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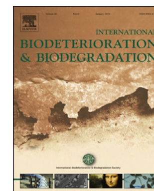

Shift in microbial population in response to crystalline cellulose degradation during enrichment with a semi-desert soil

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Addition of crystalline cellulose to semi-desert soil shifts the microbial population; this was assessed by following the 16S rRNA gene, glycosyl hydrolase, and measuring its functional diversity in the bacterial population. Quantification of the glycosyl hydrolase gene showed an increase from 1×10^4 g^{-1} of unamended soil to 3×10^4 g^{-1} of crystalline-cellulose-amended soil by the 15th day of crystalline cellulose utilization. The indigenous glycosyl hydrolase community in unamended soil was dominated by the clone families that were closely related to the glycosyl hydrolases from Betaproteobacteria and Firmicutes. The addition of crystalline cellulose induced a shift in the glycosyl hydrolase population toward an increase in the relative abundance of the glycosyl hydrolase that was consistent with those of Bacteroidetes and Flavobacteria. The population shift of glycosyl hydrolase was also supported by the comparison of the 16S rRNA gene families in unamended and crystalline-cellulose-amended soil libraries. The most abundant 16S rRNA gene sequences retrieved in the unamended soil were identical to *Pseudomonas*, *Massilia*, *Paenibacillus*, and *Bacillus* spp., while *Cytophaga* and *Flavobacterium* spp. dominated in crystalline-cellulose-amended soil.

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1. Introduction

Cellulose is the principal component of biomass on earth and its degradation and recycling is an important driver of the carbon cycle. In nature, cellulosic biomass is decomposed by complex and efficient processes, usually by microbial communities that produce cellulolytic enzymes that function synergistically to decompose plant biomass (Bayer et al., 2004). The enzymes primarily involved in plant biomass breakdown are the glycosyl hydrolases (also known as glycosidases), which catalyze the hydrolysis of the β (1–4) main chain bonds in cellulose. In the decomposition of cellulose, bacterial and fungal communities often play roles in the conversion process, depending on the microenvironment of the decay site (Yu et al., 2007; Huang et al., 2010). Bacteria have evolved and accumulated remarkable physiological and functional diversity, and thereby harbor a major reserve of genetic potential (van der Lelie et al., 2012). The classical method of tapping this reservoir of functional potential is by cultivating microorganisms and screening individuals for the requisite phenotypes. However, 95–99.9% of microorganisms are not readily cultured by standard laboratory techniques (Amann et al., 1990). Metagenomic approaches provide a powerful tool to bypass the limitation of cultivation-based methodologies and allow prediction of the biochemical potential of a particular community or the prospecting of novel biocatalysts from environmental samples for further characterization (Li et al., 2009). The importance of cellulose-degrading microorganisms in deserts was studied by the isolation of cellulose degrading bacteria (Taylor, 1982) or by directly measuring their cellulase activity dynamics (Doyle et al., 2006). However, these studies did not address the unculturable population.

Family 48 glycosyl hydrolases (GH48) are major components of some cellulase systems, occurring in free enzyme systems in actinobacteria (*Cellulomonas firmi*, *Thermobifida fusca*), multifunctional enzymes (*Caldicellulosiruptor saccharolyticus*), firmicutes (*Bacillus pumilus*, *Paenibacillus polymyxa*), and anaerobic fungi (*Piromyces equi*). So far 35 genes from various cultured species have been reported and each bacterium usually contains a single gene that codes for a GH48 enzyme, making family 48 hydrolase genes a desirable target for primer design and molecular characterization of different environmental samples for the presence of novel glycosyl hydrolases (Izquierdo et al., 2010).

The aim of this study was to examine the microbial response to crystalline cellulose degradation during enrichment with a semi-desert soil. A cultivation-independent molecular analysis targeting

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the bacterial 16S rRNA gene and glycosyl hydrolase gene was used to monitor bacterial population changes during cellulose degradation.

2. Materials and methods

2.1. Materials

Crystalline cellulose (cellulose powder, CP, purchased from Sigma) used in the present study contained 92.2% glucan, 3.4% xylan, and 3.2% mannan on a dry basis with negligible ash content; this was determined according to National Renewable Energy Laboratories (NREL) procedures (Xiang et al., 2003). All the other chemicals and reagents used in this study were of analytical grade and purchased from Sigma or HiMedia, India.

2.2. Collection of soil sample

Soil samples were collected from semi-desert soil of northern India (26°54′19″N 75°50′33″E). The soil was characterized as described by Koga et al. (2003). It consisted of 73–83% sand, 8–14% clay, and 9–13% slit. It contained 0.19–0.23% organic carbon and had a pH of 7.7–8.1 in water. Soil was collected up to the depth of 0–15 cm by means of a sterile spade from three different spots within 2- by 2-m zone in July 2011, put in sterile bags, and immediately transported to the lab under cold conditions (4 °C). All three samples were mixed thoroughly and immediately processed for the experiment.

2.3. Utilization of crystalline cellulose powder by soil community

Duplicate cultures were initiated in 250-ml Erlenmeyer flasks using 5 g of soil and 50 ml of Bushnell Haas broth (BHB) (Kumar et al., 2011) containing 0.5% (w/v) crystalline cellulose powder as the only added carbon source. Flasks were shaken at 150 rpm up to 15 days at 37 °C. At each time point, samples were removed in triplicate and were used for cell enumeration, crystalline cellulose powder analysis, and DNA extraction. Similarly, two controls were included: (a) control flask A (BHB + soil + crystalline cellulose), which was autoclaved to determine the abiotic loss of crystalline cellulose, and (b) control flask B (BHB + soil).

2.4. Cell enumeration

One milliliter of the soil sample was removed and serially diluted in saline sterilized water (0.85% NaCl, w/v). Samples were vortexed to ensure even sample distribution and 100 μl was plated on BHA (BHB + 15 g agar l^{-1}) (Bushnell Haas Agar) containing 0.5% (w/v) carboxy methyl cellulose (CMC, Sigma) as sole carbon source. The plates were incubated in a BOD (biological oxygen demand) incubator at 37 °C. After three days of incubation, colonies were scored to measure the growth.

2.5. Estimation of crystalline cellulose powder

Crystalline cellulose was estimated by the method of Velichkov (1992) by acidifying the complete content in each flask (both experimental and control) by addition of concentrated H_2SO_4 at each time point until the final concentration of H_2SO_4 reached 70% (v/v). The acidified flasks were kept at 30 °C for 1 h to complete the acid digestion of crystalline cellulose powder and an aliquot (100 μl) of the sample (in duplicate) was withdrawn and mixed with 3 ml of anthrone reagent (0.1%, w/v). The tubes were incubated in a boiling water bath for 10 min and immediately cooled to room temperature in an ice bath. Absorbance was measured at 630 nm by spectrophotometer (Hitachi, India). The concentration of the residual crystalline cellulose powder was calculated using the standard curve made with crystalline cellulose powder. The efficiency of the estimation of crystalline cellulose powder in the soil by this method was 95 ± 2%.

2.6. Extraction of soil community DNA

Total community DNA was isolated by slightly modifying the method as described by Kumar and Khanna (2010). Whole enriched cultures or control flasks were centrifuged at 10,000 g for 10 min at 4 °C (Sigma, India). The pellets were collected, suspended in 10 ml of lysozyme solution (10 mg ml^{-1} of $T_{10}E_1$, pH 8.0), and incubated at 37 °C for 1 h. Protienase K (18 mg ml^{-1}, Fermentas, India) was added to a final concentration of 1 mg ml^{-1} and further incubated at 37 °C for 1 h. An aliquot (25 μl) of SDS (10%, w/v) was added and incubated at 65 °C to complete the lysis. After 2 h of incubation, lysate was centrifuged at 12,000 g for 15 min at 4 °C and supernatant was collected and washed with 10 ml of phenol:-chloroform:isoamyl alcohol (25:24:1). The aqueous layer was collected after centrifugation at 12,000 g for 15 min at 4 °C and one volume of isopropanol was added and kept at room temperature (25 °C) for 1 h to precipitate the DNA. The DNA was collected as pellet by centrifugation and dissolved in 100 μl of $T_{10}E_1$, pH 8.0).

2.7. PCR amplification of the 16S rRNA gene

Triplicate samples of the extracted DNA were PCR amplified targeting the 16S rRNA gene, using the universal oligonucleotide primers 8F and 1492R that are designed to anneal to conserved regions of bacterial 16S rRNA gene (Kumar et al., 2011). The PCR reactions were prepared with 10× buffer, 0.2 mM deoxynucleoside triphosphate (dNTP), 1 U Taq polymerase (all from Life Technologies, India), and 0.5 μM for each primer (Fementas, India) to yield final volumes of 25 μl, using 35 cycles of 94 °C (60 s), 55 °C (60 s), and 72 °C (60 s) with an initial denaturation at 94 °C (3 min) and a final extension at 72 °C (15 min). However the yield of DNA extracted from control B was very low, and did not give any PCR result (data not shown).

2.8. Design of GHF48 primers and PCR amplification of partial GHF48 genes

The GH48 gene primers were designed using the CODEHOP approach so as to ensure a very high probability of annealing on the gene of interest with 11–12 completely degenerate core nucleotides at their 3′-end and efficient amplification with a consensus 18–25 nucleotide clamp sequence at the 5′-end (Rose et al., 1998). The GH48 amino acid sequences of the following bacteria were retrieved from the NCBI database: *Bacillus pumilus* (ABV62241), *Cellulomonas firmi* (AEE47513), *Micromonospora* sp. L5 (ADU09645), *Paenibacillus polymyxa* (ADM70135), and *Salinispora tropica* (ABP55395), and multiple alignments were generated using MEGA software version 4 (Kumar et al., 2008), then cut into blocks using the BlockMaker server (http://blocks.fhcrc.org/blocks/). Primers were designed using the default parameters of the CODEHOP server (http://blocks.fhcrc.org/codehop.html). From the primer solutions proposed by the server, the following primer set was picked for the amplification of GH48 gene fragments: GH-F_2 5′-CTACTTCTCCGCCGAGggnrtnccnta-3′ and GH-R_2 5′-GGACTGCAGC-CACGTGTAGarytcnarytg-3′. The PCR reactions were prepared in a similar manner as that described for 16S rRNA gene amplification. PCR (Applied Biosystems, 9700) was run for 35 cycles of 94 °C (60 s), 50 °C (30 s), and 72 °C (60 s) with an initial denaturation at 94 °C (3 min) and a final extension at 72 °C (15 min).

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