

Dynamic Changes in Aromatic Hydrocarbon Associated Catabolic Gene Profiles Linked to Aerobic and Anaerobic Microcosm Studies

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Abstract

The biodegradation of BTEX pollutants is often facilitated by both aerobic and anaerobic bacteria in the environment. Developing strategies to enable a rapid characterisation of related microbial populations is of importance to many industries, including the petrochemical industry. Here we use a qPCR assay to show that dynamic changes in key catabolic genes, from aerobic and facultative anaerobic bacteria involved in the breakdown of benzoate and toluene through microcosm studies, can be linked to the aerobic or anaerobic nature of these microcosms. This approach may prove useful in further studies of the biodegradation of pollutants by eubacteria *in situ*.

Keywords: qPCR; Bioremediation; Environment

Introduction

Aromatic compounds are one of the most prevalent types of compound present within our environment [1]. The BTEX family of aromatic compounds are of particular environmental importance due to their widespread occurrence, persistence in nature [2], potential to cause adverse human health effects, safety hazards, ecological and aesthetic impacts [3].

Aerobic pathways of aromatic hydrocarbon degradation employ oxygen as a terminal electron acceptor, and mono- and dioxygenase enzymes, to cleave the benzene ring which results in a loss of aromaticity [4]. Biodegradation pathways of aromatic hydrocarbons have been intensively investigated, much is known about the genes involved [2] and many related monooxygenase and dioxygenase genes have been characterised. Toluene dioxygenase, studied in detail by Gibson and colleagues [5] is an especially good illustration of a gene involved in the breakdown of one of the BTEX compounds. However, it is also the case that some microorganisms do not possess the toluene dioxygenase gene and instead transform toluene to benzoate, prior to aromatic ring cleavage by benzoate dioxygenase [6].

Under anaerobic conditions, BTEX compounds appear to be processed through the central benzoyl CoA reductase pathway-for a review readers are directed toward references [1] and [7]. Most BTEX degradation appears to be facilitated by facultative anaerobes, such as *Thauera spp* and *Azoarcus spp* [1] that functionalise the aromatic substrate via formation of a benzoyl CoA derivative before further ring degradation using reductive biochemistry [8].

Microcosms are widely used to both facilitate enrichment of pure cultures from environmental samples and allow studies of complex communities and their interactions with specific substrates to be performed. Previous studies have focused upon qualitative PCR approaches using catabolic genes as indicators of microbial degradation of compounds [9] or qPCR looking at one catabolic gene [10,11].

One key question that has not been extensively addressed in microcosm studies relates to the changes that occur in catabolic gene profiles in response to the addition of aromatic hydrocarbon substrates. If microcosms are to be used as a model for biodegradation *in situ* we need to provide evidence that such a dynamic change actually occurs.

As previously noted aerobic and anaerobic genes used by eubacteria

for aromatic hydrocarbon degradation are very different. Therefore, the aim of this study was to monitor catabolic gene levels within both aerobic and anaerobic microcosms through qPCR. Any relationship linking gene profiles to the presence or absence of oxygen within degrading microbial communities could lead to direct inferences being made using a similar approach to microbial communities in environmental samples.

Materials and Methods

Microcosm construction

Benzoate degrading microcosms were established under both aerobic and anaerobic conditions using an inoculum of anaerobic river sediment (obtained from the River Lagan in Belfast, Northern Ireland) whilst toluene microcosms were set up using sediment from a petrochemical site contaminated with BTEX based in the UK. In the latter case, the inoculum was sediment from core material recovered at a depth of 6 metres below ground and stored in an air-tight container under nitrogen at 3°C prior to use.

Anaerobic microcosms were established inside a nitrogen glove box (Cole-Parmer). The box was flushed with nitrogen and media, vials, and soil were placed within the housing for at least 24 hours prior to microcosm construction to allow the removal of dissolved oxygen. Inoculation of media, addition of resazurin and sealing of vials was carried out within the glove box.

Anaerobic and aerobic microcosms were constructed in 6 ml and 100 ml headspace vials (Supelco), respectively. 1 g of sediment was added to both microcosm types together with 4 ml of minimal salts

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media prepared as previously described [12]. Resazurin was added to the media at a concentration of 0.01 wt%, providing visual confirmation that all active anaerobic microcosms became anoxic within the first 24 hours of incubation. Control microcosms were set up exactly as described above followed by autoclaving at 15 psi and 121°C for 15 minutes prior to incubation

In total two experiments were constructed to monitor the degradation of benzoate (experiment 1) and toluene (experiment 2) under both aerobic and anaerobic conditions. In total 4 microcosm sets were constructed, in triplicate, comprising of: (a) river sediment with benzoate as a sole carbon source, at a final concentration of 4 mM, incubated under aerobic conditions, (b) river sediment with benzoate as a sole carbon source, at a final concentration of 4 mM, incubated under anaerobic conditions (experiment 1) and, (c) BTEX contaminated sediment with toluene as a sole carbon source, at a final concentration of 1 mM, incubated under aerobic conditions, (d) BTEX contaminated sediment with toluene as a sole carbon source, at a final concentration of 1 mM, incubated under anaerobic conditions (experiment 2). In each case incubations were carried out at 30°C.

Chemical analysis

Benzoate degradation was monitored by reverse phase HPLC as previously described [13]. Toluene degradation was monitored by GC/MS (Agilent) via headspace analysis where samples were injected onto a 105 m × 0.530 mm column (Agilent) and samples were heated to 120°C for 0.5 min followed by a 3°C/min ramp to 140°C and finally a 25°C/min ramp to 250°C holding until completion.

Molecular biology techniques

DNA extraction was carried out, in triplicate, as previously described using bead beating combined with phenol/chloroform extraction [14]. Total microbial DNA was quantified by UV absorption at 260 nm [15]. Primer pair BZAQ4F/R [16] were used to amplify a 484bp fragment from the, α subunit of the *bcr* gene. The *bcr* products generated were used to prepare standards for use in qPCR assays with primer BZAQ4F and PFR1 (5'-TCCTGMCCGCCSATGTCSAG`3) [17]. Primer PFR1 was designed through multiple sequence alignments (ClustalW-EBI) of *bcr* α subunits present in *T. aromatica*, *R. palustris*, *A. evansii* and *M. magneticum* and used to target a small section of the α subunit of the *bcr* gene for use in qPCR studies. For *bdo* assays primer pair BDOF and BDOF was used as described previously [18]. All qPCR standards and reactions were performed as described in our previous work [17] using an Opticon 3 real-time PCR thermo cycler (Biorad) coupled with Maxima SYBR Green Master Mix (Fermentas) fluorescent technology.

DNA sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Bio systems Big-Dye Ver 3.1 chemistry on an Applied Bio systems model 3730 automated capillary DNA sequences.

Results

Experiment 1-microbial led degradation of benzoate

In the aerobic, river sediment, microcosm studies complete degradation of benzoate occurred within 3 days of experiment initiation (from an initial concentration of 4 mM). In contrast to this ~20% of benzoate remained in the corresponding anaerobic microcosms following incubation for 14 days (Table 1) upon which point the experiment was terminated. Heat killed controls, maintained $\geq 90\%$

of the initial benzoate added to microcosms under both aerobic and anaerobic conditions. Estimated degradation rates of carbon sources in these microcosms were as follows:

Aerobic benzoate- $1.30 \pm 0.12 \text{ mM.d}^{-1}$; and

Anaerobic benzoate - $0.23 \text{ mM} \pm 0.02.\text{d}^{-1}$

Following incubation both the aerobic and anaerobic microcosms were subjected to DNA extraction followed by further analysis of total DNA. It was observed that qPCR analysis on DNA isolated from benzoate microcosms (Figure 1) indicated that total *bcr* copies were higher following incubation under anaerobic conditions ($5.62 \times 10^5 \pm 3.88 \times 10^4$) in comparison to incubation under aerobic conditions ($3.26 \times 10^5 \pm 1.9 \times 10^4$). In contrast to this observation *bdo* copies were lower under anaerobic conditions ($1.25 \times 10^5 \pm 1.22 \times 10^4$) compared to incubations under aerobic conditions ($2.81 \times 10^5 \pm 2.23 \times 10^4$). Thus differences in gene abundances were clearly influenced by the aerobic/ anaerobic conditions of incubation as the same starting inoculum was used for both aerobic and anaerobic microcosms.

Experiment 2-Microbial led degradation of Toluene

In the aerobic, petroleum site sediment, microcosms toluene was completely degraded in under 3 days. In the corresponding anaerobic microcosms ~20% of toluene remained after 6 days (Table 1). In the heat sterilised controls $\geq 80\%$ of the toluene added to the microcosms remained at the end of the test period under both aerobic and anaerobic conditions. Estimated degradation rates of toluene were determined as follows:-

Aerobic toluene- $0.40 \pm 0.00 \text{ mM.d}^{-1}$; and,

Anaerobic, toluene- $0.13 \pm 0.05 \text{ mM.d}^{-1}$

Further qPCR analysis on DNA isolated from toluene microcosms (Figure 2) indicated that *bcr* copies were higher ($1.52 \times 10^5 \pm 6.2 \times 10^3$) following incubation under anaerobic conditions compared to incubation under aerobic conditions ($9.3 \times 10^4 \pm 3.2 \times 10^3$). In contrast the *bdo* copies were lower following incubation under anaerobic conditions ($1.32 \times 10^5 \pm 1.65 \times 10^4$) compared to aerobic conditions ($1.64 \times 10^5 \pm 1.08 \times 10^4$). In both experiments measurements were made based upon triplicate microcosms. Efficiency of qPCR reactions ranged from 101-107% falling within the acceptable range of 90-110% [19].

PCR product sequencing

The nature of the qPCR Products was confirmed through gel electrophoresis and melting curve analysis as described previously [20]. Products were cloned and sequenced to confirm their identity. In each case 20 plasmids were analysed and found to contain inserts of the

Carbon source	Benzoate		Toluene	
	microcosm ¹	microcosm ²	microcosm ¹	microcosm ²
Time Point 0 Conc. mM	4	4	1	1
Time Point 1 Conc. mM	3.824 ± 0.05	2.3 ± 0.142	0.98 ± 0.16	0.435 ± 0.1
Time Point 2 Conc. mM	0.004 ± 0.003	0.92 ± 0.28	0 ± 0	0.135 ± 0.06

¹Indicates aerobic microcosms

²Indicates anaerobic microcosms

Table 1: Degradation table indicating the removal of benzoate and toluene under aerobic and anaerobic conditions at analysis time points, as described in text.

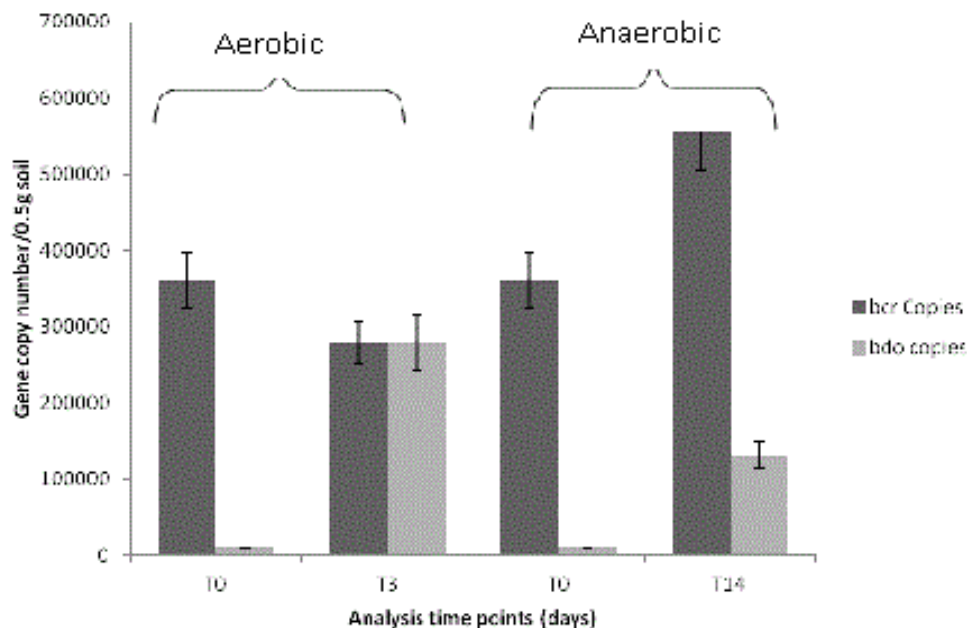


Figure 1: Copy numbers of both *bcr* and *bdo* were determined within total DNA extracted from aerobic and anaerobic microcosms where benzoate was the sole carbon source. Results are based on the average of triplicate samples and error bars represent the standard deviation from the mean.

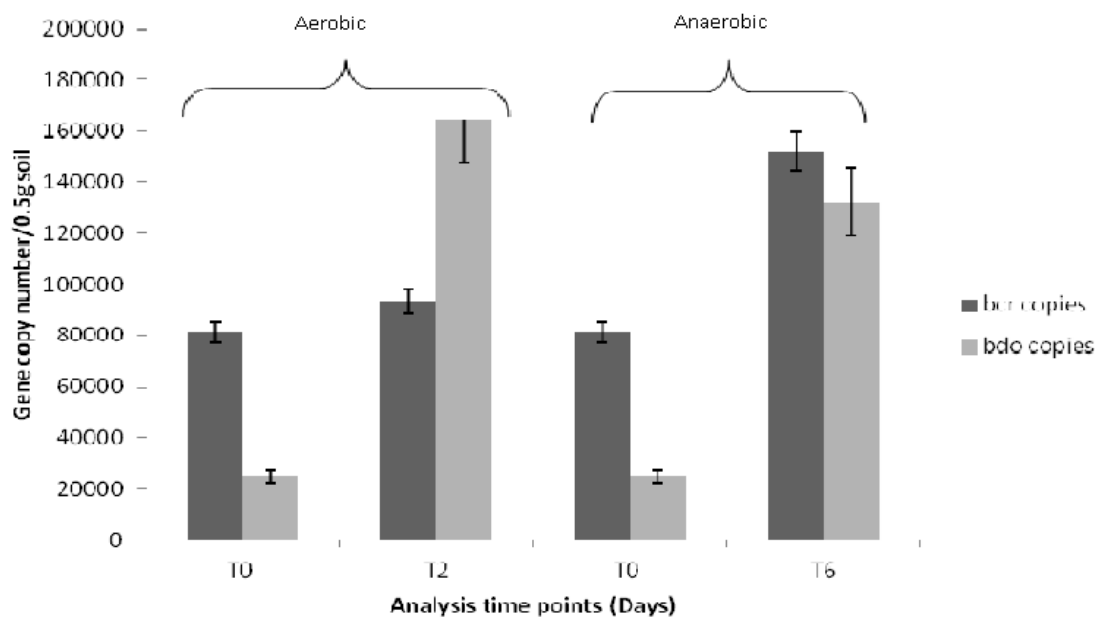


Figure 2: Copy numbers of both *bcr* and *bdo* were determined within total DNA extracted from aerobic and anaerobic microcosms where toluene was the sole carbon source. Results are based on the average of triplicate samples and error bars represent the standard deviation from the mean.

correct size, 4 of which were randomly picked for sequence analysis. Sequencing indicated that *bcr* genes obtained from anaerobic benzoate microcosms, using primer pair PFF1/R1, exhibited similarities to *Thauera spp* (1 sequence), *Geobacter spp* (2 sequences) and *Alkaliphilus spp* (1 sequence) *bcr* genes with homologies ranging from 88-90%.

Similarly *bdo* genes obtained from aerobic benzoate microcosms shared homologies ranging from 85-87% to *Acinetobacter spp* (2 sequences) and *Pseudomonas spp* (2 sequences) *bdo* genes (accession numbers JQ774497-JQ774500).

Discussion

Microbial degradation rates in microcosms amended with benzoate, under both aerobic and anaerobic conditions, were higher than those rates observed in microcosms amended with toluene. This fact may well be a result of the toxicity of toluene towards microorganisms [21] thus limiting the availability of the compound to a smaller subsection of the overall microbial population present within the toluene microcosms. Unsurprisingly aerobic rates of compound degradation were observed

to be higher than anaerobic rates, regardless of the aromatic compound tested.

The overall change in the *bcr* and *bdo* gene abundances from experimental start up (t zero) to experimental completion (t final) in both the benzoate and toluene microcosms, inoculated with river sediment and sediment from the petroleum site respectively, are documented in Table 2. In both the benzoate and toluene microcosms the catabolic genes tested in this study responded to the redox conditions of each microcosm as the degradation of the target compound proceeded. Under anaerobic conditions, *bcr* copy numbers increase by a larger percentage over the duration of each experiment relative to aerobic microcosms. In contrast, *bdo* copy numbers increase by a larger percentage in aerobic microcosms compared to their anaerobic counterparts. This data indicates that comparisons, through qPCR approaches, of the *bcr* and *bdo* catabolic genes could be used as a diagnostic tool to differentiate between areas of aerobic and anaerobic bioremediation potential in the subsurface of a polluted site. This data also suggests that studies centred on copy number observations of either *bcr* or *bdo* independently would fail to divulge information relating to the aerobic/anaerobic nature of a given site under investigation.

Results from this work also indicate that more *bcr* copies were detected overall within the benzoate microcosms compared to the toluene microcosms. This could reflect the differences in the types of microorganisms present in the two inocula. For example microorganisms present in the river sediment used in the benzoate microcosms may experience constantly fluctuating redox conditions as a result of river levels rising and dropping [22] resulting in conditions where facultative microorganisms, which harbour the class I type of *bcr*, may flourish. In contrast with the conditions which may occur in the river sediment the BTEX contaminated sediment used to construct the toluene microcosms may not have been exposed to oxygen at all for a prolonged period due to its depth and lack of aeration meaning a strictly anaerobic population predominated, capable of degrading toluene through a class II type of *bcr* enzyme absent from facultative microorganisms [23]. The primers used to detect the *bcr* gene in this study were designed to target the class I type of *bcr* enzyme therefore potentially overlooking the strictly anaerobic microorganisms present in both samples. Additional tests using a more definitive range of carbon sources coupled with other sediment types would be helpful in validating this hypothesis.

The microorganisms most closely related to the sequences obtained from this work are well documented in terms of their ability to degrade aromatic hydrocarbons [24] with both *Thaura spp* *Geobacter*

spp previously being shown to degrade aromatic compounds under anaerobic conditions [25,26].

Conclusion

The results of this work indicate that the copy number of *bcr* and *bdo* within microbial communities may be influenced by prevailing redox conditions. This, coupled with copy number changes of each gene, could be useful in determining whether (i) aromatic compound degradation is actively occurring within a given sample; and (ii) degradation of aromatics is occurring under aerobic or anaerobic conditions.

The technique could be used to provide supporting evidence for *in situ* biodegradation (natural attenuation) and also in the design of *in situ* remediation systems (e.g. involving oxygen or nitrate/sulphate injection).

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Catabolic gene target	Microcosm conditions	% Change in abundance following incubation under specified conditions
<i>Bcr</i>	Anaerobic + Benzoate	+54.8% ± 8.74%
	Aerobic + Benzoate	-16.2% ± 15.4%
	Anaerobic + Toluene	+87.6% ± 7.5%
<i>Bdo</i>	Aerobic + Toluene	+13.94% ± 0.019%
	Anaerobic + Benzoate	+1210% ± 10.8%
	Aerobic + Benzoate	+2690% ± 200%
	Anaerobic + Toluene	+428% ± 135%
	Aerobic + Toluene	+556% ± 115%

Table 2: Gene abundances upon experimental completion relative to time zero prior to incubation. Each gene studied under various conditions is shown. This data suggests a clear trend in both the benzoate and toluene microcosms, whereby under anaerobic conditions a greater increase in *bcr* copy numbers is observed, whereas under aerobic conditions a greater increase in *bdo* copy number is observed.

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