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Scientific Research

Comparison of the Bacterial Microbiota in a Bale of Collected Cardboard Determined by 454 Pyrosequencing and Clone Library

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Abstract

Biofouling, the accumulation of microorganisms, is a major problem in paper mills processing paper and cardboard. This leads to the production of lower quality recycled products. Several studies have focused on the microbial content in the paper mill and the final products. Our aim was to determine the microbial biota in a bale of collected cardboard prior to entering the paper mill. Total genomic DNA was isolated and analyzed using two different methods for comparison purposes: 454 pyrosequencing and clone library. A total of 3268 V6-V8 454 pyrosequencing reads and 322 cloned V6-V8 16S rRNA nucleotide sequences were obtained. Both methods showed the presence of three major bacterial genera: *Bacillus*, *Solibacillus* and *Paenibacillus*, all members of the spore-forming phylum Firmicutes. Pyrosequencing, however, revealed a richer and more diverse bacterial community than clone library. It showed the presence of additional minor Firmicute genera and of a small number of Proteobacteria. The sorting at the recycling plant, the storing, and the processing at the paper mill, the end uses, will all contribute to the bacterial microbiota present in a bale of collected cardboard as revealed here.

Keywords

454 Pyrosequencing, Bacterial Microbiota, Cardboard, Clone Library, Paper Mill

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1. Introduction

In a paper mill, collected paper and cardboard obtained from a recycling plant are mixed with water to form a fiber slurry. The slurry is passed through fine mesh screens, leaving sheets of wet paper and cardboard fibers on the screens, and white water, water with cloudy appearance due to the presence of fibers. The former are further dried and processed to yield the final recycled products. Owing to the large volumes of water used, the latter is re-circulated in the treatment of additional bales of collected paper and cardboard.

Bales of collected paper and cardboard, owing to their high cellulose content, provide a substrate for microbial growth during storage and processing [1] [2]. Moreover, because the white water has been enriched in cellulose and residual sugars through multiple reuses, it is a suitable substrate for microbial proliferation. These lead to the formation of biofilms, the clogging of pipes and the corrosion of equipment. Biocides can be used to control the microbial contamination. This, however, is not without drawbacks. Biocides are costly and eventually released in the environment where they cause negative impacts. Plant downtime for cleaning can occur and, in some cases, the quality of the final recycled paper and cardboard can be much lowered as evidenced by the presence of bacteria, holes, pores, undesirable odor, and decreased mechanical strength [3].

A better knowledge of the microbial contamination source is necessary to control the problems caused by the accumulation of microorganisms, biofouling, and to increase the quality of the final recycled paper and cardboard [4]. Culturable bacterial population diversity has been studied in the fiber slurry [5]-[7], white water [5] [8], biofilms [6] [9] [10], and the final recycled products [11] by 16S rRNA gene sequences. In addition, biofilms were also studied by microscopy [5] [9] [10] [12], physiological and biochemical characterization [9] [10] [12], and fatty acids analysis [10] [12]. Unculturable bacterial population diversity has been determined in the fiber slurry [13] [14], white water [14] and biofilms [13]-[15] by 16S rRNA gene sequences.

One of the most important sources of bacterial contamination in a paper mill is the cellulosic raw material [2]. However, the microbiota present in bales of collected paper and cardboard has never been characterized.

The characterization of bacterial populations traditionally relied on cultures followed by sequencing the 16S rRNA gene, the nucleotide sequence of choice in bacterial identification [16]. Most bacteria, however, cannot be cultivated [17]. Metagenomics is the study of the entire genetic material present in a sample. Here, total DNA is isolated and a 16S rRNA clone library is prepared. The nucleotide sequence of each clone is determined. More recently, high-throughput massively parallel pyrosequencing has revolutionized environmental microbiology [18]. It permits the direct sequencing of the amplified target DNA, bypassing the need for cloning.

Our aim in the present preliminary study was to determine the bacterial microbiota in a single bale of collected cardboard, prior to entering the paper mill. Two different approaches were used: 454 pyrosequencing and clone library.

2. Materials and Methods

2.1. Sampling

A bale of collected cardboard of 1.8 m × 0.7 m × 1.2 m in size was obtained from a recycling plant (QC, Canada). Fifteen samples of approximately 20 g each were randomly collected from the bale using a high-speed hole cutter (3.5 cm in diameter) coupled to an electric drill. They were pooled together, transferred in a plastic bag and frozen at −80˚C until use.

2.2. Total Genomic DNA Extraction

A 10 g cardboard sample was transferred in a 1 L flask containing 125 ml saline (0.85% NaCl) and incubated on a rotary shaker at 120 rpm, at room temperature, overnight. A 12 ml volume of mixture was transferred to a 15 ml tube, centrifuged in a Beckman-Coulter Allegra 25 R Centrifuge tabletop at 1000 rpm, 3 min, at room temperature, to pellet the fibers. The supernatant was transferred to a 50 ml tube, centrifuged in a Beckman (Model J2-21M) at 9000 rpm, 15 min, at room temperature, to pellet the cells. The supernatant was discarded and the cell pellet resuspended in 480 µl 50 mM EDTA pH 8.0. Total genomic DNA was isolated using the Wizard DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions for "Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria".

2.3. PCR Amplification of the 16S rRNA Gene V6-V8 Region and 454 Pyrosequencing

Two separate PCR amplifications were performed; a first one in which the amplicons were sequenced on a

Roche 454 GS-FLX Titanium platform, a second one in which the amplicons were cloned and sequenced.

The 16S rRNA gene V6-V8 region was amplified with the primer pair used for 454 pyrosequencing A1-B969F (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG·ACGAGTGCGT·ACGCGHNRAACCTTACC-3') and B-BA1406R (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG·ACGGGCRGTGWGTRCAA-3') as designed by Comeau *et al*. [19]. The forward primer included Roche's A adaptor, a multiplex identifier and the 16S rRNA gene V6 specific primer. This primer corresponds to nucleotide positions 969 to 985 in the *E. coli* 16S rRNA gene. The reverse primer included Roche's B adaptor and the 16S rRNA gene V8 specific primer. It corresponds to nucleotide positions 1390 to 1406.

The amplification was performed as described by Comeau *et al*. [19] at the Plate-forme d'Analyses Génomiques (PAG), Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Quebec City, QC, Canada. The amplification conditions were as follows: an initial denaturation at 98˚C for 30 s, followed by 30 cycles at denaturing temperature of 98˚C for 10 s, annealing at 55˚C for 30 s, extension at 72˚C for 30 s, followed by a final extension at 72˚C for 5 min.

The nucleotide sequences were determined on a Roche 454 GS-FLX Titanium platform at IBIS. The raw pyrosequencing reads were deposited in the NCBI Sequence Read Archive with accession number SRR961674.

2.4. PCR Amplification of the 16S rRNA Gene V6-V8 Region, Cloning and Sequencing

The 16S rRNA gene V6-V8 region was amplified with the primer pair B969F (5'-ACGCGHNRAACCTTACC-3') and BA1406R (5'-ACGGGCRGTGWGTRCAA-3') [19]. A PCR reaction contained 15 µl of Top Taq Master Mix (Qiagen Sciences, Germantown, MD, USA), 0.6 µl of each primer and 50 ng of total genomic DNA, in a final volume of 30 µl. The amplification conditions were as described above. The PCR amplicons were visualized on a 0.7% agarose gel and stored at −20˚C.

The PCR amplicons were cloned using a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The isolation of recombinant plasmids was performed using a QIAprepSpin Miniprep Kit (Qiagen Sciences) following the manufacturer's instructions. The nucleotide sequences were determined for both strands by the dideoxynucleotide-chain-termination method [20] using a capillary array automated DNA sequencer (ABI3730XL DNA Analyzer, Applied Biosystems, Foster, CA, USA) at the sequencing platform of the McGill University and Génome Québec Innovation Centre, Montreal, QC, Canada. The nucleotide sequences were deposited in GenBank under accession numbers KF589334-KF589827.

2.5. Sequence Analysis, Taxonomic Identification and Statistics

Raw 454 pyrosequencing reads and V6-V8 16S rRNA nucleotide sequences obtained following cloning were processed within Mothur v.1.30.0 (http://www.mothur.org) [21] [22] as described by Comeau *et al*. [23]. Briefly, low-quality pyrosequencing reads and low-quality V6-V8 16S rRNA nucleotide sequences obtained following cloningalong with chimeric sequences were removed. The high-quality reads and nucleotide sequences were aligned against the bacterial SILVA (http://www.arb-silva.de/) [24] reference alignmentand were improved by removing misaligned reads. The singletons were finally excluded to yield the final dataset. These final reads were clustered into Operational Taxonomic Units (OTUs) at the 97% similarity level, and taxonomic identification of the OTUs was carried out in Mothur against a modified 16S rRNA gene database based upon the "GreenGenes97" (http://greengenes.lbl.gov) [25] [26] reference files for pyrosequencing [23].

The rarefaction curves, the plot of the number of OTUs over the number of sequences sampled, were generated. The Chao 1 richness estimator, the Shannon and Simpson diversity indices, and the evenness were calculated at the 97% similarity level using Mothur.

3. Results and Discussion

3.1. PCR Amplification of the 16S rRNA Gene V6-V8 Region and 454 Pyrosequencing

A total of 3268 V6-V8 454 pyrosequencing finalreads was obtained. Their average length was 435 bp. At the 97% similarity level, these reads were clustered into 117 OTUs. As shown in **Figure 1(A)**, 97% of these OTUs belonged to the phylum Firmicutes. The remaining 3% belonged to the phylum Proteobacteria. All Firmicutes were assigned to three families: *Bacillaceae*, *Planococcaceae*, *Paenibacillaceae* with 76%, 14% and 7% of the total OTUs, respectively. These three families comprised six, two, and one genera. The unclassified *Bacillaceae*

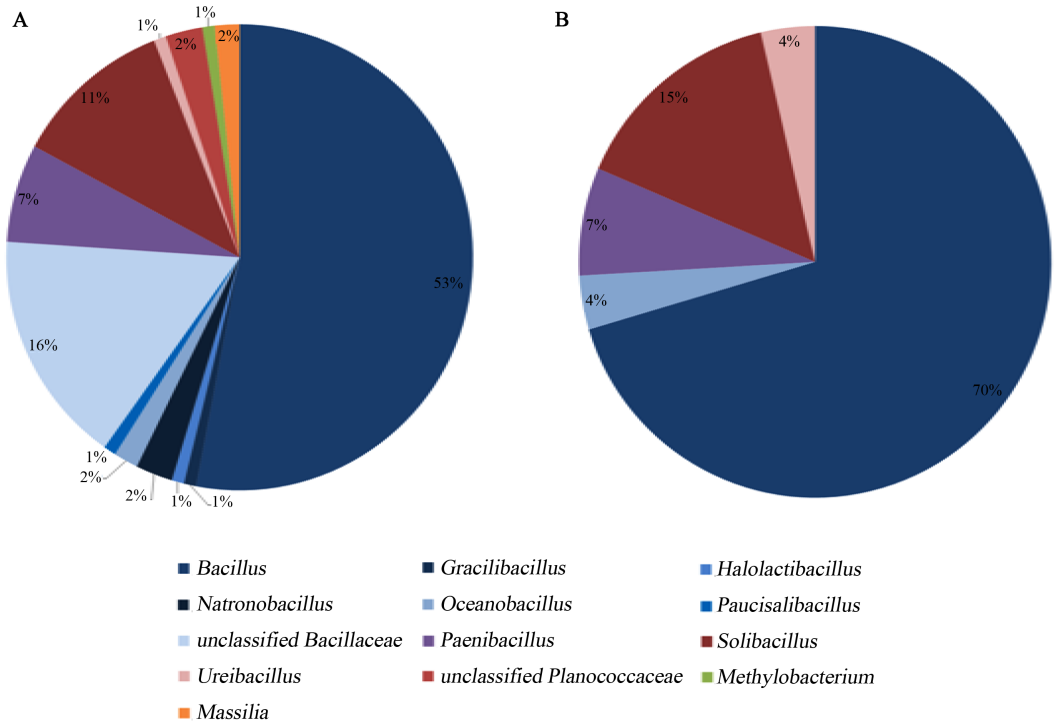

Figure 1. Taxonomic distribution of all bacterialOTUs at the 97% similarity level. (A) Distribution of bacterial genera obtained from 454 pyrosequencing; (B) Distribution of bacterial genera obtained from clone library.

and *Planococcaceae* represented 16% and 2% of the total OTUs. Interestingly, the genus *Bacillus*, a member of the *Bacillaceae* family, accounted for 53% of the total OTUs. The genera *Solibacillus* and *Paenibacillus*, members of the *Planococcaceae* and *Paenibacillaceae* families, were also predominantly represented with 11% and 7% of the total OTUs.

3.2. PCR Amplification of the 16S rRNA Gene V6-V8 Region, Cloning and Sequencing

A total of 494 V6-V8 sequences was obtained. They vary from 436 to 441 bp in length. Following processing, a total of 322 sequences was retained. Their average length was 439 bp. At the 97% similarity level, these sequences were clustered into 27 OTUs. As shown in **Figure 1(B)**, they all belonged to the phylum Firmicutes. Here also, all Firmicutes were assigned to three families: *Bacillaceae*, *Planococcaceae*, *Paenibacillaceae* with 74%, 19% and 7% of the total OTUs, respectively. These three families comprised two, two, and one genera. Here also, the genus *Bacillus* accounted for 70% of the total OTUs. The genera *Solibacillus* and *Paenibacillus* were also predominant with 15% and 7% of the total OTUs.

3.3. Comparison of the Bacterial Communities Obtained Using 454 Pyrosequencing and Clone Library

The rarefaction curves showed that 454 pyrosequencing yielded more OTUs than clone library when the same number of sequences was sampled (**Figure 2**). Pyrosequencing revealed a richer bacterial community than clone library. In addition, pyrosequencing yielded Chao 1 and Shannon indices with higher values compared to clone library (**Table 1**). This indicates a richer and more diverse bacterial community, respectively. Furthermore, pyrosequencing generated a Simpson diversity index and evenness with lower values compared to clone library. Clearly, 454 pyrosequencing revealed a much more diverse bacterial community than clone library.

Bacteria, mostly Firmicutes and Proteobacteria, are introduced to the paper mill through contaminated cellulosic raw material, fresh incoming water and paper-making chemicals [2] [5]. Physical conditions in the paper mill range from basic to acidic pH, ambient and high temperatures, aerobic and anaerobic environments, etc.

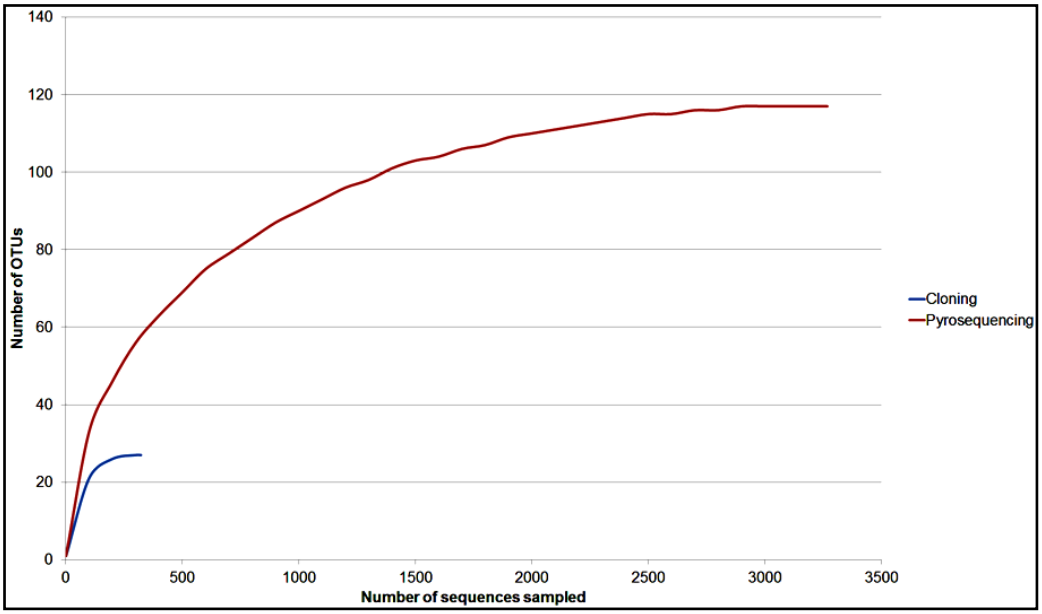

Figure 2. Rarefaction analysis of the bacterial sequences obtained from 454 pyrosequencing and clone library at the 97% similarity level.

Table 1. Comparison of the richness and diversity of the bacterial communities for the two approaches used (97% similarity level).

	454 pyrosequencing	Clone library
Accession numbers	SRR961674	KF589334-KF589827
Chao 1 richness	117	27
Shannon diversity index	3.41	2.42
Simpson diversity index	0.06	0.14
Evenness	0.26	0.42

Bacterial spores, owing to their resistance, will survive these very harsh environmental conditions and will be selected for. This leads to the accumulation and predominance of the spore-forming Firmicutes and the near absence of the much more sensitive non-spore-forming Proteobacteria. This was shown by Öqvist *et al*. [8] who found *Bacillus* and *Enterococcus* in white water. Disnard *et al*. [27] and Rättö *et al*. [28] both found *Bacillus* and *Paenibacillus* in biofilms. Suihko and Stackebrandt [29], Suominen *et al*. [30] and Väisänen *et al*. [31] all found *Bacillus* and *Paenibacillus* in final recycled food packaging paper and board. Suihkoand Stackebrandt [29] also found *Bacillus silvestris*, which has since been renamed *Solibacillus silvestris* [32], a genus present in our study. Likewise, McCusky Gendron *et al*. [11] and Namjoshi *et al*. [33] found *Bacillus* and *Paenibacillus* on unused paper towels and linerboard, respectively. Following use, some of these final recycled products will re-enter the recycling process. The bale of cardboard used in our study was a mix of cardboard collected from different sources. Most cardboard present in our bale had already gone through the recycling process several times, from the recycling plant to the paper mill, the production of the final recycled products, their various uses and back. The recycling plant is also a major source of microbial contamination. It receives paper, cardboard, glass, metal, plastic and textiles from domestic, industrial and commercial sources, some potentially soiled with food, grease and other organic residues, all substrates for microbial proliferation and accumulation. Following sorting, bales will most often be stored outdoors, under different meteorological and environmental conditions (rain, snow, wind, temperature, and the presence of small rodents and birds, etc.) for extended periods. This will most certainly lead to the formation of a much more complex and dynamic microbiota.

Ultimately, bales of cardboard ready to enter the paper mill for recycling have already been in contact with

several potential sources of contamination. This is what we found herewith the presence of Firmicutes: *Bacillus*, *Solibacillus* and *Paenibacillus*, and the near absence of Proteobacteria in our bale of cardboard.

We are planning to follow up on this study by analyzing the bacterial community in other potential sources of contamination in a paper mill such as fresh incoming water and chemicals, additives and fillers. We will also study different bales of cardboard stored under various conditions. A better knowledge of their microbiota appears necessary to improve the storing conditions in order to reduce biofouling in the paper mill and ultimately to increase the quality of the final recycled products.

4. Conclusion

In conclusion, both methods, 454 pyrosequencing and clone library, showed the predominance of spore-forming Firmicutes in the bale of collected cardboard. However, pyrosequencing revealed a richer and more diverse bacterial community than clone library. The large number of steps in the recycling process, from the paper mill to the recycling plant and back, contributes to the bacterial microbiota revealed in this study.

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