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ScienceDirect
Journal of Microbiological Methods 68 (2007) 633–638
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Variations in T-RFLP profiles with differing chemistries of fluorescent dyes used for labeling the PCR primers

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Received 16 July 2006; received in revised form 7 November 2006; accepted 20 November 2006
Available online 29 December 2006

Abstract

Culture independent molecular methods have emerged as indispensable tools for studying microbial community structure and dynamics in natural habitats, since they allow a closer look at microbial diversity that is not reflected by culturing techniques. Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis is one of the informative and widely used techniques for such studies. However, the method has a few limitations to predict microbial community structure with significant accuracy. One of the major limitations is variation in real Terminal Restriction Fragment (TRF) length and observed TRF length. In the present study we report the generation of TRF length variations using different fluorescent dyes to label the PCR primers. T-RFLP profiles generated from primers labeled with different dyes varied significantly and led to inconsistent microbial species identification. Occurrence of such variations can have serious consequences on interpretation of the T-RFLP profiles from environmental samples representing complex microbial community. Therefore, in a T-RFLP study, the primers and labeling dye system should be carefully evaluated and optimized for an individual community under investigation. Further, it would be recommended to establish a target gene library in parallel with T-RFLP analysis to facilitate the accurate prediction of microbial community structure.

Keywords: Microbial ecology; Community structure; Variation; T-RFLP analysis

1. Introduction

Microbial ecology has experienced tremendous development over the last few years (Hunter-Cevera, 1998). One of the active fields of microbial ecology is the study of the microbial community structure and dynamics within the natural habitats (Dahllof, 2002). The upsurge has been fueled by the accumulation of compelling evidences that a large number of the microorganisms are non-amenable to standard laboratory cultivation procedures and also by the emergence of several high-throughput, cultivation-independent molecular techniques for describing microbial diversity at the molecular level (Ranjard et al., 2000). By far the majority of these methods are based on use of Polymerase Chain Reaction (PCR) for amplifying some gene(s) of interest directly from the environmental sample DNA followed by the fingerprinting analysis of the amplified gene(s) (Morgan, 1991). These cultivation-independent molecular methods depend upon representing the entire microbial diversity by some of the taxonomically significant molecular marker (Hugenholtz et al., 1998). Information generated with such methods can be used qualitatively as well as quantitatively to determine species diversity and also the relative abundances of individual species within the community under observation (Liu et al., 1997; Blackwood et al., 2003).

Amongst the recently developed molecular methods, Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis is one of the most commonly used high-throughput techniques for community structure determination (Kitts, 2001; Marsh, 2005). T-RFLP analysis has also been used to investigate the community structure dynamics involving spatial and temporal changes manifested by biogeochemical alterations or human interventions (Torsvik et al., 1998). An environmentally significant act of human intervention, which requires close assessment, is bioremediation of environmental pollutants by bioaugmentation of degradative bacterial strains to the actual site

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doi:10.1016/j.mimet.2006.11.012

of contamination. Nitrophenolic compounds are an important group of pollutants, widely distributed in the environment due to their extensive use in manufacturing of insecticides, pesticides, dyes, plastics and explosives (Spain, 1995). With increasing awareness about the hazardous effects of these pollutants efforts are being made to decontaminate the polluted site by optimizing the use of available microbial potential and developing bioremediation technologies. Several bacteria belonging to genera *Rhodobacter*, *Pseudomonas*, *Moraxella*, *Arthrobacter*, *Nocardia* and *Bacillus* are known to degrade nitrophenol (Chauhan et al., 2000; Hanne et al., 1993; Spain and Gibson, 1991). However, their application for *in-situ* bioremediation of contaminated soils and sites has been very scarce, since simulating the commercial application of the biodegradation process requires several levels of stringent tests for determining the applicability and compatibility of the bioaugmentation process. One important aspect to be investigated is the treatment impact of exogenous introduction of degradative organism on existing indigenous microbial community. Community structure-dynamics analysis using 16S rRNA gene T-RFLP can be a worthwhile option to be used for such investigation. There are reports on use of T-RFLP analysis of 16S rRNA gene, as well as a few other functional genes from mercury resistance bacteria, nitrogen fixers, ammonia oxidizers and denitrifying bacteria for gaining insight into complexities of microbial community structures and their dynamics (Braker et al., 2001; Bruce, 1997; Hoshino et al., 2005). Although T-RFLP analysis is a reproducible and accurate tool for rapid characterization of microbial community (Liu et al., 1997), however, there are quite a few limitations associated with the method that can prevent it from deciphering microbial community structure with significant accuracy. One of the major limitations is variation in the observed length and real length of the TRFs (Kaplan and Kitts, 2003). A few of the earlier studies have reported possible reasons for this variation. In the present study we report generation of variations in Terminal Restriction Fragment (TRF) lengths with the use of different fluorescent dyes used for labeling the T-RFLP primers. We also studied the effects of these variations on the community structure determination.

As a part of *p*-nitrophenol (PNP) bioremediation studies, field trials for PNP degradation were performed. PNP contaminated experimental plots were bioaugmented with a PNP degrading strain viz. *Arthrobacter protophormiae* RKJ100 and impact of exogenous introduction of a degradative strain on microbial community dynamics was investigated with standard technique of 16S rRNA gene T-RFLP analysis. As a part of this study experiments were performed, wherein combinations of T-RFLP primers labeled with different fluorescent dyes have been used. The rational of using primers labeled with different dyes was to study the effects of these dyes on the T-RFLP profile of microbial community under observation. T-RFLP profile generated with primers labeled with different dyes showed significant variations and resulted in non-consistent microbial community structure prediction. Present study reports the effects of primer labeling dyes on the T-RFLP profiles and also provides an indication about the ability of small molecular tags to adversely influence the observed T-RFLP patterns and thus the identification of microbial community structure.

2. Materials and methods

2.1. Bacterium used for the bioaugmentation studies

Bacterial strain used in the present study is a PNP degrading soil isolate *A. protophormiae* RKJ100 (Chauhan et al., 2000). It was also capable of utilizing 4-nitrocatechol (4NC) and *o*-nitrobenzoate (ONB) as sole source of carbon and energy (Chauhan et al., 2000; Chauhan and Jain, 2000).

2.2. Field studies, sampling and estimation of PNP degradation

PNP degradation by bioaugmentation of strain RKJ100 was studied at field scale. Four plots measuring 1 m × 1 m and 30 cm deep were prepared and filled-up with PNP contaminated soil as reported earlier (Labana et al., 2005). Two of the four plots were inoculated with strain RKJ100 as free cells and as cells immobilized on corncob powder respectively. Soil samples were collected at different time points after bioaugmentation from a depth of ~10 cm from three different random positions. Collected samples were pooled, mixed thoroughly and used for further analysis of PNP biodegradation and microbial community structure dynamics (Labana et al., 2005; Paul et al., 2006). Soil samples from all the four plots were collected on 0 day, 3 day, 10 day and 15 day of the bioaugmentation for analysis.

2.3. DNA isolation, and T-RFLP procedure

Soil DNA from collected soil samples was isolated using soil DNA isolation kit available from Mo-Bio Laboratories Inc. (Salana-Beach, California, USA) as per manufacturer's instructions. Isolated DNA was quantified spectrophotometrically at 260 nm. Partial 16S rRNA gene was amplified for the T-RFLP analysis using eubacterial 16S primers: 27F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 926R (5′-CCG TCA ATT CCT TTG AGT T-3′) labeled either with carboxyfluorescein (6-FAM) or its hexa-chloro derivative (HEX) in combinations (Table 1); the labeled and unlabeled primers used in the study were obtained from BioBasic Inc., Canada. The reaction cocktail consisted of 150 ng of template DNA, 0.5 U of Deep Vent DNA Polymerase (New England BioLabs Inc., MA, USA), 1X ThermoPol Buffer [20 mM Tris–HCl (pH 8.8 at 25 °C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100], 200 μM dNTPs and 100 pmol of each primer in final volume of 50 μL. PCR program used for partial 16S rRNA gene amplification was as follows: initial denaturation and enzyme activation at

Table 1
Combination of T-RFLP PCR primer sets

PCR reaction	Forward primer	Reverse primer
1	5′ (FAM)-labeled 27F primer	5′ 926R unlabeled primer
2	5′ 27F unlabeled primer	5′ (FAM)-labeled 926R primer
3	5′ (HEX)-labeled 27F primer	5′ 926 R unlabeled primer
4	5′ 27F unlabeled primer	5′ (HEX)-labeled 926R primer
5	5′ (FAM)-labeled 27F primer	5′ (HEX)-labeled 926R primer
6	5′ (HEX)-labeled 27F primer	5′ (FAM)-labeled 926R primer