ELSEVIER

FEMS Microbiology Letters 233 (2004) 307–314

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

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

Gejiao Wang, Terry J. Gentry *, Gregor Grass [1], Karen Josephson, Christopher Rensing, Ian L. Pepper

Department of Soil, Water and Environmental Science, University of Arizona, Tucson, AZ 85721, USA

Received 9 December 2003; received in revised form 23 February 2004; accepted 28 February 2004

First published online 9 March 2004

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′ exonuclease 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

* Corresponding author. Present address: Environmental Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Bldg. 1505, MS-6038, Oak Ridge, TN 37831-6038, USA. Tel.: +1-865-574-7302; fax: +1-865-576-8646.

E-mail address: gentrytj@ornl.gov (T.J. Gentry).

[1] Present address: Institut für Mikrobiologie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Str. 3, 06099 Halle, Germany

0378-1097/$22.00
doi:10.1016/j.femsle.2004.02.025

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 numbers of a *Pseudomonas resinovorans* strain with carbazole and dioxin degradation in soil. The *P. resinovorans* inoculant contained the green fluorescent protein (GFP) gene which was also used to determine population numbers via culturing methods. In both of these studies, only one gene was used for RTm-PCR detection of the inoculant. Detection of multiple genes in the same microorganism would help to validate the RTm-PCR results and to also give an indication of the method's reproducibility with different genes.

For this study, we compared the use of RTm-PCR for two different genes and also culturing methods to enumerate a GFP-labeled *Pseudomonas putida* culture following inoculation into three different soils. The specific objectives of this study were to assess the ability of the engineered degrader to proliferate and degrade 2-chlorobenzoate (2-CB) in three different soils and compare RTm-PCR quantification with culturable degrader numbers and 2-CB 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 $\mu g\,ml^{-1}$) or kanamycin (Km, 50 $g\,ml^{-1}$) was added to the media when appropriate. Additionally, LB containing 3 mM $NiCl_2$ 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 *Eco*RI (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 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
E. coli DH5α	*RecA1 endA1 gyrA96 thi-1 hsdR17*(r_k-, m_k+) *supE44 relA1 deoR ΔlacZYA-argF U169*	New England Biolabs
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17*(r_k-, m_k+) *supE44 relA1 Δ*(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]	Promega
P. putida KT2440	r^-m^+	[16]; DSMZ[b]
Plasmids		
p2BG	Ap^r 2-CB$^+$; pBluescript II KS+ with ca. 7-kb insert containing *cbdSABC*	[18]
pBHR1	Km^r, broad-host-range	[17]; MoBiTech
pBHR1::2-CB	2-CB$^+$; pBHR1 with ca. 10-kb *Eco*RI fragment insert containing *cbdSABC*	This work
pEGFP	Ap^r GFP$^+$	Clontech
pEGFP::nreB	*nre*B$^+$; pEGFP with 2.1-kb *Eco*RI fragment insert containing *nreB*	This work
pGEMCII2	Ap^r *nre*B$^+$; pGEM T-Easy with 2.1-kb insert containing *nreB*	[20]
pGN2	GFP$^+$ 2-CB$^+$ *nreB*$^+$ Km^r; pBHR1::2-CB with 3.2-kb *Spe*I, PCR product containing *gfp* and *nreB*	This work

[a] 2-CB, 2-chlorobenzoate; Ap, ampicillin; Km, kanamycin; GFP, green fluorescent protein.
[b] 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 *Eco*RI, 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 *Eco*RI, purified and ligated as mentioned above for pBHR1::2-CB, and transformed into *E. coli* DH5α. Prospective positive colonies that produced GFP were selected from LB + $NiCl_2$ plates. The *nreB* insert in pEGFP::nreB was confirmed as outlined above with *Eco*RI 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′-NNN<u>ACTAGT</u>$_5$CCCAA-TACGCAAACCGCCTCTC-3′ and 5′-NNNNNAGA-TCT$_{1247}$ACACCCGCCAACACCCGCT-3′ (N = 25% of each A, C, G, and T; underlined sequence is an added *Spe*I site; subscript numbers refer to position in pEGFP sequence). The PCR product was purified and digested with *Spe*I (New England Biolabs, Beverly, MA) using the PCR-added 5′ restriction endonuclease site and the internal *Spe*I 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. Km^r, GFP^+ colonies were isolated from LB + Km plates. Plasmid DNA was purified from isolates and digested with *Spe*I and *Eco*RI 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 Coronado 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 5×10^5 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 hexametaphosphate) [25] and mixed. An aliquot of the soil-extraction solution was then centrifuged at 16,000*g* 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 C_{18} column (3.9 mm × 150 mm) and absorption measured at 235 nm. Elution was isocratic 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 spread-plating 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 (± 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 13,000*g*. 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, Foster City, CA) and synthesized by Applied Biosystems. The *gfp* probe contained fluorescent 5-carboxyfluorescein (FAM) as a reporter fluorochrome on the 5′ end and $N,N,N,'N'$-tetramethyl-6-carboxy-rhodamine (TAMRA) as a quencher on the 3′ end of the nucleotide sequence. The *cbdA* probe contained fluorescent VIC (proprietary name of Applied Biosystems, Foster City, CA) as a reporter on the 5′ end and TAMRA as a quencher on the 3′ end. The RTm-PCR was first optimized by testing different primer and probe concentrations in order to obtain the minimum cycle threshold values. We also compared the results from two separate RTm-PCR reactions for *gfp* and *cbdA* with those obtained by combining *gfp*- and *cbdA*-RTm-PCR in a single tube. Following optimization, the RTm-PCR reactions for the experimental samples were performed in single tubes (50 μl) and consisted of 450 nM of each forward and reverse primer, 125 nM of each TaqMan probe, 25 μl of 2× TaqMan Master Mix Buffer (Applied Biosystems), and 10 μl of lysed *P. putida* GN2 cells or 5 μl of isolated soil DNA. The DNAs extracted from the soil microcosms for standard curves were diluted 1:50 prior to RTm-PCR analysis. The RTm-PCR was initiated with a 2 min cycle at 50 °C, followed by 95 °C for 10 min and by 40 cycles of denaturing for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. The PCR was carried out in a spectrofluorimetric thermal cycler, ABI Prism 7000 Sequence Detection System (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 Brazito, 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* ($y = 37.63 - 2.86x$, $r^2 = 0.99$) and *gfp* ($y = 37.78 - 2.94x$,

Table 2
Sequences of primers and TaqMan probes used for RTm-PCR in this study

Primer/probe	Sequence (5′–3′)[a]	T_m (°C*)	Amplified fragment (bp)
gfp forward	CTGCTGCCCGACAACCAC	60	
gfp TaqMan	VIC-CCAGTCCGCCCTGAGCAAAGACC-TAMRA	78	94
gfp reverse	TCACGAACTCCAGCAGGAC	60	
cbdA forward	ACGGGCACATGCTTCTTTG	58	
cbdA TaqMan	FAM-ACCCGGCTCCCTAACCCGGAA-TAMRA	70	118
cbdA reverse	TGATTGACGATAGCGTCGG	58	

* T_m was calculated using the equation: $T_m = 2(A + T) + 4(G + C)$.
[a] VIC: proprietary name of Applied Biosystems; FAM: 5-carboxyfluorescein; TAMRA: $N,N,N,'N'$-tetramethyl-6-carboxy-rhodamine.

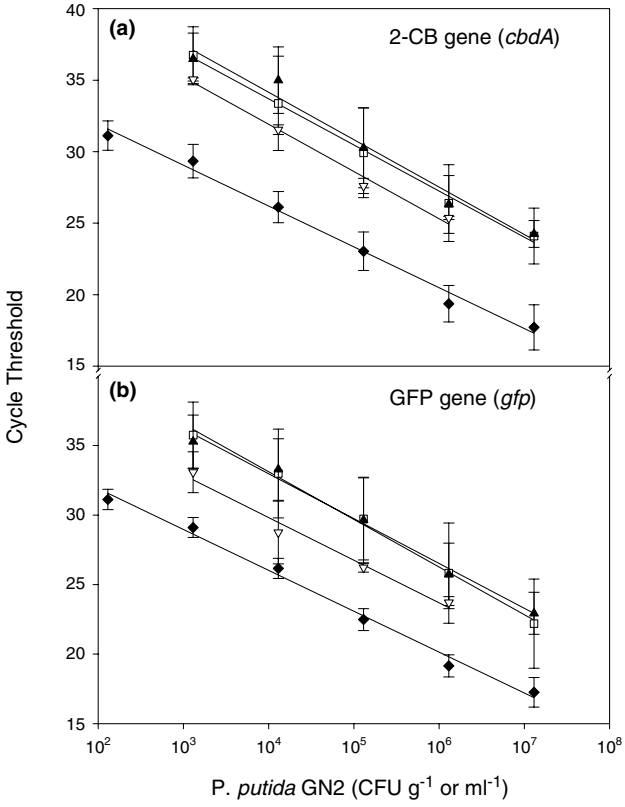

Fig. 1. Real-time PCR standard curves for the detection of *Pseudomonas putida* GN2 in pure culture (◆) or from Brazito (□), Madera (▲), or oversite (▽) soil using either (a) the 2-chlorobenzoate degradative gene, *cbdA*, or (b) the green fluorescent protein gene, *gfp*. Error bars represent the variation of two replicate samples.

$r^2 = 0.99$) [$y = y$-axis value; $x = x$-axis value; $r^2 =$ correlation coefficient] were linear over six orders of magnitude (Fig. 1). Both genes could be detected in pure culture from as few as 130 CFU ml^{-1} (1.3 CFU per PCR).

Following extraction with the MoBio kit, the *cbdA* and *gfp* genes from the Brazito and Madera soils could be amplified by PCR but not from Oversite soil. The Oversite DNA extract was further purified by passing it through a QIAquick PCR Purification kit which sufficiently cleaned the DNA for PCR amplification. Therefore, all Brazito, Madera, and Oversite soil DNA extracts were passed through the QIAquick procedure prior to PCR.

The RTm-PCR sensitivity for *P. putida* GN2 genes decreased about one order of magnitude in soil as compared to pure culture (Fig. 1). The detection limit in all soils was around 10^3 CFU of *P. putida* GN2 g^{-1} dry soil (Fig. 1). Standard curves were linear over five orders of magnitude (10^3–10^7 CFU g^{-1} dry soil) for Madera and Brazito and four orders of magnitude for Oversite (10^3–10^6 CFU g^{-1} dry soil). Linear regression values for the standard curves were: $y = 46.80 - 3.43x$, $r^2 = 1.00$ (Brazito-*gfp*); $y = 45.88 - 3.22x$, $r^2 = 0.99$ (Madera-*gfp*); $y = 42.13 - 3.07x$, $r^2 = 0.98$ (Oversite-*gfp*); $y = 46.57 - 3.23x$, $r^2 = 1.00$ (Brazito-*cbdA*); $y = 47.41 - 3.32x$, $r^2 = 0.98$ (Madera-*cbdA*); and $y = 45.16 - 3.30x$, $r^2 = 0.99$ (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).

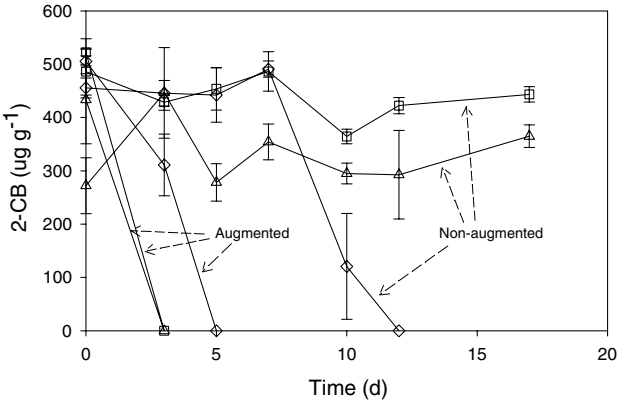

Fig. 2. Concentrations of 2-chlorobenzoate in Brazito (◇), Madera (△), and Oversite (□) soils either not augmented or augmented with *Pseudomonas putida* GN2 as indicated in the figure. Error bars represent the standard deviation of three replicate samples.

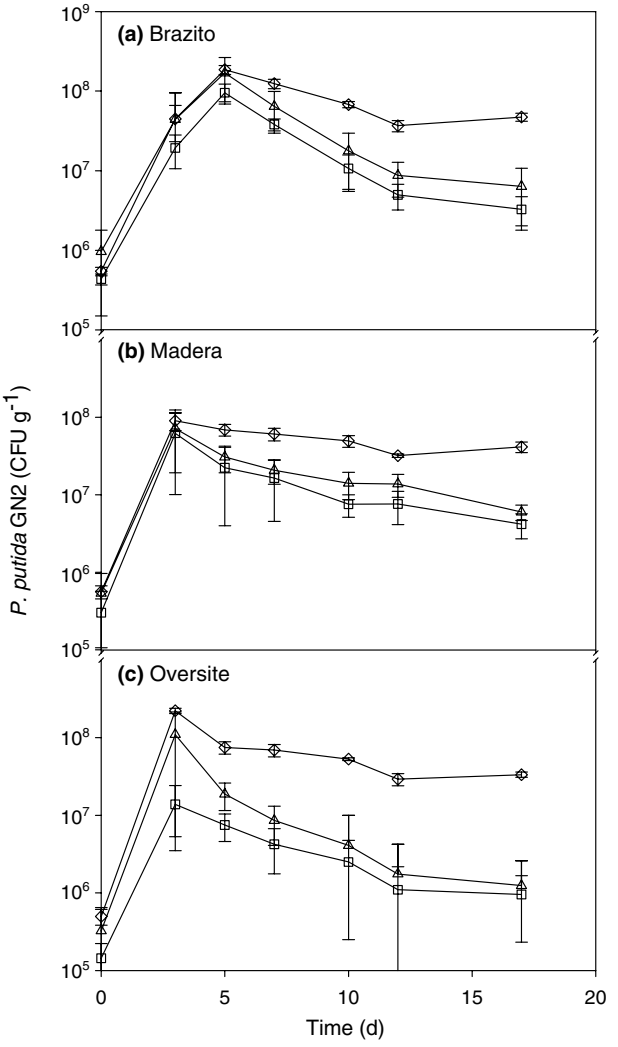

Fig. 3. Comparison of *Pseudomonas putida* GN2 numbers in (a) Brazito, (b) Madera, and (c) Oversite soils as detected by culturable methods (◇) and RTm-PCR of the 2-chlorobenzoate degradative gene, *cbdA* (△), and the green fluorescent protein gene, *gfp* (□). Error bars represent the standard deviation of three replicate samples.

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 10^4 CFU g^{-1}) of colonies developed on the 2-CB plates from the non-augmented Brazito soil after 12 d. Five of these degraders were analyzed with RTm-PCR, but the *cbdA* gene was not detected in any of the isolates. Further 16S rDNA analysis indicated that the isolates were similar to *Streptomyces hebeinensis* (GenBank Accession No. AY277529; 99% nucleotide identity based on the approximately 440 bp region of 16S rDNA amplified by PCR). A representative 16S rDNA sequence was submitted to GenBank and assigned Accession No. AY505495. About 10^5 CFU of culturable 2-CB degraders g^{-1} were detected in the non-augmented Madera soil after 17 days. 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 ($\sim 10^5$ 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 $\sim 10^7$ 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

We thank Brian Coullahan for technical support during real-time PCR analysis and Katsuhisa Suzuki and Naoto Ogawa for providing plasmid p2BG. We also thank the Coronado National Forest, USDA-Forest Service for permission to collect Madera and Oversite Canyon soils. This work was supported by NIEHS Basic Superfund Grant 5 P42 ESO4940-09.

References

[1] Fulthorpe, R.R., Rhodes, A.N. and Tiedje, J.M. (1998) High levels of endemicity of 3-chlorobenzoate-degrading soil bacteria. Appl. Environ. Microbiol. 64, 1620–1627.

[2] Gentry, T.J., Wang, G., Rensing, C., Pepper, I.L., 2004. Chlorobenzoate-degrading bacteria in similar pristine soils exhibit different community structures and population dynamics in response to anthropogenic 2-, 3-, and 4-chlorobenzoate levels. Microb. Ecol. (in press).

[3] Yi, H.-R., Min, K.-H., Kim, C.-K. and Ka, J.-O. (2000) Phylogenetic and phenotypic diversity of 4-chlorobenzoate-degrading bacteria isolated from soils. FEMS Microbiol. Ecol. 31, 53–60.

[4] Focht, D.D. and Shelton, D. (1987) Growth kinetics of *Pseudomonas alcaligenes* C-0 relative to inoculation and 3-chlorobenzoate metabolism in soil. Appl. Environ. Microbiol. 53, 1846–1849.

[5] Gentry, T.J., Newby, D.T, Josephson, K.L. and Pepper, I.L. (2001) Soil microbial population dynamics following bioaugmentation with a 3-chlorobenzoate-degrading bacterial culture. Biodegradation 12, 349–357.

[6] Bach, H.J., Tomanova, J., Schloter, M. and Munch, J.C. (2002) Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. J. Microbiol. Methods 49, 235–245.

[7] Baldwin, B.R., Nakatsu, C.H. and Niehs, L. (2003) Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl. Environ. Microbiol. 69, 3350–3358.

[8] Hermansson, A. and Lindgren, P.E. (2001) Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Appl. Environ. Microbiol. 67, 972–976.

[9] Hristova, K.R., Lutenegger, C.M. and Scow, K.M. (2001) Detection and quantification of methyl tert-butyl ether-degrading strain PM1 by real-time TaqMan PCR. Appl. Environ. Microbiol. 67, 5154–5160.

[10] Kolb, S., Knief, C., Stubner, S. and Conrad, R. (2003) Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl. Environ. Microbiol. 69, 2423–2429.

[11] Rodrigues, J.L., Aiello, M.R., Urbance, J.W., Tsoi, T.V. and Tiedje, J.M. (2002) Use of both 16S rRNA and engineered functional genes with real-time PCR to quantify an engineered, PCB-degrading Rhodococcus in soil. J. Microbiol. Methods 51, 181–189.

[12] Stubner, S. (2002) Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with SybrGreen detection. J. Microbiol. Methods 50, 155–164.

[13] Widada, J., Nojiri, H., Kasuga, K., Yoshida, T., Habe, H. and Omori, T. (2001) Quantification of the carbazole 1,9a-dioxygenase gene by real-time competitive PCR combined with co-extraction of internal standards. FEMS Microbiol. Lett. 202, 51–57.

[14] Widada, J., Nojiri, H., Yoshida, T., Habe, H. and Omori, T. (2002) Enhanced degradation of carbazole and 2,3-dichlorodibenzo-*p*-dioxin in soils by *Pseudomonas resinovorans strain* CA10. Chemosphere 49, 485–491.

[15] Kikuchi, T., Iwasaki, K., Nishihara, N., Takamura, Y. and Yagi, O. (2002) Quantitative and rapid detection of the trichloroethylene-degrading bacterium *Methylocystis* sp. M in groundwater by real-time PCR. Appl. Microbiol. Biotechnol. 59, 731–736.

[16] Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martinsdos Santos, V.A.P., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R.T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K.N., Düsterhöft, A., Tümmler, B. and Fraser, C.M. (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. 4, 799–808.

[17] Szpirer, C.Y., Faelen, M. and Couturier, M. (2001) Mobilization function of the pBHR1 plasmid, a derivative of the broad-host-range plasmid pBBR1. J. Bacteriol. 183, 2101–2110.

[18] Suzuki, K., Ogawa, N. and Miyashita, K. (2001) Expression of 2-halobenzoate dioxygenase genes (*cbdSABC*) involved in the degradation of benzoate and 2-halobenzoate in *Burkholderia* sp. TH2. Gene 262, 137–145.

[19] Sambrook, J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[20] Grass, G., Fan, B., Rosen, B.P., Lemke, K., Schlegel, H.-G. and Rensing, C. (2001) NreB from *Achromobacter xylosoxidans* 31A is a nickel-induced transporter conferring nickel resistance. J. Bacteriol. 183, 2803–2807.

[21] Atlas, R.M. (1993) Handbook of Microbiological Media. CRC Press, Boca Raton, FL. 1079 pp.

[22] Cassidy, M.B., Leung, K.T., Lee, H. and Trevors, J.T. (2000) A comparison of enumeration methods for culturable *Pseudomonas fluorescens* cells marked with green fluorescent protein. J. Microbiol. Methods 40, 135–145.

[23] Abbey, A.M., Beaudette, L.A., Lee, H. and Trevors, J.T. (2003) Polychlorinated biphenyl (PCB) degradation and persistence of a *gfp*-marked *Ralstonia eutropha* H850 in PCB-contaminated soil. Appl. Microbiol. Biotechnol. 63, 222–230.
[24] DiGiovanni, G.D., Neilson, J.W., Pepper, I.L. and Sinclair, N.A. (1996) Gene transfer of *Alcaligenes eutrophus* JMP134 plasmid pJP4 to indigenous soil recipients. Appl. Environ. Microbiol. 62, 2521–2526.
[25] Brendecke, J.W., Axelson, R.D. and Pepper, I.L. (1993) Soil microbial activity as an indicator of soil fertility; the long term effects of municipal sewage sludge on an arid soil. Soil Biol. Biochem. 25, 751–758.
[26] Furuno, S., Watanabe-Murakami, Y., Takebe-Suzuki, N. and Yamaguchi, K. (2000) Negative control of plasmid pSC101 replication by increased concentrations of both initiator protein and iterons. J. Gen. Appl. Microbiol. 46, 29–37.
[27] Venkova-Canova, T., Patek, M. and Nesvera, J. (2003) Control of rep gene expression in plasmid pGA1 from *Corynebacterium glutamicum*. J. Bacteriol. 185, 2402–2409.
[28] McLoughlin, A.J. (1994) Plasmid stability and ecological competence in recombinant cultures. Biotechnol. Adv. 12, 279–324.
[29] Frostegard, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X. and Simonet, P. (1999) Quantification of bias related to the extraction of DNA directly from soils. Appl. Environ. Microbiol. 65, 5409–5420.
[30] Kabir, S., Rajendran, N., Ameniya, T. and Itoh, K. (2003) Real-time quantitative PCR assay on bacterial DNA: in a model soil system and environmental samples. J. Gen. Appl. Microbiol. 49, 101–109.