strain JS42. This strain was previously able to grow only on 2-NT and NB but not on 4-NT.

Nitroaromatic compounds are relatively new to the environment and bacterial systems have evolved to metabolize them only recently. This was possible due to either recruitment of existing enzymes of different pathways from different organisms or mutating a promiscuous enzyme system to serve new degradation functions (Copley 2009). Genes and pathways for detoxification of these recalcitrant nitroaromatic compounds are still in their intermediate stages of evolution. Study of these genes and pathways for the degradation of nitroaromatic compounds thus serves as a role model to explain the evolution in bacterial pathways (Kivisaar 2009, 2011; Liu et al. 2011). Further genetic and biochemical characterization of nitroarene degradation pathways have not only given insights into mechanism of catabolism but also revealed the ways for creation of hybrid and novel enzymes for degradation of other recalcitrant compounds (Ju and Parales 2009). Information about pathway enzymes has also helped in creation of better biocatalysts for new substrates (Keenan et al. 2004; Leungsakul et al. 2005).

Recently we reported the isolation and characterization of three *Diaphorobacter* sp. (strain DS1, DS2 and DS3) based on their ability to use 3-NT as sole source of carbon and nitrogen from a waste water treatment plant of Hindustan Organic Chemicals Limited (HOCL), India (Singh and Ramanathan 2013). Strain DS2 alone was shown to degrade multiple substrates like 2-NT, 3-NT, NB and a limited amount of 4-NT. The proposed pathway for 3-NT degradation in the strain DS2 invoked the presence of a dioxygenase enzyme as a first critical step that converts 3-NT to methylcatechol intermediates (Fig. 1). These intermediates were further degraded by well known meta ring cleavage pathways. Here we report the cloning, sequence analysis of the dioxygenase from all *three* strains thereby allowing one to infer its evolutionary aspects. The evolutionary aspects are further strengthened by homology modeling of 3NTDO from strain DS2. A comparison of growth of these strains on nitrotoluene isomers has revealed the possible evolution of strain DS1 for more efficient degradation of 4-NT. The results presented here reveal that 3NTDO from these strains falls under naphthalene family of Rieske non-heme iron dioxygenases.

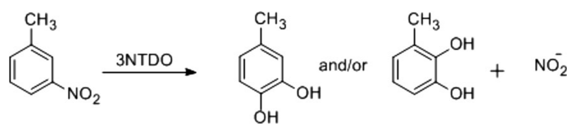

Fig. 1 First step in 3-NT degradation pathway is conversion of 3-NT to methylcatechol intermediates catalyzed by 3NTDO of *Diaphorobacter* sp. strain DS2

Materials and methods

Bacterial strains, plasmid, media and growth conditions

Diaphorobacter sp. strain DS1 (ATCC 11717), DS2 (ATCC 11718) and DS3 (ATCC 11719) were isolated on the basis of 3-NT degradation (Singh and Ramanathan 2013). *Escherichia coli* DH5α was used for transformation of plasmids and grown in Luria–Bertani (agar for solidification) medium. Primers were purchased from Bioserve Biotechnologies Pvt. Limited, Hyderabad, India. Agarose, β-agarase, Ultra Pure Genomic DNA isolation Kit, Instant TA cloning Kit and DNA ligase were purchased from M/S Bangalore Genei, Bangalore, India. Gel extraction kit was obtained from Qiagen. *Pfu* DNA polymerase was purchased from Stratagene. Nitrotoluene isomers were purchased from Qualigens, India and were of the highest purity.

Isolation and purification of the natural plasmid from strain DS2

The plasmid was isolated and separated by agarose (low melting) gel electrophoresis as reported by Singh and Ramanathan (2013). Plasmid containing gel bands were sliced out and treated with *β*-agarase and incubated for 2 h at 42 °C. This *β*-agarase treated sample was loaded to microcon ultra centrifugation filter devices [3 kDa molecular weight (MW) cut off size] and agarase enzyme was removed by centrifugation. Plasmid DNA was further purified by repeated ethanol precipitation (Sambrook and Russel 2001).

Isolation of genomic DNA

Genomic DNA was isolated from the 3-NT degrading stains by using Ultra pure genomic DNA isolation Kit. For this purpose, a minimal medium containing 200 mg/l 3-NT was inoculated with 10 % inocula and incubated at 28 °C. When biomass O.D. at 540 nm reached around 0.3, the cells were harvested, washed with STE (sodium chloride 100 mM, Tris–chloride 10 mM and EDTA 1 mM; pH 8.0) buffer and used for genomic DNA isolation (as per the kit manufacturer instructions).

Primers

In most dioxygenase gene clusters the reductase is encoded first and oxygenase small subunit is encoded last (Ju and Parales 2010). Alignment of the isofunctional dioxygenase genes revealed that primers could be designed to amplify different subunits using consensus sequences. Initially the primers to amplify different subunits as well as whole gene were designed on the basis of 2NTDO gene sequence (accession no. U49504) and worked well for amplification.

Primers used for amplification of the whole dioxygenase gene were AAF-1 (5′ATGGAACTGGTAGT AGAACCCCTC3′) and ADR-2 (5′TCACAGGAAG ACCAACAGGTTGTG3′). Initially the functional gene was PCR amplified and sequenced. Based on this sequence information, regulatory protein gene sequence with reductase gene sequence was also amplified using primer sets DS 1209-forward (5′TTATGCTTCAGAGAAAAGCTCGACG3′) DS 1201-reverse (5′TCAGACACCGATGGGATAGAA CGC3′) and sequenced. For confirmation of sequences whole 5 kb gene was also amplified and sequenced.

PCR amplification of 3NTDO genes

Composition of PCR reaction mixture was as follows: 10× PCR buffer 2.5 µl; 4 mM dNTPs 2.5 µl; 10 µM forward primer 2.5 µl; 10 µM reverse primer 2.5 µl; Template DNA 2–3 µl; Water 11-12 µl; *Pfu* DNA polymerase 1.0 µl. PCR program used was as follows: the initial temperature was kept at 95 °C for 5 min (10 min in colony PCR) to denature the DNA completely. During thermal cycles, 1 min was given for denaturation, then 1 min for annealing of the primers to the DNA strands at temperatures below 5 °C from the Tm of primer set. The extension time for *Pfu* DNA polymerase was 2 min/kb at 72 °C. After 25 cycles the reaction mixture was held for a final extension at 72 °C for 10 min. After amplification PCR product was purified by using Qiagen® gel extraction kit. The desired bands were sliced out from agarose gel and used as per kit instructions to purify the bands. The PCR amplified and purified products were extended using *Taq* DNA polymerase and dATP to add an additional adenine to the 3′ end of the PCR product and again gel purified as above.

Ligation into TA cloning vector and transformation to *E. coli* DH5α cells

The desired PCR amplified and purified gene fragments were ligated on TA cloning vector using *Instant TA cloning Kit* using the protocol given in the kit. Ligation products were transformed to *E. coli* DH5α cells by heat shock protocol (Sambrook and Russel 2001). Positive colonies having desired gene fragments were selected based on colony PCR and streaked on fresh plates. Plasmids from positive colonies were isolated, confirmed for having insert by restriction digestion and then subsequently sequenced.

DNA sequencing and sequence analysis

The entire gene cluster (~5 kb) was sequenced by using T-7, SP-6 universal and many other internal primers. Overlapping sequences were identified and removed. Probable open reading frames (ORFs) were found out using ORF finding program (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi) and its similarity with other ORFs of dioxygenases present in the database. Amino acid sequences were deduced from nucleotide sequence using the standard genetic code with the help of Expasy Translate Tool (http://web.expasy.org/translate/). Sequences similar to the deduced ORFs were retrieved from available database using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similar sequences extracted from the database were aligned using clustalw2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2).

Phylogenetic analysis

The oxygenase large subunit is the main catalytic subunit of the dioxygenase enzyme system. Sequences similar to the oxygenase large subunit (MntAc) of 3NTDO system were searched using the BLAST program of NCBI (Altschul et al. 1990) and retrieved from the data bases. A neighbor-joining phylogenetic tree (Saitou and Nei 1987) was constructed and further evaluated by bootstrap sampling (Felsenstein 1985). Phylogenetic analyses were conducted in MEGA4 software (Tamura et al. 2007).

Homology modeling of oxygenase of 3NTDO system

The oxygenase large subunit of 3NTDO from strain DS2 shares a 90 % sequence identity with NBDO from *Comamonas* sp. strain JS765. A model of 3D structure of 3NTDO was generated using MODELLAR 9.9 (http://salilab.org) using coordinates of NBDO (PDB ID, 2BMO) from strain JS765. The evaluation of the modeled structure was performed by an analysis of Ramachandran plot (Ramachandran et al. 1963), ProSA (Sippl 1993), PROCHECK (Laskowski et al. 1993), VERIFY3D (http://nihserver.mbi.ucla.edu/Verify_3D), WHATCHECK and Prove computer programs (http://nihserver.mbi.ucla.edu/SAVES/).

Growth of *Diaphorobacter* sp. strains on mononitrotoluene isomers

Growth studies of different strains on nitrotoluene isomers were performed as reported previously by Singh and Ramanathan (2013).

Detection of nitrite

Nitrite was measured by the methods as described by Haigler et al. (1994) and Singh and Ramanathan (2013).

Nucleotide sequence accession numbers

Complete nucleotide sequences of 4,997 bp fragment DNA from strain DS1, DS2 and DS3 have been submitted to the GenBank database with accession numbers KC691251, KC691252 and KC691253 respectively.

Results and discussion

Location of the 3NTDO gene

There are several reports that catabolic gene clusters are present on the plasmids in microorganisms (Kulkarni and Chaudhari 2007). However only strain DS2 revealed the presence of a large sized plasmid. The other two strains (strain DS1 and strain DS3) did not reveal the presence of a plasmid (Singh and

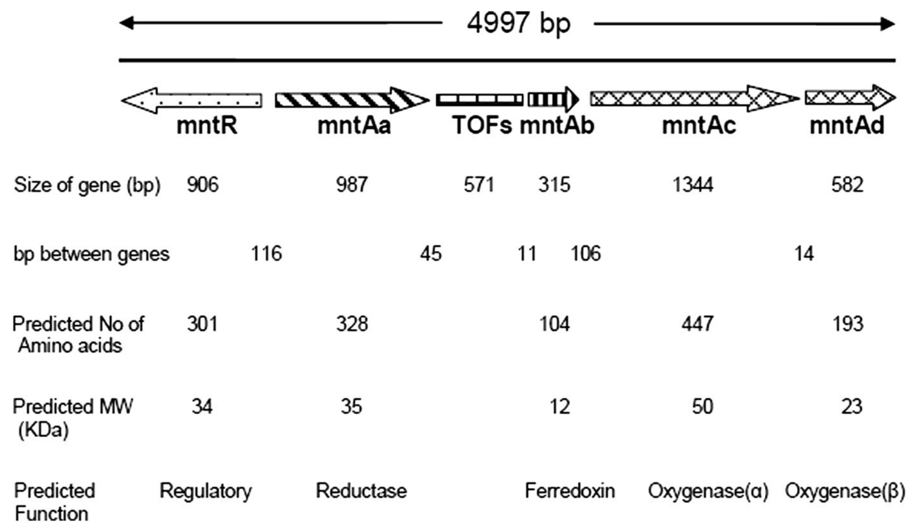

Fig. 2 Structural organization of 3NTDO genes and its regulatory protein sequence from *Diaphorobacter* sp. strain DS2. Protein designations with predicted MW and functions are also shown. *MW* molecular weight, *bp* base pairs

Ramanathan 2013). To locate the position of the 3NTDO genes in strain DS2, a PCR amplification of the oxygenase large subunit was performed using genomic DNA and plasmid DNA as a template separately. PCR amplification was observed only with chromosomal DNA and with colonies of strain DS2 but not with the plasmid DNA (Fig. S1). This indicated that the 3NTDO gene in strain DS2 is present on chromosomal DNA and not on the plasmid. Thus the role of the plasmid in the strain DS2 is still currently unknown and under investigation. The gene amplification was also observed with genomic DNA of strain DS1 and DS3.

Sequence analysis of 3NTDO

Five complete ORFs were identified by probable ORF finding program and by homology to polypeptide sequences from several previously reported multicomponent dioxygenase systems. The predicted products from ORFs were designated as a putative regulatory protein, a ferredoxin redutase subunit, a ferredoxin subunit, an oxygenase large and small subunit based on their homology. A 571 bp DNA stretch was present in between reductase and ferredoxin subunits. In its gene structure the regulatory protein is divergently transcribed from the other four ORFs. The organization of gene cluster, predicted functions and MW of the predicted polypeptides are shown in Fig. 2.

Different subunits were expressed in *E. coli* using pET21a expression vectors. 3- and 4-methylcatechols with nitrite release was observed from 3-NT when the crude extracts from the *E. coli* expressing different components (reductase, ferredoxin and oxygenase) were mixed together (details are not shown). These crude extracts of individual components alone or in combination of two were unable to form methylcatechol isomers and release nitrite from 3-NT. This confirmed that all the necessary genes have indeed been cloned.

Based on the gene structure and degree of homology, 3NTDO clearly belongs to the naphthalene dioxygenase (NDO) family of Rieske non heme iron dioxygenases (Ferraro et al. 2005). In this family of dioxygenases, the first component is the reductase which accepts the electrons from NAD(P)H and transfers them to the ferredoxin subunit which subsequently reaches the oxygenase subunits. In the gene structure reductase is followed by either two complete functional ORFs as present in Nag (NDO from *Ralstonia* sp. strain U2) or non functional ORFs (DNTDO from *Burkholderia* sp. strain DNT and *Burkholderia* sp. strain R34) or truncated part of a single ORF (2NTDO from *Acidovorax* sp. strain JS42 and NBDO from *Comamonas* sp. strain JS765). This is followed by the ferredoxin subunits and finally the oxygenase large and small subunits. A similar kind of gene arrangement as reported here for 3NTDO, has

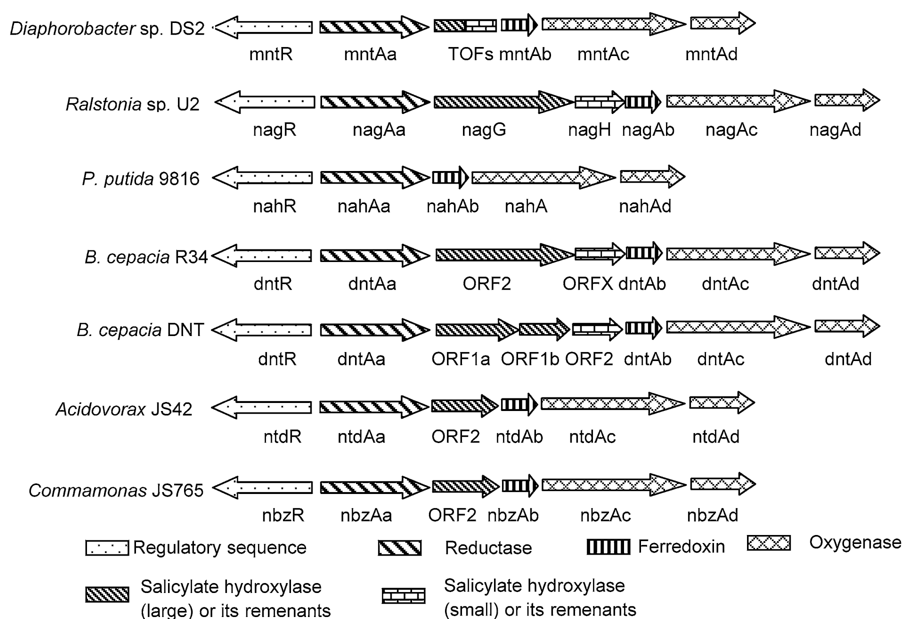

Fig. 3 Comparison of various multicomponent dioxygenases with the 3-NT dioxygenase from *Diaphorobacter* sp. strain DS2. The amino acid sequences are 66–100 % homologous affording identification of domains

also been reported earlier for 2NTDO, NBDO, DNTDO (of strain DNT) and NDO (*Ralstonia* sp. strain U2) (Fig. 3).

There is a putative regulatory protein gene sequence (*mntR*) present upstream to the functional dioxygenase gene. It is divergently transcribed from the reductase (*mntAa*) gene. The *mntR* is 906 bp long and encodes for a 301 amino acid long polypeptide. Its alignment with other closely known sequences revealed a very high level amino acid identity (Table 1) with NagR (transcriptional regulator of NDO from *Ralstonia* sp. strain U2), DntR (transcriptional regulator of DNTDO from *Burkholderia* sp. strain DNT) and NtdR (transcriptional regulator of 2NTDO from *Acidovorax* sp. JS42) (Fig. S2). Its amino acid alignment with other regulatory protein sequences showed the presence of two unique amino acids at positions 134 (Asp in strain DS2 and Asn in others) and 167 (Ser in strain DS2 and Phe in others). Additionally it differs from NagR at position 232 (Val in MntR from strain DS2 and Ile in NagR) which has been shown to be an important residue for recognition of nitroaromatic compounds by the transcriptional regulators like NtdR and Valine mutants at 232 position of NagR and DntR (Ju et al. 2009).

Reductase (MntAa from DS2) alignment with other known reductase sequences (Fig. S3) revealed that it has highest identity with DNTDO reductase followed by 2NTDO and NBDO reductase. In spite of differences, all the known sequences have conserved cysteines which coordinates [2Fe–2S] cluster. The reductase contains a $CX_4CX_2CX_{29}C$ motif that is a characteristic of plant type iron sulfur [2Fe–2S] motif (Neidle et al. 1991; Otaka and Ooi 1989; Parales et al. 1996) also present in other previously reported multi component dioxygenase systems.

Ferredoxin subunit (MntAb from DS2) alignment with other ferredoxin sequences of three component enzyme systems showed the conserved cysteines and histidines. This is present as a $CXHX_{15-17}CX_2H$ motif which is characteristic of Rieske-type iron-sulfur [2Fe–2S] proteins (Chadhain et al. 2007; Davidson et al. 1992; Neidle et al. 1991) (Fig. S4). The last 18 nucleotides of ferredoxin ORF are directly repeated just after the stop codons of ferredoxin subunits. This repeat is not present in any other reported nitroarene dioxygenase gene.

Alignment of the oxygenase large subunit (MntAc from DS2) with other homologous dioxygenases revealed that it also has two domains: a Rieske domain and a catalytic domain. The Rieske domain has a highly conserved Cys (79, 99) and His (81, 102) residues that coordinate the iron-sulfur cluster [2Fe–2S] in the iron sulfur protein (ISPα) subunit as reported in the crystal structure of NDO (Kauppi et al. 1998). Based on similarity with NDO, the catalytic center consists of a mononuclear iron coordinated by two histidine and one aspartic acid (a 2-His-1-carboxylate facial triad) present in the C-terminal

Table 1 Comparison of % amino acid identity in different subunits of gene sequence of strain DS2 and other similar enzymes

Gene	Function	Deduced no. of amino acid residue	Protein with similar sequence	% Identity (no. of. residue)	Organism	Accession no.
mntR	Regulatory	301	NtdR	98 (301)	*Acidovorax* sp. strain JS42	AAP70492
			DntR	98 (301)	*Burkholderia cepacia* R34	AAL50025
			DntR	98 (301)	*Burkholderia* sp. DNT	AAP70493
			NagR	99 (301)	*Ralstonia* sp. U2	AAG13636
mntAa	Reductase	328	NtdAa	98 (328)	*Acidovorax* sp. strain JS42	U49504
			NbzAa	98 (328)	*Comamonas* sp. strain JS765	AF379638
			DntAa	98 (328)	*Burkholderia* sp. DNT	AY936476
			DntAa	99 (328)	*Burkholderia cepacia* R34	AF169302
			NahAa	66 (328)	*Pseudomonas putida* 9816-4	AF491307
			NagAa	99 (328)	*Ralstonia* sp. U2	AF036940
			NagAa	95 (328)	*Burkholderia* sp. C3	GQ184726
mntAb	Ferredoxin	104	NtdAb	87 (104)	*Acidovorax* sp. strain JS42	U49504
			NbzAb	87 (104)	*Comamonas* sp. strain JS765	AF379638
			DntAb	100 (104)	*Burkholderia* sp. RASC	U62430
			DntAb	100 (104)	*Burkholderia cepacia R34*	AF169302
			NahAb	75 (104)	*Pseudomonas putida* 9816-4	AF491307
			NagAb	85 (104)	*Ralstonia* sp.U2	AF036940
			PahAb	85 (104)	*Comamonas testosteroni* H	AF252550
			NagAb	100 (104)	*Burkholderia* sp. C3	GQ184726
mntAc	Oxygenase (large subunit)	447	NtdAc	92 (447)	*Acidovorax* sp. strain JS42	U49504
			NbzAc	90 (447)	*Comamonas* sp. strain JS765	AF379638
			CnbAc	96 (447)	*P.Stutzeri*	GU181397
			DntAc	88 (451)	*Burkholderia* sp. RASC	U62430
			DntAc	90 (447)	*Burkholderia cepacia* R34	AF169302
			NahAc	82 (449)	*Pseudomonas putida* 9816-4	U49496
			NagAc	89 (447)	*Ralstonia* sp.U2	AF036940
			PahAc	90 (447)	*Comamonas testosteroni* H	AF252550
			NagAc	89 (447)	*Burkholderia* sp. C3	GQ184726
mntAd	Oxygenase (small subunit)	193	NtdAd	95 (194)	*Acidovorax.*sp. strain JS42	U49504
			NbzAd	90 (194)	*Comamonas* sp. strain JS765	AF379638
			CnbAd	89 (194)	*P. Stutzeri*	GU181397
			DntAd	91 (194)	*Burkholderia* sp. RASC	U62430
			DntAd	91 (194)	*Burkholderia cepacia* R34	AF169302
			NahAd	75 (194)	*Pseudomonas putida* 9816-4	U49496
			NagAd	89 (194)	*Ralstonia* sp. U2	AF036940
			PahAd	96 (194)	*Comamonas testosterone* H	AF252550

region (Ferraro et al. 2005) (Fig. S5). This triad is highly conserved in the family of Rieske nonheme iron dioxygenases. The catalytic domain of 3NTDO has been identified based on alignment of ISPα subunit of NDO (Simon et al. 1993), NBDO (Lessner et al. 2002), 2NTDO (Parales et al. 1996), DNTDO (Johnson et al. 2002) and PAH (Moser and Stahl 2001; Tittabutr et al. 2011). The oxygenase small subunit (MntAd from DS2) contains 193 amino acids. There is one deletion present in MntAd from strain DS2 whereas Thr187 is present in other dioxygenase systems (Fig. S6).

Table 2 Similarity of TOFs nucleotide sequence to selected homologs

Name of source	For first half sequences (1–273 nucleotide)			For second half sequences (274–571 nucleotide)			Accession no.
	Gene name	% Similarity	Alignment[a]	Gene name	% Similarity	Alignment[a]	
Burkholderia cepacia R34	*Orf1a*	260/261 (99)	14–274 5,753–6,013	*Orf2*	299/299 (100)	273–571 7,191–7,489	AF169302
Burkholderia sp. RASC	*Orf2*	260/261 (99)	14–274 1,697–1,957	*OrfX*	299/299 (100)	273–571 3,163–3,434	U62430
Burkholderia sp. C3 *pNag-13 catabolic gene cluster*	*Orf2*	264/274 (96 %)	1–274 1,037–1,310	*Orf3*	289/299 (97 %)	273–571 2,489–2,787	GQ184726
Comamonas testosteroni salicylate-5-hydroxylase small oxygenase subunit-like protein (pahH) gene				*pahH*	286/299 (96 %)	273–571 62–360	AF252550
Ralstonia sp. U2 plasmid pWWU2 putative	*nagG*	260/261 (99 %)	14–274 3,315–3,575	*nagH*	284/299 (95 %)	273–571 4,754–5,052	AF036940
Acidovorax sp. JS42	*Orf2*	259/261 (99 %)	14–274 1,541–1,801				PSU49504

[a] First range indicates portion of *Diaphorobacter* sp. strain DS2 derived nucleotides and second range indicates portion of similar sequences used in alignment

The different amino acid residues present near the active site of 3NTDO reveal the unique identity of the enzyme from *Diaphorobacter* sp. DS2 in comparison to other dioxygenase enzyme systems of other strains. For example- no nitroarene dioxygenase has been reported to use a histidine at position 293 but in this reported sequence such a histidine is present at this position. The two unique amino acid residues that were identified near the active site when compared to other enzyme systems are Ser249 (Gly in other enzyme systems) and Met251 (Thr in 2NTDO, Phe in NBDO, Ser in DNTDO from *Burkholderia* sp. strain DNT and Leu in NDO from *P*. sp. 9816-4). Only recently, a chloronitrobenzene dioxygense has been reported to have Met251 (Liu et al. 2011).

Apart from all five ORFs, there is a 571 bp long sequence present between the reductase and the ferredoxin subunits in the 3NTDO. Comparison of this sequence with the reported genes of similar dioxygenases revealed that this could be a remnant part of two different ORFs so are temporarily designated as truncated open reading frames (TOFs). This sequence form a probable ORFs of 312 nucleotide long leaving rest of the sequences (bp 313–571) of TOFs as non translated regions (as predicted by ORF finding server of NCBI). Comparison of nucleotide sequence of TOFs with other similar sequence homologs is shown in Table 2.

In between the reductase and ferredoxin subunits, there are two ORFs present in NDO from *Ralstonia* sp. strain U2 (*nagG* and *nagH*), DNTDO from *Burkholderia* sp. strain DNT (ORF-2 and ORF-X), and pnag from *Burkholderia* sp. strain C3 (ORF-2 and ORF-3). Both the ORFs from *Ralstonia* sp. strain U2 (*nagG* and *nagH*) encode for large and small subunits of salicylate-5-hydroxylase respectively receive electrons from a common reductase and ferredoxin subunits of NDO system. In other strains these two ORFs are either truncated or mutated to become non functional. These are not required for the catalytic action of dioxygenases. In 3NTDO system of strain DS2, the sequence present between the reductase subunit and the ferredoxin subunit is a fusion of the two truncated ORFs. Initial nucleotide sequence (1–273 bp) is identical to gene coding for N terminus of *nagG*/ORF-2/ORF-2 and rest of the nucleotides (274–571) is identical to the gene coding C terminus of *nagH*/ORF-X/ORF-3 of *Ralstonia* sp. strain U2, *Burkholderia* sp. strain DNT and *Burkholderia* sp. strain C3 respectively. In NBDO and 2NTDO, ORF-2 is truncated part of only nagG.

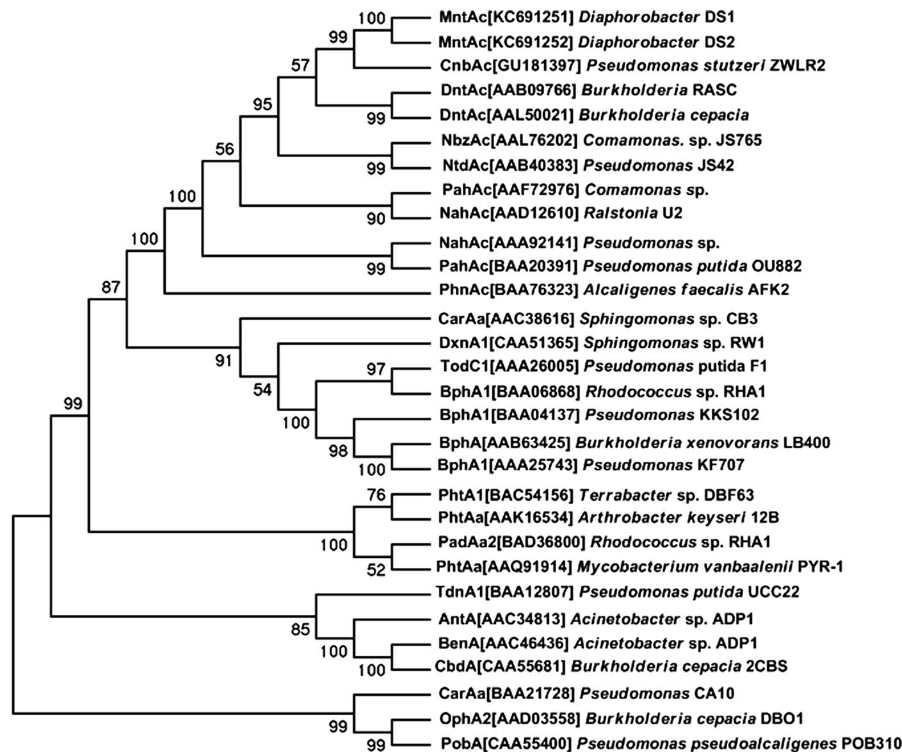

Fig. 4 Neighbor-joining phylogenetic tree based on amino acid sequences of oxygenase large subunit of 3NTDO (MntAc) with homologous sequence of other enzyme systems. Tree was made by program MEGA4. The sequence is protein-id of oxygenase followed by accession number in parentheses and then oxygenase system with source of the strain. *MntAc [AGH09221]* 3-NT dioxygenase from *Diaphorobacter* sp. DS1, *MntAc [AGH09226]* 3-NT dioxygenase from *Diaphorobacter* sp. DS2. *CnbAc [ADQ90222]* chloronitrobenzene dioxygenase from *Pseudomonas stutzeri* ZWLR2, *DntAc [AAB09766]* 2,4-dintrotoluene dioxygenase from *Burkholderia cepacia* R34, *DntAc [AAL50021]* 2,4-dintrotoluene dioxygenase from *Burkholderia cepacia*, *NbzAc [AAL76202]* NBDO from *Comamonas* sp. strain JS765, *NtdAc [AAB40383]* 2NTDO from *Acidovorax* sp. JS42, *PahAc [AAF72976]* polyaromatic hydrocarbon dioxygenase from *Comamonas testosteroni* H, *NahAc [AAD12610]* NDO from *Ralstonia* sp. U2, *NahAc [AAA92141]* NDO from *Pseudomonas* sp. 9816-4, *PahAc [BAA20391]* polyaromatic hydrocarbon dioxygenase from *Pseudomonas putida* OU882, *PhnAc [BAA76323]* phenanthrene dioxygenase from *Alcaligenes faecalis* AFK2, *CarAa [AAC38616]* carbazole dioxygenase from *Sphingomonas* sp.CB3, *DxnA1 [CAA51365]* dioxin dioxygenase from *Sphingomonas* sp. strain RW1, *TodC1 [AAA26005]* toluene dioxygenase from *Pseudomonas putida* F1, *BphA1 [BAA06868]* biphenyl dioxygenase from *Rhodococcus* sp. RHA1, *BphA1 [BAA04137]* biphenyl dioxygenase from *Pseudomonas* sp. KKS102, *BphA [AAB63425]* biphenyl dioxygenase from *Burkholderia xenovorans* LB400, *BphA1 [AAA25743]* biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707, *PhtA1 [BAC54156]* phthalate 3,4-dioxygenase from *Terrabacter* sp. DBF63, *PhtAa [AAK16534]* phthalate dioxygenase from *Arthrobacter* keyseri, *PadAa2 [BAD36800]* phthalate dioxygenase from *Rhodococcus* sp. RHA1, *PhtAa [AAQ91914]* phthalate dioxygenase from *M. vanbaalenii* PYR-1, *TdnA1 [BAA12807]* aniline dioxygenase from *P. putida* UCC22, *PhtA1 [BAC54156]:AntA [AAC34813]* anthranilate dioxygenase from *Acinetobacter* sp. ADP1, *BenA [AAC46436]* benzoate 1,2-dioxygenase from *Acinetobacter* sp. ADP1, *CbdA [CAA55681]* 2-halobenzoate 1,2-dioxygenase from *Burkholderia cepacia* 2CBS, *CarAa [BAA21728]* carbazole dioxygenase from *Pseudomanas* sp. CA10, *OphA2 [AAD03558]* phthalate dioxygenase from *Burkholderia cepacia* DBO1, *PobA [CAA55400]* phenoxybenzoate dioxygenase from *Pseudomonas pseudoalcaligenes* POB310

Phylogenetic analysis of oxygenase large subunit (MntAc) of 3NTDO

It has been shown that the oxygenase large subunit is the main catalytic subunit and is responsible for substrate specificity (Parales et al. 1998). To understand the evolutionary relationship of the oxygenase alpha subunit from *Diaphorobacter* sp. strains DS1, DS2 and DS3 with other reported oxygenase alpha subunits from the reported three component enzyme

systems, a phylogenetic analysis was performed using MEGA4 software. In the phylogenetic tree (Fig. 4), our sequences form a tight cluster with the enzymes performing similar kind of functions like NBDO, 2NTDO, DNTDO, NDO and PAH dioxygenase systems. It is more close to the α subunit of chloronitrobenzene dioxygenase. These results show that the enzymes could have evolved from common ancestors.

3NTDO sequence comparison of strains DS1, DS2 and DS3

Dioxygenases from the three *Diaphorobacter* strains were almost identical with differences at few positions. Position 326 of reductase is occupied with Ile/Ile/Ser in strains DS1, DS2 and DS3 respectively. Amino acids at positions 293, 298 and 433 in oxygenase large subunit are Ile/His, Val/Ile and Thr/Ala in strain DS1 and DS2 respectively. Oxygenase large subunit of DS2 and DS3 are identical. Oxygenase small subunits differ only at one position (124) as Thr/Ala in strain DS2 and DS3 respectively. Oxygenase small subunit of strain DS1 is identical with that of strain DS2.

Evolution of 3NTDO gene

It has been suggested in several reports that *Ralstonia* sp. strain U2 (Fuenmayor et al. 1998) is the progenitor of all the nitroarene dioxygenases, because it has the entire functional gene in its gene assembly. This seems true for the 3NTDO also. Degradation of compounds not only depends on the presence of genes for appropriate enzymes but also on their activation/repression of the expression of these genes. The regulatory protein sequence of 3NTDO (MntR) differs only at three amino acid positions from NagR of *Ralstonia* sp. strain U2 out of which two are uniquely present in strain DS2 only. DntR from DNTDO also has a regulatory sequence identical to NagR with a difference at two positions. But both these positions in DntR are in the DNA binding helix-turn-helix domain and not in the inducer binding domain (Maddocks and Oyston 2008). The sequence from NtdR and NagR differs in only five amino acids at positions 74, 169, 189, 227 and 232 (Jones et al. 2003; Lessner et al. 2003) where as the regulatory sequence of strain DS2 differs at only position 232 from NagR out of above five positions. Position 232 is important for activation of transcription by nitroaromatic compounds (Ju et al. 2009). NagR cannot recognize nitroaromatic compounds as inducer while mutant form of NagR I232 V could recognize nitrotoluenes as inducer molecules (Ju et al. 2009). Thus the regulatory protein sequence (MntR) in the *Diaphorobacter* sp. is equivalent to the NagR with a single mutation of isoleucine by a valine at 232 position in predicted inducer binding site. It provides a crucial evidence for the origin of regulatory sequence in strain DS2 from *Ralstonia* sp.U2 with a single mutation of I232 V. Moreover, nucleotide sequence comparison of the DNA sequences upstream to the reductase (downstream to transcriptional regulator gene) (Fig. S7) revealed that transcriptional regulator binding site and promoters are identical to the strains U2 and JS42 (Lessner et al. 2003).

The different components (reductase, ferredoxin and oxygenase) of 3NTDO show different levels of identity with components from similar multicomponent enzyme systems of different organisms (Table 1). Its reductase subunit (MntAa) shares a high amino acid sequence identity with those of DNTDO from *Burkholderia cepacia* (Johnson et al. 2002) and NDO of *Ralstonia* sp. strain U2 (99 %) (Fuenmayor et al. 1998) but its ferredoxin subunit (MntAb) is 100 % identical to the ferredoxin of DNTDO from *Burkholderia* sp. strain DNT and *Burkholderia* sp. strain R34. Its large oxygenase subunit (MntAc) showed more identity with chloronitrobenzene dioxygenase (CnbAc, 96 %) from *Pseudomonas stutzeri* ZWLR2-1 (Liu et al. 2011) where as the small oxygenase subunit (MntAd) with PahAd of *Comamonas testosteroni* (96 %) and NTDO from *Acidovorax* sp. strain JS42.

It is known that oxygenase large subunit controls substrate specificity. If we compare important active site residues in oxygenase large subunit (MntAc) of strain DS2 with well characterized oxygenase systems, it contains amino acid combinations of other systems, in which the sequence retains His293 which is present in NDO system of *Pseudomonas putida* 9816-4, *Ralstonia* U2, *Comamonas testosteroni* H and *Burkholderia* sp. C3, the position 350 is occupied by Valine which is reported in DNTDO of *Burkholderia* sp. strain R34. Thus, the above facts seem to indicate that 3NTDO gene in *Diaphorobacter* sp. strain DS2 came through a horizontal gene transfer from ancestors common to strains like *Ralstonia* U2 or *Burkholderia* sp. strain R34 and then its catalytic subunit

has been diversely evolved to degrade other nitroaromatic compounds.

The gene evolution in these dioxygenase systems cannot be explained by considering only one mode of evolution. All the modes of evolution (like horizontal gene transfer, selective mutation and promiscuity) are responsible for the evolution of a dioxygenase system (Kivisaar 2009, 2011). Further presence of truncated ORFs (which is not required for enzyme activity) reveals that gene evolution is in an intermediate stage of so called progressive compaction of the genes. This is in good agreement with evolution of other nitroarene dioxygese systems and merits further study.

Homology modeling of oxygenase of 3NTDO

Based on sequence identity and available crystal structure data, NBDO, PDB ID-2BMO from *Comamonas* sp. strain JS765 was taken as a template (90 % similarity) to model a three dimensional structure of the oxygenase of 3NTDO from *Diaphorobacter* sp. DS2. Initially, twelve model structures were obtained from Modeller and the best structure out of these was selected after energy minimization and Ramachandran plot analysis using all parameters of SAVES. Its PROCHECK analysis based on inspection of psi/phi Ramachandran plot (Fig. S8) showed that 91.1 % residues in core region, 8.8 % in additionally allowed region and 0.2 % in generously allowed region. Superposition of αβ heterodimer of NBDO and 3NTDO (620 Cα atoms) gave a RMS deviation of 0.31Å (α subunit RMS deviation of 0.23 Å for 437 Cα atoms, β subunit RMS deviation of 0.32 Å for 193Cα). ProSA analysis revealed that modeled structure (Fig. 5) occupied region of X-RAY predicted native protein structure of same size with Z score of -7.95. This structure was also analyzed by using the programs Verify3D, Whatcheck and Prove and all showed that the selected structure was reasonable and quite similar to the template.

According to crystal structure of NBDO, the oxygenase is an $\alpha_3\beta_3$ hetero-hexamer, where catalytic α subunits contain two domains: Rieske domain and catalytic domain. The iron–sulphur cluster [2Fe–2S] of Rieske domain is coordinated by Cys79, His81 located in between β5 and β6 and Cys99, His 103 from type I turn between β7 and β8 are conserved. In spite of having similar sequences, the β9 and α3 strands are missing from the modeled oxygenase of 3NTDO (Fig. 6a) while these are present in NBDO. The catalytic domain of non-heme Rieske family contains two histidine −1-carboxylate facial triad present in C-terminal region. On comparing the modeled structure of 3NTDO with catalytic site of NBDO template, it was observed that the structure around catalytic site was retained. Beside this, it is believed that the loop between α8 and β13 act as the lid covering the channel to the active site (221–238) (Kauppi et al. 1998) and this loop was found to be more flexible in 3NTDO than in NBDO (Fig. 6b, c). On comparing sequences, it was found that Thr223, Pro224 are uniquely present in 3NTDO while in both known nitrotoluene and NBDO, Serine is present at these positions.

Due to this change, the loop between α8 and β13 seems to be more adaptable thus allowing for access of large substrates and giving enough space for binding at the active site of the catalytic domain of oxygenase of 3NTDO. The substrate binding pocket size was also found to be larger in the modeled structure than the template (Fig. S9).

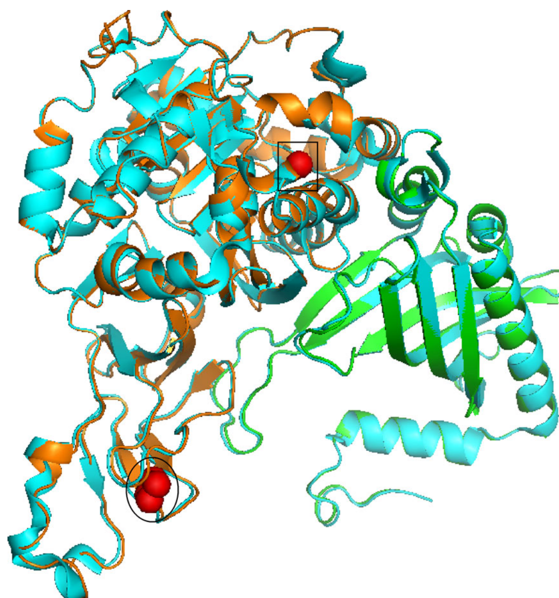

Fig. 5 Superimposed backbones of homology modeled oxygenase large (α chain turquoise) and small subunit (β chain *green*) of 3NTDO from strain *Diaphorobacter* sp. DS2 with template of oxygenase large and small subunit of NBDO (*blue*) of *Comamonas* sp. JS765. Rieske iron is *encircled* and mononuclear iron is shown under *rectangular box*. The structure was prepared using PyMOL (http://www.pymol.org). (Color figure online)

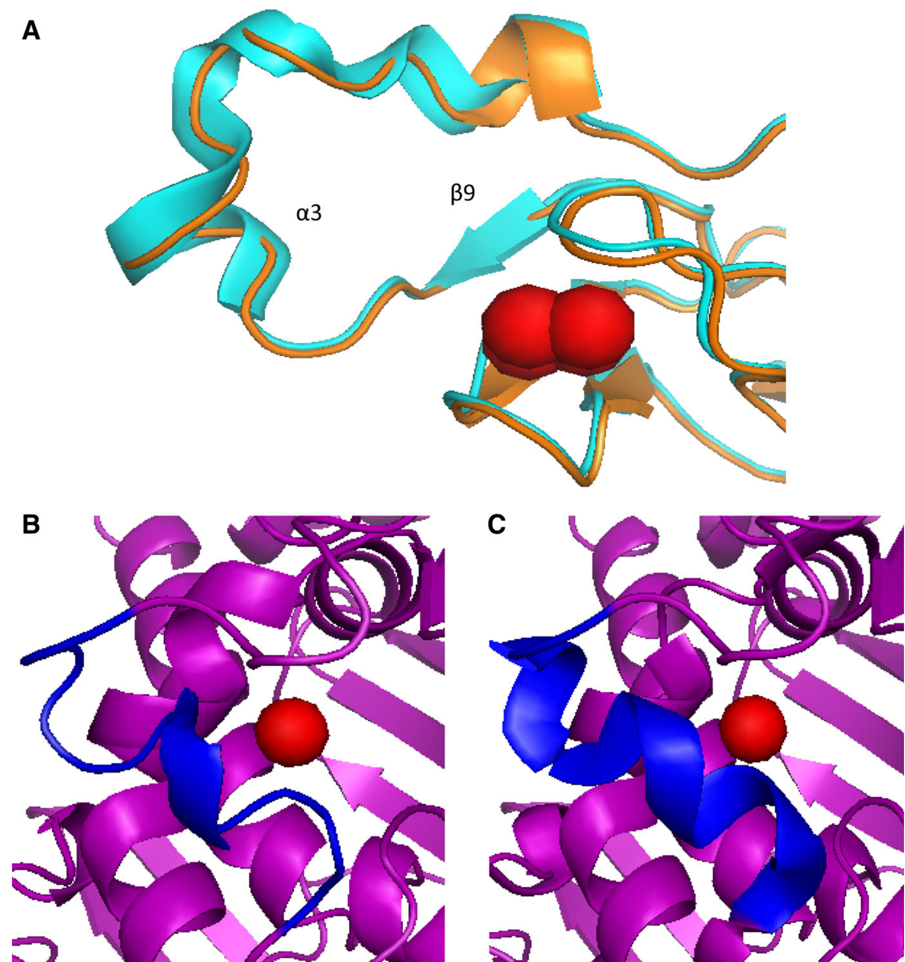

Fig. 6 **a** Cartoon representation of overlay secondary structure of Rieske cluster of NbzAc (*cyan*) of NBDO and MntAc (*orange*) of 3NTDO showing the missing β9 and α3 in a chain of oxygenase of 3NTDO; Difference near the active site lid of oxygenase of 3NTDO (**b**) and NbzAc of NBDO (**c**) are shown in *blue*. Both mononuclear and Rieske centre iron are in *red*. The structure was prepared using PyMOL (http://www.pymol.org). (Color figure online)

Growth of strain DS1, DS2 and DS3 on nitrotoluene isomers

The degradation of 3-NT along with 2-NT, NB and very small amount of 4-NT was reported by strain DS2 only. Other two isolated strains (DS1 and DS3) were evaluated for degradation of nitrotoluene isomers and compared with strain DS2. When all three strains were incubated separately at fixed concentration (150 mg/l) of 3-NT, highest amount of biomass increase was observed with strain DS2 in comparison to strain DS1 and strain DS3 (Fig. 7a). Similar results were obtained with 2-NT as a substrate too (Fig. 7b). But just reverse results were obtained when 4-NT was used as a substrate. The highest amount of biomass was observed with strain DS1 and the lowest amount was observed with strain DS2 (Fig. 7c) (Whereas with 3-NT and 2-NT as a substrate, strain DS2 showed highest biomass). This revealed that the evolution of proteins in strain DS1 is biased towards 4-NT degradation than in strain DS2 as the latter strain grows better in 3NT.

Degradation of 4-NT by a dioxygenase mechanism has not been reported by a single strain. Only recently has growth of a mutant strain of *Acidovorax* JS42 on 4-NT been reported (Ju and Parales 2011). The authors have also identified a few mutations in the catalytic subunit of 2NTDO responsible for 4-NT degradation. Wild type JS42 was not able to grow on 4-NT. Neither mutant nor wild type JS42 grew upon 3-NT. However, strain DS1 can naturally grow on all isomers of nitrotoluene with significant increase in biomass. The doubling time of all three strains in three isomers of mononitrotoluene was calculated using Roth formula (Roth V, 2006 http://www.doublingtime.com/compute.php). The doubling times of strain DS1/

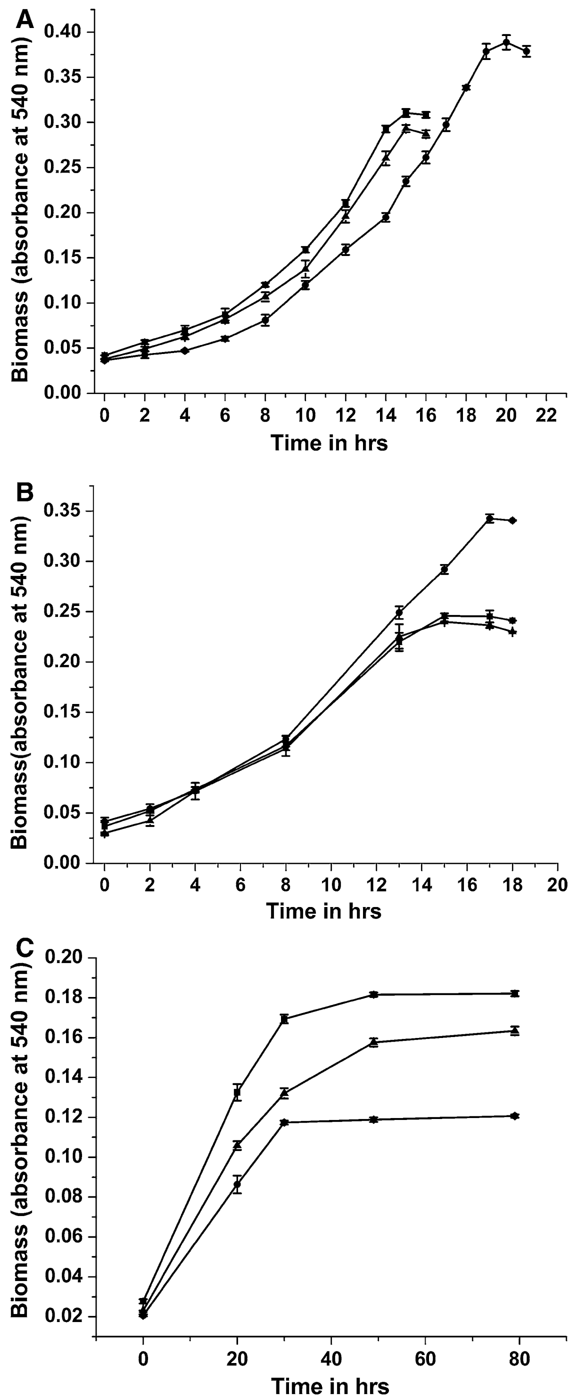

Fig. 7 Biomass growth patterns of *Diaphorobacter* strain DS1, strain DS2 and strain DS3 on 3-NT (**a**), 2-NT (**b**), and 4-NT (**c**). *Filled square*-DS1, *filled circle* DS2, *filled triangle* DS3

DS2/DS3 were 7.16/6/6.95 h on 2-NT; 5.32/5.68/5.19 h on 3-NT; and 15.02/14.49/14.96 h on 4-NT respectively.

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