Biochemical and Molecular Characterization of the Degradation Pathways of 2- and 4-Nitrobenzoates in *Cupriavidus* sp. Strain ST-14 and Construction of a Recombinant Strain ST-14::3NBA Capable of Degrading 3-Nitrobenzoate

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Text S1

Supplemental Methods

Enrichment and isolation of 2NBA-degrading bacteria. A 2NBA-degrading bacterium was isolated by enrichment culture technique by inoculating 1.0 g of soil in MSM (100 ml) supplemented with 1 g/liter of 2NBA as the sole carbon and energy source, and incubated at 28^0C on a rotary shaker (180 rpm.). When growth was observed, the enrichment process was repeated with several transfers under the same conditions. The enriched cultures were subsequently purified by plating on nutrient agar (2%, w/v) medium based on distinct colony morphology and pigmentation pattern. Each colony was then allowed to grow individually in MSM supplemented with 2NBA, as mentioned above. Finally, one strain, designated as ST-14, capable of growing in the presence of 2NBA as the sole carbon source was selected for further analysis.

Identification of strain ST-14. Strain ST-14 was identified based on its morphological, biochemical, and physiological characteristics along with 16S rRNA sequence analysis. Phase contrast microscope (Olympus IX70, Olympus Japan) was used for morphological studies whereas biochemical and physiological tests were performed according to the methods of Smibert and Krieg (1). The 16S rRNA gene was amplified using universal primers 27f and 1492r (2). The amplified product was then sequenced according to the protocol as described in the Materials and methods section while levels of 16S rRNA gene sequence identity were determined using BLAST as described earlier (main text). To explore the phylogenetic affiliation of strain ST-14, 16S rRNA sequences of the closely related representative strains were retrieved from GenBank and a phylogenetic tree was constructed using the neighbor-joining algorithm as implemented in MEGA5 (3).

Oxygen uptake. Respirometric analysis was performed by measuring O_2 uptake of whole bacterial cells in presence of MNBAs and their putative metabolic intermediates as described earlier (4). Briefly, measurements were carried out at 28ºC with a biological oxygen monitor (YSI model 5300A, Yellow Springs Instrument) comprising of Clark-type polarographic oxygen electrodes (YSI model 5331A) and sample chambers fitted within a standard bath (YSI model 5301B). 3.0 ml of sample volume was used containing a reaction mixture of 200 µl cell suspension (OD_{600} = 1), 500 µl substrate at desired concentration and 2.3 ml of potassium phosphate buffer (50 mM, pH 7.0). The reaction was performed by monitoring O_2 uptake for 5 min after injecting a suitable amount of either of the parent substrates or their probable pathway intermediates into the chamber at a final concentration of 0.1 mM. The rate of O_2 uptake was expressed as nmol of O_2 consumed per min per mg of protein which was corrected for endogenous oxygen consumption.

Rational designing of primers. Screening of fosmid clones for the presence of 3HAA dioxygenase and α-subunit of protocatechuate 3,4-dioxygenase genes were done with degenerate primers listed in Table 2. For designing of primers, nucleotide sequences of 3HAA dioxygenase gene from *P. fluorescens* KU-7 and α-subunit of PCA 3,4-dioxygenase gene from *Cupriavidus* genus present in NCBI (Gene id: 4456671 and 24154372) were used as query sequences in BLAST analysis to obtain homologous sequences of respective genes from NCBI database. Most conserved regions exhibited in the multiple sequence alignments of respective homologous gene sequences were then used to design degenerate primers.

For reverse transcription (RT) experiments, forward and reverse primers (Table 2) were obtained from the specific gene or two adjacent genes of the sequenced gene clusters in strain

ST-14 to cover intergenic spaces as well as to depict polycistronic nature of the transcripts containing the genes.

Complementation of mutant strains. In order to restore the 2NBA degradation property of the mutant strain (ST-14 ^{onb}) by complementation, a 1.6 kb fragment comprising *onbA* and *onbR* genes were PCR amplified using primers onb_comp_F and onb_comp_R (Table 2) and cloned into pBBR1MCS3_START vector, giving rise to plasmid pBBR1MCS3_START-onbAR. This construct was then introduced into ST-14 ^{onb} by electroporation. All tetracycline resistant transformants (ST-14 ^{onb}pCOMP) were selected and examined for their capability to degrade 2NBA. Similarly, for ST-14 ^{pnb} mutant strain, a 1.2 kb fragment containing *pnbA* and *pnbB* genes was PCR amplified using primers pnb_comp_F and pnb_comp_R (Table 2) and cloned into pBBR1MCS3_START vector to construct pBBR1MCS3_START-pnbAB. Introduction of this plasmid into the mutant strain ST-14 ^{pnb} was performed by electroporation. Transformants (ST-14 ^{pnb}pCOMP) selected on tetracycline plates were further checked for their ability to degrade 4NBA.

TABLE S1 Percent identity match of gene products for *onb*, *pnb* and *pob-pca* clusters of ST-14 with that of close homologous cluster/individual gene

Gene name	Close homologue (protein id, aa identity %)	Organism
onbA	2-Nitrobenzoate nitroreductase (BAF56676, 71%)	*Pseudomonas fluorescens* KU-7[a]
onbB	2-Hydroxylaminobenzoate mutase (BAF56677, 50%)	*Pseudomonas fluorescens* KU-7[a]
onbC	3-Hydroxyanthranilate 3,4-dioxygenase (BAC65311, 68%)	*Pseudomonas fluorescens* KU-7[a]
onbD	2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase (BAC65312, 69%)	*Pseudomonas fluorescens* KU-7[a]

onbE	2-Aminomuconate-6-semialdehyde dehydrogenase (BAC65304, 69%)	*Pseudomonas fluorescens* KU-7[a]
onbF	2-Aminomuconate deaminase (BAC65310, 71%)	*Pseudomonas fluorescens* KU-7[a]
onbG	4-Oxalocrotonate decarboxylase (BAC65309, 69%)	*Pseudomonas fluorescens* KU-7[a]
onbH	2-Oxopent-4-dienoate hydratase (BAC65306, 64%)	*Pseudomonas fluorescens* KU-7[a]
onbI	4-Hydroxy-2-oxovalerate aldolase (BAC65308, 78%)	*Pseudomonas fluorescens* KU-7[a]
onbJ	Acetaldehyde dehydrogenase (BAC65307, 71%)	*Pseudomonas fluorescens* KU-7[a]
onbR1	Regulatory protein (BAC65313, 55%)	*Pseudomonas fluorescens* KU-7[a]
onbX1	Patatin (WP_042887838, 85%)	*Cupriavidus necator*
onbX2	MFS transporter (WP_042305405, 74%)	*Burkholderia terrae*
onbX3	FMN reductase (WP_029043781, 81%)	*Cupriavidus* sp. WS
pnbA	*p*-Nitrobenzoate reductase (AAF01445, 78%)	*Pseudomonas pickettii* YH105[a]
pnbB	*p*-Hydroxylaminobenzoate lyase (AAF01444, 84%)	*Pseudomonas pickettii* YH105[a]
pnbR	Regulator for *p*-nitrobenzoate degradation (AAF01443, 83%)	*Pseudomonas pickettii* YH105[a]
pcaG	Protocatechuate 3,4-dioxygenase, alpha subunit (AAZ64372, 87%)	*Cupriavidus necator* JMP134[a]
pcaH	Protocatechuate 3,4-dioxygenase, beta subunit (AAZ64373, 91%)	*Cupriavidus necator* JMP134[a]
pcaB	3-Carboxy-*cis,cis*-muconate cycloisomerase (AAZ64371, 82%)	*Cupriavidus necator* JMP134[a]
pcaL	Bifunctional 4-carboxymuconolactone decarboxylase/3-oxoadipate enol-lactone hydrolase (AAZ64370, 85%)	*Cupriavidus necator* JMP134[a]
pobA	Aromatic-ring hydroxylase (AAZ64368, 86%)	*Cupriavidus necator* JMP134[a]
pcaQ	LysR-type regulator (AAZ64374, 83%)	*Cupriavidus necator* JMP134[a]
pobR	AraC family regulator (AAZ64369, 82%)	*Cupriavidus necator* JMP134[a]
pcaK	Transporter protein (AAZ64375, 85%)	*Cupriavidus necator* JMP134[a]
orf1	LysR family transcriptional regulator (WP_035876839, 96%)	*Cupriavidus* sp. amp6
orf2	Chemotaxis protein (WP_039010018, 81%)	*Cupriavidus* sp. IDO
orf3	Hypothetical protein (WP_029045346, 91%)	*Cupriavidus*sp. amp6

[a] Information of genes/proteins from these organisms were obtained from published literature.

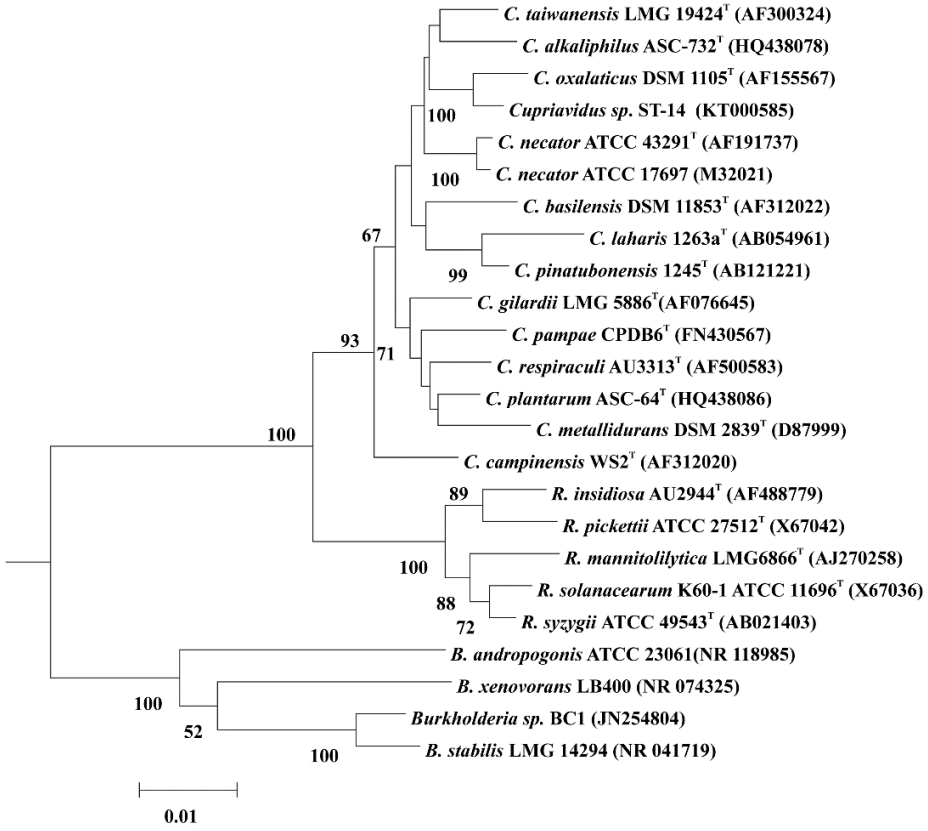

FIG S1 Phylogenetic tree based on the 16S rRNA gene, showing the relation between strain ST-14 and representative type strains of the genus *Cupriavidus* (5). The accession numbers of the sequences used are given in parentheses. Numbers at nodes indicate levels of bootstrap support based on neighbor joining analysis of 100 resampled datasets. Bootstrap values below 50% are not shown. Bar represents 0.01 substitutions per nucleotide position.

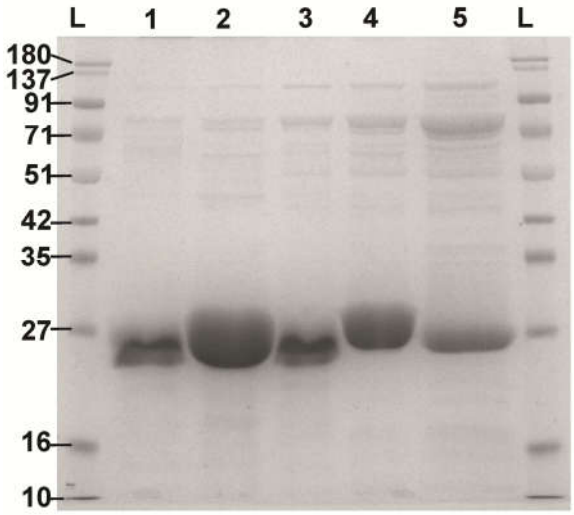

FIG S2 Heterologous expression and activity of key enzymes of 2NBA and 4NBA degradation pathways. SDS PAGE analysis of Ni-NTA column-purified overexpressed recombinant His-tagged proteins OnbA (1), OnbB (2), OnbC (3), PnbA (4), PnbB (5) and prestained protein molecular mass markers (Puregene, Genetix, India) in kDa (L).

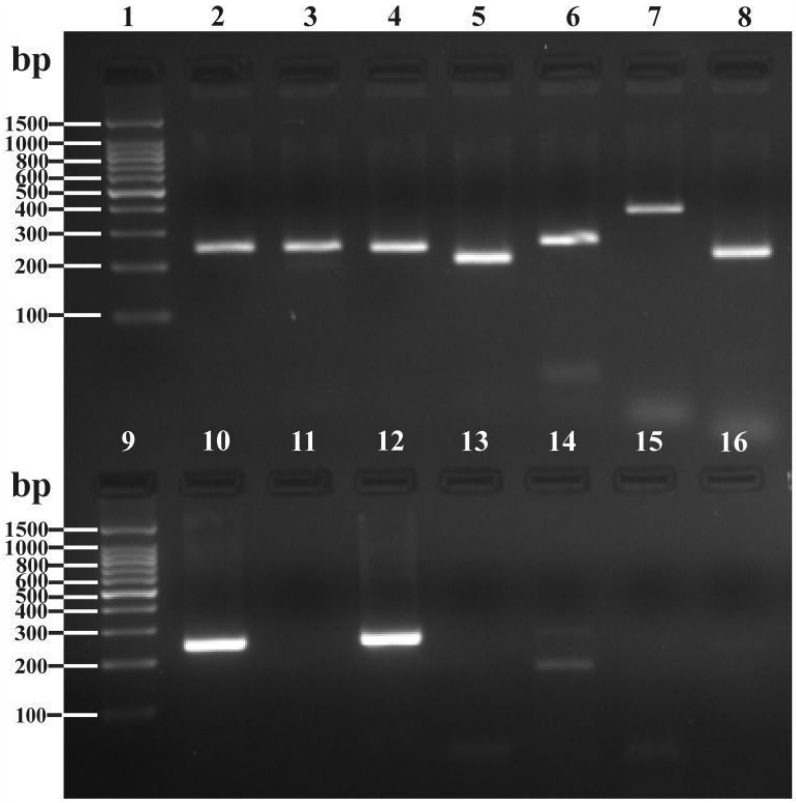

FIG S3 Agarose gel electropherogram of RT-PCR products amplified from ST-14 grown on 2NBA (lanes 4-8), 4NBA (lanes 2-3) and succinate (lanes 10-16). Lane 1 and 9: 100 bp ladder; lanes 2, 4, 10 and 12: 16S rRNA; lane 3, 11: *pnbB_pnbA*; lane 5, 13: *onbC*; lane 6, 14: *onbA_onbR1*; lane 7, 15: *onbR1_onbE* and lane 8, 16: *onbD_onbB*.

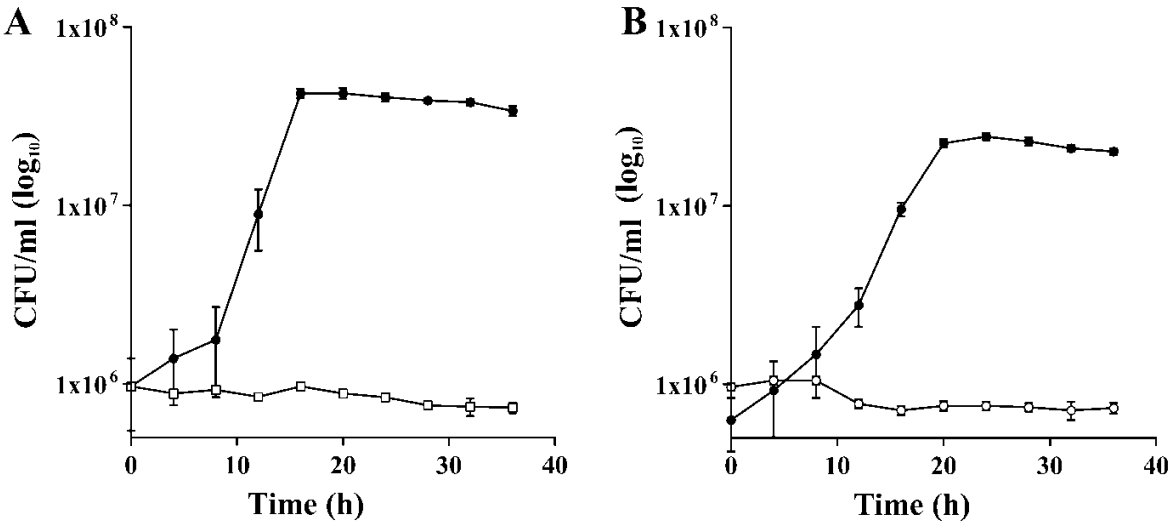

FIG S4 Growth profiles of mutant strains of ST-14 and their complemented counterparts. (A) Growth of ST-14 ^{onb} (open rectangles) and ST-14 ^{onb}pCOMP (solid circles) in presence of 2NBA (1.0 g/liter). (B) Growth of ST-14 ^{pnb} (open rectangles) and ST-14 ^{pnb}pCOMP (solid circles) in presence of 4NBA (0.5 g/liter). Vertical bars represent mean ±SD from triplicate measurements.

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