Naphthalene metabolism and growth inhibition by naphthalene in *Polaromonas naphthalenivorans* strain CJ2

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This study was designed to characterize naphthalene metabolism in *Polaromonas naphthalenivorans* CJ2. Comparisons were completed using two archetypal naphthalene-degrading bacteria: *Pseudomonas putida* NCIB 9816-4 and *Ralstonia* sp. strain U2, representative of the catechol and gentisate pathways, respectively. Strain CJ2 carries naphthalene catabolic genes that are homologous to those in *Ralstonia* sp. strain U2. Here we show that strain CJ2 metabolizes naphthalene via gentisate using respirometry, metabolite detection by GC-MS and cell-free enzyme assays. Unlike *P. putida* NCIB 9816-4 or *Ralstonia* sp. strain U2, strain CJ2 did not grow in minimal medium saturated with naphthalene. Growth assays revealed that strain CJ2 is inhibited by naphthalene concentrations of 78 µM (10 p.p.m.) and higher, and the inhibition of growth is accompanied by the accumulation of orange-coloured, putative naphthalene metabolites in the culture medium. Loss of cell viability coincided with the appearance of the coloured metabolites, and analysis by HPLC suggested that the accumulated metabolites were 1,2-naphthoquinone and its unstable auto-oxidation products. The naphthoquinone breakdown products accumulated in inhibited, but not uninhibited, cultures of strain CJ2. Furthermore, naphthalene itself was shown to directly inhibit growth of a regulatory mutant of strain CJ2 that is unable to metabolize naphthalene. These results suggest that, despite being able to use naphthalene as a carbon and energy source, strain CJ2 must balance naphthalene utilization against two types of toxicity.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment, and the toxic and carcinogenic characteristics of PAHs have motivated efforts to develop bioremediation technologies to eliminate sources of PAH exposure (Samanta *et al.*, 2002; Xue & Warshawsky, 2005). Naphthalene is commonly used as a model for studying PAH metabolism by bacteria because it is the simplest and most soluble PAH, as well as a frequent constituent of PAH-contaminated environments (Peters *et al.*, 1999). Studies of naphthalene degradation have shown that there are two primary pathways for the metabolism of naphthalene, which are distinguished by the conversion of salicylate to catechol or gentisate. Metabolism of naphthalene via catechol has been studied extensively in two *Pseudomonas* species that carry the archetypal catabolic plasmids NAH7 (in *P. putida* G7) and pDTG1 (in *P. putida* NCIB 9816-4) (Dennis & Zylstra, 2004; Yen & Serdar, 1988; Sota *et al.*, 2006). The *nah* dissimilatory genes are organized into two operons: one

encoding the enzymes involved in the conversion of naphthalene to salicylate (naphthalene degradation upper pathway) and another encoding the conversion of salicylate to catechol, followed by *ortho*- or *meta*-cleavage to TCA cycle intermediates (naphthalene degradation lower pathway) (Yen & Gunsalus, 1982). In contrast, the *nag* genes found in *Ralstonia* sp. strain U2 are organized in a single operon and encode the alternative gentisate pathway, which converts naphthalene to fumarate and pyruvate via salicylate and gentisate (Fuenmayor *et al.*, 1998; Zhou *et al.*, 2002).

Although the genetics and biochemistry of naphthalene metabolism have been studied in depth, the inhibition of naphthalene metabolism due to the toxicity of naphthalene and naphthalene metabolites has received less attention (Auger *et al.*, 1995; Murphy & Stone, 1955; Park *et al.*, 2004). Naphthalene has been reported to be directly toxic to *P. putida* G7 under oxygen- and nitrogen-limited conditions, although it is unclear if the toxicity was due to naphthalene or a naphthalene metabolite (Ahn *et al.*, 1998). Naphthalene was also shown to be toxic to non-naphthalene-degraders, as *P. putida* KT2440 showed reduced viability in soil amended with naphthalene (Park

Abbreviations: DMF, *N,N*-dimethylformamide; PAH, polycyclic aromatic hydrocarbon.

et al., 2004), and a bioluminescent strain of *Escherichia coli* showed reduced bioluminescence in the presence of naphthalene (Lee *et al.*, 2003).

Polaromonas naphthalenivorans CJ2 was isolated from coal-tar-contaminated freshwater sediment for its ability to use naphthalene as its sole carbon source, and was shown to be responsible for *in situ* naphthalene degradation by field-based stable isotope probing (Jeon *et al.*, 2003). The naphthalene catabolic genes in strain CJ2 are homologous to the *nag* operon of *Ralstonia* sp. strain U2, but the genes are arranged in two separate clusters, each with its own regulatory protein (Jeon *et al.*, 2006). In the present investigation, we show that strain CJ2 metabolizes naphthalene via the gentisate pathway using respirometry, GC-MS and cell-free enzyme assays. In addition, we explore the inhibitory effects of naphthalene and its metabolites on the growth of strain CJ2.

METHODS

Bacterial strains. *P. naphthalenivorans* strain CJ2 was isolated from freshwater sediment from a coal-tar-waste contaminated site (Jeon *et al.*, 2003). Naphthalene degraders *P. putida* NCIB 9816-4 (Yen & Serdar, 1988) and *Ralstonia* sp. strain U2 (Zhou *et al.*, 2002) were included in this study for comparison. Strain CJN110 is a mutant of strain CJ2 in which the regulatory gene *nagR* has been disrupted by a suicide vector carrying a kanamycin resistance gene (Jeon *et al.*, 2006).

Growth assays. All growth assays were conducted with Stanier mineral salts media (MSB; Stanier *et al.*, 1966) supplemented with pyruvate (18 mM), glucose (6 mM) and/or naphthalene crystals. The aqueous concentration of naphthalene was lowered with Amberlite XAD7 resin. Naphthalene binds to the XAD7 resin, which acts as a reservoir, and maintains the aqueous concentration of naphthalene below saturation (230 μM or 30 p.p.m.) while supplying enough naphthalene to support growth over the course of the experiment (Morasch *et al.*, 2001). Naphthalene (6–14 mg) was added to 6 ml MSB containing 0.1 g XAD7 before the tubes were sealed with Teflon-lined stoppers and autoclaved. To grow strain CJ2 in MSB saturated in naphthalene, a large inoculum (>7 %, v/v) from a dense starter culture was required. Viability of test bacteria was measured by enumerating c.f.u. from 10 μl drops of serially diluted cultures on R2A agar. Kanamycin was added to media at 40 $\mu\text{g ml}^{-1}$ when appropriate.

Respirometry. Bacterial cells were grown to late exponential phase in MSB with naphthalene (0.5 % w/v) and harvested (6000 g). Cells were washed twice and resuspended in 50 mM KH_2PO_4 buffer (pH 7.4). Endogenous respiration was measured with an oxygen electrode (Rank Brothers) after adding 2 ml washed cell suspension to the incubation chamber, and oxygen consumption was recorded following the addition of 20 mM substrate dissolved in *N,N*-dimethylformamide (DMF). Respiration upon addition of DMF only was included as a control treatment.

Enzyme assays. Bacterial cells were grown in 500 ml MSB with 18 mM pyruvate or 4 mM salicylate to late exponential phase, and cells were harvested by centrifugation (6000 g) and washed once with 50 mM KH_2PO_4 buffer (pH 7.4). Cells were resuspended in buffer to a concentration of 0.1 g ml^{-1} and sonicated three times for 30 s with 60 s cooling intervals. Cellular debris was cleared by centrifugation (25 000 g for 45 min) and protein concentrations were determined with the Bio-Rad Bradford assay. Enzyme assays were performed with

a minimum of 50 μg protein. The enzyme substrates, gentisate (0.19 μmol) or catechol (0.9 μmol), were added in 1 ml volumes. Gentisate 1,2-dioxygenase activity was measured spectrophotometrically by measuring the increase of absorption at 334 nm, due to the formation of maleylpyruvate, and was calculated with an extinction coefficient of 10 800 $\text{M}^{-1} \text{cm}^{-1}$ (Crawford *et al.*, 1975). Catechol 1,2-dioxygenase activity was assayed by measuring the increase in absorption at 260 nm, due to the formation of *cis,cis*-muconate, and was calculated with an extinction coefficient of 16 800 $\text{M}^{-1} \text{cm}^{-1}$ (Dorn & Knackmuss, 1978). Catechol 2,3-dioxygenase activity was measured by monitoring the increase in 2-hydroxy-*cis,cis*-muconate semialdehyde at 375 nm, and was calculated with an extinction coefficient of 33 000 $\text{M}^{-1} \text{cm}^{-1}$ (Cerdan *et al.*, 1994).

GC-MS detection of naphthalene pathway metabolites. Cells (500 ml) grown on naphthalene or pyruvate were harvested, washed twice and resuspended in 10 ml 50 mM KH_2PO_4 buffer. Fifty microlitres of 200 mM naphthalene in DMF was added to 5 ml of the concentrated cell suspension and metabolism was allowed to proceed for 15 min. Suspensions were acidified with HCl to pH 1.5 and extracted twice with 7.5 ml ethyl acetate, which was then dried over anhydrous Na_2SO_4 and concentrated under an atmosphere of N_2 to a volume of 300 μl . Extracts were derivatized with 25 μl BSTFA [bis(trimethylsilyl)trifluoroacetamide] for 5 min prior to GC-MS analysis and quantified using external standard calibration curves.

HPLC analysis of naphthalene and inhibitory compounds. Naphthalene concentrations were determined by HPLC. Samples (100 μl) were taken from culture tubes and immediately fixed in an equal volume of methanol. Samples were filtered through tightly packed glass wool prior to injection, and naphthalene was separated using a PAH-Hypersil column (150 $\times$ 4.6 mm; Keystone Scientific) and a Waters model 590 HPLC pump with a mobile phase of methanol/water (65:35) at a flow rate of 1 ml min^{-1} . Eluents were monitored by UV-visible light detection (ABI analytical absorbance detector; Spectroflow 757) at a wavelength of 270 nm and quantified using an external standard calibration curve.

Putative naphthalene metabolites were separated with a Hypersil BDS-C18 column (4.6 $\times$ 250 mm; Agilent) at a flow rate of 1 ml min^{-1} with a Spectra-Physics model SP8800 ternary HPLC pump. The mobile phase consisted of 20 % methanol and 80 % 40 mM acetic acid for 10 min, followed by a linear increase in methanol to 50 % over 10 min; after 5 min the methanol concentration was linearly increased to 90 % over 10 min and held for 15 min. Eluents were detected at 260 nm using a UV-Vis detector (SPD-10A VP; Shimadzu). Aged 1,2-naphthoquinone solution (50 μM final concn) was prepared by diluting a filter-sterilized 10 mM methanolic stock, aseptically adding it to sterile MSB and allowing the solution to shake for 48 h.

RESULTS

Respirometry

To better understand the physiology of *Polaromonas naphthalenivorans* CJ2, we compared strain CJ2 with archetypal naphthalene-degrading bacteria *Pseudomonas putida* NCIB 9816-4 and *Ralstonia* sp. strain U2. The simultaneous induction of enzymes involved in naphthalene metabolism was compared in the three strains by measuring oxygen uptake after the introduction of potential naphthalene metabolites to washed cell suspensions (Fig. 1). The simultaneous adaptation assays were aimed at confirming that strain CJ2 metabolizes naphthalene via gentisate as

Fig. 1. Respiration by three strains of bacteria (*P. putida* NCIB 9816-4, *Ralstonia* sp. strain U2 and *P. naphthalenivorans* strain CJ2) grown on naphthalene and exposed to naphthalene (NAP) and five potential metabolites [salicylaldehyde (SALD), salicylate (SAL), catechol (CAT), gentisate (GEN) and DMF]. The oxygen uptake experimental values represent the mean $\pm$ SD of triplicate experiments. All values are corrected for endogenous respiration of cells.

suggested by the sequence homology of the naphthalene catabolic operons in *Ralstonia* sp. strain U2 and strain CJ2, as well as to reveal potential physiological effects of the naphthalene catabolic gene arrangement in strain CJ2.

Consistent with the established metabolic pathway in *P. putida* NCIB 9816-4, these cells rapidly consumed oxygen after addition of naphthalene, salicylaldehyde, salicylate and catechol, while oxygen depletion was not rapid after the addition of gentisate (Fig. 1). *Ralstonia* sp. strain U2 cells consumed oxygen when exposed to naphthalene, salicylaldehyde and salicylate. *Ralstonia* sp. strain U2 cells, our positive control for the gentisate pathway, did not display enhanced respiration when exposed to gentisate (Fig. 1). Strain CJ2 consumed oxygen after addition of naphthalene, salicylaldehyde and salicylate. However, similar to *Ralstonia* sp. strain U2, neither gentisate nor catechol led to rapid depletion of oxygen. *Ralstonia* sp. strain U2 is known to lack a gentisate transporter (Xu *et al.*,

2006), and if the same is true for strain CJ2, this accounts for the absence of enhanced oxygen uptake in the presence of gentisate. Regardless, the lack of enhanced oxygen uptake in the presence of catechol by *Ralstonia* sp. strain U2 and strain CJ2 reveals a key distinction between these two strains and *P. putida* NCIB 9816-4.

Metabolite detection

We measured potential naphthalene metabolites with GC-MS to support the respirometry data and to eliminate ambiguity regarding the role of gentisate or catechol in the naphthalene metabolic pathway in strain CJ2. Again, *P. putida* NCIB 9816-4 and *Ralstonia* sp. strain U2 served as controls for the catechol and gentisate pathways, respectively. Table 1 shows the detected metabolites from washed cell suspensions that were incubated for 15 min after addition of naphthalene in DMF to a final concentration of 2 mM.

As expected for the *nah* pathway expressed by *P. putida* NCIB 9816-4, we detected *cis*-1,2-dihydroxydihydronaphthalene, 1,2-dihydroxynaphthalene, salicylaldehyde, salicylate and catechol. Likewise, as expected for the *nag* pathway expressed by *Ralstonia* sp. strain U2, we detected *cis*-1,2-dihydroxydihydronaphthalene, 1,2-dihydroxynaphthalene, salicylaldehyde, salicylate and gentisate. As suggested by the respirometry data, the metabolite profile for strain CJ2 is consistent with the genetic similarities between *Ralstonia* sp. strain U2 and strain CJ2. We detected *cis*-1,2-dihydroxydihydronaphthalene, 1,2-dihydroxynaphthalene, salicylaldehyde, salicylate and gentisate in strain CJ2 cell suspensions. The presence of gentisate and the absence of catechol provides clear evidence that strain CJ2 metabolizes naphthalene through the gentisate pathway.

Dioxygenase assays

To provide further evidence for naphthalene metabolism by the gentisate pathway in strain CJ2, dioxygenase assays were conducted with cell-free extract from induced (salicylate-grown) and non-induced (pyruvate-grown) strain CJ2, *P. putida* NCIB 9816-4 and *Ralstonia* sp. strain U2. Gentisate 1,2-dioxygenase activity was detected in

Table 1. Survey of metabolites produced by *P. putida* NCIB 9816-4, *Ralstonia* sp. strain U2 and strain CJ2

The experimental values represent the mean $\pm$ SD of triplicate experiments. 1,2-DHDN, *cis*-1,2-Dihydroxydihydronaphthalene; 1,2-DHN, 1,2-dihydroxynaphthalene; ND, not detected.

Bacteria	Naphthalene metabolites detected [$\mu\text{g (mg protein)}^{-1}$]					
	1,2-DHDN	1,2-DHN	Salicylaldehyde	Salicylate	Catechol	Gentisate
<i>P. putida</i> NCIB 9816-4	2870 $\pm$ 1280	1450 $\pm$ 600	2.3 $\pm$ 2.2	1520 $\pm$ 86	3.4 $\pm$ 2.7	ND
<i>Ralstonia</i> U2	1260 $\pm$ 560	1060 $\pm$ 90	4.0 $\pm$ 3.3	390 $\pm$ 130	ND	2.0 $\pm$ 1.3
Strain CJ2	2000 $\pm$ 1320	450 $\pm$ 300	3.6 $\pm$ 1.4	8.7 $\pm$ 6.4	ND	2.6 $\pm$ 2.2

strain *Ralstonia* sp. strain U2 when the cultures were induced by salicylate [$0.150 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$], but not when grown on pyruvate [$0.004 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]. Although gentisate dioxygenase activity was not as pronounced in strain CJ2, activity was induced by salicylate [$0.032 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$] and not by pyruvate [$0.003 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]. Neither *ortho*- nor *meta*-cleavage of catechol was observed in strain CJ2 or *Ralstonia* sp. strain U2. As expected, *P. putida* NCIB 9816-4 displayed catechol 1,2-dioxygenase activity and no gentisate dioxygenase activity.

Growth inhibition of strain CJ2 by naphthalene

Although strain CJ2 was isolated under naphthalene vapour as a carbon source, a comparison of growth on naphthalene in minimal medium (MSB) between strain CJ2, *P. putida* NCIB 9816-4 and *Ralstonia* sp. strain U2 revealed unusual growth characteristics in strain CJ2. Both

P. putida NCIB 9816-4 and *Ralstonia* sp. strain U2 grew equally well in MSB broth supplemented with 18 mM pyruvate or saturated with naphthalene crystals ($\sim 230 \mu\text{M}$). In contrast, strain CJ2 was unable to grow in MSB broth supplemented with naphthalene crystals (Fig. 2a), but grew well in MSB broth with 18 mM pyruvate (Fig. 2b), demonstrating that the inhibition of growth is substrate-specific. Additionally, the lack of growth in MSB supplemented with naphthalene was accompanied by the appearance of a light orange colour in the medium, indicating the possible accumulation of naphthalene metabolites. Incidentally, growth of strain CJ2 in MSB saturated with naphthalene was influenced by cell density, as growth occurred if a large inoculum ($>7\%$) of a dense starter culture was used (data not shown).

Naphthalene concentration and growth inhibition

To test the effect of naphthalene concentration on growth inhibition, we grew strain CJ2 in MSB broth with 0.1 g XAD7 resin that had been equilibrated with different masses of naphthalene. This procedure has been shown to buffer the aqueous naphthalene concentration at levels proportional to the added mass (Morasch *et al.* 2001). Once an equilibrium was established between XAD7-bound naphthalene and aqueous naphthalene, strain CJ2 was inoculated (initial cell density $1.56 \times 10^6 \text{ c.f.u. ml}^{-1}$) into media with initial aqueous concentrations of naphthalene of approximately 23, 40, 55, 78 or 100 μM , as determined by HPLC analysis of aqueous naphthalene in sterile medium. Growth was strongly inhibited when the initial naphthalene concentration in the medium was higher than 78 μM . However, strain CJ2 grew well when the initial naphthalene concentration of the medium was at

Fig. 2. Growth of naphthalene-degrading bacteria on mineral salts medium with naphthalene crystals (a) or pyruvate (b). Unlike *P. putida* NCIB 9816-4 (■) and *Ralstonia* sp. strain U2 (▲), which grew equally well with pyruvate and naphthalene, strain CJ2 (●) grew only in MSB with pyruvate but not in MSB saturated with naphthalene. The experimental values represent the mean $\pm$ SD of triplicate experiments.

Fig. 3. Growth of strain CJ2 in MSB medium with initial aqueous naphthalene concentrations (23–100 μM) buffered by XAD7 resin. Growth was inhibited at concentrations of 78 μM and higher. The experimental values represent the mean $\pm$ SD of triplicate experiments. □, 23 μM ; ▲, 40 μM ; ●, 55 μM ; ■, 78 μM ; ○, 100 μM naphthalene.

or below 55 μM (Fig. 3). This indicates that growth inhibition by naphthalene is concentration-dependent, with optimum growth occurring at $\sim 40 \mu\text{M}$ naphthalene and inhibitory effects manifest above $\sim 78 \mu\text{M}$ naphthalene.

Direct inhibition by naphthalene

The hypotheses for explaining the inhibitory effect of naphthalene on strain CJ2 are: (i) naphthalene itself could be directly inhibitory or toxic when present at high concentrations; (ii) the metabolism of naphthalene could lead to the accumulation of toxic or inhibitory metabolites when a high concentration of naphthalene is available to the cells; or (iii) both naphthalene and its metabolites play a role in growth inhibition.

To determine if naphthalene is directly toxic to strain CJ2, we measured the growth of CJN110, a NagR1 regulatory mutant of CJ2 that is unable to grow on naphthalene (Jeon *et al.*, 2006), in MSB supplemented with 6 mM glucose in the presence of naphthalene at concentrations that were inhibitory or non-inhibitory to the wild-type strain. Partially inhibited growth occurred on glucose in the presence of 23 μM naphthalene. However, at all other naphthalene concentrations, growth on glucose was severely inhibited (Fig. 4). Because strain CJN110 is unable to metabolize naphthalene, inhibition of growth was not due to the production of potentially toxic naphthalene metabolites. Therefore, naphthalene has a direct inhibitory or toxic effect on strain CJN110 derived from strain CJ2.

Fig. 4. Growth of a regulatory mutant of strain CJ2 (strain CJN110, NagR⁻) in glucose with or without naphthalene. The experimental values represent the mean $\pm$ SD of triplicate experiments. ■, glucose, no naphthalene; □, glucose + 23 μM naphthalene; ●, glucose + 40 μM naphthalene; ○, glucose + 55 μM naphthalene; △, 23 μM naphthalene, no glucose.

Evidence for the production of toxic metabolites

To explore the relationship between naphthalene concentration and growth inhibition, cell viability and naphthalene concentration were monitored over the course of a growth experiment. Strain CJ2 was inoculated into tubes containing MSB with 6 mM glucose or 0.1 g naphthalene-loaded XAD7 resin, resulting in initial aqueous naphthalene concentrations of 31, 47 and 86 μM , respectively.

Growth occurred as expected in cultures containing 6 or 10 mg naphthalene (Fig. 5a), with both culture conditions reaching an OD₆₀₀ near 0.68 and cultures containing 10 mg growing slightly slower than those containing 6 mg. However, in tubes containing 12 mg naphthalene, growth was severely inhibited and did not exceed an OD₆₀₀ of 0.13. The aqueous naphthalene concentration dropped below 10 μM in cultures with 6 mg naphthalene (Fig. 5b), suggesting that strain CJ2 metabolized naphthalene faster than it was diffusing from the XAD7 resin. In cultures containing 10 or 12 mg naphthalene there was an initial increase in the aqueous naphthalene concentration, indicating that a new equilibrium was established upon the addition of the cells. The subsequent decrease in naphthalene concentration shows that even the inhibited cultures were metabolizing naphthalene.

Though growth was inhibited in cultures containing 12 mg naphthalene, viable cells were detected until 34 h (Fig. 5c). However, after 34 h no viable cells were detected, and the loss of viability corresponded with the appearance of a light orange colour in the media, which appeared between 34 and 47 h. These results suggest that not only does naphthalene have an inhibitory effect, but also naphthalene metabolism by inhibited cells results in the accumulation of a naphthalene metabolite that is toxic to the cells. It should be noted that even though there was no visible colour accumulation prior to 34 h, it is possible that a low level of the toxic metabolite had accumulated and was responsible for growth inhibition.

Analysis of toxic accumulated metabolites

Previous reports have suggested that the accumulation of an orange metabolite in naphthalene-metabolizing cultures results from the abiotic oxidative transformation of 1,2-dihydroxynaphthalene to 1,2-naphthoquinone (Auger *et al.*, 1995; Davies & Evans, 1964; Murphy & Stone, 1955). Spectrophotometric scans (240–400 nm) of 1,2-naphthoquinone standards and coloured media from naphthalene-inhibited cultures of strain CJ2 suggested that 1,2-naphthoquinone was present at concentrations between 50 and 100 μM (data not shown). Therefore, we used HPLC to compare 1,2-naphthoquinone standards (dissolved in MSB) with media from strain CJ2 cultures that were either successfully grown on naphthalene or cultures that were inhibited by naphthalene and had accumulated orange-coloured metabolites.

Fig. 5. Relationship between cell growth, aqueous naphthalene concentration and cell viability. Strain CJ2 was inoculated into MSB with glucose (□) or 0.1 g XAD7 and 6 (■), 10 (○) or 12 (●) mg naphthalene. Optical density (a), aqueous naphthalene concentration (b) and viable cells (c) were monitored. Inoculum density was held constant at 9.8×10^6 c.f.u.

Analysis of 1,2-naphthoquinone dissolved in MSB revealed that it too was unstable in aqueous media. When 50 μ M 1,2-naphthoquinone was analysed by HPLC shortly after dissolution in MSB, there was one major peak with a retention time of 20 min (peak II in Fig. 6a). A probable oxidation product with a retention time of 24 min (peak III in Fig. 6a) also appeared. However, after 50 μ M

1,2-naphthoquinone was incubated for 48 h in sterile MSB, peak II was no longer detected, while new peaks (I and IV) with retention times of 15 and 26 min, respectively, were abiotically produced (Fig. 6b). Analysis of the coloured medium from a naphthalene-inhibited culture showed three peaks: one peak (II) corresponds with the primary peak of freshly dissolved 1,2-naphthoquinone, while the other two peaks, I and IV, correspond with daughter products of aged 1,2-naphthoquinone (Fig. 6c). No peaks of putative inhibitory metabolites were present in the uncoloured medium from cultures of CJ2 that successfully grew on naphthalene (Fig. 6d). Chemical instability, ineffective derivatization procedures and lack of authentic standards prevented GC-MS identification of the putative toxic compounds eluting as peaks I and IV in the inhibited culture of strain CJ2 (Fig. 6c). Support for the contribution of 1,2-naphthoquinone (peak II) to toxicity was obtained in assays showing that growth of strain CJ2 was completely inhibited in MSB-glucose medium when 1,2-naphthoquinone was present at a concentration of ~ 50 μ M (data not shown).

DISCUSSION

The isolation of strain CJ2 has provided a unique opportunity to investigate the genetics and physiology of a bacterium that is linked to the *in situ* biodegradation of naphthalene in contaminated sediment. Sequencing of the naphthalene catabolic genes has revealed a novel arrangement of structural and regulatory genes in strain CJ2 (Jeon *et al.*, 2006). In this study, we gained insight into the physiology of naphthalene metabolism in strain CJ2 by comparing it to two archetypal naphthalene-degrading bacteria. Our data from respirometry, metabolite detection by GC-MS and enzyme assays showed that strain CJ2 metabolizes naphthalene via the gentisate pathway. Growth assays revealed that strain CJ2 cannot grow on naphthalene at concentrations above 78 μ M, and that metabolic imbalances may lead to inhibition and toxicity.

Bacteria that metabolize aromatic hydrocarbons face the challenge of acquiring carbon and energy from compounds that are potentially toxic (Ramos *et al.*, 2002; Sikkema *et al.*, 1995). The inability of potential biodegrading populations to tolerate aromatic hydrocarbon toxicity may contribute to the persistence of pollutants in the environment. The mechanisms of toxicity are generally believed to be disruption of biological membranes (Sikkema *et al.*, 1995) and the production of toxic metabolites (e.g. Park *et al.*, 2004). The lipophilic character of aromatic hydrocarbons can alter membrane fluidity, permeabilize the membrane and cause swelling of the lipid bilayer. Alteration of membrane structure can disrupt energy transduction and the activity of membrane-associated proteins (Sikkema *et al.*, 1995). Additionally, metabolites of aromatic compounds, such as catechols and quinones, can be more toxic than the parent compound due to an increase in solubility, production of reactive oxygen

Fig. 6. Metabolite analysis by HPLC. Chromatograms of 50 μM 1,2-naphthoquinone freshly dissolved in MSB medium (a), 50 μM 1,2-naphthoquinone aged in sterile MSB medium for 48 h (b), culture medium from naphthalene-inhibited strain CJ2 (c) and culture medium from naphthalene-grown strain CJ2 (d). Note that the vertical scale in (a) is twice that of the other panels.

species, or adduct formation with DNA and proteins (Penning *et al.*, 1999; Schweigert *et al.*, 2001).

In the present investigation we have shown that *Polaromonas naphthalenivorans* CJ2 is susceptible to both (i) direct naphthalene inhibition and (ii) formation of toxic intermediate metabolites. Naphthalene inhibited the growth of strain CJ2 at concentrations of 55 μM (Fig. 4), which is well below the aqueous saturation point of naphthalene (230 μM). Naphthalene has been reported to be toxic to the archetypal naphthalene degraders *P. putida* G7 and *P. putida* NCIB 9816-4, but only under nitrogen- or oxygen-limiting conditions (Ahn *et al.*, 1998) or during incubation in soil amended with a high concentration (0.2% w/v) of naphthalene crystals (Park *et al.*, 2004), respectively. We found that inhibition of strain CJ2 by naphthalene was independent of metabolism, and, based on the study of Sikkema *et al.* (1994), we speculate that the mechanisms of direct inhibition are likely to be related to impaired membrane function.

In addition to growth inhibition, naphthalene metabolism by strain CJ2 at inhibitory concentrations resulted in the accumulation of toxic oxidation products derived from 1,2-naphthoquinone, which resulted in a complete loss of viability (Figs 5c and 6c). Davies & Evans (1964) showed that a 25 μM solution of 1,2-dihydroxynaphthalene is

converted by non-enzymic oxidation to 1,2-naphthoquinone at a rate of approximately 20% min^{-1} at pH 6.5. 1,2-Naphthoquinone has been reported to accumulate and inhibit both growth and naphthalene metabolism when ferrous and magnesium salts are omitted from growth media (Murphy & Stone, 1955) or when naphthalene bioavailability is increased by the addition of surfactant (Auger *et al.*, 1995). Our analysis by HPLC suggests that 1,2-naphthoquinone (presumably produced abiotically from 1,2-dihydroxynaphthalene) and two abiotic transformation products of 1,2-naphthoquinone accumulate when strain CJ2 is exposed to inhibitory concentrations of naphthalene.

Strain CJ2 was shown to metabolize naphthalene *in situ* by stable isotope probing, which suggests that strain CJ2 is an active member of the naphthalene-degrading population in the sediment (Jeon *et al.*, 2003). Strain CJ2 has evolved, apparently successfully, to occupy a niche as a naphthalene degrader, even though naphthalene has a strong inhibitory effect and subsaturation levels of naphthalene can result in toxic metabolite accumulation. It is possible that strain CJ2 never experienced selective pressure to develop greater tolerance to naphthalene because adsorption to soil and metabolism of naphthalene by other bacteria kept naphthalene concentrations well below inhibitory levels. If this is the case, strain CJ2 may not have evolved

adaptation mechanisms frequently associated with tolerance to aromatic compounds, such as membrane isomerization and efflux pumps (Ramos *et al.*, 2002). Furthermore, the accumulation of 1,2-naphthoquinone-related oxidation products might be due to unrealistic naphthalene concentrations imposed in laboratory incubations combined with the slow growth of strain CJ2. An enzyme in the naphthalene catabolic pathway may have a low specific activity that is only problematic when the concentration of naphthalene exceeds a threshold. Another possibility is that if strain CJ2 is adapted to low naphthalene bioavailability in soil, the bacterium has evolved to accumulate as much naphthalene as possible, whether through active uptake or through modifications of the cell membrane and envelope. Thus, when presented with naphthalene at the concentrations used in this study, strain CJ2 accumulated naphthalene to inhibitory and toxic quantities.

This study also showed that strain CJ2 metabolizes naphthalene via gentisate using the *nag*-type pathway found in *Ralstonia* sp. strain U2. Metabolism of aromatics via gentisate has been studied less extensively than metabolism via catechol, and it is not clear whether one pathway has an advantage over the other. A study investigating bacteria that metabolize 3-chlorobenzoate suggested that micro-organisms using the gentisate pathway have lower maximum specific growth rates and lower apparent half-saturation constants for oxygen and 3-chlorobenzoate; thus, they may be well adapted to substrate- and/or oxygen-limited conditions (Krooneman *et al.*, 2000). If these same characteristics are applicable to strain CJ2, they could help explain why it is successful in naphthalene-contaminated sediments despite being sensitive to inhibitory effects of naphthalene.

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