Real-time PCR quantification of a green fluorescent protein-labeled, genetically engineered *Pseudomonas putida* strain during 2-chlorobenzoate degradation in soil

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Abstract

The potential for real-time PCR (RTm-PCR) detection of the genetically engineered strain *Pseudomonas putida* GN2 was studied during 2-chlorobenzoate (2-CB) degradation in three different soils. The strain contained the constructed plasmid pGN2 which encoded genes for 2-CB oxidation (*cbdA*) and the green fluorescent protein (*gfp*). *P. putida* GN2 numbers were assessed by plating onto 2-CB minimal media and also by RTm-PCR detection of *cbdA* and *gfp*. Addition of *P. putida* GN2 decreased the time required to degrade 2-CB in all tested soils by more than 7 days. The RTm-PCR estimations of *P. putida* GN2 numbers strongly correlated with those obtained from plate count methods during active 2-CB degradation. However, after 2-CB degradation in the soils had ceased, RTm-PCR estimations of *cbdA* and *gfp* genes were generally one order of magnitude lower than those from plate counts. These results indicate the potential for RTm-PCR to rapidly determine degrader numbers in soil following bioaugmentation but also the need to exercise caution when attempting to determine cell numbers of degraders from the RTm-PCR quantification of plasmid encoded genes after substrate is depleted.

Keywords: Bioaugmentation; *cbdA*; Chlorobenzoate; GFP; Plasmid; Real-time PCR

1. Introduction

Chlorobenzoate (CB) contamination occurs at many polluted sites due to the partial metabolism of polychlorinated biphenyls by microorganisms. Although CB degraders are widely distributed and microbially diverse even at uncontaminated sites [1–3], adequate degrader populations may not be present or metabolically active in some soils [4,5]. In these instances, it may be necessary to augment the contaminated soil with an exogenous degrader. One factor that may limit the successful establishment of such degraders is the inability to rapidly assess changes in microbial populations at the site and to adjust environmental conditions accordingly. Traditionally, introduced degraders have been enumerated via culturing methods, such as on selective media which takes several days to complete. Data may not be obtained in time to adjust environmental conditions before the augmentation attempt has failed.

Recently, quantitative real-time PCR (RTm-PCR) technology has been used to rapidly quantify genes and microorganisms in complex environmental media including soils [6–14]. The TaqMan RTm-PCR procedure uses the 5' exoribonuclease activity of the *Taq* DNA polymerase to digest an internal fluorogenic TaqMan probe that anneals to DNA template during primer elongation. The digestion results in the release of a fluorescent...
signal. The cycle at which a significant increase in fluorescence emission occurs compared to the background baseline is termed the cycle threshold. A greater initial amount of DNA template results in a smaller cycle threshold value. Thus, quantification of the original template level can be calculated using cycle threshold values in comparison with those obtained from a standard curve of the DNA template.

To date, most studies on biodegradative microorganisms using RTm-PCR have focused on the quantification of the introduced microorganisms [11,13,15]. Relatively few studies have correlated RTm-PCR results with contaminant biodegradation [9,14]. Hristova et al. [9] used RTm-PCR to quantify a methyl tert-butyl ether-degrading bacterium in groundwater and sediment and correlated population increases with contaminant degradation. Widada et al. [14] used RTm-PCR to correlate population increases with contaminant degradation. To date, most studies on biodegradative microorganisms using RTm-PCR have focused on the quantification of the introduced microorganisms [11,13,15]. Relatively few studies have correlated RTm-PCR results with contaminant biodegradation [9,14]. Hristova et al. [9] used RTm-PCR to quantify a methyl tert-butyl ether-degrading bacterium in groundwater and sediment and correlated population increases with contaminant degradation. Widada et al. [14] used RTm-PCR to correlate population increases with contaminant degradation.

2. Materials and methods

2.1. Construction of GFP-labeled 2-CB degrader

The bacterial strains and plasmids used are listed in Table 1. Escherichia coli strains and P. putida KT2440 [16] were grown in Luria-Bertani (LB) broth or agar (Difco, Detroit, MI). Ampicillin (Ap, 100 µg·ml⁻¹) or kanamycin (Km, 50 g·ml⁻¹) was added to the media when appropriate. Additionally, LB containing 3 mM NiCl₂ was used during plasmid construction to confirm the presence of the nickel resistance gene nreB. The E. coli strains were grown at 37 °C, and P. putida KT2440 was grown at 27 °C.

To insert the 2-CB degradative genes into the medium copy number, broad-host-range plasmid, pBHR1 [17], the plasmid p2BG (containing an approximately 7 kb insert including the 2-CB genes cbdSABC) [18] and pBHR1 were digested with EcoRI (Promega, Madison, WI). Following incubation, the pBHR1 digest was treated with alkaline phosphatase (Fermentas, Hanover, MD) and purified with a QIAquick PCR Purification kit (Qiagen, Valencia, CA). The pBHR1 and p2BG digests were ligated with T4 DNA Ligase (Promega), and the ligation mixture was then transformed into E. coli strain JM109 using standard

<table>
<thead>
<tr>
<th>Object of study</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Bacterial strains and plasmids used in this study</strong></td>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em> DH5α</td>
<td>RecA1 endA1 gyrA96 thi-1 hsdR17(t₈ - , m₅ +) supE44 relA1 deoR ΔlacZYA-argF U169</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>recA1 endA1 gyrA96 thi hsdR17(t₈ - , m₅ +) supE44 relA1 Δ(lac-proAB) [Vₜ traD36 proAB lacI²ZAM15] r⁻ m⁻</td>
<td>Promega</td>
</tr>
<tr>
<td><em>P. putida</em> KT2440</td>
<td></td>
<td>[16]; DSMZ&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>p2BG</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; 2-CB&lt;sup&gt;+&lt;/sup&gt;; pBluescript II KS+ with ca. 7-kb insert containing <em>cbdSABC</em></td>
<td>[18]</td>
</tr>
<tr>
<td>pBHR1</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;, broad-host-range</td>
<td></td>
</tr>
<tr>
<td>pBHR1::2-CB</td>
<td>2-CB&lt;sup&gt;+&lt;/sup&gt;; pBHR1 with ca. 10-kb <em>EcoRI</em> fragment insert containing <em>cbdSABC</em></td>
<td>[17]; MoBiTech</td>
</tr>
<tr>
<td>pEGFP</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; GFP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Clontech</td>
</tr>
<tr>
<td>pEGFP::nreB</td>
<td>nreB&lt;sup&gt;+&lt;/sup&gt;; pEGFP with 2.1-kb <em>EcoRI</em> fragment insert containing <em>nreB</em></td>
<td>This work</td>
</tr>
<tr>
<td>pGEMCI2</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; nreB&lt;sup&gt;+&lt;/sup&gt;; pGEM T-Easy with 2.1-kb insert containing <em>nreB</em></td>
<td>[20]</td>
</tr>
<tr>
<td>pGN2</td>
<td>GFP&lt;sup&gt;+&lt;/sup&gt; 2-CB&lt;sup&gt;+&lt;/sup&gt; nreB&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;; pBHR1::2-CB with 3.2-kb SpeⅠ, PCR product containing <em>gfp</em> and <em>nreB</em></td>
<td>This work</td>
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</table>

<sup>a</sup>2-CB, 2-chlorobenzoate; Ap, ampicillin; Km, kanamycin; GFP, green fluorescent protein.

<sup>b</sup>German Collection of Microorganisms and Cell Cultures (www.dsmz.de).
procedures [19]. Presumptive positive colonies were selected after 24 h growth on LB + Km. The 2-CB insert in pBHR1::2-CB was confirmed by purifying plasmid DNA from selected isolates using Wizard Plus SV Minipreps (Promega), digesting with EcoRI, and analyzing the digests with gel electrophoresis and ethidium bromide staining. To clone nreB into pEGFP for use in future experiments, plasmid pGEMCII2 with a 2.1-kb insert containing nreB [20] and pEGFP were digested with EcoRI, purified and ligated as mentioned above for pBHR1::2-CB, and transformed into E. coli DH5α. Presumptive positive colonies that produced GFP were selected from LB + NiCl₂ plates. The nreB insert in pEGFP::nreB was confirmed as outlined above with EcoRI digest. To clone the GFP and nickel resistance genes into pBHR1::2-CB, the GFP gene and nreB insert were amplified by PCR from pEGFP::nreB using the Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) with the primer pair 5′-NNNACTAGTGCTCCCAA-TACGCAAACCGCTCTC-3′ and 5′-NNNNNGAT-TCT2347CACCGCCACACCCGCT-3′ (N = 25% of each A, C, G, and T; underlined sequence is an added SpeI site; subscript numbers refer to position in pEGFP sequence). The PCR product was purified and digested with SpeI (New England Biolabs, Beverly, MA) using the PCR-added 5′ restriction endonuclease site and the internal SpeI restriction site at position 1090 in pEGFP to result in a 3.2 kb-fragment containing the genes for GFP and nickel resistance. The digest was purified and ligated using procedures outlined above and transformed into E. coli JM109. Km², GFP⁺ colonies were isolated from LB + Km plates. Plasmid DNA was purified from isolates and digested with SpeI and EcoRI in order to confirm the insertion of the 2-CB, GFP, and nickel resistance genes into the constructed plasmid, pGN2.

The pGN2 plasmid was electroporated into P. putida KT2440 using standard procedures [19] to generate P. putida GN2. Activity of the 2-CB genes in P. putida GN2 was confirmed by degradation of 2-CB in modified M9 broth (without glucose or thiamine) [21] containing 100 μg 2-CB ml⁻¹ (98%; Sigma–Aldrich, St. Louis, MO). Production of GFP by P. putida GN2 was determined using epifluorescence microscopy [22,23].

2.2. Microcosms

Three different sandy loam soils were utilized to prepare the microcosms. Brazito soil was collected from the surface horizon (0–15 cm depth) at the University of Arizona’s Campbell Avenue Agricultural Experiment Station in Tucson, AZ. Madera and Oversite soils were collected from the surface horizon of sites in the Coro-nado National Forest near Green Valley, AZ and Sierra Vista, AZ. The Brazito, Madera, and Oversite soils had pH values of 8.5, 6.5, and 6.8, respectively. Percent organic C, sand, silt, and clay values were 0.7, 76, 12, and 12, respectively, for the Brazito soil; 2.6, 76, 20, and 4, for the Madera soil; and 2.8, 74, 21, and 5 for the Oversite soil. Following collection, field-moist soil was passed through a 2-mm sieve, and 100 g dry weight equivalent was added to polypropylene jars.

Microcosms were amended with 500 μg of 2-CB g⁻¹ dry soil using a 2-CB stock solution prepared similarly to a previously described method [24]. Microcosms were also either not augmented or augmented with approximately 5 × 10⁶ colony-forming units (CFU) of P. putida GN2 g⁻¹ dry soil. The inoculant was from a late-log phase culture grown at 27 °C in the modified M9 minimal salts medium containing 100 μg 2-CB ml⁻¹. Treatments were conducted in triplicate. After addition of all amendments, the soils were brought to approximately field capacity (175% of −0.3 MPa soil water potential) with sterile deionized water and incubated at 27 °C.

2.3. Chlorobenzoate levels and enumeration of degraders

The 2-CB was extracted from soil and quantified using HPLC as previously described [2]. Briefly, 1.2 g moist soil was placed into 9.5 ml of extracting solution (6 μM Zwittergent detergent and 0.2% sodium hexa-metaphosphate) [25] and mixed. An aliquot of the soil-extraction solution was then centrifuged at 16,000g for 10 min, and the supernatant was filtered (0.2 μm, Pall Gelman, Ann Arbor, MI). The filtered sample was injected into a Waters Associates LC Module HPLC system with a Waters C₁₈ column (3.9 mm × 150 mm) and absorption measured at 235 nm. Elution was iso-cratic with an acetonitrile-acidified water (50:50, v/v) mobile phase at a flow rate of 1 ml min⁻¹. Acidified water, pH 2.6, was prepared with phosphoric acid. The 2-CB degrader numbers were determined by serially diluting the initial soil extraction solution and spreading the appropriate dilutions onto Bushnell-Haas minimal salts agar containing 500 μg of 2-CB ml⁻¹ [2,21]. Degrader colonies were enumerated after incubation of plates at 27 °C for 14 days. Additionally, degrader colonies on Bushnell-Haas agar with 2-CB were screened for GFP expression using a Dark Reader Spot Lamp (Clare Chemical Research, Inc., Dolores, CO). Five dominant indigenous 2-CB degraders were isolated from each the non-augmented Brazito and Madera soils and characterized based on partial 16S rDNA sequences using previously described methods [2].

2.4. DNA extraction for RTm-PCR and generation of standard curves

DNA was isolated from the soil microcosms using the UltraClean Soil DNA Kit (MoBio Laboratories, Inc.,
Solana Beach, CA) and further purified with QIAquick PCR Purification Kit (Qiagen). For the RTm-PCR standard curves, duplicate cultures of *P. putida* GN2 were grown to a cell density of 6.3 \((\pm 4.6) \times 10^7\) CFU ml\(^{-1}\) (log phase) in modified M9 broth (without glucose or thiamine) [21] containing 500 µg 2-CB ml\(^{-1}\) at 28 °C and 150 rpm. The cultures were serially diluted in 0.85% NaCl. Fifty microliters aliquot of each dilution was boiled at 100 °C for 5 min and centrifuged for 2 min at 13,000g. Standard curves were also determined for *P. putida* GN2 DNA extracted from each of the three different soils. Duplicate samples of each soil (0.25 g dry wt.) were amended with 50 µl of *P. putida* GN2 with the same cell density as described above. DNA was extracted and purified from the soils, and 10-fold serial dilutions were made for RTm-PCR analysis.

2.5. Primer/probe pairs and RTm-PCR

The RTm-PCR primers and probes were designed for the enhanced green fluorescent protein gene (*gfp*) (Clontech, Palo Alto, CA) and the 2-halobenzoate dioxygenase gene (*cbdA*) (Table 2) [2,18]. Both *gfp* and *cbdA* genes were introduced into *P. putida* GN2 as described above. Probe sequences for RTm-PCR were developed using the Primer Express software (version 2, Applied Biosystems) in the Arizona Research Laboratory, Division of Biotechnology, at the University of Arizona. The cycle threshold values obtained from triplicate samples were converted to CFU of *P. putida* GN2 g\(^{-1}\) dry soil using the standard curves generated from Brazilito, Madera, and Oversite soils spiked with the different dilutions of *P. putida* GN2.

3. Results

3.1. Gene expression in *P. putida* GN2

*Pseudomonas putida* GN2 demonstrated activity of the introduced plasmid genes, *cbdSABC* and *gfp*. *P. putida* GN2 degraded 100 µg of 2-CB ml\(^{-1}\) within 45 h in contrast to insignificant degradation by *P. putida* KT2440 without the pGN2 plasmid (data not shown). The *P. putida* GN2 cells also produced enough GFP to be easily detected by epifluorescence microscopy and the Dark Reader Spot Lamp.

3.2. RTm-PCR standard curves

The cycle threshold values for *cbdA* and *gfp* amplified from separate tubes were similar to those obtained when the two primer/probes were amplified in the same tube (data not shown); therefore, *cbdA* and *gfp* were quantified in the same tube in order to reduce the amount of reagents used. Using the pure culture of *P. putida* GN2 (without DNA isolation), the standard curves for *cbdA* (*y* = 37.63 – 2.86*x*, \(r^2 = 0.99\)) and *gfp* (*y* = 37.78 – 2.94*x*,

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5′-3′)</th>
<th>(T_m) (°C)</th>
<th>Amplified fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gfp</em> forward</td>
<td>CTGCTGCCCAGCAACCAC</td>
<td>60</td>
<td>94</td>
</tr>
<tr>
<td><em>gfp</em> TaqMan</td>
<td>VIC-CCAGTCCCGCCTGAGCAAGACC-TAMRA</td>
<td>78</td>
<td>94</td>
</tr>
<tr>
<td><em>gfp</em> reverse</td>
<td>TCACGAACTCCAGCAGGAC</td>
<td>60</td>
<td>94</td>
</tr>
<tr>
<td><em>cbdA</em> forward</td>
<td>ACGGGACATGCTTCTTCTTG</td>
<td>58</td>
<td>118</td>
</tr>
<tr>
<td><em>cbdA</em> TaqMan</td>
<td>FAM-ACCCGCGTCTCTAACCAGGAA-TAMRA</td>
<td>70</td>
<td>118</td>
</tr>
<tr>
<td><em>cbdA</em> reverse</td>
<td>GTATTGACGATAGCGTGCCG</td>
<td>58</td>
<td>118</td>
</tr>
</tbody>
</table>

\(T_m\) was calculated using the equation: \(T_m = 2(A + T) + 4(G + C)\).

* VIC: proprietary name of Applied Biosystems; FAM: 5-carboxyfluorescein; TAMRA: \(N,N,N',N'\)-tetramethyl-6-carboxy-rhodamine.

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Table 2
Sequences of primers and TaqMan probes used for RTm-PCR in this study
bars represent the variation of two replicate samples.

\[ y = 46.57 - 3.23x, \quad r^2 = 1.00 \text{ (Brazito-cbdA); } y = 47.41 - 3.32x, \quad r^2 = 0.98 \text{ (Madera-cbdA); and } y = 45.16 - 3.30x, \quad r^2 = 0.99 \text{ (Oversite-cbdA). RTm-PCRs for the standard curves were performed multiple times yielding similar results.}

### 3.3. Chlorobenzoate degradation

Augmentation of the three soils with *P. putida* GN2 increased the rate of 2-CB degradation. The 2-CB was eliminated from the inoculated Madera and Oversite microcosms within 3 days and from the inoculated Brazito microcosms within 5 days (Fig. 2). In contrast, the microbial populations in the non-augmented Brazito microcosms required 12 days to degrade the 2-CB while the non-augmented Madera and Oversite microcosms showed negligible degradation even after 17 days.

### 3.4. Comparison of RTm-PCR results with culturable *P. putida* GN2 numbers

In the augmented soils, the *P. putida* GN2 cells increased from their initial numbers to about 10^8 CFU g\(^{-1}\) following inoculation and then decreased slightly after the 2-CB was degraded. Based on the *P. putida* GN2 standard curves in soil, the estimated numbers of *P. putida* GN2 measured by the two TaqMan probes were similar to the values determined by plate counts during active 2-CB degradation (Fig. 3). In all three soils, the maximum degrader numbers corresponded to the highest level of 2-CB degradation (5 days for Brazito and 3 days for Madera and Oversite). Following 2-CB degradation, the number of RTm-PCR-estimated degraders decreased more rapidly than the number of culturable degraders, with the RTm-PCR-estimated degraders being about one order of magnitude lower in all soils by 17 days (Fig. 3).
3.5. RTm-PCR detection and characterization of indigenous degraders

The RTm-PCR for cbdA gave no indication of indigenous 2-CB degraders in any of the non-augmented soils even though the 2-CB was degraded in the Brazito soil. However, relatively low numbers (about 10⁴ CFU g⁻¹) of colonies developed on the 2-CB plates from the non-augmented Brazito soil after 12 d. Five of these degraders, having colony morphologies similar to the 2-CB degrader Burkholderia sp. 2CBA previously found in the Madera soil [2], were tested with RTm-PCR and found to contain the cbdA gene.

4. Discussion

RTm-PCR was used with two different primer/probe sets to quantify a genetically engineered bacterium during contaminant degradation in soil. The RTm-PCR accurately determined P. putida GN2 numbers during active 2-CB degradation in all three tested soils as indicated by the similarity between the RTm-PCR results and plate count data. The RTm-PCR results for both tested genes were also very comparable with each other throughout the study indicating the reproducibility of the detection method. However, RTm-PCR appeared to underestimate P. putida GN2 numbers, as compared to culturable numbers, following 2-CB degradation. If these results were reversed, with the RTm-PCR results being higher than the culturable numbers, the most likely hypothesis would be that some of the P. putida GN2 had died following 2-CB degradation. While RTm-PCR would detect all of the P. putida GN2, including dead cells, the culturable method would only detect live cells [14]. However, since the culturable P. putida GN2 numbers were higher than those determined by the RTm-PCR, the results seem to indicate a decrease in the copy number of the gfp and cbdA genes, both of which were plasmid encoded. The reduction may have been due to decreased selective pressure for maintenance of the pGN2 plasmid following removal of the 2-CB [26–28]. This decrease would result in an underestimation of the number of P. putida GN2 by RTm-PCR since the standard curves were determined by spiking the soils with actively growing cells which appear to have a higher plasmid copy number. RTm-PCR did not detect the cbdA gene in the non-augmented Madera soil community DNA even though the gene was detected in bacteria isolated from the Madera soil in the current and previous (Burkholderia sp. 2CBA) studies [2]. The inability to detect the gene was probably due to low 2-CB degrader numbers in the soil (~10⁵ CFU g⁻¹). The cbdA gene in Burkholderia sp. 2CBA, is thought to be chromosomally encoded and would therefore be contained at a lower copy number than the medium copy number, plasmid encoded cbdA gene in P. putida GN2 [2,17]. The DNA from the inoculated P. putida GN2 may have also been more readily extracted than the DNA from the...
indigenous microorganisms [29,30]. These differences would potentially explain why P. putida GN2 was detected at $10^3$ CFU g$^{-1}$ but the indigenous Burkholderia sp. 2CBA could not be detected at $10^5$ CFU g$^{-1}$. Analysis of Madera soil from a previous study [2] indicated that the cbdA gene could be detected in samples where $\sim10^7$ CFU of Burkholderia sp. 2CBA g$^{-1}$ were present (data not shown).

Overall, these results indicate the ability of the constructed strain, P. putida GN2, to proliferate and actively degrade 2-CB in different soils. With the incorporated GFP marker, this strain should be readily applicable to other augmentation experiments. This study also demonstrates the utility of RTm-PCR to rapidly and accurately assess the numbers of an introduced microorganism. However, the gene(s) used for RTm-PCR detection should be carefully chosen since the copy number of plasmid-borne genes may vary with the metabolic status of the cell and thus potentially lead to inaccurate population estimations.

Acknowledgements

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References


