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Comparative assessment of next-generation sequencing, denaturing gradient gel electrophoresis, clonal restriction fragment length polymorphism and cloning-sequencing as methods for characterizing commercial microbial consortia

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ABSTRACT

Characterization of commercial microbial consortia products for human and environmental health risk assessment is a major challenge for regulatory agencies. As a means to develop an approach to assess the potential environmental risk of these products, research was conducted to compare four genomics methods for characterizing bacterial communities; (i) Denaturing Gradient Gel Electrophoresis (DGGE), (ii) Clonal-Restriction Fragment Length Polymorphism (C/RFLP), (iii) partial 16S rDNA amplification, cloning followed by Sanger sequencing (PRACS) and (iv) Next-Generation Sequencing (NGS) based on Ion Torrent technology. A commercially available microbial consortium, marketed as a remediation agent for degrading petroleum hydrocarbon contamination in soil and water, was assessed. The bacterial composition of the commercial microbial product was characterized using the above four methods. PCR amplification of 16S rDNA was performed targeting the variable region V6 for DGGE, C/RFLP and PRACS and V5 for Ion Torrent sequencing. Ion Torrent technology was shown to be a promising tool for initial screening by detecting the majority of bacteria in the consortium that were also detected by DGGE, C/RFLP and PRACS. Additionally, Ion Torrent sequencing detected some of the bacteria that were claimed to be in the product, while three other methods failed to detect these specific bacteria. However, the relative proportions of the microbial composition detected by Ion Torrent were found to be different from DGGE, C/RFLP and PRACS, which gave comparable results across these three methods. The discrepancy of the Ion Torrent results may be due to the short read length generated by this technique and the targeting of different variable regions on the 16S rRNA gene used in this study. *Arcobacter* spp. a potential pathogenic bacteria was detected in the product by all methods, which was further confirmed using genus and species-specific PCR, RFLP and DNA-based sequence analyses. However, the viability of *Arcobacter* spp. was not confirmed. This study suggests that a combination of two or more methods may be required to ascertain the microbial constituents of a commercial microbial consortium reliably and for the presence of potentially human pathogenic contaminants.

1. Introduction

The use of microorganisms in commerce for environmental and industrial purposes is a growing business in Canada and globally. Microorganisms isolated from natural environments are used as an individual microbial strain or microbial blends to produce commercial products such as compost starters, septic tank treatments, bio-surfactants, and bio-remediators (Nowak et al., 2008). A comprehensive list of microbial strains in a product is typically not available. Therefore, a human and environmental risk assessment of those products is required before they are released to the environment. Moreover, the New Substances Notification Regulations (NSNR) pursuant to the Canadian Environmental Protection Act (CEPA 1999) requires all biotechnology products imported or produced in Canada, which are not listed under the Domestic Substances List (DSL), to be notified to the New Substance Division of Environment Canada, prior to import or manufacture. However, complete characterization of microorganisms in these products is a major challenge for regulatory agencies to ensure that they are safe for human and environmental health (Masson et al, 2011).

It is estimated that traditional culture-based techniques are only capable of isolating less than 1% of the total microbial community (Amann et al., 1995; Hugenholtz et al., 1998; Torsvik et al., 1998). Conversely, molecular-based approaches are culture-independent, and

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robust, which has led to an increased understanding of complex microbial communities (Amann et al., 1995). A wide range of molecular approaches such as Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993; Green et al., 2009), 16S rDNA amplification, cloning and Sanger sequencing (Dowd et al., 2008), Restriction Fragment Length Polymorphism (RFLP) (Hiraishi et al., 1995; Urakawa et al., 1998), DNA microarray (Dubois et al., 2004; Masson et al., 2011; Spiegelman et al., 2005), Length Heterogeneity Polymerase Chain Reaction (LH-PCR) (Ritchie et al., 2000), Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett, 1999) etc., have been developed to characterize microbial communities or mixed-microbial consortia. Specifically, culture independent 16S rRNA gene sequencing has been increasingly used to examine microbial diversity in complex environments (Simon and Daniel, 2011). Due to the inherent limitations of conventional methods, microbiologists typically focus on dominant microorganisms in complex microbial communities. However, the rapid development of next generation sequencing (NGS) technologies has laid the foundation for novel approaches in characterizing complex microbial consortia particularly with massively parallel DNA sequencing of short hypervariable regions of small subunit (SSU) which, make it possible to detect relatively low abundant microorganisms in a consortium (Sogin, et al., 2006; Huse, et al., 2008; Petrosino et al., 2009; Shokralla et al., 2012). NGS has not only increased the throughput of analysis, but it has also lowered the cost per sequence read (Kircher and Kelso, 2010).

Over the last decade, advances over Sanger-type sequencing have driven costs down dramatically. Indeed, short read length 'sequencing by synthesis' (Roche-pyrosequencing, Illumina/Solexa-dye-terminator and ABI-SOLiD) has rendered genome and deep metagenomic sequencing affordable (Trevors and Masson, 2010). Further advances have been made with the introduction of Roche-454 GS technologies and Illumina MiSeq exploiting pyrosequencing approach reducing run time and cost (Loman et al., 2012). The Ion Torrent sequencing approach (Personal Genome Machine), in contrast, relies on semiconductor chip and hydrogen ion sensor, to detect H^+ ion release during hydrolysis of the triphosphate moiety as nucleotides are incorporated into DNA (Loman et al., 2012; Rothberg et al., 2011). The introduction and advancement of NGS have provided a cost effective opportunity to comprehensively characterize complex microbial consortia compositions over conventional molecular techniques such as DGGE, RFLP and cloning followed by Sanger sequencing.

The objective of this research was to compare DGGE, Clonal-Restriction Fragment Length Polymorphism (C/RFLP), partial 16S rDNA amplification, cloning followed by Sanger sequencing (PRACS) and Ion Torrent semiconductor sequencing technologies to characterize a commercially formulated microbial consortium for stability and microbial composition.

2. Methodology

2.1. Source of microorganisms, sampling and DNA extraction

A commercially available microbial consortium, used for the bioremediation of petroleum hydrocarbon contaminated environments, was obtained from an anonymous supplier. The product was received in a 200 L drum container. Samples from the bulk container were taken within 24 h of receiving the product and after two months of storage. During each sampling, the container was vigorously shaken manually using a drum dolly followed by stirring with a sterilized steel rod to obtain homogeneous samples. The samples were aspirated from the container to sterile sampling bottles using a vacuum pump. Tubing used to extract representative samples from the bulk container were sterilized by autoclaving at 121 °C for 15 min. Samples were taken so as to minimize external contamination. Initially, two time points were chosen to evaluate the stability of the microbial consortium over time; however, for this method comparison study, data obtained from these two time points were pooled in the analysis. A total of 12 sample replicates, 150 mL each, were drawn from the bulk container at each sampling time.

DNA extraction was performed using a MOBIO Power Water DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). Twenty to thirty milliliters of a sample was filtered through a 0.45 μm mixed cellulose membrane filter (Millipore Corporation, MA, USA) and the filter was transferred to a tube with beads. The rest of the procedure was followed as per the manufacturer's instructions. Extracted DNA was quantified using a Nanodrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA). DNA extracted from 12 sample replicates at each time point were combined for further analysis.

2.2. Methods of characterization

The bacterial composition of the commercial product was characterized using the following methods: (a) Ion torrent sequencing, (b) DGGE followed by Sanger sequencing of excised bands, (c) C/RFLP followed by Sanger sequencing of representative clones with unique fingerprints and (d) partial 16S rDNA amplification, cloning followed by Sanger sequencing (PRACS). Samples drawn at two time points were separately analyzed by each method; however, data from the two time points were pooled for the final analysis.

2.2.1. Ion torrent sequencing

16S universal bacterial primers E786F (5 -GATTAGATACCCTGGTAG-3) and U926R (5 -CCGTCAATTCCTTTRAGTTT-3) (Baker et al., 2003) were used to amplify a 140 bp region of the 16S rRNA gene as previously described (Yergeau et al, 2012; Bell et al, 2013). Briefly, each 16S primer was fused with unique multiplex identifier (MID) tags and Ion Torrent adapters at the 5 end. Total genomic DNA of samples taken at two time points was multiplexed with each MID (5 -CAGAAGGAAC-3 and 5 -CTGCAAGTTC-3). The PCR reaction mixture contained 1 μL of template DNA, 0.3 μmol/L of each primer, 0.4 mg/mL of BSA, 200 μmol/L of dNTP, 0.05 units/μL of rTaq DNA polymerase (GE Healthcare, Canada) and sterile ddH_2O in a final volume of 25 μL. The PCR cycling protocol included, initial denaturation at 95 °C for 5 min followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s followed by a final extension for 3 min at 72 °C. Each PCR reaction was performed with a minimum of 3 replicates per reaction.

The emulsion PCR was performed using an Ion OneTouch 200 Template Kit (Life Technologies, Carlsbad, CA, USA) and the OneTouch ES instrument (Life Technologies) according to the manufacturer's instructions. The sequencing was performed using the Personal Genome Machine (PGM) system (Life Technologies) and 316 chip with an Ion Sequencing 200 kit according to the manufacturer's instructions.

2.2.2. Denaturing gradient gel electrophoresis (DGGE)

16S rDNA specific universal primers, F984 (968–984) 5 -AACGCGAA GAACCTTAC-3 and R1378 Bacteria (1378–1401), 5 -CGGTGTGTACAA GGCCCGGGAACG-3 (Novinscak et al., 2009), were used for PCR amplification of a 440 bp segment of the V6–V8 region of the 16S rDNA. The forward primer, F984 was fused with a 40 bp GC clamp (5 -CGCCCG GGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3). The PCR reaction mixture contained 1x reaction buffer, 1.5 mmol L^{-1} $MgCl_2$, 0.5 μmol L^{-1} of each primer, 200 μmol L^{-1} of dNTP, 1.25 units of Taq DNA polymerase and sterile ddH_2O in a final volume of 25 μL. The PCR cycling protocol included, initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min followed by a final extension of 5 min at 72 °C. Each PCR reaction was performed with a minimum of 3 replicates per reaction.

The resulting 16S rDNA PCR amplicons were used for DGGE analysis using the DCode Universal Mutation Detection system (Bio-Rad, USA). DGGE analysis was performed with 8% polyacrylamide (m/v) gels in