Metabolism of 2-hydroxy-1-naphthoic acid and naphthalene via gentisic acid by distinctly different sets of enzymes in *Burkholderia* sp. strain BC1

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*Burkholderia* sp. strain BC1, a soil bacterium, isolated from a naphthalene balls manufacturing waste disposal site, is capable of utilizing 2-hydroxy-1-naphthoic acid (2H1NA) and naphthalene individually as the sole source of carbon and energy. To deduce the pathway for degradation of 2H1NA, metabolites isolated from resting cell culture were identified by a combination of chromatographic and spectrometric analyses. Characterization of metabolic intermediates, oxygen uptake studies and enzyme activities revealed that strain BC1 degrades 2H1NA via 2-naphthol, 1,2,6-trihydroxy-1,2-dihydropyranthalene and gentisic acid. In addition, naphthalene was found to be degraded via 1,2-dihydroxy-1,2-dihydropyranthalene, salicylic acid and gentisic acid, with the putative involvement of the classical nag pathway. Unlike most other Gram-negative bacteria, metabolism of salicylic acid in strain BC1 involves a dual pathway, via gentisic acid and catechol, with the latter being metabolized by catechol 1,2-dioxygenase. Involvement of a non-oxidative decarboxylase in the enzymic transformation of 2H1NA to 2-naphthol indicates an alternative catabolic pathway for the bacterial degradation of hydroxynaphthoic acid. Furthermore, the biochemical observations on the metabolism of structurally similar compounds, naphthalene and 2-naphthol, by similar but different sets of enzymes in strain BC1 were validated by real-time PCR analyses.

**INTRODUCTION**

Salicylic acid or salicylate-type metabolic intermediates, namely 1-hydroxy-2-naphthoic acid (1H2NA), 2-hydroxy-1-naphthoic acid (2H1NA) and 3-hydroxy-2-naphthoic acid (3H2NA), are commonly detected in the bacterial degradation of low molecular mass polycyclic aromatic hydrocarbons (Mallick *et al.*, 2011). Diversification of metabolic pathways occurs largely with the degradation of this class of structurally homologous metabolites. Salicylic acid has been studied most widely and was reported to be metabolized by the action of either salicylate 1-hydroxylase via catechol (Pinyakong *et al.*, 2003; Simon *et al.*, 1993), salicylate 5-hydroxylase via gentisic acid (Zhou *et al.*, 2001) or direct ortho ring-cleavage dioxygenase via 2-oxohepta-3,5-dienedioic acid (Hintner *et al.*, 2001). Salicylic acid was also metabolized through gentisic acid via salicyl-CoA (Ishiyama *et al.*, 2004) and through catechol via the decarboxylase-mediated transformation product phenol (Iwasaki *et al.*, 2010). By contrast, hydroxynaphthoic acids were metabolized by the action of hydroxynaphthoic acid hydroxylase via dihydroxynaphthalene (Pinyakong *et al.*, 2000), by direct ortho ring-cleavage of 1H2NA via trans-2’-carboxybenzalpyruvate (Iwabuchi & Harayama, 1997) or by direct meta ring-cleavage of 1H2NA and 2H1NA via 2,2-dicarboxyphromene and 2-hydroxycromene-2-glyoxylic acid, respectively (Mallick *et al.*, 2007; Roy *et al.*, 2012). Nevertheless, additional catabolic pathways in the bacterial metabolism of hydroxynaphthoic acids remain to be explored.

Analogous to non-oxidative decarboxylase-mediated salicylic acid metabolism, no enzymic activity was reported in the transformation of 2H1NA. However, non-oxidative decarboxylation of various other aromatic acids such as vanillic acid (Chow *et al.*, 1999; Lupa *et al.*, 2008), 4-hydroxybenzoic acid (Lupa *et al.*, 2008; Matsui *et al.*, 2006), 3,4-dihydroxybenzoic acid (He & Wiegel, 1996) and 2,6-dihydroxybenzoic acid (Yoshida *et al.*, 2004) has been studied extensively. Some of these non-oxidative decarboxylases were shown to catalyse reversible carboxylation reactions as well (Matsui *et al.*, 2006; Yoshida *et al.*, 2004). Nevertheless, 1-naphthol was detected in the metabolism of phenanthrene, as the decarboxylated product of 1H2NA (Feng *et al.*, 2012, and references therein). By contrast,

**Abbreviations:** 1H2NA, 1-hydroxy-2-naphthoic acid; 2H1NA, 2-hydroxy-1-naphthoic acid; 3H2NA, 3-hydroxy-2-naphthoic acid; DOC, dissolved organic carbon; NBB, n-butylboronic acid.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA, nagG, nagH and nagAc gene sequences of *Burkholderia* sp. strain BC1 are JN254804.1, AB762071, AB762072 and AB762073, respectively.

One supplementary table and two supplementary figures are available with the online version of this paper.
oxygenation of the unsubstituted ring of naphthalene derivatives has occasionally been reported. 1-Naphthoic acid was transformed to 1,2-dihydroxy-1,2-dihydro-8-carboxynaphthalene by the action of 1-naphthoate-7,8-dioxygenase, a homologue of naphthalene dioxygenase (Phale et al., 1995). Likewise, 2-naphthol has been suggested to yield 1,2,6-trihydroxy-1,2-dihydroxynaphthalene by the action of a ring-hydroxylating dioxygenase (Walker & Lippert, 1965). Similarly, dioxygenation of both 1-naphthol and 2-naphthol was reported by a mutant strain of naphthalene-degrading Pseudomonas fluorescens N3 (NCIMB 40530) (Bianchi et al., 1997). Dioxygenation of the unsubstituted ring was also reported for bromonaphthalenes in Pseudomonas putida NCIB 9816/11 (Hudlicky et al., 1996), 2-methylnaphthalene in Sphingomonas paucimobilis 2322 (Dutta et al., 1998) and Pseudomonas putida NCIB 9816/11 (Deluca & Hudlicky, 1990), and 2-methoxynaphthalene by the actions of naphthalene, toluene and biphenyl dioxygenases (Whited et al., 1994).

Among the Betaproteobacteria, the genus Burkholderia has the most impressive potential for the catabolism of aromatic compounds, including polycyclic aromatic hydrocarbons (O’Sullivan & Mahenthiralingam, 2005; Pérez-Pantoja et al., 2011). Metabolic robustness of Burkholderia is attributed due to the huge coding capacity of their large multireplicon genomes, while the presence of an array of insertion sequences is considered to enhance genome plasticity and general adaptability (Lessie et al., 1996). In the present study, we describe the isolation of a Burkholderia strain capable of degrading 2H1NA via 2-naphthol with the involvement of a non-oxidative decarboxylase. Moreover, the strain exhibits capabilities for degradation of naphthalene via gentisic acid with the putative involvement of the nag operon and that of 2-naphthol via gentisic acid by employing another distinct set of enzymes of similar ability, providing insight into the diversity of catabolic pathways.

METHODS

Chemicals. Naphthalene, phenanthrene, 2H1NA, 1H2NA, 3H2NA, salicylic acid, catechol, gentisic acid, salicylaldehyde, gentisaldehyde, 2,4-dihydroxybenzoic acid, 1-naphthol, 2-naphthol, 1,6-dihydroxynaphthalene, N-butylboronic acid, diethylpyrocatechol, N-ethylmaleimide and iodoacetamide were purchased from Sigma-Aldrich. Unless stated otherwise, all other chemicals and reagents used in this study were of analytical grade and were used without further purification.

Enrichment and isolation of 2H1NA-degrading bacteria. Initially, a consortium was enriched from a soil sample collected from a naphthalene balls manufacturing waste disposal site (Panhati, West Bengal, India) in liquid mineral salt medium (MSM; Mallick et al., 2007) at 28 °C on a rotary shaker (180 r.p.m.) supplemented with 2H1NA (0.5 g L⁻¹) as the sole carbon and energy source. When growth was observed, the enrichment process was repeated with several transfers (3–4 weeks) under the same conditions and the enriched culture was subsequently purified by plating on Luria–Bertani agar medium [10 g tryptone L⁻¹, 5 g yeast extract L⁻¹, 5 g NaCl L⁻¹ and 2% (w/v) agar]. Each type of colony was isolated based on a distinct morphology and pigmentation pattern and was then allowed to grow individually in MSM supplemented with 2H1NA, as discussed above. Finally, one strain, designated BC1, capable of growing in the presence of 2H1NA as the sole carbon source was selected for further analysis.

Identification of strain BC1. Morphological features were studied using a phase-contrast microscope (Olympus IX70, Olympus Japan). Conventional biochemical tests were performed using standard methods (Smibert & Krieg, 1994). The 16S rRNA gene was amplified using universal primers 27F and 1492R (Johnson, 1994). The amplified product was sequenced according to the manufacturer’s specifications for Taq DNA polymerase-initiated cycle sequencing reactions using fluorescently labelled dyeoxynucleotide terminators with an ABI PRISM 377 automated sequencer (Perkin-Elmer Applied Biosystems). Levels of 16S rRNA gene sequence similarity were determined using BLAST version 2.2.12 of the National Center for Biotechnology Information (Altschul et al., 1990). The 16S rRNA gene sequences of representative strains of the genus Burkholderia were retrieved from GenBank and a phylogenetic tree was reconstructed using the neighbour-joining algorithm as implemented in MEGA 5 (Tamura et al., 2011) to explore the phylogenetic affiliation of strain BC1.

Culture conditions and isolation of metabolites. Cells were routinely grown at 28 °C on a rotary shaker (180 r.p.m.) in 100 ml Erlenmeyer flasks containing 25 ml MSM (pH 7.0) supplemented with (per litre) either 0.5 g 2H1NA, 1.0 g naphthalene or 0.1–0.5 g of possible pathway intermediates individually as the sole carbon source. Growth of strain BC1 was monitored by measuring the optical density at 660 nm as well as the dry weight of the culture. For measurement of cell dry weight, the culture was initially allowed to stand for 20 min to settle insoluble substrate crystals, if present at all. Then, 20 ml of culture was removed carefully from the top of the settled culture and passed through a 0.22 µm pre-weighted cellulose nitrate membrane (Whatman). For the naphthalene-grown culture, before passing through a 0.22 µm membrane, the culture was passed through glass wool to filter out remaining naphthalene crystals followed by washing with deionized water to collect any adhering cells present in the glass wool. Finally, the 0.22 µm membrane was dried at 90 °C to constant weight. To measure substrate consumption and formation of metabolite(s), 25 ml culture, incubated for different periods, was centrifuged at 8000 g for 10 min. The supernatant was then extracted three times with an equal volume of ethyl acetate and analysed by HPLC (see below).

For resting cell transformations, cells were harvested in the late exponential phase by centrifugation (8000 g, 10 min), washed twice with an equal volume of potassium phosphate buffer (50 mM, pH 7.0) and finally resuspended in the same buffer to yield an OD₆₆₀ of 1.0. 2H1NA, naphthalene or possible pathway intermediates were added at 0.1–0.5 g L⁻¹ individually to washed cell suspensions, and incubated at 28 °C for different periods up to 48 h. After incubation, the resting cell culture was centrifuged (8000 g, 10 min) and the supernatants were extracted three times with an equal volume of ethyl acetate under both neutral and acidic conditions. The organic extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. A portion of the evaporated neutral fraction residue was dissolved in 100 µl N,N-dimethylformamide, 100 µl n-butylboronic acid (NBB; 500 µg ml⁻¹ in N,N-dimethylformamide) was added and the mixture was heated to 70 °C for 15 min to form the NBB derivatives (Raschke et al., 2001). Unless stated otherwise, each experimental set was performed in triplicate.

Oxygen uptake. Respirometric measurements of whole cells in the presence of 2H1NA and various probable metabolic intermediates were carried out at 28 °C with a YSI model 5300A0 biological oxygen monitor (Yellow Springs Instrument) equipped with Clark-type polarographic oxygen electrodes (YSI model 5331A) and sample
chambers fitted within a YSI model 5301B standard bath. The sample size was 3.0 ml, and the reaction mixture contained 200 μl cell suspension (25 mg cells, wet weight), substrate (500 μl) and 2.3 ml potassium phosphate buffer (50 mM, pH 7.0). The reaction was initiated by injecting a suitable amount of the assay substrate and oxygen uptake was monitored for 5 min. The aqueous solutions of the possible intermediates of the 2H1NA or naphthalene degradation pathway were added to give a final concentration of 0.1 mM. The oxygen uptake rate was expressed as nmol min⁻¹ (mg protein)⁻¹. Rates were corrected for endogenous oxygen consumption.

**Chemical analyses.** To determine the amount of dissolved organic carbon (DOC) in the culture medium, cells and undissolved substrate, if any, were initially pelleted down by centrifugation (10 000 g, 10 min). The culture supernatant (20 ml) was then filtered through a 0.22 μm cellulose nitrate membrane followed by washing with deionized water. The combined filtrate was directly analysed using a Shimadzu TOC VCPH Total Organic Carbon Analyser, with high-temperature catalytic combustion and platinum/aluminium oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively. The depletion of 2H1NA and naphthalene by strain BC1 during its growth on the respective substrates as well as formation of 2-naphthol from 2H1NA was measured quantitatively by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C18 reversed-phase column attached to a model SIL-oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively. The depletion of 2H1NA and naphthalene by strain BC1 during its growth on the respective substrates as well as formation of 2-naphthol from 2H1NA was measured quantitatively by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C18 reversed-phase column attached to a model SIL-oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively. The depletion of 2H1NA and naphthalene by strain BC1 during its growth on the respective substrates as well as formation of 2-naphthol from 2H1NA was measured quantitatively by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C18 reversed-phase column attached to a model SIL-oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively. The depletion of 2H1NA and naphthalene by strain BC1 during its growth on the respective substrates as well as formation of 2-naphthol from 2H1NA was measured quantitatively by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C18 reversed-phase column attached to a model SIL-oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively. The depletion of 2H1NA and naphthalene by strain BC1 during its growth on the respective substrates as well as formation of 2-naphthol from 2H1NA was measured quantitatively by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C18 reversed-phase column attached to a model SIL-oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively. The depletion of 2H1NA and naphthalene by strain BC1 during its growth on the respective substrates as well as formation of 2-naphthol from 2H1NA was measured quantitatively by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C18 reversed-phase column attached to a model SIL-oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively. The depletion of 2H1NA and naphthalene by strain BC1 during its growth on the respective substrates as well as formation of 2-naphthol from 2H1NA was measured quantitatively by HPLC using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C18 reversed-phase column attached to a model SIL-oxide catalysts. For calibration, potassium hydrogen phthalate and anhydrous sodium carbonate were used as organic and inorganic standards, respectively.

**GC-MS analysis of 2H1NA and naphthalene degradation products.** was performed by using a model TraceGC Ultra column (Thermo Fischer Scientific) with a model PolarisQ mass spectrometer equipped with a DB-5MS capillary column (0.25 mm inner diameter × 30 m length × 0.25 μm film thickness). The ion source was kept at 230 °C and both the inlet temperature and the transfer line temperature were kept at 280 °C. The temperature programme comprised a 2 min hold at 70 °C, increase to 200 °C at 10 °C min⁻¹, followed by 1 min hold at 200 °C, further increase to 325 °C at 5 °C min⁻¹, and 15 min hold at 325 °C. The injection volume was 1 μl, and the carrier gas was helium (1 ml min⁻¹). The mass spectrometer was operated at an electron ionization energy of 70 eV.

**Preparation of cell-free extract.** Suspensions of cells grown individually on 2H1NA, 2-naphthol, gentisic acid, naphthalene, salicylic acid and succinate were prepared as described by Mallick et al. (2007) and were loaded into a pre-cooled French press (Constant Cell Disruption System, One Shot model) fitted with a 8.0 ml cell followed by lysis at 30 000 p.s.i. (207 MPa) for one cycle. Cell-free extracts were obtained by centrifuging the cell lysates at 20 000 g for 30 min at 4 °C. The supernatant was used as cell-free enzymes for further studies. Protein was measured by using the Lowry method with BSA as the standard (Lowry et al., 1951).

**Enzyme assays.** The enzymic transformations of various substrates were carried out by recording cell-free extract-catalysed changes in UV-visible spectra on a Cary 100 Bio UV–visible spectrophotometer (Varian Australia) using 1 cm path length quartz cuvettes. Data were analysed with the Varian Cary Win UV Scan application software. 2H1NA decarboxylase activity was monitored by measuring time-dependent spectral changes of 2H1NA to the characteristic spectrum of 2-naphthol. In addition, 2H1NA decarboxylase activity was quantitatively determined by HPLC based on the formation of 2-naphthol in a reaction mixture (final volume, 1 ml) containing 150 nmol 2H1NA, 50 mM potassium phosphate buffer (pH 7.0) and crude cell-free extract (50 μg). The reaction was started by the addition of enzyme and was incubated at 30 °C for 30 min with shaking (70 r.p.m.). For the decarboxylase inhibition assay, crude cell-free extract (50 μg), pre-incubated with diethylpyrocarbonate, N-ethylmaleimide or iodoacetamide (1 mM) for 10 min, was used as the enzyme preparation. Gentisaldehyde dehydrogenase activity was monitored spectrophotometrically in the presence of NAD⁺ by measuring the shift in absorbance maxima from 365 to 320 nm (Raison et al., 1966). Gentisate dioxygenase activity was assayed by measuring the formation of maleyl pyruvate at 330 nm (Lack, 1959) using molar extinction coefficients of 10.2 mM⁻¹ cm⁻¹ for maleyl pyruvate. Similarly, spectral changes of various possible metabolites of the naphthalene degradation pathway(s) were tested individually with cell-free extract to monitor the activities of catechol 2,3-dioxygenase (Kojima et al., 1961), catechol 1,2-dioxygenase (Dorn & Knackmuss, 1978) and salicylaldehyde dehydrogenase (Eaton & Chapman, 1992). One unit of enzyme activity is defined as the amount of activity required for the production of 1 μmol of product or disappearance of 1 μmol of substrate per minute. Specific activities were expressed as units per milligram of protein.

**Rational designing of primers for real-time analysis.** Reported nag gene sequences for the alpha subunit of naphthalene dioxygenase (nagAc), alpha subunit of salicylate 5-hydroxylase (nagG) and gen- tisate dioxygenase (nagd) were retrieved from NCBI and degenerate primers were designed on the basis of their sequence alignments. For 16S rRNA gene quantification, primers were designed based on the 16S rRNA gene sequence of strain BC1. Sequences of the PCR primers are described in Table S1 (available in the online Supplementary Material).

**DNA isolation and PCR conditions.** Bacterial DNA was isolated from 10 ml culture using a genomic DNA isolation kit (Sigma) according to the manufacturer’s instructions. PCR was carried out in a 50 μl reaction volume with the primers designed for real-time analysis, using an MJ Mini Gradient Thermal Cycler (Bio-Rad) with the following reaction conditions: 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. The PCR products were gel purified using a QIAquick Gel Extraction kit (Qiagen) and cloned in TA vector (Fermentas). The PCR products were sequenced using universal M13f and M13r primers as described above.

**RNA isolation and real-time PCR analysis.** Total RNA was isolated using Trizol (Life Technologies) reagent from mid-exponential phase cultures of strain BC1 grown on salicylic acid, 2H1NA, gentisic acid or succinate as sole carbon source, according to the manufacturer’s instructions. Residual DNA was removed by additional treatment with RNase-free DNase I (Fermentas). Total RNA was quantified spectrophotometrically and its integrity was checked by electrophoresis in 1.5% agarose gels. Reverse transcription was performed using 1 μg of hot denatured DNA-free RNA in a final volume of 20 μl with Revert Aid M-MuLV reverse transcriptase (Fermentas) and primers for the nagG, nagAc, nagd or 16s rRNA gene (Table S1), according to the manufacturer’s instructions. To estimate nag gene expression quantitatively, real-time PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems) with the individual set of RT primers using SYBR Green mix and cDNAs prepared from different sets of cells. The reaction conditions consisted of an initial activation step (5 min at 95 °C) and cycling step (denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C, for 40 cycles), after which a melt curve analysis was performed. Detection of the de-quenched probe, calculation of threshold cycles and analysis of data were performed.
using Sequence Detector software (version 1.4; Applied Biosystems). Relative changes in \( nagG \), \( nagAc \) and \( nagI \) mRNA expression were compared with succinate as control, normalized to 16S rRNA, and quantified by the 2\(-\Delta\Delta Ct \) method (Schmittgen & Livak, 2008). Mean values were obtained from triplicate experiments.

**RESULTS**

**Isolation and identification of 2H1NA-degrading bacteria**

Using an enrichment culture technique, a 2H1NA-degrading bacterial strain, designated BC1, was isolated from a naphthalene balls manufacturing waste disposal site. Cells of strain BC1 were rod-shaped, non-pigmented, non-spore-forming and Gram-negative. Tests for catalase and oxidase were positive and the isolate was capable of reducing nitrate to nitrite. The partial 16S rRNA gene sequence (1380 bp) of the isolate was determined. The phylogenetic position of strain BC1 among closely related strains in GenBank was analysed based on levels of 16S rRNA gene sequence similarity (Fig. S1). The 16S rRNA gene sequence of strain BC1 showed 99.99 and 99.42 % similarity to \( Burkholderia \) multivorans LMG 13010\(^T \) and \( Burkholderia \) latens R-5630\(^T \), respectively. Based on the biochemical tests and phylogenetic relationships, the isolate was tentatively identified as a member of the genus \( Burkholderia \).

**Growth characteristics**

The optimum temperature and pH for growth of strain BC1 on naphthalene and 2H1NA were 28 °C and pH 7.0. Optimum growth of strain BC1 in MSM was observed when 2H1NA and naphthalene were individually supplemented at a concentration of 0.5 and 1.0 g l\(^{-1} \), respectively, under most favourable growth conditions. Growth on 2H1NA was observed when a higher dose of inoculum (10 %, v/v, 5.2 \( \times \) 10\(^7 \) c.f.u. ml\(^{-1} \)) of an overnight 2H1NA-grown culture was used, 2H1NA was degraded completely within 30 h of incubation and maximum growth (225 mg l\(^{-1} \), cells dry weight) was reached at 24 h. During metabolism of 2H1NA, accumulation of 2-naphthol was evident during initial growth, and this was progressively degraded during subsequent incubation (Fig. 1a). Growth on 2H1NA was poor when a lower dose of inoculum was used, which may be explained by the toxicity of 2-naphthol on strain BC1. Apart from 2H1NA, strain BC1 was able to utilize 2-naphthol as the sole source of carbon and energy provided it was supplemented at a lower concentration, and optimum growth was observed at 0.1 g 2-naphthol l\(^{-1} \). Naphthalene, by contrast, was completely degraded and a maximum growth was reached at 20 h in MSM-naphthalene medium using 2 % inoculum (1.92 \( \times \) 10\(^7 \) c.f.u. ml\(^{-1} \)) of an overnight naphthalene-grown culture (Fig. 1b). From the growth experiments a direct association between the DOC of the culture supernatants and substrate depletion was observed: DOC contents decreased by more than 95 % at the end of 30 h for both cultures with a subsequent increase in bacterial biomass.

In addition, salicylic acid, gentisic acid and catechol, the known pathway intermediates of naphthalene metabolism, served as growth substrates for strain BC1. Although 2H1NA was reported to be one of the intermediates in the bacterial metabolism of phenanthrene (Mallick et al., 2011), strain BC1 was unable to utilize phenanthrene or its well-studied metabolite 1H2NA. Similarly, both anthracene and 3H2NA could not support growth of strain BC1.

**Oxygen uptake**

To evaluate the role of oxygenase-mediated catabolism in the 2H1NA and naphthalene pathways, the probable

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**Fig. 1.** Growth of \( Burkholderia \) sp. BC1 upon utilization of 2H1NA (a) and naphthalene (b) as the sole carbon and energy sources under optimal growth conditions. \( \bullet \), Dry weight of cell with substrate; \( \circ \), dry weight of cell without substrate; \( \checkmark \), DOC in culture supernatant; \( \bullet \), remaining substrate concentration; \( \circ \), remaining substrate concentration in uninoculated control; \( \checkmark \), 2-naphthol concentration during 2H1NA utilization. Vertical bars represent mean ± SD of triplicate measurements.
metabolic intermediates were tested for oxygen uptake ability individually by cells grown on 2H1NA, 2-naphthol, gentisic acid, naphthalene and salicylic acid (Table 1). Both 2H1NA- and 2-naphthol-grown cells revealed high oxygen uptake in the presence of 2-naphthol and gentisic acid, whereas, effectively, no oxygen consumption was noted in the presence of salicylic acid and only very low oxygen uptake was observed in the presence of catechol. By contrast, gentisic acid-grown cells respired only on gentisic acid. These results suggest the involvement of multiple operons in strain BCI for the metabolism of 2H1NA, where 2H1NA, 2-naphthol and gentisic acid are the possible inducers. Similarly, respirometric studies on naphthalene- and salicylic acid-grown cells demonstrated positive oxygen uptake on naphthalene, salicylic acid, gentisic acid and catechol, indicating salicylic acid as the inducer for the naphthalene degradation pathway in strain BCI. Moreover, oxygen uptake on both gentisic acid and catechol suggests the presence of multiple pathways for the metabolism of salicylic acid in strain BCI. Succinate-grown cells, in turn, failed to show oxygen uptake on any of the metabolic intermediates, indicating the inducible nature of both the 2H1NA and the naphthalene metabolic pathways. Interestingly, naphthalene- and salicylic acid-grown cells showed high oxygen uptake on 2-naphthol whereas both 2H1NA- and 2-naphthol-grown cells showed relatively low levels of oxygen uptake on naphthalene. A higher oxygen uptake rate on 2-naphthol with respect to naphthalene by naphthalene-grown cells may be explained by the enhanced transport of relatively polar 2-naphthol compared with naphthalene. Based on the respirometric data, the naphthalene and 2H1NA degradation pathways in strain BCI are suggested to be distinct from each other, and the respective oxygenases involved in the metabolism of naphthalene and 2-naphthol have broad substrate specificities to metabolize both of these compounds.

**Metabolite identification**

Major metabolites that were identified by GC-MS from resting cell incubation cultures (10 h) in the presence of 2H1NA are listed in Table 2. The presence of 2-naphthol (II) gentisaldehyde (I) and gentisic acid (III) in the acidified organic extract suggests a similar pathway for 2-naphthol metabolism in strain BCI as observed by Walker & Lippert (1965). In addition, 1,6-dihydroxynaphthalene (IV) and 2,6-dihydroxynaphthalene (V) were identified in the neutral organic extract, but these are reported to be produced not biochemically, but rather by abiotic dehydration of unstable 1,2,6-trihydroxy-1,2-dihydronaphthalene, a dioxygenase-mediated transformed product of 2-naphthol. Likewise, GC-MS analyses of resting cell incubation cultures (24 h) in the presence of naphthalene identified 1,2-dihydroxy-1,2-dihydronaphthalene (XVI, NBB derivative) in the neutral organic extract, and salicylaldehyde (VI), salicylic acid (VIII), gentisic acid (III) and catechol (VII) in the acidic fraction (Table 2). Unfortunately, we could not detect 1,2,6-trihydroxy-1,2-dihydronaphthalene as such or as an NBB derivative. Identification of the metabolites was based on the comparison of retention times and mass spectra (fragmentation pattern and peak intensity) of reference compounds as well as library matches of MS data and results of the published literature.

**Enzyme assays**

To decipher the pathway enzymes involved in the degradation of 2H1NA, cell-free extract of 2H1NA-grown cells was tested for 2H1NA decarboxylase, gentisaldehyde dehydrogenase and gentisate dioxygenase activities. Enzymic conversion of 2H1NA was monitored spectrophotometrically and, based on the transformation profile, formation of 2-naphthol was observed, validating the involvement of a non-oxidative aromatic decarboxylase (Fig. 2a). The 2H1NA decarboxylase activity leading to the formation of 2-naphthol was further validated by HPLC analysis. The specific activity calculated for 2H1NA decarboxylase in the crude cell-free extract was 0.0183 U mg\(^{-1}\). Interestingly, activity of this oxygen-insensitive decarboxylase was inhibited by a histidine residue-specific inhibitor, diethylpyrocarbonate, while thiol group inhibitors such as N-ethylmaleimide and iodoacetamide failed to inhibit the decarboxylase activity.

Again, the cell-free extract of 2H1NA-grown cells exhibited NAD\(^{+}\)-dependent transformation of gentisaldehyde to

<table>
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<tr>
<th>Substrate</th>
<th>Naphthalene</th>
<th>Salicylic acid</th>
<th>2H1NA</th>
<th>2-Naphthol</th>
<th>Gentisic acid</th>
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<td>Naphthalene</td>
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</tbody>
</table>

Table 1. Oxygen uptake rates with various compounds by resting-cell suspensions of *Burkholderia* sp. strain BC1 grown on different substrates

All values are corrected for endogenous O\(_2\) uptake. ND, Not detected.
gentisic acid with a shift of \( \lambda_{\text{max}} \) from 365 to 320 nm due to the formation of gentisic acid (\( \lambda_{\text{max}} 320 \) nm), suggesting the presence of gentisaldehyde dehydrogenase (Fig. 2b). As gentisaldehyde itself has absorbance around 340 nm, formation of NADH (\( \lambda_{\text{max}} 340 \) nm) from NAD\(^+\) during this transformation could not be exclusively detected. Again, when the cell-free extract of 2H1NA-grown cells was incubated with gentisic acid, a rapid shift of the absorption maximum from 320 to 330 nm was observed (Fig. 2c), confirming the ring-cleavage of gentisic acid with the formation of maleyl pyruvate (\( \lambda_{\text{max}} 330 \) nm). Cell-free extract of gentisic acid and 2-naphthol-grown cells exhibited both gentisate dioxygenase and gentisaldehyde dehydrogenase activities, together with very weak salicylaldehyde dehydrogenase activity. When 2,4-dihydroxybenzoic acid (one of the possible intermediates which may be generated from the degradation of 2-naphthol via 1,2,7-trihydroxy-1,2-dihydronaphthalene by the action of 2-naphthol-7,8-dioxygenase) was incubated with 2H1NA-grown cell-free extract, no change in spectral pattern was observed. This confirms the metabolism of 2-naphthol in strain BC1 via dioxygenation at the 5,6-positions of the unsubstituted ring rather than that at the 7,8-positions (Walker & Lippert, 1965).

### Table 2. GC-MS data for the metabolites of 2H1NA and naphthalene obtained from the organic extracts of the resting-cell incubation of *Burkholderia* sp. strain BC1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention time (min)</th>
<th>( m/z ) of major ion peaks (%)(^*)</th>
<th>Suggested compound(^†)</th>
</tr>
</thead>
<tbody>
<tr>
<td>From 2H1NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11.35</td>
<td>138 (M(^+), 100), 137 (74), 81 (33), 92 (14), 57 (15)</td>
<td>Gentisaldehyde</td>
</tr>
<tr>
<td>II</td>
<td>12.98</td>
<td>144 (M(^+), 100), 116 (34), 115 (58), 89 (8), 63 (7)</td>
<td>2-Naphthol</td>
</tr>
<tr>
<td>III</td>
<td>12.16</td>
<td>154 (M(^+), 52), 136 (100), 108 (32), 52 (44), 51 (30), 53 (27)</td>
<td>Gentisic acid</td>
</tr>
<tr>
<td>IV</td>
<td>16.95</td>
<td>160 (M(^+), 100), 131 (36), 132 (21), 103 (12), 77 (6)</td>
<td>1,6-Dihydroxynaphthalene</td>
</tr>
<tr>
<td>V</td>
<td>16.82</td>
<td>160 (M(^+), 100), 131 (40), 132 (29), 134 (16) 103 (14), 77 (9)</td>
<td>2,6-Dihydroxynaphthalene</td>
</tr>
<tr>
<td>From naphthalene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>6.31</td>
<td>122 (M(^+), 100), 121 (92), 104 (18), 93 (29), 76 (27), 65 (42), 50 (11)</td>
<td>Salicylaldehyde</td>
</tr>
<tr>
<td>VII</td>
<td>8.45</td>
<td>110 (M(^+), 100), 92 (10), 81 (11), 64 (33), 63 (13)</td>
<td>Catechol</td>
</tr>
<tr>
<td>VIII</td>
<td>11.04</td>
<td>138 (M(^+), 55), 120 (100), 92 (60), 64 (26)</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>III</td>
<td>12.16</td>
<td>154 (M(^+), 52), 136 (100), 108 (32), 52 (44), 51 (30), 53 (27)</td>
<td>Gentisic acid</td>
</tr>
<tr>
<td>XVI</td>
<td>14.82</td>
<td>228 (M(^+), 100), 171 (22), 144 (21), 128 (20), 116 (30)</td>
<td>1,2-Dihydroxy-1,2-dihydronaphthalene(^‡)</td>
</tr>
</tbody>
</table>

*Ion abundance percentages are shown in parentheses.  
†Identification was based on the match of mass spectra (fragmentation and peak intensity) and GC retention times with data for authentic compounds.  
‡Analysis was performed after derivatization of neutral organic extract with NBB.

**Fig. 2.** Spectral changes during transformation of 2H1NA (a), gentisaldehyde (b) and gentisic acid (c) by cell-free extract of *Burkholderia* sp. strain BC1 grown on 2H1NA. The sample and reference cuvettes contained 50 mM potassium phosphate buffer (pH 7.0) in 1 ml volume. The sample cuvette also contained 150 nmol 2H1NA (a), 220 nmol gentisaldehyde and 10 nmol NAD\(^+\) (b), and 156 nmol gentisic acid (c). Spectra were recorded every minute after the addition of 50 \( \mu \)g protein to both cuvettes. The up and down arrows indicate increasing and decreasing absorbance, respectively.
Both 1H2NA and 2H1NA have been described as the metabolic intermediates in the pathway of bacterial degradation of phenanthrene (Mallick et al., 2011) where the hydroxynaphthoic acids are metabolized either by the actions of hydroxynaphthoic acid hydroxylase or by direct ring-cleavage dioxygenases. However, there is no report on the mono- or dioxygenation of the unsubstituted ring of hydroxynaphthoic acids, leading to a productive catabolic pathway. Although in the degradation of phenanthrene by Sphingomonas sp. strain P2 1,5-dihydroxy-2-naphthoic acid was identified as the dead-end product, reportedly formed by specific loss of water from the cis-5,6-dihydroxylated derivative of 1H2NA, it was assumed to be catalysed by phenanthrene dioxygenase in strain P2 (Pinyakong et al., 2000).

Strain BC1, characterized as a member of the genus Burkholderia, although incapable of utilizing phenanthrene, was found to assimilate 2H1NA by a non-conventional pathway via 2-naphthol and gentisic acid. The enzymic transformation of 2H1NA to 2-naphthol is attributed to the action of a non-oxidative decarboxylase. Similar enzyme activity was noted for the decarboxylation of other aromatic acids (Gu et al., 2011; Matsui et al., 2006; Yoshida et al., 2004). Formation of 1-naphthol from 1H2NA has previously been documented in the metabolism of phenanthrene (Feng et al., 2012, and references therein), but this is the first report on the bacterial metabolism of 2H1NA via 2-naphthol. As 2-naphthol is toxic to various bacterial species (Balashova et al., 1999; Mallick & Dutta, 2008), less is known regarding its metabolism in bacteria compared with its non-hydroxylated counterpart. However, there are few reports on the bacterial utilization of 2-naphthol as sole carbon source (Tao et al., 2007; Walker & Lippert, 1965), but due to its dose-dependent toxicity, the bacterial metabolism of a high concentration of 2-naphthol appears to be complicated. Recently, degradation of 2-naphthol in wastewater has been proposed by the synergistic activities of Fusarium proliferatum and Bacillus subtilis (Zang & Lian, 2009). Hydroxylation of 2-naphthol at both the substituted
and the unsubstituted ring was reported for toluene/\-xylene monooxygenase (Tao et al., 2005). In the present study, strain BC1 utilized 2-naphthol as a sole carbon source and only grew at a substrate concentration of 0.25 g l\(^{-1}\) or less, exhibiting optimum growth at 0.1 g 2-naphthol l\(^{-1}\). Nonetheless, strain BC1 can efficiently utilize naphthalene as sole carbon source. Based on the identification of metabolites by GC-MS, oxygen uptake and enzymic studies, the respective pathways of degradation of 2H1NA and naphthalene by strain BC1 have been proposed (Fig. 4).

In the 2H1NA metabolic pathway, 2-naphthol is metabolized via a dioxygenase attack at the 5,6-carbon positions of the unsubstituted aromatic ring, yielding 1,2,6-trihydroxy-1,2-dihydronaphthalene. This is supported by the GC-MS-based identification of its dehydrated products, namely 1,6- and 2,6-dihydroxynaphthalenes (Table 2), formed in the ratio of 8 : 2. 1,2,6-Trihydroxy-1,2-dihydronaphthalene could not be detected as the hydroxylic group of the compound is considered to act as an acid self-catalyst leading to the elimination of water (Bianchi et al., 1997).

The region-specific dioxygenation of 2-naphthol as mentioned above is similar to that proposed for the metabolism of 2-naphthol by a \textit{Pseudomonas} species (Walker & Lippert, 1965). The 2H1NA pathway then proceeds via the formation of 1,2,6-trihydroxynaphthalene, which is a substrate for ring-cleavage dioxygenase, subsequently yielding gentisaldehyde and gentisic acid. The presence of 2H1NA decarboxylase, gentisaldehyde dehydrogenase and gentisate dioxygenase in the cell-free extract of 2H1NA-induced cells further supports this observation. The inability of 2H1NA- or 2-naphthol-grown cells of strain BC1 to respire on salicylic acid rules out the metabolism of 2-naphthol via 1,2-dihydroxyaphthalene and convergence with the classical naphthalene pathway.

Nevertheless, 2H1NA decarboxylase activity was solely observed in the cell-free extract of 2H1NA-grown cells but not in preparations grown/induced in the presence of 2-naphthol, gentisic acid or the structural analogues of 2H1NA, namely 1H2NA, 3H2NA and salicylic acid, suggesting the decarboxylase is 2H1NA-inducible in strain BC1. Inhibition of enzyme activity by a histidine residue-specific inhibitor indicates that 2H1NA decarboxylase in strain BC1 possibly belongs to the metal-dependent hydrolase superfamily as reported in \(\gamma\)-resorcylic decarboxylase (Ishii et al., 2004; Yoshida et al., 2004), 2,3-dihydroxybenzoate decarboxylase (Kamath et al., 1989) and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (Li et al., 2006). Decarboxylases

\[ \text{Fig. 4. Proposed pathways for the degradation of 2H1NA and naphthalene by \textit{Burkholderia} sp. strain BC1. The transient intermediates or ring cleavage products which have not been detected are shown in square brackets. Chemical designations: I, gentisaldehyde; II, 2-naphthol; III, gentisic acid; VI, salicylaldehyde; VII, catechol; VIII, salicylic acid; IX, 2H1NA; X, 1,2,6-trihydroxy-1,2-dihydronaphthalene; XI, 1,2,6-trihydroxynaphthalene; XII, 2-hydroxy-4-(2\'-oxo-5-hydroxy-3,5-cyclohexadienyl)-buta-2,4-dienoate; XIII, cis-2,5-dihydroxybenzaldehyde; XIV, cis-2,5-dihydroxybenzylic acid; XV, naphthalene; XVI, 1,2-dihydroxy-1,2-dihydronaphthalene; XVII, 1,2-dihydroxynaphthalene; XVIII, 2-hydroxy-4-(2\'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate; XIX, cis-o-hydroxybenzylic acid; XX, cis,cis-muconic acid.} \]
belonging to this superfamily are insensitive to oxygen and their subunit molecular masses are in the range 28–39 kDa (Ishii et al., 2004; Li et al., 2005; Yoshida et al., 2004). A 12.5% SDS-PAGE analysis revealed a differentially over-expressed ~32 kDa intense band in the crude enzyme preparations of 2H1NA-grown cells in comparison with that in the 2-naphthol- or succinate-grown preparations (Fig. S2), supporting the inducible characteristic and the type of decarboxylase.

Naphthalene, by contrast, was degraded via salicylic acid using the traditional naphthalene assimilation pathway in strain BC1 (Fig. 4). Degradation of naphthalene has been extensively studied in both Gram-negative and Gram-positive bacteria and is dissimilated via either catechol or gentisic acid (Mallick et al., 2011). However, identification of both gentisic acid and catechol in the spent medium of naphthalene-grown culture suggests the simultaneous presence of both salicylate 1-hydroxylase and salicylate 5-hydroxylase in strain BC1, similar to that observed in Burkholderia sp. C3 (Tittabutr et al., 2011). The presence of isofunctional genes may be advantageous for proper funnelling of metabolite, such as salicylate to central metabolism, thereby preventing its toxic accumulation (Lanfranconi et al., 2009). Biochemical studies showed that naphthalene is degraded via the salicylic acid–gentisic acid route, using a putative nag pathway, induced by salicylic acid, similar to that found in Ralstonia sp. U2 and Polaromonas sp. CJ2 (Jeon et al., 2006; Zhou et al., 2001). However, in the presence of salicylic acid at 1 g L⁻¹, salicylate 1-hydroxylase and catechol ortho cleavage pathway enzymes are induced to a much higher level than observed during naphthalene degradation, where salicylic acid is present as a transient intermediate. These observations point towards the concentration-dependent expression of salicylate-inducible pathways in the metabolism of salicylic acid in strain BC1. Inducer concentration-dependent differential expression of metabolic pathways has also been described in a number of studies on bacterial metabolism of aromatic compounds (Murray & Williams, 1974; Song et al., 2000). Bacteria degrading naphthalene via the gentisate route are relatively rare, with few strains reported (Grund et al., 1992; Jeon et al., 2006; Zhou et al., 2001), while adding a repertoire of bacteria to this group may help in understanding the abundance of nag-like genes in nature, in contrast to its most frequently available nah counterpart.

Metabolism of the structurally similar compounds naphthalene and 2-naphthol in strain BC1 seems to be analogous to the involvement of the sets of degradative enzymes. Biochemical results suggest that these two pathways are mutually exclusive in this strain, which was confirmed by real-time PCR analysis, where nag gene expression was observed only in the presence of salicylic acid but not with 2H1NA or gentisic acid. Moreover, real-time analysis suggests the presence of multiple gentisate dioxygenases in strain BC1 apart from NagI, similar to that observed in Polaromonas sp. CJ2 (Lee et al., 2011). Further characterization of the catabolic operons will shed light on the regulatory mechanisms involved in the metabolism of 2H1NA and naphthalene/salicylic acid in this strain.

ACKNOWLEDGEMENTS

Financial support for this work was provided by Bose Institute, Kolkata, India. P. P. C. and J. S. were supported by fellowships from the Council of Scientific and Industrial Research, Government of India, while S. B. was supported by fellowships from Bose Institute.

REFERENCES

characterization and gene cloning of a novel enzyme catalyzing carboxylation of resorcinol, 1,3-dihydroxybenzene, from Rhizobium radiobacter. Biochem Biophys Res Commun 324, 611–620.


Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using


Edited by: E. Madsen