Microbial Ecology

Using Real-Time PCR to Assess Changes in the Hydrocarbon-Degrading Microbial Community in Antarctic Soil During Bioremediation

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Abstract

A real-time polymerase chain reaction (PCR) method to quantify the proportion of microorganisms containing alkane monooxygenase was developed and used to follow changes in the microbial community in hydrocarbon-contaminated Antarctic soil during a bioremediation field trial. Assays for the *alkB* and *rpoB* genes were validated and found to be both sensitive and reproducible (less than 2% intrarun variation and 25–38% interrun variation). Results from the real-time PCR analysis were compared to analysis of the microbial population by a culture-based technique [most probable number (MPN) counts]. Both types of analysis indicated that fertilizer addition to hydrocarbon-contaminated soil stimulated the indigenous bacterial population within 1 year. The proportion of *alkB* containing microorganisms was positively correlated to the concentration of *n*-alkanes in the soil. After the concentration of *n*-alkanes in the soil decreased, the proportion of alkane-degrading microorganisms decreased, but the proportion of total hydrocarbon-degrading microorganisms increased, indicating another shift in the microbial community structure and ongoing biodegradation.

Introduction

The ability to accurately quantify functional genes in the environment is an important step in understanding many ecological processes. Existing techniques include hybridization methods such as dot blots, and end-point polymerase chain reaction (PCR) methods such as competitive PCR and most probable number (MPN)-PCR. Although these methods are useful, hybridization methods are often only semiquantitative and end-point PCR methods require many reactions and a post-PCR analysis step, making them both labor-intensive and expensive. Real-time PCR is fast becoming the most popular alternative (see [11, 16, 32] for a detailed description). Originally developed for gene expression studies, it has found many applications in microbial ecology. These include the measurement of 16S rRNA genes as a measure of total and specific cell populations [21], the measurement of catabolic genes such as *bssA* [2] and *atz* [5] and the measurement of genes for important ecological processes such as denitrification [13] and ammonia oxidation [17].

The advantages that real-time PCR offers include speed, sensitivity, accuracy, simplicity, and the possibility of robotic automation. As with all new technologies, there is a need to explore and define the limitations of the method in terms of its sensitivity, accuracy, and reproducibility. Although the sensitivity and accuracy of real-time PCR assays are usually well characterized (e.g., [2]), few studies provide any data on the reproducibility of the method. Dionisi *et al.* [6] reported intra- and interrun variability and Hristova *et al.* [14] reported the reproducibility per sample. A recent study by Smith *et al.* [22] with the 16S rRNA gene found that the absolute gene copy numbers were influenced by a number of factors including the DNA isolation method and template concentration. Their observation that the intrarun variability was low (3–5%) but interrun variability was higher (11–26%) agrees with the conclusions of Dionisi *et al.* [6].

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Information on the reproducibility of real-time PCR methods is particularly important given that the relationship between the signal produced (the threshold cycle C_T) and concentration values for the amount of the target is a log-linear one described by the equation:

$$C_T = M * \log(\text{target concentration}) + B$$

where M is the slope of the standard curve and B is the Y intercept. This log-linear relationship has the effect of producing large variations in concentrations between replicates despite the fact that the raw signal is very similar. This suggests that caution should be used when drawing conclusions from small differences, especially when the number of replicates (n) is small. This effect was noted by Dionisi *et al.* [6], who reported that coefficient of variation (CV = standard deviation/mean) values calculated on the C_T were much lower than those calculated on copy number of the target gene (2% compared to between 20% and 36%).

As part of our investigations into the biodegradation of diesel spills in Antarctica, we wished to measure the activity of alkane-degrading microbes in soil samples. The alkane monooxygenases are a group of enzymes that catalyze the first step in the aerobic degradation of alkanes. The genetics of these enzymes are well characterized, particularly in *Pseudomonas* [25, 26] and *Rhodococcus* [28]. The *alkB* gene in particular has been found in isolates from a variety of genera, including *Pseudomonas* [25], *Rhodococcus* [29], *Alcanivorax* [10], and *Xanthobacter* [27], and it has also been detected by hybridization methods in environmental samples [30]. Alkane monooxygenases have been detected using hybridization methods in Antarctic and Brazilian soils [15] and in both contaminated and pristine Arctic and Antarctic soils [30]. A more quantitative MPN-PCR method was used to follow populations of alkane-degrading microbes in seawater microcosms [20], and Heiss-Blanquet *et al.* [12] used a competitive PCR method to correlate *alkB* levels to *n*-heptane degradation in freshwater and soil samples. All these studies, however, utilized several primer sets to target different types of alkane monooxygenases. To the best of our knowledge, no simple and quantitative method of measuring *alkB* in complex environmental samples using one assay has been reported. In this work, we present the development and validation of a real-time PCR method for the *alkB* and *rpoB* genes.

We examined the effect of nutrient addition on microbial communities in a bioremediation field trial of hydrocarbon-contaminated, nutrient-poor Antarctic soil. We hypothesized that hydrocarbon-degrading bacteria, having a source of carbon not available to other heterotrophic bacteria, would be preferentially stimulated by the addition of nutrients. To test this, we used a combination of molecular (real-time PCR) and culture-based techniques to examine changes in the function of microbial communities in this bioremediation field trial.

Materials and Methods

Bioremediation Field Trial. A landfarming bioremediation field trial was carried out at Australia's Old Casey Station, East Antarctica, from 1998 to 2004, and has been previously described in detail [8]. Soil in the area was contaminated with 36,000 L of Special Antarctic Blend (SAB) diesel in 1982 [23]. The field trial consisted of sieved (4.75 mm) and homogenized soil in clean metal containers. The containers were free-draining, but partially buried to simulate natural field conditions while mitigating the effects of lateral fuel migration. Each treatment was applied to four containers. In the current study, we were interested in the effects of fertilization treatments [addition of two amounts of controlled release nutrients: a low concentration (LCRN) to yield ca. 200 NO_3^- kg^{-1}, 300 NH_4^+ kg^{-1}, 100 PO_4^{-3} kg^{-1} or a high concentration of fertilizer (HCRN) to yield ca. 700 mg NO_3^- kg^{-1}, 900 NH_4^+ kg^{-1}, 400 PO_4^{-3} kg^{-1}] on the microbial population. Both fertilization treatments were compared to a control, which did not receive any fertilizer. The samples used in this study were collected in early 2000, approximately a year after the trial was initiated, and again (from the same plots) in 2004, after the trial had been running for 5 years. Soil samples were stored frozen at –18°C until analysis. DNA was extracted from approximately 0.5 g of each soil sample by using the PowerSoil DNA kit (MoBio) following the manufacturer's directions. The DNA quality was checked on agarose gels and quantified on a SmartSpec 3000 spectrophotometer (Bio-Rad). The chemical analysis of these samples by GC-FID is described by Powell *et al.* [18]. For this study, the sum of *n*-alkanes is the sum of *n*-alkanes from octane to octadecane.

Design of Primers to Detect the Alkane Monooxygenase Gene alkB. Primers were designed to amplify a fragment of the alkane monooxygenase gene alkB. Sequences (accession numbers are shown in Table 1) were obtained from the GenBank database and aligned. Regions of similar nucleotide composition were identified, and the NetPrimer program (http://www.premierbiosoft.com/netprimer/netprimer.html) was used to assist in choosing the best primer pair that resulted in a PCR product of approximately 100 bp. This gene is not well conserved, and to compensate several degenerate bases were incorporated in the primers. The forward primer f(alkBFd) is 5′-AAC TAC MTC GAR CAY TAC GG-3′ and the reverse primer (alkBRd) is 5′-TGA MGA TGT GGT YRC TGT TCC-3′ (where M = AC, R = AG and Y = CT). The specificity of this primer pair was initially tested by using the "search for short nearly exact matches" option of the Blast func-

Table 1. Accession number of sequences used in constructing the *alkB* primers and the theoretical melt temperature of the amplification product

Strain	*Accession number*	*Theoretical T_m of alkB product (°C)*
Rhodococcus erythropolis	AJ009586	83
Rhodococcus erythropolis	AJ297269	82
Rhodococcus erythropolis	AJ301874	80
Rhodococcus sp. 1BN	AJ401611	84
Rhodococcus sp. Q15	AF388179	80
Rhodococcus sp. Q15	AF388180	84
Rhodococcus sp. Q15	AF388181	82
Rhodococcus sp. Q15	AF388182	82
Rhodococcus fascians	AJ301873	84
Burkholderia cepacia	AJ293344	85
Thalassolituus oleivorans	AJ431700	73
Alcanivorax borkumensis	AJ577851	79
Pseudomonas fluorescens	AJ009579	84
Pseudomonas aeruginosa	AJ009581	83
Pseudomonas putida	AJ233397	77
Pseudomonas aureofacians	AJ250560	77
Xanthobacter flavus	AJ418063	78

tion [1]. When this pair of primers was blasted together, the closest matches returned were sequences from alkane monooxygenases.

The primers were synthesized by Geneworks (Hindmarsh, South Australia) and initially tested against two strains of alkane-degrading Rhodococci (*Rhodococcus fascians* JCM 10002 and *R. rhodochrous* JCM 3202) and an *Escherichia coli* strain (as a negative control) using the HotStar PCR kit (Qiagen). Amplification from genomic DNA preparations resulted in a single band of approximately 100 bp from the two rhodococci, but no bands were obtained from the *E. coli*. DNA from a soil sample (BR106 from the field trial described above) was subjected to the same amplification and the resulting product was cloned using the TOPO-TA cloning kit (Invitrogen). Plasmids were extracted by using the UltraClean miniplasmid prep kit (MoBio) and the clones were sequenced with the provided M13 primer using the DTCS QuickStart kit (BeckmanCoulter) and a CEQ8000 genetic analysis system (Beckman-Coulter).

An additional set of nondegenerate primers was developed based on the results of this clone library. These primers (AlkBF: 5′-AAC TAC ATC GAG CAC TAC GG-3′ and AlkBR: 5′-TGA AGA TGT GGT TGC TGT TCC-3′) were used for all other real-time PCR experiments.

Standards. Standards for the *alkB* gene were constructed from one of the clones described above. The plasmid was subjected to amplification with M13 primers to obtain a PCR product approximately 300 bp long containing the 100-bp *alkB* fragment. Use of the plasmid itself may result in an incorrect determination of the amount of DNA present due to the presence of supercoiled DNA, and it is recommended that there is at least 20 bp outside of the primer binding sites when constructing standards from a PCR product (Quantitect SYBRGreen PCR Handbook, Qiagen). The PCR product was purified by using the UltraClean PCR cleanup kit (MoBio) and measured spectrophotometrically at 260 nm. This was diluted in a 10-fold series to create the standards for a five point standard curve (5.8×10^4 to 5.8×10^8 copies) that was run in duplicate with each set of samples. The number of copies per microliter was calculated as follows:

- Molecular weight of fragment = 300 bp × 660 Da = 1.98×10^5 g
- 1 molecule or 1 copy of fragment = $1.98 \times 10^5/6.02 \times 10^{23} = 3.23 \times 10^{-19}$ g
- therefore 10 ng contains $10 \times 10^{-9}/3.23 \times 10^{-19}$ copies = 3.04×10^{10} copies

Quantification of AlkB in Soil Samples. Real-time PCR assays were carried out on a Rotorgene 3000 (Corbett) by using Quantitect SYBR green PCR kits (Qiagen). The primer concentrations were optimized to 1 pmol of both the forward and reverse primer in each 20 μL reaction. Two microliters of template DNA (whether sample or standard) was added to each reaction. Cycling conditions were as follows: hold for 15 min at 94°C, followed by 35 cycles of denaturing at 84°C for 20 s, annealing at 50°C for 30 s, extension and acquiring signal at 72°C for 45 s, a second acquiring step at 77°C for 15 s; hold at 45°C for 1 min followed by a melt curve from 45°C to 95°C. In addition to melt curve analysis, PCR products were checked on a 2% agarose gel to ensure they were the expected size.

Quantification of Total Bacteria in Soil Samples. The primers described by Dahlloff *et al.* [4] for the ribosomal polymerase B subunit (*rpoB*) gene 1698F (5′-CAA CAT CGG TTT GAT CAA C-3′) and 2041R (3′-CGT TGC ATG TTG GTA CCC AT-3′) were used to quantify the total number of bacterial genomes in each sample. Reaction conditions were the same as described above except that 1.5 pmol of each primer was used. Cycling conditions with these primers were as follows: hold for 15 min at 94°C, followed by 35 cycles of denaturing at 88°C for 30 s, annealing 50°C for 30 s, extension and acquiring signal at 72°C for 30 s; hold at 45°C for 1 min followed by a melt curve from 45°C to 95°C. A set of standards (3.8×10^4 to 3.8×10^8 copies) was created for this assay in the same manner as that described above for the *alkB* assay.

Most Probable Number Counts. Cultivable bacteria were enumerated using tailored versions of the Wrenn and Venosa MPN count method [31]. Initial soil

dilutions of ca. 0.5 g soil to Bushnell–Haas broth (Difco) were used for a four-tube MPN, with 1:10 serial dilutions in a 96-well microtiter plates. Samples were analyzed in triplicate. After serial dilution, 5 μL filter-sterilized hydrocarbons (either SAB or an alkane mix containing 9.1 g decane and 4.8 g pentadecane) were applied to the surface of the broth. The last row of wells were not inoculated and served as a negative control for growth. After 14 days incubation at 10°C, 50 μL of a 3% iodonitrotetrazolium violet (Sigma) solution was added to each of the wells and incubated overnight. Tubes were scored as positive on the formation of a pink precipitate. Total heterotrophic bacteria were also enumerated, in triplicate, in a similar manner except that half-strength tryptone soya broth was used instead of Bushnell–Haas plus hydrocarbons and positive tubes were indicated by turbidity after 7 days incubation.

Results

Validation of Real-Time PCR Methods. Thirty-six sequences were obtained from the clones (created with the degenerate primer set), and of these 29 (80%) were related to alkane monooxygenase genes, mostly from *Pseudomonas* species. These sequences were used to design a set of nondegenerate primers by replacing the degenerate bases with the base most frequently found in that position in our clones. Although this increases the specificity of the assay and probably results in some variations of the gene not being detected, it is necessary to avoid the problem of differing amplification efficiencies created by the use of degenerate primers.

Melt-curve analysis was used to check for the production of secondary products such as primer dimers in the assays. It was observed in early runs with low DNA template concentrations that there were sometimes two peaks in the melt curves of both the *alkB* and *rpoB* gene assays: one with a melt temperature (T_m) at around 81°C or 86°C, respectively, and a second, smaller peak at 87°C or 91°C (e.g., Fig. 1A). The peak with the lower T_m was consistently present and on 2% agarose gels corresponded to a band of the expected size. The other peak was only present when the DNA template concentration was low or primer concentration was high and showed up as a faint fuzzy band of less than 100 bp on 2% agarose gels. A second primer dimer with a T_m of 72°C was also observed in some samples (Fig. 1B). Melt curves from optimized *rpoB* assays showing the presence of a small amount of product in the negative control are shown in Fig. 1C.

Differences in the T_m of the *alkB* PCR product (Fig. 1D) were observed between pure cultures (e.g., *Pseudomonas aeruginosa*, 81°C), soil samples (80.5°C), marine sediment samples (83°C), and the PCR product standard (82°C). Although the peaks were at slightly different temperatures, there was only a single peak for each sample, and when run on a 2% agarose gel the PCR products were the same size. The theoretical melt temperature of the expected product from each of the strains listed in Table 1 was calculated by using the Biomath calculator for melting temperature available at http://www.promega.com/biomath/default.htm (Promega). The range of melt temperatures calculated was quite large—up to 4°C within the same species and 12°C over different species (Table 1).

The standard curves for both assays were linear ($r^2 > 0.95$) over 8 orders of magnitude for the *alkB* assay and 6 orders of magnitude for the *rpoB* gene assay. This equated to a lower limit of 3 copies μL^{-1} for the *alkB* assay and 1880 copies μL^{-1} for the *rpoB* assay. The r^2 values were consistently greater than 0.95 and usually greater than 0.99 for both assays. Once the primer concentration was optimized, the efficiency of the reactions was between 0.9 and 1.1 (or 90–110%). Runs that fell outside these parameters ($r^2 < 0.95$ and efficiency <0.9 or >1.1) were repeated. There was usually no signal in the no-template control for the *alkB* assay, but for the *rpoB* assay the signal in the no-template control was generally between 1×10^3 and 5×10^3 copies μL^{-1}.

To assess the within-run reproducibility of the assays, the same sample was analyzed six times within the same run. The CV for both assays was low when calculated on the C_T values: 1.4% for the *alkB* gene assay and 1.2% for the *rpoB* gene assay, but much higher when calculated on the number of copies μL^{-1}: 16% and 18%, respectively.

The between-run reproducibility was assessed by including duplicates of the same sample with each run. As the selected threshold value varied for each run, the CV could only be calculated on the concentration of each target gene. The variability measured in this way was higher than the within-run variation: 25% for the *alkB* gene and 38% for the *rpoB* gene.

Field Trial Results. Student's *t* test was used to determine which treatments and/or times were significantly different from each other (Table 2). The MPN results were very variable, much more than the real-time PCR results. This variability meant that none of the differences between groups are significant at $p < 0.05$ although differences between groups could be detected at the $p < 0.15$ level for the MPN results. All the real-time PCR results were tested for significance at $p < 0.05$.

In the samples from 2000, which were taken approximately 1 year after the addition of fertilizer to the soil, only the LCRN treatment has significantly more copies of the *alkB* gene g^{-1} soil (Fig. 2A and Table 2). Both the LCRN and HCRN treatments have significantly more copies of *rpoB* than the control (Fig. 2C and Table 2). There are no significant differences, for any of the MPN

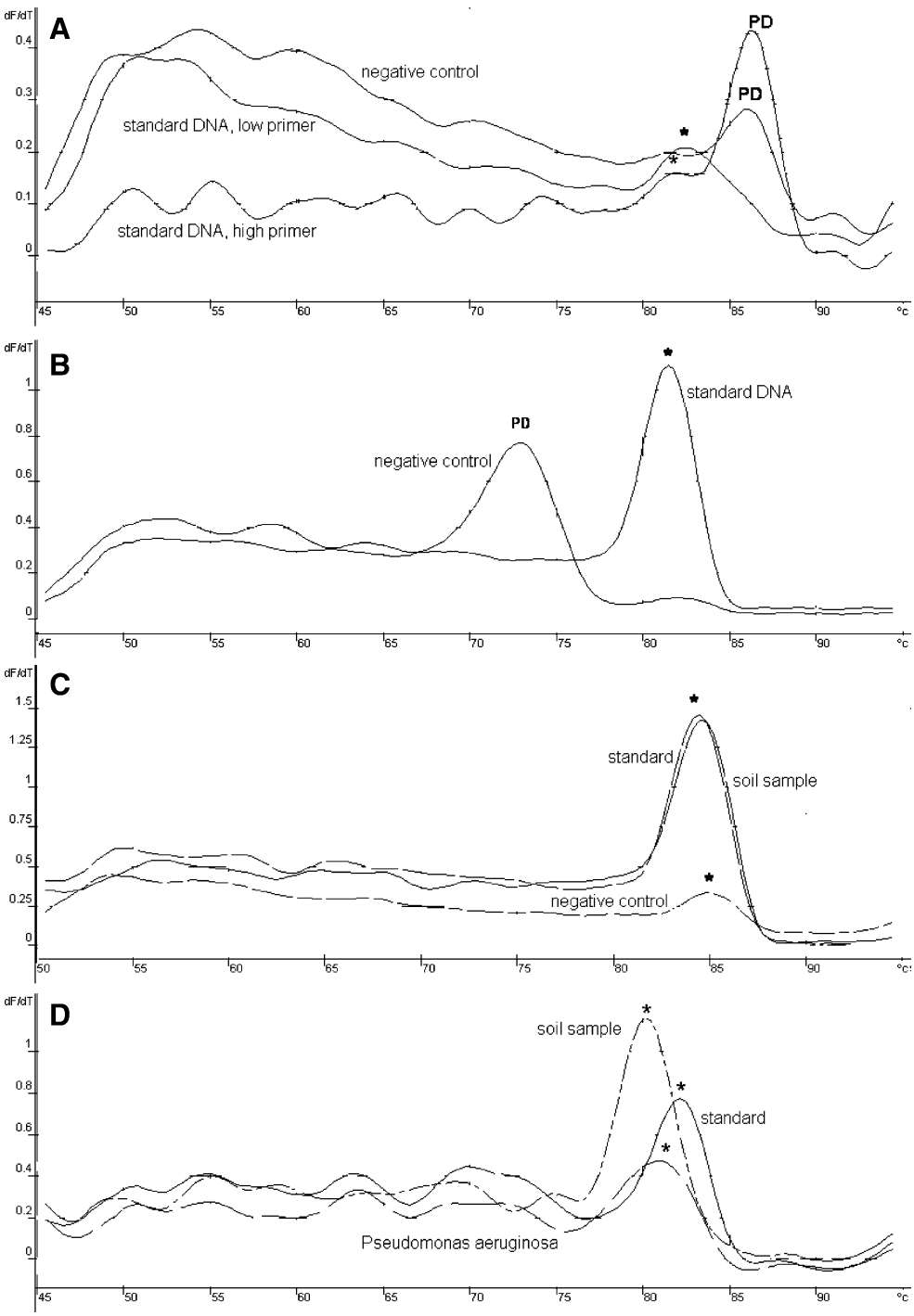

Figure 1. Melt curves from the *alk*B gene assay (A, B, D) and *rpo*B gene assay (C). The product is marked with an * and primer dimers are marked PD. (A, B) Occurrence of primer dimers formed when the primer concentration was high and the amount of template DNA low or absent. The temperature of denaturing step in the real-time PCR protocol was 94°C for (A) and 84°C for (B). Variation in T_m for different sample types is shown in a composite figure in panel (D).

results, between any of the groups in 2000 (Fig. 2B, D, H and Table 2). The ratio of number of copies of the *alkB* gene (specific to some bacteria) to the *rpoB* gene (present in all bacteria) was used to look for changes in the microbial population structure (Fig. 2E). This ratio was significantly lower in the HCRN treatment than in the control (Table 2).

After a further 4 years *in situ*, more differences could be observed between the control and the fertilization treatments. The number of copies of both the *alkB* gene and the *rpoB* gene g^{-1} soil are significantly lower in the treatments than in the control (Fig. 2A and C and Table 2), as is the ratio between the two genes (Fig. 2E and Table 2). The number of total heterotrophs is significantly greater in the HCRN treatment than in the control (Fig. 2D and Table 2). Differences in total heterotrophs and alkane-degrading bacteria are not significantly different from the control at $p < 0.15$ (Fig. 2B and D

Table 2. Significant differences between microbial communities

	2000		*2004*		*2000–2004*		
	LCRN	*HCRN*	*LCRN*	*HCRN*	*Control*	*LCRN*	*HCRN*
Copies *alkB* g^{-1} soil	↑ 0.004	– (0.98)	↓ 0.004	↓ 0.001	↑ 0.001	↓ 0.007	– (0.10)
Copies *rpoB* g^{-1} soil	↑ 0.003	↑ 0.002	↓ 0.008	↓ 0.03	↑ 0.002	– (0.29)	– (0.88)
alkB:*rpoB*	– (0.60)	↓ 0.017	– (0.66)	↓ 0.004	↓ 0.024	↓ 0.002	↓ 0.011
Alkane degraders	– (0.24)	– (0.21)	– (0.53)	– (0.19)	– (0.20)	– (0.20)	– (0.19)
SAB degraders	– (0.24)	– (0.37)	↑ 0.11	↑ 0.13	↑ 0.15	↑ 0.13	↑ 0.13
Total heterotrophs	– (0.49)	– (0.22)	– (0.49)	– (0.17)	– (0.34)	– (0.51)	↑ 0.13

Comparisons are between the control and either the LCRN or HCRN treatments for the first two columns and between the 2000 and 2004 samples for the last column. *p* values from Student's *t* tests are shown.
↑: significant increase; ↓: significant decrease; –: not significantly different.

and Table 2) for the LCRN treatment. However, the SAB-degrading bacteria are significantly higher in both the LCRN and HCRN than in the control (Fig. 2H). The percentage of the bacterial population that is SAB-degrading is also higher ($p < 0.15$) in the HCRN treatment than in the control (not shown).

The comparison between samples taken in 2000 and 2004 show that the total number of bacteria, measured via both real-time PCR and MPN, has increased for the control but has remained at about the same level for both the LCRN and HCRN treatments. The number of copies of the *alkB* gene g^{-1} soil, however, has decreased (significantly for LCRN) in the two treatments but increased in the control. No significant differences could be detected between the 2000 and 2004 samples for the MPN of alkane-degrading bacteria (Table 2). The ratio of *alkB*:*rpoB* gene copy numbers is significantly less (Table 2) for all three groups in the samples from 2004. The abundance of SAB-degrading microorganisms (as determined by MPN counts) has increased significantly for both treatments but decreased in the control.

The concentrations of *n*-alkanes present in the field trial samples in 2000 and 2004 are shown in Fig. 2G. In the 2000 samples, the concentration of *n*-alkanes are significantly lower in the two treatments; in the 2004 samples *n*-alkanes were only detectable in the control samples. The concentration of *n*-alkanes remaining in the soil is positively correlated ($r^2 = 0.87$) with the ratio of the *alkB*:*rpoB* gene copy numbers.

Discussion

Real-Time PCR Assay Validation. At the time the *alkB* primers were designed, there were only 20 sequences for the alkane monooxygenase gene available in Genbank. Even with this small number it was obvious that the sequences were divergent and finding a suitable region where primers could be placed approximately 100 bp apart (optimal for real-time PCR) was difficult. For this reason, some degenerate bases were incorporated in the primers and this set was used to create a clone library that allowed us to then design primers that would be specific for our site.

The clone library constructed with the degenerate primer set demonstrates that these primers are specific for the alkane monooxygenase gene *alkB*. The non-*alkB* sequences obtained did not return any significant similarities from BLAST searches in GenBank. The presence of these sequences may be attributable to the fact that the system used (TOPO-TA from Invitrogen) recommended not purifying the PCR product prior to the ligation step.

The T_m of the product of interest in both assays was identified by correlating the size of the fragment on 2% agarose gels with single peaks in melt curves. A peak was observed in the melt curves from both the *alkB* and *rpoB* gene assays that was identified as primer dimer. This is a particular problem when using the SYBRGreen chemistry as the SybrGreen dye produces a signal from any double-stranded DNA, which can result in false positives or elevated readings. To prevent the formation of the secondary product with a higher T_m, the denaturing temperature during the cycling steps was changed to 84°C or 88°C. This temperature was still high enough to denature the product with the lower T_m but not the secondary product (primer dimer) with the higher T_m, thereby preventing it from being amplified. Another peak at 72°C appeared in the negative controls (Fig. 1B) resulting in a high signal for the negative controls. As suggested in the Quantitect SYBRGreen PCR Handbook (Qiagen), by adding a 15-s acquiring signal step at a higher temperature at which the primer dimer has denatured (77°C in this case), the signal due to primer dimer was removed as SYBRgreen does not bind to denatured DNA.

Variations of approximately 3°C in the T_m were observed for different samples in the *alkB* gene assay (Fig. 1D). The theoretical T_m were calculated for a range of sequences from GenBank (Table 1) and varied by 12°C. A similar phenomenon was observed by Stubner [24], who concluded that the diversity of sequences present in their samples resulted in melt curves with broad flat peaks unlike the melt curves from the standards, which had a single, sharp peak.

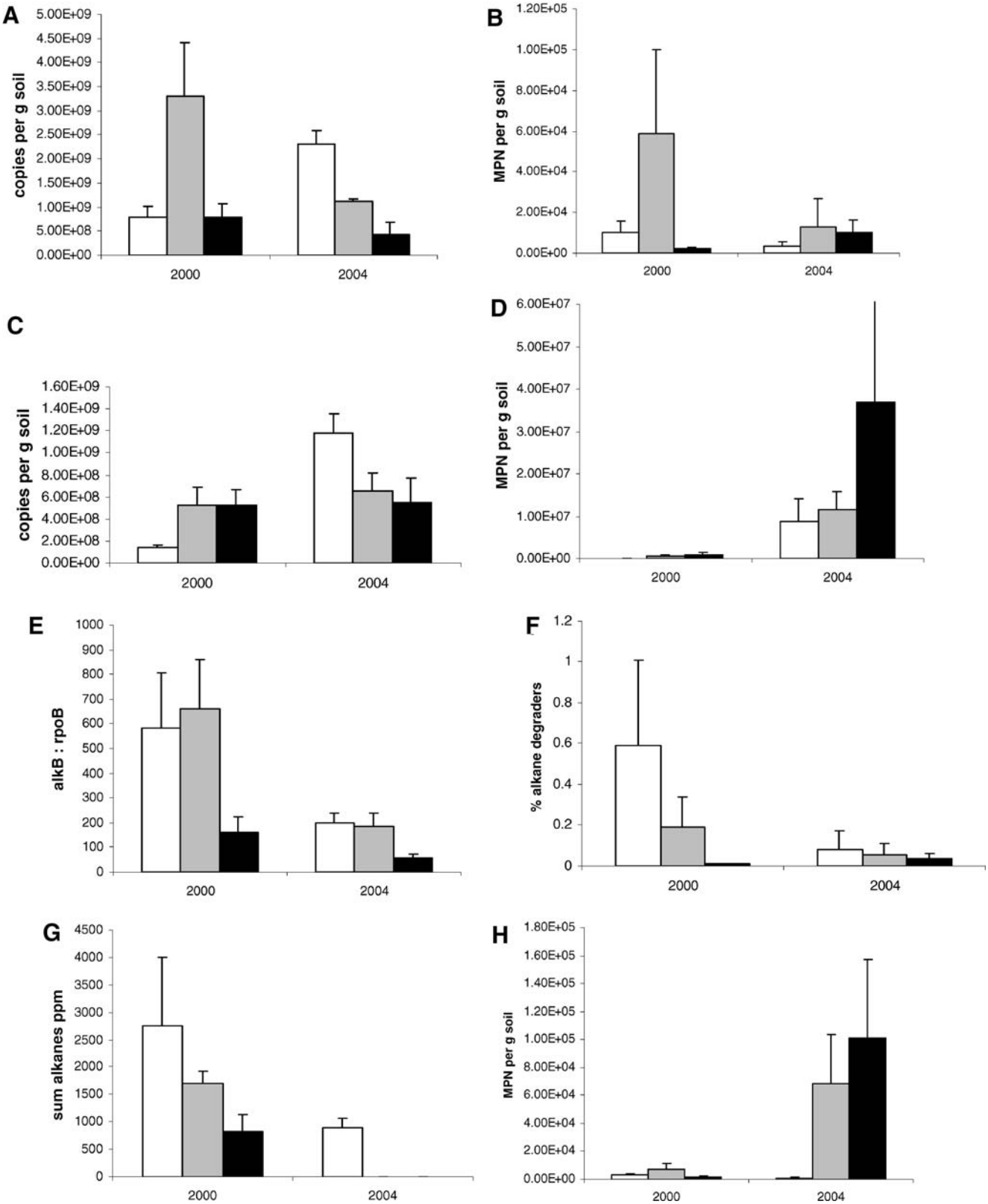

Figure 2. Changes in the control (*white boxes*) and fertilized treatments LCRN (*grey boxes*) and HCRN (*dark boxes*). Copy numbers g^{-1} soil of the *alkB* gene (A), *rpoB* gene (C), ratio of *alkB*:*rpoB* (E); most probable number count g^{-1} soil of alkane degraders (B), total heterotrophs (D) and SAB degraders (H); alkane degraders as a percentage of total heterotrophs (F); and the concentration of the sum of *n*-alkanes mg kg^{-1} soil (G). Bars indicate standard deviations.

The limit of detection for the two assays described here was defined in different ways. Although the *rpoB* gene standard curve was linear to 1880 copies μL^{-1}, the negative control often had close to 5×10^3 copies μL^{-1} (Fig. 1C). As our samples always contained over 1×10^6 copies μL^{-1}, this was not significant. However, we defined the lower limit of detection as three times the standard deviation of the negative control taken over four different runs (2.7×10^3), which resulted in the lower limit for reliable quantification of the amount of *rpoB* genes in a sample as 1×10^4 copies μL^{-1}. Other studies have also reported signal in the negative control (e.g., [9]). We observed that the *alkB* standard curve was linear down to 3 copies of *alkB* μL^{-1}, but at this level adhesion of DNA to tubes is likely to be a problem. When serial dilutions of samples were run alongside a standard curve, it was observed that the dilutions of the samples were not linear below 200–300 copies μL^{-1} (data not shown). For this reason, we would suggest that the lower limit for reliable quantification for *alkB* is 300 copies μL^{-1}. This is similar to other studies that have reported detection limits of around 100 copies per PCR reaction [9, 17].

The CV values from this study (based on concentrations rather than C_T values) compare well to those calculated by Dionisi *et al.* [6]. In that study, the within-run CV was 17% (compared to 16% and 18% in our assays) and the between-run CV was 37.5% (compared to 25% and 38% in our assays). The high between-run CV is partially attributable to the log-linear relationship between the raw signal (C_T) and the concentration values. To minimize this effect we assayed all the samples, except those that required repeating, in one run. Naturally, this may not be practicable for large studies and caution should be used when interpreting small differences between groups.

Many factors—including the amount of sample used for DNA extraction, the efficiency of the extractions, and the presence of PCR inhibitors—affect the number of copies of any genes measured in a sample. To avoid these problems, the ratio of number of copies of the *alkB* gene (specific to some bacteria) to the *rpoB* gene (present in all bacteria) was used to look for changes in the microbial population structure. This is analogous to the use of a housekeeping gene in gene expression studies, and indeed the 16S rRNA gene was previously used for this purpose [5]. The proportion of *alkB:rpoB* genes observed was high: between 50% and 600% (Fig. 2E). This is not a reflection of the actual gene numbers in the samples, but is more likely a result of the differences in amplification efficiencies between the two assays. The average efficiency of the *rpoB* assay (92%) was lower than the average efficiency of the *alkB* assay (95%). As the efficiency of each assay was consistent, comparisons between samples are still valid; however, the ratios between the *alkB* and *rpoB* genes should be considered as indicative of the community structure rather than absolute percentage values.

Although the variability of the MPN results made it more difficult to detect significant differences between groups, where differences were detected, they reflected the same trends as the real-time PCR data. For example, both methods suggest that addition of fertilizer to the soil increased the total number of bacteria present (see Fig. 2C and D).

Changes in the Microbial Community after Fertilization. Various studies have shown that the addition of fertilizer to hydrocarbon-contaminated soil increases hydrocarbon degradation (e.g., [3, 7, 19]). However, few studies have followed the microbial population dynamics in any detail, often only showing an increase in the number of total or hydrocarbon-degrading bacteria. Here we show that there is a link between the decrease in the amount of alkanes present with the number of copies of *alkB* and changes in the microbial community structure.

The sum of the *n*-alkanes remaining in each treatment is shown in Fig. 2G. It is clear that fertilization has stimulated the biodegradation of alkanes as 1 year after the application of fertilizer, both treatments have significantly less *n*-alkanes remaining than the control, and the higher concentration of fertilizer has the least remaining *n*-alkanes. After a further 4 years, *n*-alkanes were only detectable in the control but the concentration was significantly reduced from that in 2000. Although some of the *n*-alkanes will have been lost through evaporation, the increased loss in the fertilized treatments compared to the control is attributable to increased biodegradation.

Our data indicate that fertilization has stimulated the total numbers of bacteria present in these soils as both the *rpoB* real-time PCR and the total heterotrophic MPN data show that in 2000, the treatments showed significantly higher levels than the control. In addition, the 2004 samples are generally higher than the 2000 samples.

Fertilization had a more complex effect on the hydrocarbon-degrading population. Initially, the fertilization significantly increased the number of copies of *alkB* g^{-1} soil: the LCRN samples from 2000 were significantly higher than the control. However, by 2004 the control had significantly more copies of *alkB* g^{-1} soil. Between 2000 and 2004, there has been a significant increase in the number of copies of *alkB* g^{-1} soil in the control and also a significant decrease in both the treatments. The correlation between the number of copies of *alkB* g^{-1} soil and the concentration of *n*-alkanes suggests that this is probably because the control treatment is the only one with *n*-alkanes remaining. Sei *et al.* [20] measured the degradation of alkanes in seawater microcosms over 60 days and observed that an increase in the level of *alk*

genes preceded the degradation of alkanes. This was followed by a decrease in the level of *alk* genes after the alkanes had been degraded in a manner similar to the observations made in this study. In Fig. 2E, it can be seen that the proportion of the population that carries the *alkB* gene has decreased significantly in all treatments, most particularly in the HCRN treatment 5 years after the fertilizer was applied. In the 2000 samples (1 year after application), the HCRN treatment has the smallest proportion of *alkB*-containing organisms and also the least amount of *n*-alkanes remaining.

The addition of fertilizer has most likely stimulated the growth of bacteria that have an available carbon source and are able to compete for the nutrients. After 1 year the HCRN treatment contained very low levels of *n*-alkanes; the most easily utilized hydrocarbon source has been depleted. The microbial community may have been dominated by alkane degraders earlier in the year, but by the time the samples were collected, the community had already adapted to the use of other hydrocarbon as a carbon source. This demonstrates the ability of microorganisms to quickly respond to changing conditions even in this environment, which is frozen for much of the year. It would be interesting to follow the levels of *alkB* over much smaller time intervals to gain a better appreciation of the speed at which the microbial community adapts.

Although there are no significant differences ($p > 0.15$) between any groups in the alkane-degrading MPN data, there are significantly more SAB-degrading bacteria in the fertilized treatments than in the control in 2004 (Fig. 2H). This continued increase in the numbers of SAB-degrading bacteria suggests that a population capable of degrading nonalkane components of the diesel are able to successfully compete for nutrients after the easily degradable alkanes have been consumed and are now increasing in number. In terms of overall bioremediation of the site, this is very encouraging as the total petroleum hydrocarbon content of the soil 5 years after the addition of fertilizer is still approximately 2000–6000 mg kg^{-1} soil in 2004 [18].

The fact that the proportion of *alkB* containing microbes is highest in the treatments with the most *n*-alkanes present ($r^2 = 0.87$) also suggests that the alkane monooxygenase pathway is important in the degradation of *n*-alkanes in Antarctic soils. These genes have been previously detected in both pristine and contaminated Antarctic soils from areas distant from Casey Station [15], and so would appear to be widespread in the Antarctic terrestrial environment. However, this does not mean that this is the only degradative pathway operating. Work by van Beilen *et al.* [28] showed that cytochrome P450 belonging to the CYP153 family could confer the ability to degrade medium-chain length alkanes on recombinant strains. Although hydrocarbon degradation by anaerobic processes does occur in this soil [18], we did not consider these processes in this study as they are unlikely to contribute significantly to biodegradation in the surface portion of the soil profile.

Conclusions

The examination of the microbial community in the bioremediation field trial revealed that fertilization stimulated the different groups of investigated bacteria. Initially, the alkane-degrading bacteria are able to dominate the hydrocarbon-degrading population, but after the easily degraded alkanes have become scarce, other bacteria able to utilize different hydrocarbons dominate the population. This suggests that fertilization will continue to promote biodegradation of the SAB diesel even after the most easily degraded components have disappeared.

In this study, we found that real-time PCR was more reproducible, less labor-intensive, and quicker than culture-based techniques once the assays had been developed. Real-time PCR is an ideal technique for ecological studies as a single DNA extract can be analyzed for many genes, allowing a quantitative approach to analyzing both community structure and function. However, it is important that real-time PCR assays are tested thoroughly for specificity and reproducibility and that the limitations of the method are recognized.

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