Biodegradation of alkyl branched aromatic alkanoic naphthenic acids by

*Pseudomonas putida* KT2440

Running Title: Characterisation of aromatic NA degradation by *Pseudomonas putida* KT2440

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Abstract:
The majority of the world’s crude oil reserves consist of highly biodegraded heavy and super heavy crude oils and oil sands that have not yet been fully exploited. These vast resources contain complex mixtures of carboxylic acids known as naphthenic acids (NAs). NAs cause major environmental and economic problems, as they are recalcitrant, corrosive and toxic. Although aromatic acids make up a small proportion of most NA mixtures, they have demonstrable toxicities to some organisms (e.g. some bacteria and algae) and ideally need to be removed or reduced by remediation. The present study analysed the ability of *Pseudomonas putida* KT2440 to degrade highly recalcitrant aromatic acids, as exemplified by the alkyl phenyl alkanoic acid (4′-t-butylphenyl)-4-butanoic acid (t-BPBA) and the more degradable (4′-n-butylphenyl)-4-butanoic acid (n-BPBA). n-BPBA was completely metabolized after 14 days, with the production of a persistent metabolite identified as (4′-n-butylphenyl)ethanoic acid (BPEA) which resulted from removal of two carbon atoms from the carboxyl side chain (beta-oxidation) as observed previously with a mixed consortium. However, when n-BPBA concentration was increased two-fold, degradation decreased by 56% with a concomitant six-fold decrease in cell numbers, suggesting that at greater concentrations, n-BPBA may be toxic to *P. putida* KT2440. In contrast, *P. putida* KT2440 was unable to degrade the highly recalcitrant t-BPBA even after 49 days. These findings have implications for NA bioremediation in the environment.

Keywords: beta-oxidation/ naphthenic acids/ bioremediation.
The largest single crude oil accumulations in the world are found as partially biodegraded oil sands deposits in Alberta (Canada, 900 billion barrels) and Eastern Venezuela (Venezuela, 1,200 billion barrels) (Head et al. 2003). The extraction of bitumen from the vast highly degraded oil sands deposits in Northern Alberta, Canada has resulted in the accumulation of ≥ 840 million m$^3$ of oil sands tailings and oil sands process water (OSPW), which are stored in large tailings ponds (Siddique et al. 2011). Concerns have been raised about the possible environmental impacts of tailings ponds due to the presence of recalcitrant, toxic organic acids, collectively known as naphthenic acids (NAs) (Headley and McMartin 2004; Whitby 2010; Siddique et al. 2011).

Naphthenic acids (NAs) are complex mixtures of cycloaliphatic and alkyl-substituted acyclic carboxylic acids present in petroleum. NAs also contain smaller amounts of other compounds such as aromatic, olefinic, hydroxyl and dibasic acids (Headley and McMartin, 2004). Although aromatic NAs make up a small percentage of some NA mixtures (e.g. Rowland et al. 2011a,b,c), they may contribute disproportionately to the overall toxicity and recalcitrance of NAs (Headley and McMartin 2004; Johnson et al. 2011a). One solution for the removal of these recalcitrant, toxic compounds is to apply microorganisms for bioremediation. However, the mechanisms by which this is achieved are poorly understood.

A previous study isolated both *Pseudomonas putida* and *Pseudomonas fluorescens* from OSPW and demonstrated >95 % degradation of both commercial and environmental NA mixtures by a co-culture of *P. putida* and *P. fluorescens* (Del Rio et al. 2006). Johnson et al. (2011a) also identified an increase in abundance of 16S rRNA gene sequences relating to *Pseudomonas* spp. (among others) during the
degradation of alkyl branched aromatic NAs (Johnson et al. 2011a). Furthermore, *Pseudomonas putida* KT2440 has been shown to metabolise a wide variety of substrates including phenylacetic acid and other phenylalkanoic acids (Jimenez et al. 2002; Gilbert et al. 2003).

Although to date, there is no information regarding the ability of *P. putida* KT2440 to metabolise alkyl branched aromatic NAs, the diverse metabolism of *P. putida* KT2440 appears to make it an excellent potential candidate for NA degradation. The overall aims of this study were to investigate the catabolic ability of *P. putida* KT2440 to degrade different alkyl branched aromatic NAs and to identify any metabolites produced during degradation. Such information would enable the elucidation of the functional genes and metabolic pathways involved in aromatic NA degradation, thus facilitating the bioremediation of these recalcitrant aromatic NAs in the environment.

2. MATERIALS AND METHODS

2.1 Bacterial Strain

*Pseudomonas putida* KT2440 (DSMZ 6125) was obtained from the DSMZ culture collection (Braunschweig, Germany). Exponential cultures were grown at 30°C for 24 h with shaking (120 rpm) on Luria-Bertani (LB) medium (Sambrook et al., 1989) to a cell density of 1 x 10^8 cells mL⁻¹.

2.2. Aromatic carboxylic acid synthesis

The two butylphenyl butanoic acid (BPBA) isomers used in this study were: (4'-n-butylphenyl)-4-butanoic acid (n-BPBA) and (4'-t-butylphenyl)-4-butanoic acid (t-
Both BPBAs were synthesized using a modified Haworth synthesis as described previously (Smith, 2006; Smith et al. 2008).

2.3. Degradation of $n$-BPBA and $t$-BPBA by *P. putida* KT2440

Degradation experiments were set up by inoculating *P. putida* KT2440 (2% v/v) into 25 mL minimal salts medium (MSM) containing either $n$- (at final concentrations of either 2, 3 or 4 mg L$^{-1}$) or $t$-BPBA (final concentration of 4 mg L$^{-1}$) as described previously (Johnson et al. 2011a). Killed controls (to determine whether any abiotic loss had occurred) were prepared by Tyndallization of the inoculum before BPBA addition, and were checked by growth on LB agar and incubating overnight at 30 °C prior to inoculation. Abiotic controls containing MSM with the individual BPBA isomer (dissolved in 0.1 M NaOH and added to a final concentration of 4 mg L$^{-1}$) were also prepared. Procedural blanks containing either *P. putida* KT2440 inoculated (2% v/v) in MSM only or MSM supplemented with 0.1M NaOH (10 μl) were also performed. Destructive sampling was carried out at days 0, 11, 31 and 49. Cell counts were performed on cultures grown on $n$- and $t$-BPBA as well as procedural blanks by dilution plating onto LB agar, with phosphate buffered saline (pH 7.4; Sambrook et al. 1989) as the diluent and incubated at 30 °C for 24 h.

2.4. BPBA extraction and gas chromatography mass spectrometry analysis

BPBA extraction was performed as described previously (Johnson et al., 2011a). Briefly, all glassware was soaked overnight in Decon$^90$ (Decon Laboratories, Pennsylvania, USA), rinsed three times with distilled water, baked until dry at 110 °C, rinsed three times with acetone (Fisher Scientific, Massachusetts, USA) and autoclaved. The internal standard, 4-phenylbutyric acid (Acros Organics, Thermo
Fisher Scientific, Massachusetts, USA) (10 mg) dissolved in 1 mL methanol (HPLC grade, Fisher Scientific) was added to each supernatant from above (final concentration 2 mg L\(^{-1}\)). Each BPBA isomer from the supernatant was extracted by acidifying to pH <2.0 (using conc. HCl) and extracted three times with 15 mL HPLC-grade ethyl acetate (Fisher Scientific) as described by Smith et al. (2008). Solvent extracts were pooled, dried with 5-10 g anhydrous Na\(_2\)SO\(_4\) (Fisher Scientific) for > 90 min and the organic acids concentrated by rotary evaporation (Buchi/Rotavapor, Flawil, Switzerland) at 40 °C. Samples were transferred to a GC vial (Chromacol, Welwyn Garden City, UK), sparged with nitrogen and reacted with 50 µl N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco, Missouri, USA) at 60°C for 20 min to form trimethylsilyl derivatives. Derivatized samples were resuspended in 1 mL dichloromethane (HPLC grade, Acros Organics) and separated by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890 GC interfaced with an Agilent 5975C MS. Run conditions were a 1 µL splitless injection (injector temperature of 250 °C) onto a 30 m x 250 µm x 0.25 µm HP-5MS column. Oven temperatures were increased from 40 to 300 °C at 10 °C min\(^{-1}\) followed by a 10 min hold at 300 °C. Helium was used as the carrier gas at a constant flow rate of 1 mL min\(^{-1}\). The transfer line and source were held at 230 °C. The mass spectrophotometer was a quadrupole operated in full-scan mode (scan range 50-550 Da).

2.5. Statistical Analysis

Statistical analysis was performed using SPSS PASW statistics version 18.0.0.
3. RESULTS AND DISCUSSION

Degradation of \( n \)- and \( t \)-BPBA by \( P. \) putida KT2440 was investigated and the results are presented in Figs. 1 & 2. With both \( n \)- and \( t \)-BPBA incubations, killed controls (Fig. 1 A, circled) behaved accordingly and showed no abiotic losses. \( P. \) putida KT2440 was able to metabolise \( n \)-BPBA after 14 days incubation and this is the first report of the partial degradation of butyl substituted aromatic butanoic acids by \( P. \) putida KT2440. Although \( P. \) putida KT2440 is known to degrade a variety of other aromatic compounds (Nelson et al. 2002), it was unable to transform \( t \)-BPBA following 49 days incubation (Fig. 1A). The inability of \( P. \) putida KT2440 to degrade \( t \)-BPBA may be due to the toxicity of \( t \)-BPBA. It has been shown previously that \( t \)-BPBA is two-fold more toxic to the bacterium, Vibrio fisheri, compared to \( n \)-BPBA (Johnson et al. 2011a). In addition, the recalcitrance of \( t \)-BPBA by \( P. \) putida KT2440 may also be due to other factors such as the more branched side chain of \( t \)-BPBA (compared to \( n \)-BPBA) causing possible steric hindrance, \( P. \) putida KT2440 may not have the enzymes required to degrade \( t \)-BPBA, or there could be a missing enzymatic co-factor, such as iron, in the media.

During degradation of \( n \)-BPBA, a major metabolite was produced at day 14, which increased in abundance following a decrease in \( n \)-BPBA (Fig. 1B). Gas chromatograms of \( n \)- and \( t \)-BPBA degradation (Figs 2A & B) showed that the metabolite produced during \( n \)-BPBA degradation had a retention time of 15.10 min and persisted at day 49 (Fig. 2A). By contrast \( P. \) putida KT2440 was unable to metabolise \( t \)-BPBA by day 49 and no metabolites were detected during the course of the experiment (Fig. 2B). Interpretation of the mass spectrum of the TMS ester of the major metabolite produced during \( n \)-BPBA degradation by \( P. \) putida KT2440 (Fig. 3) showed that it was identical to that previously identified (Johnson et al. 2011a), as
(4'-n-butylphenyl)ethanoic acid (n-BPEA) by comparison with a synthetic sample. The production of the n-BPEA metabolite, which persisted at day 49, suggests that beta-oxidation was the initial route for n-BPBA metabolism, as has been previously described by a mixed microbial consortium (Johnson et al. 2011a) and an environmental isolate (Johnson et al., 2011b). Beta-oxidation is a ubiquitous pathway and has been found in many organisms including *P. putida* (De Waard et al. 1993; Eggink et al. 1992; Nelson et al. 2002).

Both aromatic and alicyclic compounds containing side chains with odd numbers of carbons are much more easily biodegraded as the ring structure can be cleaved through beta-oxidation (Quagraine et al. 2005; Whitby 2010). However, although beta-oxidation is capable of oxidising alkanoic side chains, it stops at the ethanoic group if the number of carbon atoms in the alkanoic side chain is even (Beam and Perry 1974; Rontani and Bonin 1992). The two BPBAs studied here contain butanoic side chains with an even number of carbon atoms and therefore, they and their BPEA metabolites have the ability to accumulate in the environment (Quagraine et al. 2005).

In contrast, other microorganisms such as *Alcaligenes* sp. PHY 12 have been shown to overcome the preference for odd carbon chains during the degradation of cyclohexylacetic acid, by initially alpha-oxidising the substrate to cyclohexylcarboxylic acid before using beta-oxidation to open the cyclohexyl ring (Rontani and Bonin 1992). In the absence of an alpha-oxidation pathway the ring structure has been shown to be cleaved through phenylacetic acid in *Pseudomonas putida* U using the phenylacetyl-CoA catabolon (phA) which contains a transport system, a phenylacetic acid activating enzyme, a ring hydroxylation complex, a ring opening enzyme, a beta-oxidation like system and regulatory genes (Olivera et al. 197...
A phA catabolon homologous to that found in *P. putida* U has been identified in *P. putida* KT2440, suggesting that the enzymes are present. It is unlikely that *n*-BPEA toxicity resulted in the inability of *P. putida* KT2440 to further transform this metabolite, since Johnson et al. (2011a) has previously showed that *n*-BPEA is over two-fold less toxic than the parent compound *n*-BPBA (by Microtox™, Azur Environmental, Fairfax, USA). It is therefore possible that other factors such as bioavailability or incubation time may have played a role in the recalcitrance of *n*-BPEA.

In addition to BPEA metabolites, previous studies using a microbial consortium (Johnson et al. 2011a) and *Mycobacterium aurum* IS2.3 (Johnson et al., 2011b) demonstrated the presence of an additional metabolite identified as (4′-carboxybutylphenyl)ethanoic acid) during BPBA degradation, which showed carboxylation of the butyl side chain. Furthermore, Johnson et al. (2011b) showed that (4′-carboxy-tert-butyphenyl)ethanoic acid was produced via two different pathways (i.e. either omega oxidation of *t*-BPEA to oxidise the tert-butyl side chain, or initial omega oxidation of the tert-butyl side chain of *t*-BPBA to produce (4′-carboxy-tert-butyphenyl)-4-butanoic acid followed by beta-oxidation of the alkanoic acid side chain. However, in the present study, these metabolites were not found when *P. putida* KT2440 was grown in the presence of either *n-* or *t*-BPBA.

Several *P. putida* strains are well known for the degradation of aromatic hydrocarbons and their degradation pathways have been mapped (Jimenez et al., 2002). *P. putida* KT2440 contains the enzymes involved in the ortho aromatic degradation pathway but not the meta pathway, as it does not contain the TOL plasmid (Jimenez et al. 2002) which contains the genes for meta cleavage pathway including an alkyl monooxygenase (Franklin et al. 1981). As *P. putida* KT2440 is
unable to break down the aromatic ring, it would seem that the method of cleavage would not be through ortho or intradiol cleavage. This would seem evident as the less abundant meta pathway has a wider substrate specificity. The meta pathway is able to utilise substituted aromatic compounds such as xylene, toluene and polyaromatic hydrocarbons (PAHs), whereas the ortho pathway is more specific and is involved in the degradation of catechol and protocatechuate (Van Der Meer et al. 1992; Worsey and Williams 1975). If dioxygenation of the (4'-carboxybutylphenyl)ethanoic acids and subsequent meta cleavage were to ensue, then this compound could be converted into malonate, acetate and 2-oxoglutarate, which can be fed into central metabolism, leading to complete mineralisation of n-BPBA. It would therefore be advantageous for NA bioremediation to utilise microorganisms containing the TOL plasmid and therefore be capable of meta ring cleavage. As well as monoaromatic compounds, P. putida strains have also been shown to utilise these pathways in the degradation of polyaromatic hydrocarbons such as phenanthrene, fluorene and naphthalene (Yang et al. 1994).

The effect of increased substrate concentration on n-BPBA degradation by P. putida KT2440 was also investigated and the data are presented in Fig. 4. Degradation of n-BPBA decreased by 70, 57 and 14% for n-BPBA concentrations of 2, 3 and 4 mg L⁻¹ respectively at day 49 (Fig. 4). However, for n-BPBA at concentrations of 2 and 3 mg L⁻¹, the differences in the percentage degradation were not statistically significant (p=0.716). In contrast, there was a statistically significant difference in the degradation for n-BPBA concentrations at 4 mg L⁻¹ compared to either 3 mg L⁻¹ (p=0.021) or 2 mg L⁻¹ (p=0.010).

In addition, viable cell counts of P. putida KT2440 from day 31, performed by dilution plating are presented in Figure 5. These showed a significant decrease in
cell numbers (from $7.67 \times 10^6$ cfu mL$^{-1}$ to $1.27 \times 10^6$ cfu mL$^{-1}$) with increasing concentration of $n$-BPBA from 0 to 4 mg L$^{-1}$ ($p=0.022$) (Fig. 5). This suggested that at greater concentrations, $n$-BPBA had a greater toxic effect.

4. CONCLUSION:

In conclusion, this study investigated the catabolic ability of *P. putida* KT2440 to degrade different alkyl branched aromatic NAs. Whilst *n*-BPBA was readily degraded within days via beta-oxidation of the alkanoic acid side chain, *P. putida* KT2440 was unable to metabolise *t*-BPBA. A major metabolite was identified (*n*-BPEA) that was produced during *n*-BPBA degradation. In addition, increasing concentrations of *n*-BPBA resulted in a decrease in the degradative ability of *P. putida* KT2440 and a decrease in cell numbers, suggesting that there was a toxic effect. Information obtained from this study is important as it would enable the elucidation of the functional genes and metabolic pathways involved in aromatic NA degradation and thus facilitate the bioremediation of these recalcitrant aromatic NAs in the environment.

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**Legends to Figures**

**Fig. 1.** Degradation of \( n \)- and \( t \)-BPBA and production of a major metabolite by *Pseudomonas putida* KT2440. (A): Percentage Recovery of \( n \)-BPBA (■) and \( t \)-BPBA (●) by *P. putida* KT2440 relative to the abiotic controls. Killed controls are circled. (B): Production of a metabolite identified as that corresponding to the ethanoic acid equivalent of \( n \)-BPBA, with a retention time of 15.10 min. Error bars represent standard error of the mean (\( n=3 \)).

**Fig. 2.** Example gas chromatograms showing degradation of \( n \)-BPBA (A) and \( t \)-BPBA (B) over time. The internal standard was 4-phenylbutyric acid.

**Fig. 3.** Mass spectrum of a trimethylsilylated metabolite of \( n \)-BPBA degradation, eluting at 15.10 mins. The metabolite was identified as \( n \)-BPEA, by comparison of the mass spectrum with those of synthetic \( n \)-BPEA (TMS ester).

**Fig. 4.** Recovery of \( n \)-BPBA after 49 days incubation with *P. putida* KT2440. Shown are the mean percentage recoveries of \( n \)-BPBA after 49 days incubation with either 2, 3 or 4 mg L\(^{-1} \) of \( n \)-BPBA. Bars represent the mean of triplicate cultures and error bars represent standard error of the mean (\( n = 3 \)).

**Fig. 5.** The Relationship between \( n \)-BPBA concentration and viable cell counts of *P. putida* KT2440 after day 31. Error bars represent standard error of the mean (\( n=3 \)).
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