Application of Denaturing Gradient Gel Electrophoresis as an Ecotoxicological Tool to Investigate the Effects of aqu- Fullerene on a Bacterial Community

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Abstract—Denaturing gradient gel electrophoresis (DGGE) was utilized to investigate the toxicity of hydrocarbon pollutant mixtures on a soil-derived microbial consortium incubated in separate microcosms under varied conditions. At different points over a 15-day exposure period, biomass was sampled and prepared for analyses by DGGE to examine the population dynamics of microbial communities during exposure to aqu-fullerene (aqu-C\(_{60}\)) and aqu-C\(_{60}\) plus a complex hydrocarbon non-aqueous phase liquid (NAPL). Nine bands representing different sequence types were shown to occur in the consortium overall. Within 48 hours, up to three populations appeared to become less dominant in microcosms that were exposed to aqu-C\(_{60}\) plus NAPL when compared with controls that were exposed only to aqu-C\(_{60}\) or only to NAPL in separate microcosms. Comparisons of DGGE analyses of amplified 16S rRNA gene fragments from bacterial isolates to profiles from whole consortium revealed that bacterial populations from the genera Burkholderia, Sphingobium, Achromobacter, and Sphingobacterium may have been inhibited by exposure to aqu-C\(_{60}\) while populations belonging to Pseudomonas aeruginosa appeared unaffected or less affected under these conditions. DGGE served as a tool for the identification of populations susceptible to toxic effects after which further examination of vulnerable organisms to determine the mechanisms of toxicity may be conducted.

Keywords: microbial ecology, DGGE, population dynamics, fullerene

INTRODUCTION

Denaturing gradient gel electrophoresis (DGGE) is a well-established molecular technique used in microbial ecology that has been used for many years to monitor bacterial population dynamics both temporally and spatially in natural and laboratory settings (Muyzer et al., 1993). Bacterial communities from soil and marine environments that are capable of biodegrading hydrocarbon pollutants
such as crude and refined fuels are valuable contributors to ecosystem health that perform vital functions in carbon cycling and detoxification of the environment (Hamamura et al., 2006). It is understood that organisms in these bacterial communities compete with each other but at the same time they may also rely on different types of cooperation. Cooperation among microorganisms appears to occur in the case of the biodegradation of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs; Kanaly and Harayama, 2010) and DGGE has been applied to study PAH-degrading bacterial populations in the past (Kanaly et al., 2000; Lafortune et al., 2009). It is thought that perturbations in the ecology of microbial ecosystems that perform vital functions such as these may lead to wider ecological effects but this is an area of study that is not well understood.

DGGE may be applied in the field of ecotoxicology as a tool by which to determine the susceptibility of bacterial populations to environmental toxicants under certain circumstances. Following identification of susceptible populations, focused analyses may be conducted to determine the potential mechanisms of toxicity. Our groups are currently developing methods along these lines to investigate the effects of potentially genotoxic environmental pollutants on bacteria through combinations of microbial ecology and -omics approaches including metatranscriptomics and DNA adductomics (Kanaly et al., 2006, 2007).

Carbon nanomaterials such as C_{60} fullerene are projected to be produced on a large scale and they will also most likely be released into environments throughout the world. The effects of C_{60} fullerene on living organisms are not well investigated and this has become a field of active research. For example, C_{60} fullerene has been reported to cause oxidative stress in fish (Oberdorster, 2004) and rats (Folkmann et al., 2009) including cytotoxicity and genotoxicity in human cell lines (Sayes et al., 2005; Dhawan et al., 2006; Matsuda et al., 2011). It has also been shown to inhibit bacterial respiration and elicit antibacterial activity (Fortner et al., 2005; Lyon et al., 2006; Fang et al., 2007; Lyon and Alvarez, 2008; Chae et al., 2009; Matsuda et al., 2011) and may have changed microbial community structure in soil (Johansen et al., 2008). At the same time, reports of relatively little or undetectable toxicity of fullerene in various test systems have been published (Velzeboer et al., 2008; Matsuda et al., 2011).

Fullerene C_{60} is sparingly soluble in water and will most likely partition into organic materials when released into the environment (Li et al., 2008). Indeed, it has been shown to associate through adsorption to other hydrophobic materials such as PAHs (Yang et al., 2006) and may partition into organic phases such as hydrocarbon NAPLs. In the environment, hydrophobic hydrocarbons such as PAHs are often times found associated with NAPL-type matrices such as in the cases of oil and creosote pollution but also in the cases of non-point source releases on parking areas and roadways, etc., and it is anticipated that fullerenes may preferentially associate with such matrices in nature after their release. Although it is not clear about the potential biological effects of nanomaterials...
such as fullerenes, near unanimous opinion indicates that the full potential of nanotechnology requires attention to issues of safety (Nel et al., 2006). Considering these points, the potential effects of aqu-C$_{60}$ exposure in a NAPL to a soil-derived microbial community known to grow on hydrocarbon pollutants was investigated by DGGE.

**MATERIALS AND METHODS**

**Chemicals**

Toluene (99% purity) was purchased from Wako Pure Chemical Industries, Tokyo, Japan. C$_{60}$ fullerene (99.9+% purity) was purchased from Strem Chemicals, Newburyport, MA, USA. A high-boiling distillation product of diesel fuel, designated HF (Jomo Oil Co., Kyoto, Japan) was prepared by heat distillation and represented 68% of the diesel fuel by weight.

**Bacterial consortium**

A bacterial consortium, consortium BPC, was recovered from soil and was investigated previously for its ability to grow on diesel fuel and mineralize high-molecular-weight PAHs (Kanaly et al., 1997, 2000). The consortium was maintained on 0.2% (w/v) HF plus 50 mg/L benzo[a]pyrene in Stanier’s Basal Medium (SBM; Atlas, 2004) by rotary shaking at 200 rpm at 30°C in the dark.

**Preparation of aqu-C$_{60}$ for exposure experiments**

Aqu-C$_{60}$ was prepared by addition of C$_{60}$ to sterilized MilliQ water in a 250-ml serum bottle followed by constant stirring in the dark at 40°C (MIR-254, Sanyo Biomedical, Tokyo, Japan) for 3 months. One ml of this suspension was extracted with four times the volume of toluene plus 0.4 g NaCl (20% w/w) and the concentration of aqu-C$_{60}$ was determined by UV analyses at 335 nm (V-530 model UV-visible spectrophotometer, Jasco, Tokyo, Japan) by comparison to a standard curve of C$_{60}$ prepared in toluene.

**Preparation of exposure microcosms**

Fifty milliliter-volume consortium exposure microcosms in 500-ml size conical flasks were incubated on a rotary shaker at 150 rpm, 30°C in the dark for 15 days. Inocula, 100 ul each, were transferred to the microcosms after cell growth on 0.2% (w/v) HF plus 50 mg/L benzo[a]pyrene in SBM for 7 days. The treatments consisted of: 1) a negative control, consortium BPC only, 2) consortium BPC plus HF, 3) consortium BPC plus 10 mg/L aqu-C$_{60}$, 4) consortium BPC plus 100 mg/L aqu-C$_{60}$, 5) consortium BPC plus 10 mg/L aqu-C$_{60}$ plus HF, and 6) consortium BPC plus 100 mg/L aqu-C$_{60}$ plus HF. At sampling times, 1.5 ml of culture were removed from each microcosm, centrifuged at 15,000 × g, the supernatant liquid removed and cell pellet frozen at –20°C for future DGGE analyses.
DGGE analyses and identification of consortium community members

16S rRNA gene fragments were amplified directly from consortium DNA by PCR using Bacteria-specific primer 1070F and the universal primer 1392R which contained a GC clamp. PCR products were separated by DGGE analyses and individual bands were sequenced as described previously (Ferris et al., 1996).

Bacterial strains from the consortium were isolated by various means on different media types and their 16S rRNA genes were sequenced as described previously (Kanaly et al., 2000). Individual strains were grown on various types of media and their cell pellets were subjected to PCR and DGGE as described above. Gel migration patterns were then compared to identify consortium members, and sequence identities were confirmed by sequencing of selected consortium DGGE bands as described above.

RESULTS

Results of DGGE analyses over the first 48 hours of incubation are given in

Fig. 1. DGGE profiles of 16S rDNA fragments from 0 to 48 hours. Treatments consisted of a negative control (inoculum without carbon source) [N]; Inoculum plus a high boiling fraction of diesel fuel [HF]; Inoculum plus aqu-C60, final concentration 10 mg/L [C60 10] or 100 mg/L [C60 100]; Inoculum plus HF plus aqu-C60, 10 mg/L [C60 10 HF] or 100 mg/L [C60 100 HF]. In all cases HF was administered at 0.2% (w/v) and microcosms consisted of 500-ml size conical flasks that contained 50 ml of Staniers Basal Medium.
Fig. 1. At $T = 0$ hrs, all samples showed similar banding profiles exhibiting three bands labeled as 1, 2 and 4 in Fig. 1. Band 1 most likely consisted of at least two comigrating bands as determined from subsequent analyses as indicated in Fig. 3. Within 24 hours clear changes in the communities in different microcosms were observed whereby a third band labeled 3 in Fig. 1 appeared in all three control microcosms: (1) the negative control, consortium BPC only, (2) consortium BPC plus HF, and (3) consortium BPC plus 10 mg/L aqu-C$_{60}$ but appeared less so in the microcosm exposed to HF plus 10 mg/L aqu-C$_{60}$ and was not detected.

Fig. 2. Results of DGGE analyses after 5, 10 and 15 days of 10 mg/L aqu-C$_{60}$ plus HF exposure compared to a positive control that consisted of only HF. Treatments consisted of consortium inoculum plus a high boiling fraction of diesel fuel (HF); and consortium inoculum plus HF plus aqu-C$_{60}$, 10 mg/L [C60 10 HF]. HF was administered at 0.2% (w/v) and microcosms consisted of 500-ml size conical flasks that contained 50 ml of Staniers Basal Medium.
Fig. 3. Identification of consortium community members by DGGE through comparisons of amplified consortium DNA with amplified DNA from isolated and purified strains of bacteria. Designations as follows: [HF 24]: Consortium inoculum plus HF after 24 hours; [HF 48]: Consortium inoculum HF after 48 hours; [R1]: *Ralstonia* sp.; [O5]: *Ochrobactrum* sp.; [ACH]: *Achromobacter* sp.; [TK2]: *Pseudomonas aeruginosa*; [AK1]: *Pseudomonas aeruginosa*; [S2]: *Sphingobium* sp.; [TK1]: *Mycobacterium* sp.; [RZ1]: *Rhizobium* sp. Strains ACH and S2, *Achromobacter* sp. and *Sphingobium* sp. respectively, migrated to the same positions under these conditions. Similarly, strains TK2 and AK1, both *Pseudomonas* spp., also migrated to the same positions. Bands 2 and 4 were determined to represent a *Burkholderia* sp. and *Sphingobacterium* sp. respectively.

in the microcosm exposed to HF plus 100 mg/L aqu-C<sub>60</sub>. These results may indicate a dose-dependent effect on this population. At the same time, bands 1 and 2 occurred at decreased intensities when compared to the controls as indicated by the arrows in Fig. 1 (*T* = 24 hrs) and a dose-dependent effect may also have occurred in the case of the population represented by band 2 after exposure to 100 mg/L aqu-C<sub>60</sub>. Finally, band 4 appeared to increase in intensity when compared
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to \( T = 0 \) hrs in all three controls but decreased in intensity in both microcosms that were exposed to aqu-C\(_{60}\) plus HF, also indicated by the arrows in Fig. 1 (\( T = 24 \) hrs).

After 48 hours, further differences were observed among microcosms whereby band 3 became dominant in all microcosms that contained HF but was much diminished in intensity in control microcosms that contained only aqu-C\(_{60}\) (indicated by circles in Fig. 1, \( T = 48 \) hrs) and this was most likely due to a lack of hydrocarbon substrate as evidenced by the results from the negative control. As indicated by the arrows at \( T = 48 \) hrs, the relative intensities of bands 1, 2 and 4 were again shown to be diminished in the microcosms that were exposed to aqu-C\(_{60}\) plus HF indicating a continuation of possible toxic effects. The population(s) represented by band 3 recovered compared to \( T = 24 \) hrs indicating a temporary inhibitory effect or that they were replaced by new populations that migrated to the same position on the gel.

Figure 2 shows the results of DGGE analyses on \( T = 5, 10 \) and \( 15 \) days of exposure of 10 mg/L aqu-C\(_{60}\) plus HF compared to a positive control that consisted of only HF addition. Four additional bands, labeled 5 through 8 in Fig. 2, plus a very faint band indicated by the arrow, appeared at different times through \( T = 15 \) days. After five days of growth by the consortium on HF, at least 6 bands were detected and represented at least eight different types of organisms. In two cases, indicated by the circled areas at \( T = 5 \) days, the presence of aqu-C\(_{60}\) may have been inhibitory. After 10 days of incubation, these populations did not recover and at least three more bands (band 6, 7, and 8, Fig. 2) appeared in the HF-treated microcosm that did not appear or appeared weakly in the microcosm exposed to aqu-C\(_{60}\). The result that these bands were not detected or weakly detected in the presence of aqu-C\(_{60}\) further indicated the possibility of aqu-C\(_{60}\) inhibition of the organisms represented by these bands. Finally, after 15 days, eight bands were detected in the positive control while only half as many bands were detected in the aqu-C\(_{60}\)-containing microcosm and their absences are indicated by the circles in Fig. 2, \( T = 15 \) days.

Eight organisms were isolated from the consortium and their 16s rRNA gene sequences were determined. Following, DGGE analyses were also conducted to examine their presence in the consortium by comparing the gel band migration positions. From these data, the identities of four organisms were estimated by this technique, two Pseudomonas aeruginosa sp. strains, Sphingobium sp. and Achromobacter sp. as shown in Fig. 3. In addition, 16S rRNA gene sequences of two additional DGGE bands, which did not correspond to migration patterns of bands from isolates, were determined to be affiliated with Burkholderia and Sphingobacterium spp.

**DISCUSSION**

In this study, DGGE was utilized to investigate the toxicity of the carbon nanomaterial aqu-C\(_{60}\) on a microbial consortium when it was mixed in a hydrocarbon NAPL. Analyses of the bacterial populations in the consortium over
15 days indicated that some populations appeared to have been inhibited by the presence of aqu-C\textsubscript{60} when it was mixed with a NAPL. Eleven distinguishable bacterial populations were detected in the consortium overall. Within 48 hours up to three populations appeared to become less dominant in microcosms that were exposed to aqu-C\textsubscript{60} plus NAPL when compared with controls that were exposed only to aqu-C\textsubscript{60} or only to NAPL in separate microcosms. The identities of some consortium members were revealed through comparative analyses with organisms isolated from the consortium and at the same time, band extraction from DGGE gels followed by direct 16s rRNA sequencing also revealed the identities of a further two consortium members.

Overall, the comparisons of DGGE analyses of amplified 16S rDNA from bacterial isolates to whole consortium amplified 16S rDNA combined with DNA sequencing of DGGE gel bands revealed that bacterial populations from the genera \textit{Burkholderia}, \textit{Sphingobium}, \textit{Achromobacter} and \textit{Sphingobacterium} may have been inhibited by exposure to aqu-C\textsubscript{60} while populations belonging to \textit{Pseudomonas aeruginosa} appeared to have been less affected under these conditions. There appear to be many factors that may influence the toxicity of fullerenes under different conditions including aggregate size, mode of aqu-C\textsubscript{60} preparation and the properties of the liquid medium into which the fullerene is suspended. Fullerenes may elicit toxic responses in organisms by the generation of reactive oxygen species (ROS) through light-induced excitation and through contact with reducing agents such as NADH for example. In this study, fullerene was mixed with a hydrophobic NAPL that a group of organisms are known to use as a carbon source for growth. The presence of fullerene appeared to inhibit certain populations during their growth. Further research will be necessary to confirm these results and to determine the potential mechanism(s) by which these organisms may have been inhibited. In nature, sparingly soluble environmental pollutants such as fullerenes shall be expected to associate with NAPL hydrocarbon pollutants and depending upon their form and location they may facilitate more extensive exposure and subsequent toxic effects to certain types of microorganisms.

CONCLUSIONS

DGGE analyses served as an ecotoxicological tool to determine if exposure to an environmental pollutant may have inhibited specific members of a bacterial consortium and for the identification of those members that were vulnerable. Continuation of this work based upon these DGGE results through different analysis types may allow for the intensive investigation of the mechanisms by which bacteria are affected by potentially toxic agents such as aqu-C\textsubscript{60}.

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