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Direct PCR Detection, Cloning, and Characterization of Bacterial β-Glucosidase Genes from Temperate Soils

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Abstract

β-glucosidases are cellulases responsible for the final transformation of cellobiose to glucose. Cellobiose is a by-product of cellulose digestion by endoglucanases and exoglucanases. Microbial DNA was extracted from temperate soils using the Zymo Microbe DNA MiniPrep protocol. β-glucosidase gene sequences were amplified by PCR using degenerate primers βgluF2 and βgluR4. DNA fragments of approximately 200 base pair were detected in all positive soil samples. The amplified DNA fragments were purified and ligated to vector pCR®4-TOPO. Transformations were performed using competent Mix and Go *Escherichia coli* cells. Plasmids were isolated from each clone and inserts were screened by PCR. DNA sequencing and BLAST analysis determined the identity of the cloned fragments. DNA sequencing of clone libraries showed the predominant presence of Proteobacteria (80%), Actinobacteria (16%) and Deinococcus-Thermus (4%). *Rhodanobacter* sp. was found to be the most frequently detected bacterial species carrying β-glucosidase genes. Direct PCR detection and cloning β-glucosidase genes from soils provided a more comprehensive assessment of the potential of different bacterial species to degrade cellobiose to glucose.

Keywords: *β-glucosidase genes; Temperate soils; Cloning; Bacteria; PCR*

Introduction

Microorganisms perform important biogeochemical activities in the environment by recycling different types of organic and inorganic compounds. Most environmental microorganisms are reluctant to grow on plate media or enrichment cultures [1,2]. The cloning and sequencing of 16S rRNA genes from environmental samples have provided a better understanding of microbial community structure and distribution but do not reveal any information about the function of microorganisms in the environment [2,3]. There are studies demonstrating the use of functional genes to study the distribution and diversity of ammonia oxidizers, nitrate reducers, and photosynthetic bacteria in soils [4-6]. Furthermore, detection and monitoring of functional genes have been used to determine the activity of bacteria during process optimization in bioremediation and anaerobic bioreactors [7,8]. However, the activities of these bacterial functional groups are not limited to specific bacterial phyla or species. There are situations where functional groups represent a small fraction of the microbial community [5]. Furthermore, gene sequences can be different but code for the same activity. This is the case with different cellulases present in soil [9-12]. Soil processes play an important part in the cycling of carbon with cellulose as one of the most important carbon sources for microbial respiration [13]. Recent studies have shown that even several bacterial species obtaining their carbon from cellulose were previously not recognized as cellulolytic.

Cellulose degradation involves the synergistic action of endoglucanases, exoglucanases, and β-glucosidases. The first two enzymes attack cellulose internally and externally resulting in the release of small oligosaccharides of cellobiose. The last enzyme, β-glucosidase completes the last step of hydrolysis by converting these small oligosaccharides into glucose monomers. Endo and exoglucanases are

inhibited by cellobiose. However, β-glucosidase is often inhibited by its product glucose. Therefore, β-glucosidase is the rate-limiting enzyme. Maintaining a high hydrolysis rate of cellulose will require efficient β-glucosidases tolerating high levels of glucose.

Glucosidases are widely distributed in bacteria, fungi, plants, and animals. They play vital roles in other biological processes such as terpenols and flavonoid production from glycoside precursors [14]. Furthermore, the transglycosylation activity of some β-glucosidase enzymes makes them potentially useful to industrial processes and pharmaceutical applications.

There are several types of bacteria with β-glucosidase enzymes. These bacteria belong to different phyla, genus, and species. However, few studies have ascertained the distribution and identity of bacterial β-glucosidase genes in soils. β-glucosidase activity has been measured in temperate soils and correlated to the microbial community structure using 16S rRNA pyrosequencing [15]. However, there was no direct assessment of the genetic diversity of β-glucosidase genes. A recent study was completed on the diversity of β-glucosidase genes in semiarid European soils [10]. PCR and denaturing gradient gel electrophoresis analysis (DGGE)-based community analysis reported the presence of 5 bacterial phyla with β-glucosidase genes. Most of the sequences were related to the phylum Proteobacteria.

Bergen Community College (BCC) is a 2-year institution located in Paramus, New Jersey. The state of New Jersey is located in the Northeastern region of the United States. All soils tested were surface soils from the main college campus at BCC. The soils in the sampling area are classified as temperate soils. The major objective of this study was to directly detect bacterial β-glucosidase genes in temperate soils by DNA extraction and PCR and clone the amplified DNA fragments to understand the gene diversity and structure.

Materials and Methods

Samples

Samples were taken from surface soils at the Bergen Community College campus located in Paramus, New Jersey. Paramus, New Jersey is located in the Northeastern region of the United States. The soil samples were taken during the months of November and December. These were the months when the sampling sites were accessible to the analysts.

DNA extractions

DNA extractions from soils were performed as described in the ZR Soil Microbe DNA MiniPrep protocol (Zymo Research, Irvine, CA). Samples of 300 milligrams were added to ZR BashingBeadTM Lysis Tube containing 750 µl of Lysis Solution. Different aliquots of extracts were used in the PCR reactions.

PCR reactions

The extracted microbial DNA was used in the PCR reactions. The reactions were targeting a fragment of approximately 200 base pair (bp) of the β-glucosidase gene using degenerate primers βgluF2 (5'- TTC YTB GGY RTC AAC TAC TA-3') and β-gluR4 (5'-CCG TTY TCG GTB AYS WAG A-3'). The reaction conditions were previously described [11]. After cycling, the final extension time was modified from 7 minutes to 10 minutes.

Ready-To-Go (RTG) PCR beads (GE Healthcare, Buckinghamshire, UK) were used for each PCR reaction volume as previously described [16]. Reaction mixtures were added to the T100TM Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) or Mastercycler thermal cycler (Eppendorf Scientific, Westbury, NY). After PCR amplification, the amplified fragments were purified as described in the DNA Clean and ConcentratorTM-25 protocol (Zymo Research, Irvine, CA).

Clone libraries of β-glucosidase genes

The purified DNA fragments from the PCR amplification of β-glucosidase genes in soils were ligated onto the plasmid pCR®4-TOPO (Life Technologies, Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's instructions. Ligation reactions were incubated for 30 minutes at 25°C.

Transformations were performed using Mix and Go Competent *E. coli* strains (Zymo 5α) (Zymo Research, Irvine, CA). Transformation reactions were incubated on ice for 10 minutes. After incubation, aliquots were plated on Luria Bertani (LB) Agar with ampicillin (50 μg/ml). After, overnight incubation at 37°C, white colonies were transferred to LB broth containing ampicillin (50 μg/ml). Samples were incubated overnight at 37°C with shaking, (200 rpm). Plasmids were isolated from each clone using the Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine, CA). Cloned inserts were reamplified using the βglu or M13 primers.

Amplicon detection was carried out by gel electrophoresis using the FlashGelTM system (Lonza Inc., Rockland, ME) with FlashGel DNA Cassettes containing either 1.2% or 2.2% agarose. A Flash Gel DNA Marker (Lonza Inc., Rockland, ME) with fragment sizes ranging from 100 bp to 4 kilobases (kb) was used to determine the presence of the correct DNA gene fragments. DNA sequencing reactions of the clone fragments using either βglu or M13 Reverse primers were performed by Genewiz, Inc. (South Plainfield, New Jersey). Homology searches were performed using the GenBank server of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the BLAST algorithm [17].

Results and Discussion

Bacterial populations with cellulase activity provide an important function during the biodegradation of cellulose. Cellulose biodegradation is an important component of the recycling of carbon in soils. β-glucosidases make glucose available by digesting cellobiose helping soil microbes to continue the recycling of cellulose to carbon dioxide and/or organic acids.

Using previously reported degenerate primers we analyzed microbial DNA extracted from temperate soils [10]. A DNA fragment of approximately 200 bp was amplified in all soil samples showing a positive reaction. The amplified fragments were successfully ligated to vector plasmid pCR®4-TOPO. Transformation reactions with Mix and Go Competent *E. coli* strains yielded more than 80 different clones. After plasmid extraction, each clone was screened by PCR to determine the presence of the cloned β-glucosidase fragment. Of the more than 80 clones, a total of 51 clones were found to show similarities with different β-glucosidase genes (Table 1). Homology values ranged from 71 to 96%. The average homology value for all 51 clones was 75%. The highest value, 96%, was found with the bacterial species *Amycolatopsis orientalis* and the lowest, 70%, with *Rhodoferax ferrireducens*.

Based upon the similarity values found by BLAST analysis, three different bacterial phyla were found in the analyzed temperate soils carrying β-glucosidase genes. The phyla found were Proteobacteria, Actinobacteria, and Deinococcus-Thermus. No other bacterial phyla were detected in the clone libraries. Previous studies in semiarid soils reported a higher diversity of β-glucosidase genes with six bacterial phyla described. The reported phyla were Proteobacteria, Actinobacteria, Firmicutes, Chlorofexi, Deinococci, and Thermotogae [10]. The largest fraction of β-glucosidase sequences detected in temperate soils was mostly related to the Proteobacteria (Table 1) (Figure 1). Similar results were found in semi-arid European soils [10].

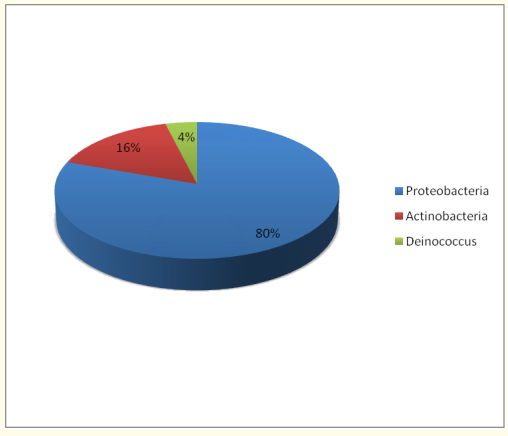

Figure 1: *Bacterial phyla of β-glucosidase genes in temperate soils.*

Clone	Genera Species	Phylum	Accession Number	% Identity
1	*Methylococcus capsulatus*	Proteobacteria	NC 002977.6	74
2	*Methylococcus capsulatus*	Proteobacteria	NC 002977.6	77
3	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	74
4	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	76
5	*Myxococcus xanthus*	Proteobacteria	NC 008095.1	76
6	*Thermomonospora curvata*	Actinobacteria	NC 013510.1	75
7	*Kitasatospora setae*	Actinobacteria	NC 016109.1	76
8	*Ketogulonigenium vulgarum*	Proteobacteria	NC 017384.1	75
9	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	74
10	*Thermomonospora curvata*	Actinobacteria	NC 013510.1	73
11	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	74
12	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	75
13	*Saccharothrix espanaensis*	Actinobacteria	NC 019673.1	80
14	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	74
15	*Arthrobacter aurescens*	Actinobacteria	NC 008711.1	73
16	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	75
17	*Streptomyces* sp.	Actinobacteria	NC 015953.1	73
18	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	72
19	*Jannaschia* sp.	Proteobacteria	NC 007802.1	73
20	*Edwardsiella tarda*	Proteobacteria	NC 013508.1.	71
21	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	75
22	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	73
23	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	77
24	*Edwardsiella tarda*	Proteobacteria	NC 013508.1.	73
25	*Burkholderia phytofirmans*	Proteobacteria	NC 010676.1	75
26	*Burkholderia xenovorans*	Proteobacteria	NC 007952.1	74
27	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	73
28	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	73
29	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	74
30	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	71
31	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	76
32	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	74
33	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	71
34	*Amycolatopsis orientalis*	Actinobacteria	NC 021252.1	96
35	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	70
36	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	81
37	*Pelagibacterium halotolerans*	Proteobacteria	NC 016078.1	75
38	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	73
39	*Serratia plymuthica*	Proteobacteria	NC 021591.1	85
40	*Truepera radiovictrix*	Deinococcocus-Thermus	NC 014221.1	75

41	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	76
42	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	76
43	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	73
44	*Jannaschia* sp.	Proteobacteria	NC 007802.1	73
45	*Burkholderia phytofirmans*	Proteobacteria	NC 010676.1	73
46	*Verrucosispora maris*	Actinobacteria	NC 015434.1	85
47	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	79
48	*Meiothermus ruber*	Deinococcocus-Thermus	NC 013946.1	83
49	*Rhodoferax ferrireducens*	Proteobacteria	NC 007908.1	77
50	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	71
51	*Rhodanobacter* sp.	Proteobacteria	NC 020541.1	71

Table 1: *Genetic analysis of bacterial β-glucosidase clones from temperate soils. N = 51.*

Eighty percent of the sequences found in temperate soils belonged to the Proteobacteria. The lowest similarity, 70%, was found with a sequence from *R. ferrireducens* while the highest was detected with *Serratia plymuthica*, 85%. The other Proteobacteria species detected in temperate soils were *Rhodanobacter* sp., *R. ferrireducens, Myxococcus xanthus, Jannaschia* sp., *Edwardsiella tardia, Methylococcus capsulatus, Burkholderia phytofirmans, Burkholderia xenovorans, and Pelagibacterium halotolerans*. Among the Proteobacteria, *Rhodanobacter* sp*. and R. ferrireducens* showed the highest and second highest numbers of sequences in the clone libraries, 37% and 18%, respectively (Figure 2).

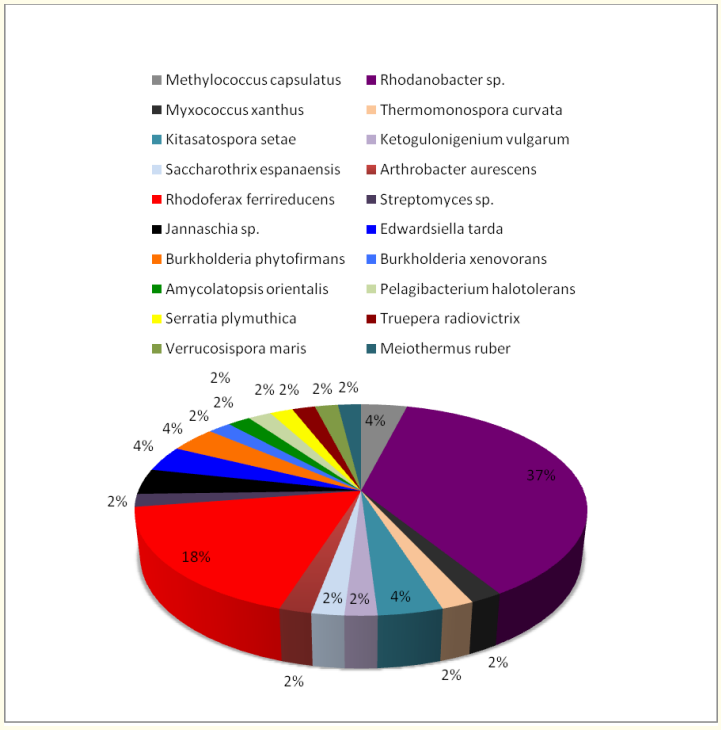

Figure 2: *Bacterial species detected in clone libraries of β-glucosidase genes in temperate soils.*

Actinobacterial sequences were the second most abundant phylum in temperate soils with 16%. The lowest similarity values, 73%, were found with sequences belonging to *Arthrobacter aurescens* and *Thermonospora curvata*. Other Actinobacteria species found were *T. curvata, Kitasatospora setae, Streptomyces* sp.*, Verrucosispora maris, A. orientalis, and Saccharotrix espanaensis. T. curvata* was the predominant Actinobacteria found in the clone libraries.

The third bacterial phylum found in temperate soils was the Deinococcus-Thermus with only two clones (4%). One clone showed 75% similarity with *Truepera radiovictrix* and the other clone showed 83% homology with *Meiothermus ruber*.

When compared to temperate soils, Proteobacteria in semi-arid soils showed a lower percentage of β-glucosidase sequences [10]. They accounted for only 64% of the clones. The most common Proteobacteria species in semiarid soils were *Alteromonas* sp. with 44% of the sequences reported. However, in temperate soils 80% of the sequences were related to Proteobacteria with 37% having a closer similarity with *Rhodanobacter* sp. *Rhodanobacter* sp. has been previously shown to inhabit surface and subsurface soils with a diverse metabolic activity ranging from denitrification and cellulose biodegradation [18]. The only common bacteria found in both semi-arid and temperate soils were *M. capsulatus. M. capsulatus* is capable of various metabolic pathways such as methane oxidation, carbon and nitrogen fixation.

Semiarid soils showed a lower number of glucosidase sequences related to Actinobacteria than temperate soils (16%). Only 5% of the sequences found were related to the species *Micromonospora* sp. and *Amycolatopsis mediterranei*. On the other hand the phylum Deinococcus-Thermus showed higher frequencies in semiarid soils. Although we detected only 4% of this phylum in temperate soils, the numbers of β-glucosidase sequences in semiarid soils were much higher with 8% of the clones showing a close similarity with Deinococcus-Thermus. All the species detected in semiarid soils were related to *Deinococcus deserti* [10].

Although more different types of bacterial phyla were found in semiarid soils, when it came to the number of bacterial species found with β-glucosidase genes, semiarid soils showed less species diversity than temperate soils. The number of bacterial species in temperate soils carrying β-glucosidase genes was 20 while semiarid soils were found to have only 15.

When compared to temperate and semiarid soils, compost samples showed completely different bacterial β-glucosidase gene diversity. Most of the bacterial β-glucosidase genes in food compost samples were found to belong to the phyla Actinobacteria and Firmicutes with a small fraction of Bacteroidetes species [9,19]. No sequences related to Firmicutes were found in temperate soils. Actinobacterial bacterial species found with the highest numbers of β-glucosidase genes in compost were *Thermobifida fusca* and *Sphaerobacter thermophillus* [19]. Other studies showed *Paenibacillus* sp. and *Clostridium thermocellum* as the most abundant bacterial species with β-glucosidase genes in cow manure and straw compost [9]. None of these bacterial species were found in temperate soils.

Metagenomic analysis of compost samples also reported the presence of a Deinococcus-Thermus bacterial species with high glucose tolerance and transglycosylation activity [14]. BLAST analysis revealed a closer similarity with β-glucosidase genes from *M. rubber*. Other metagenomic studies in soils reported clones related to the phylum Chlorofexi, species *Chlorofexus auranticus* and the phyla Actinobacteria and Cyanobacteria with the species *Cellulomonas pachnodae* and *Synechococcus* sp., respectively [20, 21]. In all these metagenomic studies the limited number of clones analyzed did not allow a deeper understanding of the genetic diversity of β-glucosidase genes. However, our study analyzed a total of 51 clones, which provided a better understanding of the diversity and structure of the bacterial populations with β-glucosidase genes. To date this is the study with the highest numbers of clones analyzed to understand the structure and identity of bacterial β-glucosidase genes in temperate soils. This study provided new information on the bacterial phyla, genera, and species involved in the degradation of cellobiose to glucose leading to a better understanding of the bacterial populations responsible for cellulose biodegradation in temperate soils. Direct PCR detection and cloning provided a faster assessment of the genetic potential of temperate soils to breakdown cellobiose to glucose. When compared to previous studies in temperate soils, the clone libraries developed in this study provided a higher number of bacterial species carrying β-glucosidase genes. Several bacterial species are not culturable or are difficult to isolate and their contributions to environmental processes have been underestimated because of the lack of resolution and sensitivity by using growth media to detect the wide diversity and structure of

the predominant functional genes [4,5,7, 19]. Direct DNA extraction and cloning of β-glucosidase genes unraveled the genetic diversity and composition in temperate soils providing a better understanding of the functional bacterial population. The predominant bacterial phylum seems to be the same in semi-arid and temperate soils [10]. Both soil types showed the highest numbers of gene sequences related to the Proteobacteria.

However, in temperate soils β-glucosidase gene sequences were predominantly related to *Rhodanobacter* sp. This might indicate the high catabolic diversity of the genus to utilize cellobiose and other organic and inorganic compounds. Several reports indicated the versatility of *Rhodanobacter* species to dominate highly contaminated subsurface areas and other environmental samples [22-25]. This versatility ranges from cellulose biodegradation, denitrification, aromatic compound biodegradation, and tolerance of low pH environments with high concentrations of uranium processing waste. Future studies will determine the culturability of bacteria carrying β-glucosidase genes to determine the possible transglycosylation activity of the isolated enzymes and their tolerance to high concentrations of glucose.

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