QUANTIFICATION OF BACILLUS CEREUS AND BURKHOLDERIA CEPACIA
WITHIN SOIL BY REAL TIME POLYMERASE CHAIN REACTION

A RESEARCH PAPER
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BY
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INTRODUCTION

During the investigation of arson cases, soil samples are often analyzed for ignitable fluid residues such as gasoline. Due to time constraints, soil samples often cannot be processed immediately and are therefore placed in storage for later processing (9). During this period of storage, biodegradation of ignitable residues by soil microorganisms commonly occurs (9, 10). In a study conducted observing biodegradation, a 58.2% decrease in detectable petroleum residue occurred in potting soil within one year, indicating the great effect microorganisms can have on ignitable residues in soil (4). Specific ignitable residues prone to degradation by microorganisms include n-alkanes, toluene, octane, and heptane (9, 10). While many scientists are interested in promoting bacteria-mediated bioremediation of such chemicals in natural environments, biodegradation of compounds in soil samples by microorganisms can invalidate forensic specimens (9). Plant species, soil type and location dictate microorganism populations (1). Agricultural, Industrial, and Residential soils are often analyzed for ignitable residues during an arson investigation.

Many microorganisms have been documented to degrade ignitable residues. *Burkholderia cepacia* and *Bacillus cereus* are of specific interest due to their ubiquitous nature in soil and known ability to degrade ignitable compounds. *B. cepacia*, originally *Pseudomonas cepacia*, is a Gram negative aerobe found in a variety of environments including freshwater, soil and vegetation (3, 6). *B.*
cepacia has been implicated in the degradation of polycyclic aromatic hydrocarbons (11), herbicides, antibiotics, and industrial waste (3). B. cereus is a Gram positive rod known for its ability to produce enterotoxins and incite foodborne illness (7). All strains of B. cereus are capable of forming endospores and some strains are psychrotrophic (2, 5) therefore easily capable of withstanding the refrigeration of arson soil samples. B. cereus has been documented to degrade of a variety of hydrocarbons (8).

MATERIALS AND METHODS

Pure culture growth and DNA Isolation

Pure Cultures of Burkholderia cepacia (USDA ARS NRRL, Peoria, IL USA) and Bacillus cereus ATCC14579 were used for this study. Strains were inoculated into 10ml of Trypticase Soy Agar (Difco, Detroit, MI) and incubated at 32°C (with shaking) for 24 h. To isolate DNA, 1ml of TSA was used with the MO-BIO Ultraclean Microbial DNA Isolation Kit (Carlsbad, CA) according to kit instructions with the addition of 20µl lysozyme to aid cell lysis during step two.

Quantification of Bacterial strains by RT-PCR

The concentration and purity of DNA samples were assessed using a Smart Spec 3000 spectrophotometer (Bio-Rad, Hercules, CA). PCR reactions included 1µl Taq DNA polymerase (BioGene, Limotton, UK), 1µl MgCl₂ (50mMol; BioRad, Hercules, CA), 12.5µl Sensimix (Bioline, Tauton, MA), 1µl SYBR Green
(BioRad), 6.5μl nuclease free water, and 1μl of each primer (Table 1; Integrated DNA technologies, Coralsville, Iowa). For standard curve construction, 0.1μg of DNA was diluted from $10^0$ to $10^{-7}$ concentrations and added to PCR’s. For soil samples, 0.1μg of DNA was used. The PCR reaction total volume was 25μl.

The Smart Cycler II (Cepheid) system was utilized for all RT-PCR reactions. For reactions, an initial denaturation step of 94°C (120s) was followed by 35 cycles of 94°C for 20s, 52°C for 60s, and 72°C for 60s and a final elongation step of 72°C for 8min. A melt curve was then performed on reactions with increments of 0.7°C from an initial temperature of 40°C to 95°C. Triplicate trials of each sample and concentration level were run.

**Isolation and Quantification of DNA in Soil**

Three types of soil were utilized including; Agricultural, Industrial, and Residential soils. Agricultural soil was collected from a long-standing crop near Gaston, IN. Industrial soil was retrieved from New Venture Gear site in Southeast Muncie and residential soil was collected from a private home-owners property in Muncie, IN. All soil samples were stored at -4°C between sampling. To extract DNA from soil samples, a phenol-chloroform method was used. An initial 15g of soil was added to 25ml TE buffer (10mM Tris-HCl, 1mM EDTA) and shaken for 6 minutes. Samples were allowed to settle (5min), and 1.5ml of surface liquid was transferred to a 2ml tube. Tubes were centrifuged (3min, 2000rpm, RT) and 750μl was decanted into a new tube. Proteinase K (5μl) and 2μl of 5mg/ml lysozyme (Amresco, Solon, OH) were added and samples were placed in a
water bath (37°C, 25min) with samples vortexed gently every 5 minutes. Phenol chloroform isoamyl alcohol (Amresco; 250μl) was then added and samples were repeatedly inverted. Samples were then centrifuged (10min, 12k rpm, RT) and the top phase removed to a new tube. Cold ethanol (95%) was added in an amount equal to the sample volume, and cold 3M sodium acetate (pH 7) was added in an amount equal to 10% of the sample volume. Samples were then centrifuged at maximum speed for 20min with the liquid phase decanted and the DNA pellet left to dry. DNA pellets were reconstituted with 30μl of nuclease free water and analyzed by spectrophotometer for concentration and purity. PCR reactions were set up as stated above. Ct values were cross-references against the standard curves created to determine bacterial density in soil.

RESULTS

To calculate the concentration of *B. cepacia* and *B. cereus* DNA in soil, a standard curve was formed using data collected from the PCR analysis. The mean Ct values were plotted against DNA concentrations to yield a standard curve (Fig. 1). High DNA concentrations were removed from standard curves due to over amplification. Soil Ct values were then substituted into the respective slope equations to determine relative DNA concentration in PCR reactions.

After calculating DNA concentration of soil PCR’s, the CFU/g was calculated using the following equation:

\[
\frac{\left(\frac{\text{mg}}{\text{ul}}\right) \text{DNA} \times 1\mu l \times 6.022 \times 10^{23}}{\# \text{ bp}} 
\times (650 \text{ daltons}) = x \text{ CFU/g}
\]
Triplicate samples were averaged, and the quantity of soil used for reactions was adjusted to 1g (Table 2).

**DISCUSSION**

In the three soil types examined, detectable levels of *B. cereus* greatly exceeded those of *B. cepacia*. This may be attributed to the higher level of ubiquity of *B. cereus*. In a previous study, only 82% of soil samples tested positive for *B. cepacia* further indicating the decreased presence of *B. cepacia* in soils (6). Bacterial concentrations of both species were highest in agricultural soil which would indicate a higher potential for ignitable residue biodegradation. *B. cepacia* has been linked strongly to crop plant rhizospheres which would explain the observed high population densities in agricultural soil (6). High levels of *B. cepacia* were also observed in the industrial soil, which can be attributed to its versatility in metabolizing a variety of carbon sources and ability to degrade industrial waste (3, 6). Extremely low densities of *B. cereus* were observed in industrial soils indicating an inability to metabolize carbon sources available in the soil. The analysis of additional microorganisms capable of degrading ignitable residues and their capability of biodegradation are warranted to further understand arson sample degradation.

**ACKNOWLEDGEMENTS**

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REFERENCES


8. Salem LB, Obayori OS, Akashoro OS, Okogie GO. 2011. Biodegradation of Bonny Light Crude Oil by Bacteria Isolated from


**Table 1.** Sequences, amplicon sizes and target designations of primers used to examine *Bacillus cereus* and *Burkholderia cepacia*.

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Sequence (5’ → 3’)</th>
<th>Amplicon size</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BACF</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>1500 bp</td>
<td><em>Bacillus cereus</em> (16s rRNA intergenic spacer region)</td>
<td>This study</td>
</tr>
<tr>
<td>BACR</td>
<td>TACGGCTACCTTGTTACGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPF</td>
<td>TACACATGCAAGTCAACGG</td>
<td>445 bp</td>
<td><em>Burkholderia cepacia</em> (23s rRNA intergenic spacer region)</td>
<td>This study</td>
</tr>
<tr>
<td>BPR</td>
<td>ACGTAGTACGCGGTGCTTA</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Table 2. Values represent CFU/g obtained for the corresponding species from RT-PCR using 0.5 μg/μL DNA concentrations from each soil type.

<table>
<thead>
<tr>
<th>Soil Type</th>
<th><em>Bacillus cereus</em></th>
<th><em>Burkholderia cepacia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Agricultural</td>
<td>8.19E+14</td>
<td>9.717E+10</td>
</tr>
<tr>
<td>Industrial</td>
<td>1.01E+17</td>
<td>9.198E+12</td>
</tr>
<tr>
<td>Residential</td>
<td>4.095E+16</td>
<td>1.254E+12</td>
</tr>
</tbody>
</table>

Figure 1. RT-PCR standard curves for bacterial type strains.