Tracking Microbial Populations Effective in Reducing Exposure

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Microbial ecology provides a link between two important fields of National Institute of Environmental Health Sciences (NIEHS) research—toxicity reduction and exposure (Figure 1). Examples of important microbiological and environmental topics in each of these fields are also shown. Microorganisms have a great diversity of capacities to biotransform and, in some cases, to completely destroy toxic chemicals in our environment. Since these transformations alter the chemistry of the hazardous chemical, they may also alter toxicity, environmental fate, and bioaccumulation potential. Most toxicology studies focus only on the parent chemical and not on the possible metabolites that might occur in nature. A more comprehensive understanding of environmental fate, human exposure, and toxicity reduction requires understanding the role of microbes as a key environmental mediator of these chemicals.

The microbial role in toxicity reduction is usually approached through basic studies on individual strains. Examples include the elucidation of pathways of biotransformation, clarification of the enzymatic mechanisms of transformation, and molecular characterization of the responsible genes such as gene organization, gene evolution, gene regulation, and analysis of the active site of the enzyme. This information is most useful in environmental toxicology when it can be verified as relevant to the actual transformation of that chemical in nature. This can most easily be done if the genes or strains in which the laboratory studies were done can be shown to be the important genes or strains in nature. The knowledge and tools of microbial ecology can be used to do this.

Environmental exposure includes those environmental processes that modify human exposure. These include chemical transport, sorption, volatilization, and transformations catalyzed by chemical, light, or biological means. For most chemicals, the most significant transformations are catalyzed by microorganisms. These transformations are a product of natural communities; however, the information from pure-culture laboratory studies may or may not be relevant. Again, it is the knowledge and tools of microbial ecology that make it possible to understand the function and behavior of natural populations that are important to these chemical transformations.

The topics within microbial ecology that are relevant to understanding environmental toxicology of hazardous chemicals include tracking important remediation organisms, understanding natural selection and population succession, understanding the importance and role of diversity and community resilience, discovering new forms of microbial diversity responsible for pathways or mechanisms not previously known, and determining the environmental limitations that express themselves through altered biotransformations. Since the theme of this article is microbial ecology, and, more specifically, tracking populations important in reducing exposure, we will illustrate several principles with examples from our research.

Tracking Bioaugmentation in Aquifers

If the indigenous microbial population is inadequate because of limited diversity, previous inhibitory chemicals at the site, or
in the case of genetically engineered microorganisms, the transformation is not known in nature, it would be desirable to inoculate the site with the appropriate biodegrading organism. We have participated in testing a prototype of aquifer bioaugmentation in a cooperative study with the German Biotechnology Institute and the U.S. Geological Survey (USGS). The field portion of the study was conducted at the USGS study site in Cape Cod, Massachusetts. A full description of the field design, microbial injection, and monitoring of the added organism are published elsewhere (1,2). We have studied approaches to successful tracking of a specific organism added to an aquifer over a 14.5-month period. The most successful approach that we found for tracking such organisms was to identify a genomic fragment by screening random clones that could uniquely distinguish this organism from the background population of the aquifer. A primer set for this anonymous region was used with the polymerase chain reaction (PCR) to amplify this fragment; an internal primer set was used to generate a probe to detect and confirm this gene segment and thus the added organism. This method was successfully used to detect the injected organism in DNA extracted from cores drilled from the aquifer and to confirm the identity of isolates from the aquifer. We were able to detect the added organism by the PCR method in some aquifer samples taken from the field 14.5 months after injection. The positive hybridization bands were detected in samples in which the population was too low to estimate by most probable number (<10 organisms/g) but was detectable by enrichment. It is difficult to place a quantitative estimate on the detection limit of the method or the numbers of organisms found in the field study. Based on quantitative dilution studies of samples spiked with target DNA or with the added organism, the detection limits were 17 organisms/g and 1000 organisms/g, respectively. The higher detection limit for the organism versus the DNA is probably due to materials that interfere with the PCR amplification or to the loss of the small quantity of DNA recovered during handling and cleanup. New methods of DNA purification and new strategies of PCR amplification are now available that should help reduce the detection limit of this method for such environmental studies.

To monitor the organism, we attempted to isolate the injected organism (and perhaps others) that could grow on the selective medium. The substrate used for selection was 3-chlorobenzoate since the added organism could grow on this substrate as a sole carbon source, but the aquifer community members could not. The isolates were then confirmed to be the added organism by using the *Pseudomonas* sp. B13-specific probe on Southern blots of isolate DNA. Since the digested DNA of the isolates from the indicated depths hybridized to this probe in the same position as the inoculated strain, the survival of the added organism for 14.5 months was confirmed (Figure 2). Probes for unique, preferably noncoding, regions appeared to be the best approach for specifically tracking an added organism. This approach appears necessary to distinguish the inoculum from its closest relatives that may be present. Another approach is to clone into the organism a gene known only in eucaryotes and to track this gene. The stability of the foreign construct in nature over a long period, however, is uncertain.

### Tracking Community Succession

Microbial community structure shifts in response to competition within a niche. This is important in the human food chain is in bioreactors that treat groundwater. We studied microbial succession in a 4-l aerobic fluidized bed reactor developed on granular activated carbon. The reactor was inoculated with three well-characterized *Pseudomonas* species, each carrying a different pathway for toluene degradation (strains G4, PKO1, and PaW1). These organisms have also been the subject of detailed biochemical and genetic studies on toluene degradation. The reactor was fed groundwater containing 3 mg/l toluene at 0.9 l/min. At a loading rate of 5.4 kg chemical oxygen demand/m³-day, effluent toluene averaged 0.012 mg/l. Initially, the influent was filter sterilized to allow the establishment of the three inoculant strains. After 17 days, the filter was removed to allow organisms present in the groundwater to compete with the established microorganisms in the biofilm. We studied the composition of the microbial community at several time points by using both standard culture and PCR-generated 16S rDNA libraries. In addition to providing the material for cloning, the 16S rDNA was used for restriction fragment length polymorphism (RFLP) analysis as a quick assessment of community changes. All three strains colonized the carbon particles prior to filter removal (Figure 3). An increase in microbial diversity was observed immediately (72 hr) after removal of the filters. Two of the three inoculated strains were detected by

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**Figure 2.** Southern hybridization analysis of *Pseudomonas* sp. B13 (the organism injected into the aquifer), five isolates obtained from 3-chlorobenzoate enrichments of aquifer material 14.5 months after *Pseudomonas* B13 injection, and several organisms that served as negative controls. The isolates were obtained from the depths indicated by mbs (meters below surface). The DNA extracted from each strain was digested by EcoRI and probed with a 344-bp anonymous probe specific for strain B13. The arrow indicates a 2.6-kb fragment specific for this strain.

**Figure 3.** Total nucleic acid was extracted from pure bacterial cultures and granular activated carbon from a fluidized bed reactor inoculated with the same strains of bacteria. The nucleic acid was subjected to PCR using universal primers complementary to the 16S ribosomal RNA gene. The PCR product was digested with *Haell*, electrophoresed on 4% (wt/vol) NuSieve (FMC) agarose, and stained with ethidium bromide. Lane MW: molecular weight DNA markers (bp): 2645, 1198, 676, 517, 350, 222, 126. Lane Blank: PCR amplification of water. All other lanes are restriction endonuclease digest from *Pseudomonas cepacia* G4, *P. pickettii* PKO1, and *P. putida* PaW1; GAC bioreactor samples taken on day 0, 13, 29, 65.
both culture and nucleic-acid methodologies. At least three new strains were also detected, one of which was a yeast. After a 10-fold increase in toluene, the reactor achieved a new steady state, but only one of the three inoculated strains, PaW1, was detected. In this experiment, the inoculum from groundwater outcompeted the added strains. Such a shift then raises the question of whether the biochemical information known for the inoculum is relevant for the new community. Since reactors like these are now in commercial operation to treat groundwater, our ability to understand the microbial community structure and its shifts is particularly relevant to human health.

Diversity Discovery

One of the most exciting areas of microbial ecology is the discovery of new important organisms in nature. It is estimated that only a small fraction, perhaps less than 1%, of the microbial species have been discovered. One new tool for diversity discovery is the use of DNA probes to help recognize functionally important organisms that were not being recovered by normal enrichment and isolation procedures and then to determine which strains were the dominant organisms in nature. We have used this approach to isolate a previously unrecognized group of organisms that degrade the herbicide 2,4-D and to determine which 2,4-D-degrading organism was dominant in Michigan soils. For two decades, 2,4-D has been a model for basic biochemical and genetic studies of biodegradation. The structure and key tfd genes from plasmid pJP4 have been identified and many are sequenced. In some soils, these genes account for most, if not all, 2,4-D degradation (3); in other soils, organisms with sequences showing no hybridization to tfd genes account for 2,4-D degradation (4). We sought these organisms using a probe that we found would specifically track the population dynamics of the newly recognized 2,4-D degraders. Once isolates from groups were obtained, we determined which was dominant in nature by searching for the RFLP band patterns of isolates that matched those of the soil DNA. Figure 4 shows that isolate K13 had an identical hybridization band pattern to the band pattern produced by soil DNA using three different restriction digestions. Thus strain K13 is a dominant 2,4-D degrading population at this site. Once identified, we further characterized this organism and determined that it was *Sphingomonas paucimobilis* (5), a very distinctive group of Gram-negative environmental strains. This study is particularly interesting because there is no sequence homology detectable at low stringency to any of the tfd genes. Virtually all of the biochemical information about 2,4-D metabolism is derived from the tfd operon.

Characterizing Diversity of Functional Communities

Because microbial diversity generally seems to be high, one would expect diversity to also be high for many natural populations that have the capacity to carry out a particular biotransformation. When a strong selection is imposed, such as for the toluene- or 2,4-D-degrading communities discussed above, then the most competitive strains become dominant, and diversity is less apparent. In many cases of aquifer remediation, selection is not that strong, so there may be many indigenous organisms contributing to the community function with each strain having slightly different ecological features. As part of a collaborative study with Stanford University, we have analyzed the diversity of phenol- and toluene-degrading isolates from an aquifer in which those cosubstrates are being used to stimulate co-oxidation of trichloroethene (TCE) (6). In this case it is particularly important to understand diversity because not all organisms that respond to these two substrates would co-oxidize TCE effectively. Thus this treatment technology may fail in some cases because the population is different. Figure 5 shows the profiles of genomic patterns of 13 isolates from attached aquifer material or filtered from water pumped from the aquifer. This method, termed REP-PCR for repeated extragenic palindromic sequences amplified by PCR, is a quick way to determine identical or closely related strains. None of the isolates in this set of strains is identical, but many are probably related because some band patterns match. This suggests that the diversity of strains carrying the toluene/phenol monooxygenase is high. It remains to be seen how this information relates to the organisms’ ability to co-oxidize TCE and to withstand the toxicity of the epoxide intermediate that is produced from TCE co-oxidation.

Conclusions

Molecular tools now available to microbial ecology are essential to establish the link between toxicity reduction and exposure. Without these tools it would be very difficult or impossible to characterize and identify important biodegrading populations in nature and to determine whether the mechanism, biochemistry, and genetic information from the elaborate laboratory studies have any relevance in nature. The examples illustrate some, but certainly not all, topics of microbial ecology important to NIEHS’s goal of reducing the risk of hazardous chemicals to humans.

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