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16S rRNA Identification of Microorganisms and Direct Detection of Functional Genes in Waste Material Generated by an In-Vessel Rotating Compost System

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Abstract

Background: The microbial community in material produced after food biodegradation by the Rocket® Composter System (RCS) was characterized by 16S rRNA analysis of isolates and clone libraries. Functional microbial genes were ascertained by direct DNA extraction and PCR.

Methods: Bacterial isolates were identified by 16S rRNA sequencing. Microbial DNA was extracted using the ZymoMicrobe DNA MiniPrep protocol. PCR amplification targeted the presence of 16S rRNA, mold intergenic transcribed space region sequences, cellulases, and ammonia monooxygenase genes. Clone libraries were developed to determine the identity of the isolated eubacteria 16S rRNA genes. Characterization of each clone was performed by DNA sequencing.

Results: 16S rRNA identification of bacterial isolates showed the phylum Firmicutes with the highest numbers of species, 42%. Lower frequencies of Actinobacteria (26%), Proteobacteria (24%), and Bacteroidetes (8%) were detected. Clone libraries of eubacteria 16S rRNA genes showed the Firmicutes comprising 86% of the sequences while Proteobacteria accounted for 14%. Molds were not culturable from the samples neither any mold ITS sequences were detected in clone libraries. Direct DNA extraction and PCR detection showed that β-glucosidase genes had the highest frequency of all functional genes.

Conclusions: The RCS produced finished material with a vigorous heterotrophic bacterial community. However, the absence of mold and other bacterial phyla might indicate that the material produced is not degraded enough to be classified as mature compost and needed further processing. Most of the β-glucosidase sequences were related to members of the phyla Actinobacteria and Firmicutes while *amoA* genes were related to *Nitrosomonas* sp.

Keywords: *16S rRNA; Bacillus; cellulases; ammonia monooxygenase; food waste; cloning; compost*

Introduction

Composting of organic waste is a process driven by microorganisms under aerobic conditions. After treatment, compost can be used as a fertilizer and soil amendment. Several studies have been published on the microbial communities of composting systems [1,2]. Because of the thermophilic conditions present during composting, most bacterial populations are predominantly gram-positive spore formers belonging to the bacterial phyla Firmicutes and Actinobacteria [3,4]. Bacterial species belonging to the phyla Proteobacteria and Bacteroidetes were found to be present during the initial composting stages but lower number were found in late stages [1,5]. Therefore, microbial composition of compost phases changed based upon increases in temperature and availability of different organic substrates [3].

Traditional methodologies rely on the cultivability of microorganisms to isolate and describe phenotypes of bacteria and mold. Molecular analyses rely on the extraction of nucleic acids from compost to understand the microbial composition of non-culturable communities and the presence of catabolic genes [2,3]. Nevertheless, a combination of cultivation-based and molecular methods will provide a better understanding of the structure and genetic capabilities of microbial populations in compost because all methods have limitations to overcome. Furthermore, pure culture studies provide a measure of the enzymatic capabilities of individual microbial populations to breakdown different types of organic compounds and support development of biofertilizers and soil amendments.

The Rocket® Composter System (RCS) is an enclosed, in vessel, continuous system for composting of food waste (Figure 1). The food and organic waste is placed into the hooper on top of the system. The RCS is fed daily with equal volumes of cafeteria waste and woodchips. The process from then on is automated. As the blades of the internal shaft turn, the material is aerated, moving it along the body of the machine providing extra loading space at the input end of the machine and pushing finished material from the exit. The RCS holds materials for around 14 days in total. After a 2-week treatment, food waste was converted into finished product. Although the RCS is constantly mixing and turning the waste, two weeks is a very short time for the maturation of compost. Further treatment of the finished material is recommended to continue the decomposition of waste leading to mature compost. However, no studies have been reported on the microbial community composition and functional genes of the finished material produced by the RCS. Since all the feed going into the system is food waste combined with woodchips, cellulases and other functional genes related to food decomposition will be present in the RCS. What types of microorganisms are present in the material digested by the RCS? Do they resemble microbial communities previously reported to be present in composted samples? What types of cellulose and ammonia monooxygenase genes are present in the finished material?

Figure 1: *The Rocket Composter system*

The purpose of this study was to describe the microbial communities in RCS finished product and to determine the presence of different functional genes responsible for the biotransformation of carbon and nitrogen.

Materials and Methods

Microbial isolation and identification

After 2 weeks of processing of the food waste by the RCS, the finished material was sampled from the system's outlet. Samples were taken from October 6, 2011 to July 24, 2013. Ten-gram aliquots were added to 90 ml of sterile water and shaken, e.g., 150 rpm, for an average of 15 minutes at room temperature. After shaking, serial dilutions in sterile water were placed on the following media: Trypticase Soy Agar (TSA), Sabouraud Dextrose Agar (SDA), or the OxyPlate™ anaerobic system containing Schaedler Blood Agar (SBA) (Oxyrase® Inc., Mansfield, OH). TSA plates were incubated at 37°C and 55°C. SBA plates were incubated at 37°C. SDA plates were incubated at 25°C

and 35°C. After incubation for 48-72 hours, different bacterial colonies based upon color and morphology were analyzed using Gram staining. Bacterial16S rRNA sequencing was performed by Accugenix, Inc. (Newark, DE). After suspensions were shaken as described above, 10 ml aliquots of each suspension were transferred to triplicate tubes of double strength lactose broth. Aliquots of 1.0 and 0.1 ml of soil suspensions were added to triplicate tubes of single strength lactose broth. All samples were incubated at 37°C for a minimum of 48 hours. Lactose tubes demonstrating lactose fermentation were transferred to eosin methylene blue agar (EMB). The plates were incubated at 37°C for 48 hours. If a green metallic sheen reaction was observed by any colony on EMB, the isolate was confirmed to be *E. coli* by 16S rRNA gene sequencing as previously described. A standard *E. coli* strain obtained from Ward Science (wardsci.com) was used as positive control for all the tests.

DNA Extractions

DNA was extracted from 26 samples obtained from July 2012 to July 2013. DNA extractions were performed as described in the ZR Soil Microbe DNA MiniPrep protocol (Zymo Research, Irvine, CA). Samples of 300 milligrams were added to ZR Bashing Bead™Lysis Tube containing 800 µl of Lysis Solution. For microbial DNA extractions, two sterile loopfuls of growth from agar plates were added to 750 µl of Lysis Solution in a ZR Bashing Bead™Lysis Tube. Different aliquots of extracts were used in the PCR reactions.

PCR Reactions

The PCR reactions and DNA primer sequences for eubacteria 16S rRNA, actinobacteria 16S rRNA, β-glucosidase, *cel48, cel5,* and *amoA* genes were previously described [6-10]. To detect mold DNA sequences in waste material and mold samples, universal intergenic transcribed space region (ITS) sequences were analyzed as previously described [11]. Ready-To-Go (RTG) PCR beads (GE Healthcare, Buckinghamshire, UK) were used for each PCR reaction volume as described by Jimenez., *et al*. [12].

A 300-µl aliquot of Biospira (Instant Ocean, Blacksburg, VA), a blending of *Nitrosomonas* spp., *Nitrosospira* spp., and *Nitrosococcus* spp., was added to 700 µl of lysis solution. Bacterial DNA was extracted using the ZR Soil Microbe DNA MiniPrep protocol. This DNA solution was used as a positive control for the amoA PCR reaction. Reaction mixtures were added to the T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) or Master cycler thermal cycler (Eppendorf Scientific, Westbury, NY).

Amplicon detection was carried out by gel electrophoresis using the FlashGel™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 kb was used to determine the presence of the correct DNA gene fragments. DNA sequencing reactions of the amplified PCR fragments from waste material and bacterial isolates 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 [13].

Cloning libraries of 16S rRNA genes

The DNA fragments from the PCR amplification of eubacteria and actinobacteria 16S rRNA genes were cloned using plasmids pJet1.2 blunted vector (Bio-Rad Laboratories, Hercules, CA) and pCR®4-TOPO (Life Technologies, Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's instructions. Transformations were performed using Mix and Go Competent *E. coli* strains (Zymo Research, Irvine, CA). White colonies grown on Luria Bertani (LB) Agar with ampicillin (50 µg/ml) were transferred to LB broth containing ampicillin (50 µg/ml). Samples were incubated overnight at 37°C.

Plasmids were isolated from each clone using the Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine, CA). Cloned inserts were reamplified using the eubacterial and actinobacterial DNA primers. DNA sequencing reactions of the amplified PCR fragments 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 [13].

ITS Mold Cloning Libraries

The amplified products from the PCR analysis of ITS genes were cloned using plasmid pCR®4-TOPO (Life Technologies, Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's instructions. Transformations were performed using Mix and Go Competent *E. coli* strains (Zymo Research, Irvine, CA). White colonies grown on Luria Bertani (LB) Agar with ampicillin (50 µg/ml) were transferred to LB broth containing ampicillin (50 µg/ml). Samples were incubated overnight at 37°C.

Plasmids were isolated from each clone using the Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine, CA). Cloned DNA inserts were reamplified using ITS1 and ITS4 primers. DNA sequencing reactions of the amplified PCR fragments were performed as described above.

Results

Identification of Bacteria Isolated From Growth Media

Gram stain analyses of randomly picked bacterial colonies isolated from October 6, 2011 to July 24, 2013 demonstrated the dominant presence of Gram-positive bacteria. Sixty eight percent of the bacterial isolates were Gram-positive with only 32% of Gram-negatives. Genetic analyses of the isolates showed that Gram-positive species belonged to either the Firmicutes or Actinobacteria phyla (Table 1). Gram-negative species were found to be members of either the phyla Proteobacteria or Bacteroidetes. Overall, the Firmicutes accounted for 42% of the bacteria isolated from the RCS. The dominant family and genus within the Firmicutes were Bacillaceae and *Bacillus*, respectively. The species isolated from the RCS compost were *Bacillus* sp.*, Bacillus oceanosedimidis, Bacillus firmus, Bacillus shackletonii, Bacillus thermoamylovorans, Bacillus fortis, Bacillus thermolactis,Bacillus horneckiae, Bacillus farraginis, Bacillus marisflavi, Bacillus smithii*, and *Bacillus humi* (Table 1). Several bacterial species were repeatedly isolated through time. The most frequently isolated species from the *Bacillus* genus were *B. thermoamylovorans,* e.g., 5 times, and *Bacillus* sp., e.g., 4 times.

Bacteria belonging to the phylum Actinobacteria were found to account for 26% of isolates. The most isolated Actinobacteria genus was *Microbacterium*. The species were *Microbacterium gubbeenense, Microbacterium barkeri, Microbacterium sp.,* and *Microbacterium halotolerans* (Table 1).

Gordonia paraffinivorans and *Cellulomonas cellasea* were the second most isolated Actinobacteria in waste material. Other species of Actinobacteria isolated were *Kroppenstedtia eburnean, Thermobifida fusca, Dietzia* sp.*, Corynebacterium freneyi, Brachybacterium* sp.*, Brachybacterium phenoliresistens, Propionibacterium acnes,* and *Propionibacterium acidopropionici* (Table 1).

The phylum Proteobacteria represented 24% of the isolates. However, most of the Proteobacteria detected were from anaerobic plates, SDA plates, or lactose enrichment media. A few species grew on TSA plates. The species were *Acetobacter pasteurianus, Acinetobacter baumanni, E. coli, Paenalcaligenes hominis, Proteus mirabilis, Pantoea* sp.*, Paracoccus solventivorans, Acinetobacter* sp.*, Klebsiella pneumoniae pneumoniae, Pseudomonas thermotolerans, Pseudoxanthomonas taiwanensis, Pusillimonas* sp.*, and Enterobacter hormaechei hormaechei* (Table 1). The Bacteroidetes were found to be only 8% of isolated bacteria. The species detected on plate media were *Sphingobacterium* sp., *Sphingobacterium thermophilum,* and *Dysgonomonas* sp. (Table 1). No other bacterial phylum was detected by plate counts or lactose enrichments. However, the phyla Firmicutes and Actinobacteria accounted for 68% of bacteria isolated on agar media.

No mold colonies were found on any of the TSA plates incubated at 25°C or 35°C. Several samples were plated on Sabouraud Dextrose Agar (SDA) to ascertain mold densities. However, all colonies growing on SDA plates incubated either at 25°C or 35°C were found to be bacteria. Four samples from different dates, 10/17/12, 11/12/12, 7/2/13 and 7/24/13, were analyzed for anaerobic bacteria. Genetic identification of randomly selected anaerobic bacteria colonies using 16S rRNA sequencing demonstrated the presence of *Pantoea* sp.*, E. hormaechei hormaechei, Enterococcus faecium, B. thermoamylovorans, Dysgonomonas* sp., *P. acnes,* and *P. acidipropionici* (Table 1).

Genera Species	Phylum
Bacillus oceanosedimidis	Firmicutes
Bacillus firmus	Firmicutes
Bacillus shackletonii	Firmicutes
Kroppenstedtia eburnean	Actinobacteria
Bacillus fortis (2)	Firmicutes
Bacillus thermolactis	Firmicutes
Cohnella xylanilytica	Firmicutes
Acetobacter pasteurianus	Proteobacteria
Acinetobacter baumannii	Proteobacteria
Ureibacillus thermosphaericus	Firmicutes
Escherichia coli (3)	Proteobacteria
Ureibacillus suwonensis (2)	Firmicutes
Cellulomonas cellasea (2)	Actinobacteria
Micrococcus luteus (2)	Firmicutes
Bacillus horneckiae	Firmicutes
Bacillus farraginis	Firmicutes
Dietzia sp.	Actinobacteria
Bacillus sp. *(4)*	Firmicutes
Paenalcaligene shominis	Proteobacteria
Streptococcus salivarius	Firmicutes
Bacillus humi	Firmicutes
Lysinibacillus sp.	Firmicutes
Oceanobacillus caeni	Firmicutes
Corynebacterium freneyi	Actinobacteria
Sphingobacterium thermophilum (2)	Bacteroidetes
Sphingobacterium sp. *(4)*	Bacteroidetes
Micrococcus luteus	Firmicutes
Proteus mirabilis (2)	Proteobacteria
Enterococcus casseliflavus	Firmicutes
Enterococcus avium	Firmicutes
Enterococcus gallinarum	Firmicutes
Microbacterium gubbeenense (4)	Actinobacteria
Brachybacterium sp.	Actinobacteria
Paracoccus solventivorans	Proteobacteria
Gordonia paraffinivorans (2)	Actinobacteria
Microbacterium barkeri (3)	Actinobacteria
Microbacterium halotolerans	Actinobacteria
Brachybacterium phenoliresistens	Actinobacteria
Bacillus marisflavi	Firmicutes

Genera Species	Phylum
Bacillus smithii	Firmicutes
Acinetobacter sp.	Proteobacteria
Klebsiella pneumoniae pneumoniae (3)	Proteobacteria
Pseudomonas thermotolerans	Proteobacteria
Pseudoxanthomonas taiwanensis (2)	Proteobacteria
Microbacterium sp.	Actinobacteria
Geobacillus thermodenitrificans	Firmicutes
Pusillimonas sp.	Proteobacteria
Thermobifida fusca (2)	Actinobacteria
Enterococcus faecium	Firmicutes
Bacillus thermoamylovorans (5)	Firmicutes
Dysgonomonas sp.	Bacteroidetes
Propionibacterium acnes	Actinobacteria
Propionibacterium acidipropionici	Actinobacteria
Pantoea sp.	Proteobacteria
Enterobacter hormaechei hormaechei (2)	Proteobacteria

Table 1. *Genetic identification of bacterial isolates from RCS finished material grown on TSA, SDA, OxyPlates, and lactose enrichments. Number between parentheses represents the isolation frequency. N=84.*

Identification of 16S rRNA clones

The composition of the non-culturable bacterial community was ascertained by DNA extraction of 26 samples dating from July 2012 to July 2013. To determine if the extracted DNA can be amplified by PCR, ribosomal eubacteria gene sequences were targeted for assay validation. Samples with a positive reaction showed the amplification of the 1.5 kb DNA fragment encoding for the 16S rRNA gene. All DNA samples indicated the presence of eubacteria genes (Figure 2).

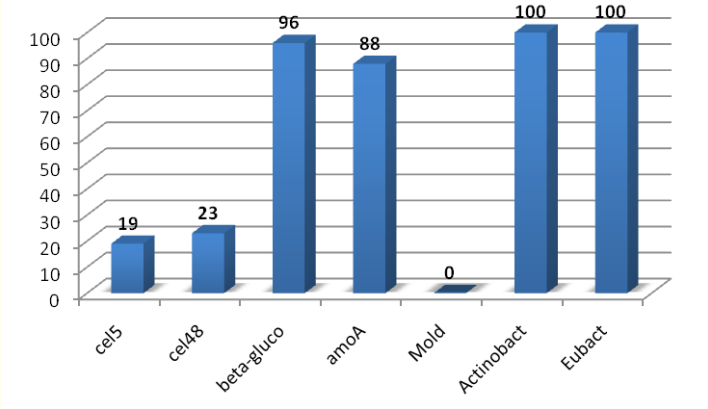

Figure 2. *Distribution of functional genes in RCS finished material. Percent of positive samples showing amplification with functional genes. N=26.*

cel5= glycoside hydrolase family 5
cel48 = glycoside hydrolase family48
beta-gluco=beta-glucosidase
amoA=ammonia monooxygenase gene A
Mold = universal intergenic transcribed space region (ITS) sequences
Eubac= eubacteria 16S rRNA gene
Actinobact= actinobacteria 16S rRNA gene

To determine the diversity of the bacterial community by 16S rRNA sequencing of eubacteria genes, clone libraries were constructed using two different vectors. Eighty six percent of the sequenced clones were found to belong to the phylum Firmicutes while 14% showed a high similarity with the phylum Proteobacteria (Table 2). No other eubacterial sequences were found related to any other phylum.

The dominant family and genus within the Firmicutes were Bacillaceae and *Bacillus*, respectively. *Bacillus* was the most common genus with 32% of the 16S rRNA sequences showing a minimum of 80% homology (Table 2). *B. infantis* was the most common species detected. *Geobacillus* was the second most common genus with 30% of the sequences having at least a 80% homology. *Geobacillus* sp. was the most common species detected. Within the Proteobacteria the genera *Bordetella* was the predominant type.

Clone/16S rRNA	Genera Species	Phylum	Accession Number	% Identity
1 Eubacteria	*Geobacillus* sp.	Firmicutes	NC 014206.1	93
2 Eubacteria	*Bacillus* sp.	Firmicutes	NC 021171.1	90
3 Eubacteria	*Pseudoxanthomonas suwonensis*	Proteobacteria	NC 014924.1	97
4 Eubacteria	*Anoxybacillus flavithermus*	Firmicutes	NC 011567.1	89
5 Eubacteria	*Geobacillus kaustophilus*	Firmicutes	NC 006510.1	86
6 Eubacteria	*Bacillus* sp.	Firmicutes	NC 021171.1	87
7 Eubacteria	*Bacillus pumilus*	Firmicutes	NC 009848.1	89
8 Eubacteria	*Bordetella petrii*	Proteobacteria	NC 010170.1	95
9 Eubacteria	*Bordetella bronchiseptica*	Proteobacteria	NC 019382.1	90
10 Eubacteria	*Geobacillus thermoglucosidasius*	Firmicutes	NC 015660.1	93
11 Eubacteria	*Geobacillus* sp.	Firmicutes	NC 014915.1	92
12 Eubacteria	*Bacillus infantis*	Firmicutes	NC 022524.1	88
13 Eubacteria	*Bacillus infantis*	Firmicutes	NC 022524.1	91
14 Eubacteria	*Bacillus infantis*	Firmicutes	NC 022524.1	80
15 Eubacteria	*Bacillus* sp.	Firmicutes	NC 021171.1	93
16 Eubacteria	*Geobacillus* sp.	Firmicutes	NC 012793.1	89
17 Eubacteria	*Bacillus infantis*	Firmicutes	NC 022524.1	89
18 Eubacteria	*Geobacillus* sp.	Firmicutes	NC 012793.1	94
19 Eubacteria	*Thermoanaerobacter brockiisubsp.finnii*	Firmicutes	NC 014964.1	88
20 Eubacteria	*Geobacillus* sp.	Firmicutes	NC 012793.1	94
21 Eubacteria	*Thermobacillus composti*	Firmicutes	NC 019897.1	93
22 Eubacteria	*Geobacillus thermodenitrificans*	Firmicutes	NC 009328.1	94
23 Eubacteria	*Geobacillus* sp.	Firmicutes	NC 012793.1	94

Clone/16S rRNA	Genera Species	Phylum	Accession Number	% Identity
24 Eubacteria	*Geobacillus* sp.	Firmicutes	NC 012793.1	94
25 Eubacteria	*Paenibacillus* sp.	Firmicutes	NC 012914.1	83
26 Eubacteria	*Oceanobacillus iheyensis*	Firmicutes	NC 004193.1	96
27 Eubacteria	*Anoxybacillus flavithermus*	Firmicutes	NC 011567.1	96
28 Eubacteria	*Pseudomonas aeruginosa*	Proteobacteria	NC 002516.2	98
29 Eubacteria	*Geobacillus thermoglucosidasius*	Firmicutes	NC 015660.1	94
30 Eubacteria	*Bacillus halodurans*	Firmicutes	NC 002570.2	92
31 Eubacteria	*Thermoanaerobacter marianensis*	Firmicutes	NC 014831.1	92
32 Eubacteria	*Alkaliphilusoremlandi*	Firmicutes	NC 009922.1	90
33 Eubacteria	*Bacillus infantis*	Firmicutes	NC 022524.1	95
34 Eubacteria	*Anoxybacillus flavithermus*	Firmicutes	NC 011567.1	93
35 Eubacteria	*Psychrobacter cryohalolentis*	Proteobacteria	NC 007969.1	99
36 Eubacteria	*Bacillus infantis*	Firmicutes	NC 022524.1	92
37 Eubacteria	*Bacillus coagulans*	Firmicutes	NC 015634.1	94
38 Actinobacteria	*Corynebacterium halotolerans*	Actinobacteria	NC 020302.1	94
39Actinobacteria	*Intrasporangium calvum*	Actinobacteria	NC 014830.1	95
40 Actinobacteria	*Corynebacterium halotolerans*	Actinobacteria	NC 020302.1	94
41 Actinobacteria	*Corynebacterium halotolerans*	Actinobacteria	NC 020302.1	94
42 Actinobacteria	*Thermobifida fusca*	Actinobacteria	NC 007333.1	99
43 Actinobacteria	*Aeromicrobium marinum*	Actinobacteria	NZ CM0011024.1	99
44 Actinobacteria	*Actinoplanes friuliensis*	Actinobacteria	NC 022657.1	91
45 Actinobacteria	*Thermobifida fusca*	Actinobacteria	NC 007333.1	99

Table 2. *Phylogenetic analysis of eubacteria and actinobacteria clones generated from cloning libraries based upon the 16S rRNA sequencing. N = 45.*

Actinobacteria 16S rRNA sequences were found in all samples analyzed using Actinobacteria-specific 16S rRNA primers (Figure 2). Clone libraries of actinobacterial 16S rRNA sequences from one sample analyzed indicated the presence of *Corynebacterium halotolerans, Intrasporangium calvum, T. fusca,* and *Actinoplanes friuliensis.*

Identification of mold ITS clones

Of the 26 samples analyzed for the presence of mold ITS sequences, two were found to show a DNA fragment of the expected molecular weight, e.g. 681 bp. The DNA fragments of the two samples showing amplification with the ITS primers were cloned to determine the identity of the DNA fragment. None of the clones analyzed showed any resemblance to mold genetic sequences. All cloned sequences were found to be bacteria related to the phylum Proteobacteria. The bacterial species were identified as *Sphingobium japonicum*.

Identification of functional genes

A glycoside hydrolase gene, *cel5*, was found by PCR analysis to be present in RCS finished product. The 362 bp DNA fragment was detected in all the positive samples. Of 26 samples analyzed, 19% exhibited a positive reaction (Figure 2). The DNA fragment detected in a sample from March 19, 2013 was excised and purified from the gel. BLAST analysis of the sequenced DNA fragment showed the highest homology, e.g., 73%, with a cellulase gene from *Bacteroides salanitronis* (Table 3). Another sample from April 23, 2013 was found to have a 79% homology with an endoglucanase celB gene from *Bacillus licheniformis*.

Sample Source	Gene	%Identity	Microorganism/Gene	Accesion Number
R1 compost DNA 3/19/13	cel5	73	*Bacteroides salanitronis/ Cellulase*	NC 015164.1
R1 compost DNA 4/23/13	cel5	79	*B. licheniformis/ Endoglucanase celB*	NC 006322.1
Bacillus sp. compost isolate	cel5	76	*B. licheniformis/ Endoglucanase celC*	NC 006322.1
S. thermophillum compost isolate	cel5	79	*B. licheniformis/ Endoglucanase celB*	NC 006322.1
R2 compost DNA 6/12/13	cel48	77	*T. fusca/ Cellulase 1,4-beta-cellobiosidase*	NC 007333.1
R1 compost DNA 7/9/13	cel48	95	*T. fusca/ Cellulase 1,4-beta-cellobiosidase*	NC 007333.1
R2 compost DNA 7/9/13	cel48	84	*B. licheniformis/ Endoglucanase celA*	NC 006322.1
R1 compost DNA 7/24/13	cel48	90	*T. fusca/ Cellulase 1,4-beta-cellobiosidase*	NC 007333.1
Bacillus sp. compost isolate	cel48	84	*B. licheniformis/ Endoglucanase celA*	NC 006322.1
T. fusca compost isolate	cel48	99	*T. fusca/ Cellulase 1,4-beta-cellobiosidase*	NC 007333.1
M. barkeri compost isolate	Gluco	74	*Sphaerobacter thermophilus/ beta-galactosidase*	NC 013523.1
Dietzia sp. compost isolate	Gluco	77	*Sphaerobacter thermophilus/ beta-galactosidase*	NC 013523.1
Compost DNA 7/11/12	Gluco	94	*T. fusca/ Cellulase 1,4-beta-cellobiosidase*	NC 007333.1
R1 compost DNA 5/22/13	Gluco	75	*Sphaerobacter thermophilus/ beta-galactosidase*	NC 013523.1
R2 compost DNA 6/19/13	Gluco	95	*T. fusca/ Cellulase 1,4-beta-cellobiosidase*	NC 007333.1
R2 compost DNA 7/9/13	Gluco	78	*T. fusca/ Cellulase 1,4-beta-cellobiosidase*	NC 007333.1
Biospira	Eubac	92	*Nitrosomonas* sp./*rRNA 16S ribosomal*	NC 015731.1
Biospira	amoA	84	*Nitrosomonas* sp./ *Ammonia monooxygenase subunit A*	NC 015731.1
R1 compost DNA 6/19/13	amoA	86	*Nitrosomonas* sp./ *Ammonia monooxygenase subunit A*	NC 015222.1

Table 3. *DNA sequencing results from PCR amplification of microbial genes.*

A glycoside hydrolase gene, *cel48*, was found by PCR analysis to be present in the RCS finished product. The *cel48* gene was found in 23% of samples analyzed (Figure 2). Amplification of the 430 bp DNA fragment indicated a positive reaction. PCR amplified DNA fragments detected in samples from June 12, 2013, July 9, 2013, and July 24, 2013 were excised from the gel, purified, and sequenced. BLAST analysis of 3 out of the 4 sequences exhibited a minimum of 77% homology with a cellulase 1,4-beta-cellobiosidase gene from *T. fusca* (Table 3). The other sample from July 9, 2013, was found to have a 84% homology with an endoglucanase celA gene present in *B. licheniformis*.

Twenty six percent of bacterial isolates showed amplification of the 430 bp DNA fragment. DNA sequencing and BLAST analysis of the amplified fragment from a *Bacillus* sp. isolate demonstrated the closest homology, e.g., 84%, with an endoglucanase celA gene from *B. licheniformis* (Table 3). Another bacterial isolate identified as *T. fusca* was found to have the same 430 bp fragment. However, BLAST analysis showed a 99% similarity with a cellulase 1,4-beta-cellobiosidase from *T. fusca.*

The presence of β-glucosidase genes was also analyzed. Ninety six percent of the samples exhibited a positive PCR amplification reaction (Figure 2). All samples showed the presence of the 300 bp DNA fragment. Table 3 shows the BLAST analysis of the sequences of the amplified DNA fragments in samples from July 11, 2012, May 22, 2013, June 19, 2013, and July 9, 2013. Three of the 4 samples showed the highest homology values to a cellulase 1,4-beta-cellobiosidase gene found in *T. fusca*. The other sample showed a 75% resemblance to a beta-galactosidase gene from *Sphaerobacter thermophilus.*

When DNA samples from bacterial isolates were analyzed by PCR using the β-glucosidase primers, 74% reacted positively. BLAST analysis of the sequences obtained from the amplified 300 bp fragments in *M. barkeri* and *Dietzia* sp. showed 74% and 77% similarities with a beta-galactosidase gene from *S. thermophilus* (Table 3).

In the absence of viable cultures of *Nitrosomonas* sp. or *Nitrosospira* sp., to use as a positive control in the amoA PCR reactions, a commercial product, e.g., Biospira, was analyzed by DNA extraction. The extracted DNA was amplified by PCR with the eubacteria primer pair to determine the composition of the product. BLAST analysis of the sequence obtained from the amplified 1.5 kb fragment showed a close resemblance, e.g., 92%, to a *Nitrosomonas* sp. 16S rRNA gene (Table 3). When the same DNA sample was analyzed using the amoA primers, a 491 bp DNA fragment was detected. BLAST analysis of the sequenced fragment demonstrated an 84% match with *amoA* genes from *Nitrosomonas* sp.

When DNA samples extracted from finished product were analyzed with the *amoA* primers, 88% showed the amplification of the 491 bp amoA DNA fragment (Figure 2). The sample taken on June 19, 2013 showed a positive amplification with the *amoA* primers. Based upon BLAST analyses of the sequenced fragment, an 86% similarity was found with the *amoA* gene from *Nitrosomonas* sp. (Table 3).

Discussion

Treatment of food waste at Bergen Community College (BCC) is based upon the use of the RCS. BCC produces large quantities of food waste from cafeteria outlets on campus and in-house catering. In the summer of 2011, the college purchased the RCS to establish a sustainable organic waste-recycling program on campus. Equal volumes of woodchips and food waste were added to the system to maintain an optimal Carbon: Nitrogen ratio. The temperature of the system never went below 52°C with a recorded high of 67°C.

Since the system is closed, samples of the treated material were taken from the outlet of the system after the end of the thermophilic phase. We combined cultivation-based and cultivation independent methods to understand the microbial composition of the material produced after two weeks treatment. Based upon 16S rRNA sequencing of bacterial isolates and clone libraries, the Firmicutes and *Bacillus* were the dominant bacterial phylum and genus. A high concentration of *Bacillus* species indicated very active thermophilic conditions inside the RCS. *B. thermoamylovorans* and *B. infantis* showed the highest detection frequencies on plate media and clone libraries. Similar results were shown in compost samples from different sources. The Firmicutes were previously found to be the most numerous bacterial phylum in household compost [2]. *B. thermoamylovorans* were reported to be commonly isolated in 16S rRNA clone libraries from food compost [14]. Studies from agricultural compost showed 78% of bacterial isolates to be Firmicutes with *Bacillus* as the predominant genus [1]. Samples from static windrow compost piles also showed *Bacillus* isolates to be the majority of the bacterial community [15].

The percentage of Actinobacteria found in RCS samples was higher than in agricultural compost [1]. Actinobacteria accounted for 9% of culturable bacteria with *Microbacterium* as the most isolated species [1]. *Microbacterium* species were the dominant Actinobacteria in the RCS samples. *Microbacterium* also dominated composts of a mixture of sewage and yard waste with intermediate curing times of 41-30 days [3]. Although Actinobacteria were frequently isolated on media plates, none of the eubacteria 16S rRNA sequences from clone libraries was related to that phylum. Proteobacteria were the second most abundant bacterial phylum in clone libraries. However, when Actinobacteria-specific 16S rRNA primers were used, clone libraries of the amplified fragments showed the presence of different Actinobacteria species. PCR detection of Actinobacteria 16S rRNA genes showed they were present in all 26 samples analyzed but the numbers might have not been high enough when compared to other bacterial phyla to be detected in the eubacteria clone libraries. Another explanation is that the universal eubacteria primers did not amplify Actinobacteria 16S rRNA genes. Previous studies reported the inability of universal bacterial 16S rRNA primers to amplified different types of Actinobacteria in environmental samples [16]. Future studies will ascertain the presence of Actinobacteria species in samples from other dates by developing clone libraries of amplified fragments using Actinobacteria-specific 16S rRNA primers.

Overall, the phyla Firmicutes, Actinobacteria, and Proteobacteria dominated the heterotrophic bacterial community in the RCS finished product. Their ability to breakdown complex polymers such as proteins, fats, starch, and cellulose is very important to optimize compost maturity [4,5,17]. The presence of these bacterial phyla and species indicates that a very efficient biodegradation treatment and maturation might be taking place after a two-week processing of food waste. However, the absence of other bacterial phyla in the clone libraries might indicate that the maturity of the material is at the early stages and further management of the RCS finished product is needed. Preliminary chemical analysis of one finished product sample confirmed the need for further processing of the samples. The sample maturity index was determined to be 4. This value indicated that the compost produced by the RCS was active compost but not finished compost. A maturity index of 8 is considered to be the best indication for compost to be fully cured. Previous studies using 16S rRNA clone libraries and next generation sequencing reported a more diverse bacterial community by the presence of more than 5 different phyla in mature compost samples [3, 18,19]. More bacterial phyla were found on plate media and enrichments than in the clone libraries, which indicated media enrichment, enhanced the detection of some of the low abundance species. However, it is unlikely that bacteria growing on rich media at temperatures much lower than in the system are representative of what is active within the system. The absence of other bacterial phyla in the eubacteria clone libraries, previously reported in compost, such as Bacteroidetes, Actinobacteria, Chlorofexi might have occurred because the numbers of clones analyzed were insufficient to cover the bacterial diversity in the RCS material [3,18,19]. Previous studies analyzed more than 500 clones to develop a deeper understanding of the diversity present in compost samples [2,3,18]. The number of clones analyzed in this study might have not been high enough to detect all the bacteria present. Furthermore, no mold was found to be culturable or present in clone libraries. Molds were previously reported to be absent from composting samples when temperatures were higher than 55°C [4,20]. Temperatures recorded by the RCS never went below 52°C. Therefore, temperature might have been a limiting factor for the growth and survival of significant mold populations inside the RCS during processing. Molds are very important microbial components for the maturation of compost and when absent will delay the further biodegradation of recalcitrant organic compounds negatively impacting the quality of the composted material [19]. When composting temperatures decrease, molds are usually reintroduced by the germination of survival spores or the introduction of environmental isolates [19].

Based upon the direct DNA extraction and PCR amplification results, the most abundant cellulase gene in the RCS material was the β-glucosidase gene. When cellulose fragments are broken down by exoglucanases, cellobiose is produced. Glucosidases complete the final step of cellulose hydrolysis by converting cellobiose to glucose. Glucosidases are a very diverse type of enzymes present in several microbial species [8]. After PCR amplification of gene sequences, the DNA fragments were excised from the gel and purified to determine the sequence identity. Our intention was not to ascertain the diversity of the microbial population carrying the gene but to determine the dominant population sequence. Therefore, cloning of the amplified fragments was not performed. DNA sequencing and

BLAST analysis demonstrated that the Actinobacteria were the dominant phylum containing β-glucosidase gene sequences. BLAST analysis indicated that *T. fusca* related sequences were the most abundant. Of the 4 DNA samples successfully analyzed, 3 exhibited genetic sequences similar to *T. fusca* and 1 to *Sphaerobacter thermophilus*. Both bacteria are members of the phylum Actinobacteria. *T. fusca* is commonly found in compost material with a diverse catabolic potential to breakdown lignin and cellulose [14,17,20].

DNA sequencing of the 300 bp fragments from two bacterial isolates identified as *M. barkeri* and *Dietzia* sp. demonstrated a closer homology with β-galactosidase genes related to *S. thermophilus. S. thermophilus* is also a member of the phylum Actinobacteria previously isolated from aerobic thermophilic sludge treatment systems [21]. Compost samples of cow manure and straw analyzed with the same primer pair we used in this study, e.g., BGH1F and BGH1R, exhibited β-glucosidase gene sequences related to *Thermosbispora bispora, Clostridium thermocellum, Paenibacillus* spp., and *Bacillus* spp. [8]. In that study Li., *et al*. [8] reported differences in the bacterial community composition between early and late phases of composting. Late compost stages exhibited high β-glucosidase activities. In the early phases easily degradable compounds such as sugars and proteins are abundant and low β-glucosidase activity is present. However, during the later stages more resistant carbon compounds such as cellulose and lignin are degraded. All the samples analyzed for functional genes came from post-thermophilic stages, which might explain the high frequency of β-glucosidase genes in finished material and bacterial isolates.

The second most abundant cellulase genes were found to be the *cel48*. Glycoside hydrolases such as cellulases hydrolyze glycosidic bonds between two or more carbohydrates. Family 48 contains enzymes with endo-β-1,4-glucanase, chitinase, endo-processive cellulase and cellobiohydrolase activity. BLAST analysis of the DNA sequences obtained after PCR amplification and sequencing showed that *T. fusca* was the dominant microbial population carrying *cel48* gene sequences. Izquierdo *et al*. [22] showed the presence of *cel48* genes in enrichment cultures from compost materials by DNA sequencing of PCR amplified cloned fragments. They reported that all the detected sequences were found to belong to members of the genus *Clostridium*. Similar results were reported in bioreactor samples where *cel48* genes were found to be predominantly related to different *Clostridium* species [9]. Lower frequencies of *cel5* genes were found in RCS material and bacteria. Family 5 comprises enzymes with endoglucanases, beta-mannanases, exoglucanases, and xylanase activities. Of the 17 DNA sequences of cellulases randomly selected and analyzed, 10 were related to species belonging to the Actinobacteria, 6 to the Firmicutes, and 1 to the Bacteroidetes. The phylum Actinobacteria showed the highest numbers of *cel48* and glucosidase genes while Firmicutes showed more *cel5* genes. Evidently the microbial populations carrying *cel5* genetic sequences were not present at the same levels of the other two cellulase genes. Nevertheless, their presence indicated that a diverse microbial community carrying different types of cellulases performed the degradation of cellulose in the RCS. Cloning of the different cellulase genes found in RCS material will provide a better understanding of the diversity and complexity of the cellulose bio degradative communities.

The high frequency of *amoA* sequences demonstrated that RCS materials are providing a vigorous ammonia oxidizing bacterial (AOB) population. The *amoA* gene encodes for a membrane associated active site polypeptide of AMO. The gene is highly conserved and present in AOB such as *Nitrosomonas* spp. and *Nitrosospira* spp. One of the major objectives of composting is to provide a stable product containing available carbon and nitrogen to plants [23,24]. Organic compound biodegradation by proteolysis and rapid hydrolysis of urea produce large amounts of ammonia [23]. However, ammonia cannot be used by plants directly and must be converted to a more available nitrogen form such as nitrate. BLAST analysis of DNA sequences obtained from the sequencing of PCR amplified *amoA* genes showed high similarities with an *amoA* gene from *Nitrosomonas* sp. Previous studies detected AOB in sewage, manure, bio waste, and green compost by real time PCR with reported numbers ranging from 1 x10^7 to 7 x 10^7 cell/g. AOB frequency using 16S rDNA PCR testing showed 38% of samples yielding a positive reaction with direct PCR and 88% with nested PCR [23]. Kowalchuk., *et al*. [24] also detected *amoA* genes in experimental and natural compost. They concluded that not all AOB were equally active. Most of the *amoA* sequences were found to cluster with the genera *Nitrosospira* and *Nitrosomonas*. Further studies will determine the genetic diversity of the amplified *amoA* genes found in RCS compost by cloning of the amplified amoA sequences.

Conclusion

On the basis of the microbiological and genetic data, the material produced after 2 weeks treatment of food waste by the RCS contained a vigorous heterotrophic bacterial community. The Firmicutes were the dominant bacterial phylum in clone libraries and isolates indicating that the processing of food waste was effective and biodegradation was heavily mediated by *Bacillus* species. The high frequency of endospores producers in the finished product indicated the highly thermophilic conditions inside the RCS.

High frequencies of cellulase genes related to the phyla Actinobacteria and Firmicutes, and *amoA* genes related to *Nitrosomonas* sp. were found in RCS finished material suggesting a very active biotransformation of cellulose and nitrogenated compounds. Nevertheless, the absence of other bacterial phyla and mold sequences in the clone libraries indicated that the maturation process was incomplete. The RCS material must be further processed to achieve the level of maturity necessary to provide stable, optimal, and cured compost.

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