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Original article

Molecular analyses of β-glucosidase diversity and function in soil

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ABSTRACT

The incongruence of genetic potential and apparent functions associated with enzyme production has attracted research attempts to elucidate the environmental influences on the molecular underpinnings of soil biogeochemistry. β-glucosidase is a phylogenetically conserved enzyme that plays an important role in carbon cycling; and its involvement in the final stages of cellulose degradation suggests an important role for soil prokaryotes in moderating enzymatic diversity and function in environmentally dynamic niches. Therefore, detailed assessment of the molecular diversity of this enzyme in soils is critical for refining models of terrestrial carbon fluxes. Here, we tested the hypothesis that the availability of specific carbon substrates for β-glucosidase constraint the diversity of phylogenetic groups that are enzymatically active in soils. To test this hypothesis, we constructed microcosms consisting of vegetation covered and bare soils amended either with cellobiose or glucose. We then proceeded to directly assess the responses of genomic and proteomic contexts of β-glucosidase diversity over an incubation period. We monitored the relative population densities of bacteria capable of degrading cellulose, and we successfully designed and used a set of degenerate primers for real-time PCR to quantify genetic determinants of β-glucosidase in composite bacterial DNA extracts. We also developed a complementary proteomic strategy for electrophoretically resolving β-glucosidase activities in-gel. A pure culture of *Pseudomonas putida* capable of degrading cellobiose that we isolated from the natural soil was used as a positive control throughout the culture-independent experiments. We demonstrated that this organism contains three distinct proteins of molecular sizes 120, 300, and 669 kDa exhibiting β-glucosidase activity, and we detected proteins in these size ranges in direct protein extracts from soil. Our results also show that there is a narrow range of bacteria capable of processing cellobiose in soil, but there is a broader versatility of enzyme action in the dominant organisms advantageously selected by amendment of soil with specific carbon sources. This study contributes to the strategies for molecular-level understanding of soil enzyme diversity and function in composite systems.

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1. Introduction

β-glucosidase cleaves β-1,4 bonds to produce glucose from β-glucosides. This is an important reaction in terrestrial C cycling involving the recycling of plant cellulosic matter. The enzyme's involvement in the degradation of cellulose to glucose suggests a tight correlation between its activity and the bioavailability of soil C [1]. However, reliance on this putative correlation for constructing numeric models of carbon cycling will require better understanding of the diversity of β-glucosidase-producing organisms and how their activities are influenced by environmental conditions, including the variability and bioavailability of carbon sources. Organic matter decomposition in soil ecosystems is mediated by a phylogenetically broad group of soil organisms across the eukaryotic and prokaryotic domains, although the role of bacteria and fungi are better characterized than the others [2]. Sustained degradation of cellulose and other readily decomposable organic carbon sources is associated primarily with the activities of soil bacteria whose cellulases can be cell-bound or excreted, in contrast to the distinctively extracellular cellulases of fungi [3–5].

While the rate at which soil organic matter is metabolized is controlled by the quality of the available C [5], the extent of C limitation to microorganisms is controlled by the dynamics of extracellular enzyme activity [6]. Most of the studies on extracellular β-glucosidase in environmental samples have focused on fungi cultivated in the laboratory [7,8], whereas less is known about bacterial β-glucosidase in this context [9,10].

Metagenomic approaches have proven effective in exploring the diversity of indicator genes used to test hypotheses about the structural composition of soil-bacterial communities [11,12]. However,

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these approaches have not been very fruitful in understanding β-glucosidase diversity in composite environmental samples, in part because of the paucity of information on the molecular genetics of the wide range of organisms involved. To circumvent the limitations associated with DNA-based approaches, proteomics offer a strategy to assess the outcome of the expression of genetic potential and the resulting phenotypes as influenced by environmental conditions [13]. Further, metaproteomics facilitates culture-independent analyses of the diversity of microbial community responses to external stimuli. These responses are potentially reliable biomarkers of soil health [14].

In this research we used artificial growth culture, genomic analyses, and proteomic approaches to investigate the molecular diversity and response of β-glucosidase to C stimulation. We followed the population densities of cellobiose-degrading bacteria, and we designed selective nucleotide primers to assess the presence of genes encoding for β-glucosidase in DNA extracted directly from soil. We also implemented a proteomic strategy for resolving and locating β-glucosidase activities in soil metaproteome directly in electrophoresis gels by detecting a fluorescent product based on methylumbelliferyl β-D-glucoside as substrate. Our results advance the understanding of the molecular underpinnings of community enzymes role in soil carbon cycling.

2. Materials and methods

2.1. Soil microcosms

Soil samples representing two different environments (vegetation cover and no cover soils) were collected from the arboretum research facility of the University of California (Irvine, CA, USA). Samples were collected up to a depth of 50 cm and then transported to the laboratory, sieved (<2 mm) and immediately conserved at 4 °C. Soil samples were subjected to bacteria enumeration and DNA and protein extraction within 2 days after their collections.

Each type of soil (100 g) was dispensed into 500 ml capacity soil Teflon microcosm. Triplicate microcosms were amended with cellobiose (CB+) and glucose (GLU+) at a concentration of 420 μg/g of soil, according to Steinweg et al. [15]. Unamended control microcosms (C) received 4.2 ml of distilled water, estimated on the basis of the water holding capacity, to maintain soil moisture content equivalent to the amended soils.

Soils analyses were done in triplicate at time 0 and after 15 days of incubation in the amended and control soil microcosms.

2.2. Total bacterial population and specific cellobiose degraders

Total bacteria and specific cellobiose degraders were evaluated through viable cell counts on Tryptic Soy Agar plates (TSA, Difco) and Cellobiose Polymyxin Colistin Agar medium plates (CPC, Fluka), respectively. One g of soil of each treatment was mixed into 10 ml of phosphate buffer saline pH 7.0 (0.14 M NaCl, 0.002 M KCL, 0.01 M NA_2HPO_4, 0.001 M KH_2PO_4), followed by serial dilution.

A pure culture of a bacterium was isolated from the CPC medium in the control soil and then identified through fatty acid methyl esterase (FAME) analysis. The highest FAME similarity index (0.819) matched the profile of *Pseudomonas putida*. This bacterium was used as a positive control throughout the experiments.

2.3. Soil DNA analyses

2.3.1. Design of oligoucleotide primers to detect β-glucosidase genes

To identify β-glucosidase genes, a set of oligonucleotide primers was designed based on the evaluation of a broad spectrum of soil bacteria. Nucleotide and amino acid sequences were retrieved from the National Center for Biotechnology Institute (NCBI), European Molecular Biology Laboratoy (EMBL) and PUMA2. Sequences were analysed using the BLAST program and the GenBank databases (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were conducted with Clustal W algorithm [16]. Sequences with high similarity and low E-values were selected for a final multiple sequence alignment. Two degenerate primers βgluF and βgluR2 (Table 1) were then designed around conserved motifs presents in soil-bacterial β-glucosidase genes, and tested for accuracy through pilot PCR amplification of soil DNA extracts. The PCR reaction mixture (25 μl) contained 10 ng of DNA extracted from soil, 400 mM each primer and 10 μl Eppendorf Master Mix (2.5×) and sterile Milli-Q water to a final volume. Amplification was conducted with an initial denaturation at 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 45 °C, extension for 1 min at 72 °C, and a final primer extension for 7 min at 72 °C.

Next, the oligonucleotide sequences were aligned to facilitate the design of a new degenerate sense primer βgluF2 and antisense primer βgluR4 (Table 1) amplifying a smaller DNA region (180 bp), in order to perform real-time PCR strategy. For this strategy, each 21 μl PCR reaction contained 2–5 ng of the DNA, 400 nM of each primer and 10.5 μl 2× IQ SYBER Green Supermix (Bio-Rad, Munich, Germany). The real-time PCR program employed was as follows: 2 min at 50 °C for carry-over prevention, 5 min at 95 °C for enzyme activation, followed by 45 cycles of 1 min at 95 °C, 1 min at 53 °C and 45 s at 72 °C, followed by a final extension for 7 min at 72 °C. PCR amplification procedure was checked with a heat dissociation protocol (from 70 °C to 100 °C) after the final cycle of the PCR. For each extracted DNA, real-time experiments were carried out three times with the threshold cycle (*Ct*) determined in triplicate.

Melting curve analyses and quantification of the gene copy number was performed on Eppendorf Mastercycle Epgradient Realplex 4 version 2.2 (Westbury, NY, USA). The amplification procedure was checked with a heat dissociation protocol (from 70 °C to 100 °C) after the final cycle of the PCR. Curves were generated by using a recombinant plasmid containing one copy of β-glucosidase gene from the positive control bacterium *P. putida*. The *Ct* values were plotted against the logarithmic transformation of the copy number, estimated through serial dilution plasmid DNA concentration. As *Ct* values may vary slightly between experiments, three dilution series of pure standard DNA were run parallel in all experiments. The relationship between *Ct* and the target gene copy number, and the copy number of the real-time standard were calculated as described by [17]. Melting curves were drawn by plotting the melting point values as a function of the dI/dT, where dI is the change in fluorescence intensity and dT is the change in temperature. As melting curve values may vary slightly between experiments, three dilution series of pure standard DNA were run parallel in all experiments.

2.3.2. Subcloning and sequencing of PCR products

PCR products obtained with degenerate primers were resolved electrophoretically on 1.6% agarose, and bands in the range of the expected size of the PCR product (900 pb or 180 pb) were purified from the gel using the MBL Agarosa Quickclean kit (Dominion-MBL

Table 1
Primers used for PCR amplifications in this study.

Primer	Sequence (5′–3′)*	Amplicon (bp)
βgluF	ACNYTNTAYCAYTGGG	900
βgluR2	GAYAAYTTYGARTGG	900
βgluF2	TTCYTBGGYRTCAACTACTA	180
βgluR4	CCGTTYTCGGTBAYSWAGA	180

*Degenerated bases: B = G + T+ C, R = A + G, S = G + C, W = A + T, Y = C + T.

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