Analysis of the fungal, archaeal and bacteriophage diversity in the human distal gut

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The composition and role of bacteria in the human gut has been studied intensely and is a burgeoning field of scientific research. However, there is a relative lack of research on other microorganisms which compose our gut flora such as bacteriophage, archaea and fungi. The aim of our study was to begin to fill this gap. The archaeal, fungal and bacteriophage diversity in the gut was analyzed using a PCR-DGGE fingerprinting method on fecal samples from 3 healthy donors. These samples were inoculated into chemostats and the microbes were grown in continuous culture to model the interactions of our flora *in vitro*. Norepinephrine was also added to the chemostats to test the microbial community's reaction to stress. Here we report that, relative to bacteria, fungal and archaeal diversity in the gut is low. The archaeal populations seemed stable over time varied depending on the individual. Fungal populations were more variable over time and changes in the community structure were observed after the addition of norepinephrine. DNA sequence analysis confirmed the presence of fungal species that are not yet cultured, yet are residents of the gut. Species of Podophage can also be detected as residents of the gut based on sequence analysis. It is clear that there is a core set of archaeal and fungal species living as residents in the gut. Bacteriophage are also present but their ecological role and effect on the microbial community in the gut is unknown.

From the very first moments of birth, to our inevitable death, humans live in close association with the microbes that constantly surround us. There are a large number of bacteria, archaea, fungi and bacteriophage that make up what is referred to as our normal human flora or microbiota [1, 2]. Over the past few years, the diversity, composition and role of the bacterial part of our flora has been studied intensely and is a burgeoning field of scientific research [2]. However, the roles of fungal, archaeal and bacteriophage communities in the human gut are not as well studied. For example, many of the previous surveys of fungal species in the human gut used culture-dependent approaches, which result in a culture-bias since many gut organisms have been found to be refractory to anoxic culture [3]. Recently, there have been some studies, using culture-independent approaches, confirming that archaeal, fungal and bacteriophage populations are an important part of our gut flora [3, 4, 5]. Yet there have been a very limited number of studies; and the roles of archaeal, phage and fungal flora in human health and disease are not well known to date.

The microbial ecology of the human gastrointestinal tract (GI tract) has significant clinical interest due to the key role the GI tract plays in digestion, nutrition and maintenance of host physiology [6]. It is estimated that the GI tract contains 70% of all microbes in the human body, making it an extremely complex ecosystem harbouring more than 100

trillion microorganisms [7]. That is about 10 times the number of human cells per individual [1], making us a walking ecosystem of prokaryotic cells, eukaryotic cells and the viruses or bacteriophage that infect them. Maintenance of a healthy GI tract is important, and there are diseases associated with an imbalance or altered state of gut microbiota collectively called inflammatory bowel disease (IBD). These diseases affect millions of people worldwide, giving an even greater importance to understanding the function and role of our gut microbiota.

To study the microbiota of the GI tract, our lab uses continuous culture vessels called chemostats to model the conditions of our gut (Figure 1). In this system, microbes that are refractory to anoxic culture can be modelled *in vitro*.

Figure 1 Picture of 3 chemostat vessels running on continuous culture in order to model conditions seen in the human gut

There is evidence from our lab that the use of chemostats can support the growth of populations of bacterial species from feces. The central purpose of this study was to see if the vessels could also support growth of fungal, archaeal and bacteriophage species, and find out which species are represented in the GI tract flora of healthy donors. It is important to note that fecal samples do not represent the microbial communities found the entire GI tract, just the distal colon region. Preliminary studies show a high correlation between bacterial populations in a fecal sample when compared the population found in a chemostat run of up to 2 months. Chemostats also provide a good model to study the effects of exogenous stimuli on a complex microbial population. Recently, there has been a hypothesized link to how stress hormones may lead to a greater susceptibility of infection by our resident microbes [8]. To follow up on these studies, the stress hormone norepinephrine was added to the chemostat vessels, and changes were observed in bacterial community structure. Thus another reason for this study was to see what effects norepinephrine has on fungal, bacteriophage and archaeal community structure.

In this study, we assessed the bacteriophage, archaeal and fungal diversity and overall shape of communities in the intestines of healthy donors using PCR and denaturing gradient gel electrophoresis (DGGE) on the 16S rRNA, 18S rRNA genes and DNA polymerase genes of bacteriophage. Fungal DGG products were cut out, cloned and sequenced. The composition of archaeal and fungal species in each individual was then characterized by unweighted pair group method and arithmetic averages clustering algorithm (UPGMA) analysis of the DGGE banding patterns.

RESULTS

PCR of 18S rRNA, 16S rRNA and Phage DNA Polymerase gene

PCR was used on target genes to analyze the diversity of fungal, archaeal and bacteriophage species from fecal chemostat samples of healthy donors. For the first round of amplification, 18S and 16S rRNA amplicons were examined by agarose gel electrophoresis to the correct molecular size of ~1.6 kbp for 18S and ~950 bp for 16S rRNA genes (see Figures 2A and 3A). Target genes amplified well from the mixed sample of isolated chemostat DNA (which contained DNA from phage, fungal, archaeal and bacterial populations); however the amplified archaeal 16S rRNA genes were not always visible on the gel. The second round of amplification yielded ~1.5 kbp 18S rRNA amplicons and ~400 bp 16S rRNA amplicons visible on a 0.8% agarose gel (Figures 2B and 3B). The HECTOR and PARIS primers targeted the DNA polymerase gene of bacteriophage from the Podophage family, to amplify a ~600 bp region of the gene. Isolated phage DNA and a mixed sample of chemostat DNA were used as templates for PCR

(see Figure 4A and B respectively). When the reaction was electrophoresed on a 0.8% agarose gel, multiple products could be seen, indicating possible non-specific DNA amplification. Amplification of phage DNA direct from chemostat sample seemed to work well when using the PARIS primer set but not the HECTOR set (Figure 4A). Gradient PCR reaction with isolated phage DNA as a template, amplified 2 products of similar length (Figure 4B). Phage DNA was consistently amplified by the PARIS primer set, but with possible non-specific annealing of the primers.

Figure 2 PCR products, using primer pair s NSO/EF3 (A), and NS1/FR1-GC (B) from Costa *et al.*, 2005, yielding 1.6 kbp amplicons were electrophoresed on a 0.8% agarose gel. Each reaction mixture in lanes F1-F6 consisted of ~25 ng DNA template, DNA Pol. Buffer (NEB), 0.2 mM dNTPs, 0.2 μ M each primer and 5U *Taq* DNA polymerase. After 8 min of denaturation at 94°C, 30 cycles of 30 sec at 94°C, 45 sec at 48°C and 3 min at 72°C were performed on each reaction, followed by a 10 min extension step at 72°C. The resulting PCR product was approximately 1kb. Molecular size markers are as indicated on the MW lane.

Figure 3 PCR products using primer pairs 21F/958R (A), and Parch519-Arch915-GC (B) from Vissers *et al.*, 2009, yielding 400 bp amplicons (lanes A1-A6) were electrophoresed on an 0.8% agarose gel. Reaction mixtures analogous to fungal 18S PCR. After 2 min of denaturation at 94°C, 30 cycles of 1 min at 94°C, 30 sec at 57°C and 1 min at 72°C were performed on each reaction, followed by a 5 min extension step at 72°C. The positive control (lane +) indicated amplification of archaeal 16S rRNA to the correct molecular weight.

Figure 4 PCR products using primer sets HECTOR and PARIS from Breitbart *et al.*, 2004, on maxwell prepped DNA (A), and isolated phage DNA (B) were electrophoresed on an 0.8% agarose gel. In Gel A, inoculum samples from feces of a healthy donor were loaded in lanes P1 and P4, samples from day 36 in the chemostat were loaded in lanes P2 and P5 and samples from day 48 in the chemostat were loaded in lanes P3 and P6. Lanes P1-P5 in gel B were inoculums from donor 2. Each reaction mixture consisted of ~25ng DNA template, DNA Pol. Buffer (NEB), 0.2 mM dNTPs, 1 mM MgCl₂, 0.2 μ M each primer and 5U *Taq* DNA polymerase. After 5 min of denaturation at 94°C, 35 cycles of 1 min at 94°C, 1 min at 52°C (for A) and 1 min at 50-57°C (for B) and 1 min at 72°C were performed on each reaction, followed by a 10 min extension step at 72°C.

Fungal DGGE Pattern Analysis

The fungal community in the human distal gut was profiled by performing DGGE analysis on amplified 18S rRNA genes (Figure 5). This resulted in DGGE profiles of 3 healthy donors (D2, D3 and D4) from fecal sample inoculums and across different time points of a chemostat run (Figure 5A). There were 5 prominent products found in all 12 samples (Figure 5A) and some that were widely distributed and found in more than half the samples. A DGGE profile of the fungal population in fecal chemostat samples from a single healthy donor (D2) across different time points revealed a relatively stable fungal population (Figure 5B). Six prominent products were found in all 6 samples, but over time in the chemostat runs, the amount of products observed tended to increase over time.

To analyze the DGGE profiles of the fungal communities in the human distal gut more clearly, the profiles were subjected to UPGMA cluster analysis (Figure 5). The different lanes were compared numerically with respect to the entire spectra of products seen by calculating the Dice correlation coefficients (Table 1). When these coefficients equalled 100, this indicated that the banding pattern of the two respective lanes was equally distributed, and it could be said that they are 100% similar. The 12 fungal DGGE profiles across 3 different donors and different chemostat time points were between 43% and 94% similar with an average similarity value of 75%. Across a single

Figure 5 DGGE analysis of the fungal community in the human distal guts of healthy donors. Dendrograms constructed using Dice correlations and the UPGMA clustering method on the DGGE banding patterns from three different donors (D2, D3 and D4), from different chemostat vessels (V1, V3 V6) at different time points (A). Differences in the DGGE banding pattern over time from a single donor (D2) were also compared (B). Norepinephrine was added to the vessels on Day 37. The cluster analysis on DGGE gel C is shown by DGGE gel A. Three bands were removed for sequencing from D2 and D3 as shown by the arrows (C). DGGE gels contained a denaturing gradient of 18 to 38% denaturants and 6% acrylamide. Electrophoresis was performed in 1X TAE buffer at 58°C and voltage of 180V for 18 hours. Dendrograms were produced by SynGene software.

donor over different chemostat time points, the profiles were between 55% and 95% similar with an average similarity value of 73% (Figure 5B). The inoculum samples from donor 2 and 3 were clustered together, representing 3 of the 4 inoculum samples tested, and formed a separate branch of the clustering tree along with a sample from day 24 from donor 4 (Figure 5A). DGGE profiles of samples from donor 2 after 36 days in the chemostat were clustered together and samples from donor 2 after the addition of norepinephrine (day 48) were also clustered together. However, in the DGGE profiles of donor 2 in Figure 5B, clustering of certain time points was not observed although day 48 samples were both outliers to the rest.

Archaeal DGGE Pattern Analysis

The archaeal community in the human distal gut was also profiled using DGGE analysis, on the amplified 16S rRNA genes (Figure 6). This resulted in DGGE profiles of 2 healthy donors (D3 and D4) from fecal matter inoculums and across 2 different time points in the chemostat run from each donor. There were 7 prominent products found in all 6 samples, with little change in banding pattern over time or between donors (Figure 6). To back this up, the DGGE profiles were clustered using UPGMA and different lanes were compared numerically by calculating the Dice correlation coefficients analogous to the fungal clustering method. The archaeal DGGE profiles were between 83% and 96% similar, with an average similarity value of 91% across different donors and chemostat time points (Figure 6). The 3 DGGE profiles from each donor were also clustered together, and were approximately 92% similar for donor 3 and 88% similar for donor 4 (Figure 6).

Figure 6 Dendrogram was constructed using Dice correlations and the UPGMA clustering method on the DGGE banding patterns from two different donors (D3 and D4), from different chemostat vessels (V1 and V3) at different time points. DGGE gels contained a denaturing gradient of 30 to 55% denaturants and 6% acrylamide. Electrophoresis was performed in 1X TAE buffer at 60°C and voltage of 120V for 5 hours. Dendrograms were produced by SynGene software.

Cloning and Sequence analysis of Fungal DGGE Products

Three products from two different donors (D2 and D3) were excised from the DGGE gel (Figure 5C), for a total of 6 products. The 3 chosen products from each donor ~~ran~~ migrated the same length down the gel so theoretically represented the same species. When the transformations were plated on LB/kan/X-gal/IPTG plates, 3 of the 6 cloning reactions were successful represented by white colonies. The 3 ligations that worked were product 2 from D3 and D2 and product 3 from D2 (Figure 5C). These 3 plasmids with ligated DNA from the 18S rRNA region were successfully sequenced, and their sequence was analyzed using BLAST (Table 1). Product B from each donor (D2B and D3B) turned out to be from an uncultured fungal species, while product C from donor 2 (D2C) showed similarity to a gene found in *Bacteroides fragilis*.

Cloning and Sequence analysis of Phage PCR Products

PCR product of amplified phage from Lane P4 (Figure 4A) and Lane P6 (Figure 4B) were ligated successfully and transformed into *E. coli* DH5α cells as indicated by the presence of white colonies on the LB/kan/X-gal/IPTG plates. Three colonies were chosen from each plate (P4E1-E3 and P61-63) and the resulting plasmids were sent for sequencing. The sequencing reaction did not work for clones P62 and P63, however the rest of the clones were sequenced successfully (Table 1). All four sequences were analyzed using BLAST and all found to be most similar to an uncultured Podophage species from the previous Breitbart *et al.*, 2004 study (Table 1). The difference was these ligated products were only ~50 bp long, while the actual products in the database there were similar to were ~600 bp long. However most showed 100% sequence identity.

DISCUSSION

PCR and Primer Design

A dearth of information exists on the population structure and diversity of fungal, archaeal and bacteriophage species in the GI tract of humans, and the primary aim of this study was to use a PCR-DGGE method to address this concern. The primers used in this study were adopted from previously published studies, but in this case we use them on chemostat samples of fecal matter. In those studies, they observed a nested PCR approach worked well to amplify fungal and archaeal DNA templates which have a low population density in a mixed chemostat sample compared to bacterial and phage DNA. Results from this study confirm that, with good, clean amplicons observed in final amplification step of the expected sequence in each case (Figures 2 and 3). It is important to note that two previous studies [3, 4] reported that the protozoan *Blastocystis spp.* is a major component in the micro-eukaryotic community in out gut, and while the goal of this study is to assess fungal diversity, the 18S rRNA primers may pick up 18S genes from other micro-eukaryotes. The bacteriophage primers

were specifically for the Podophage family, as no gene target exists that is conserved through all phage species. The family Podophage are characterized as T7-like lytic phages, and a previous study demonstrated them to a significant part of the human intestinal virome [9].

Fungal Diversity in the Human Distal Gut

The human GI tract is a complex environment populated by bacterial, archaeal and fungal species [1]. The density of the microbial community in this environment is higher than in any other ecosystem on earth, where there exists upwards of approximately 1000 bacterial species [2, 7]. However based on our DGGE data, the diversity of fungal species in the human GI tract seems to be low compared to prokaryotes (Figure 5). There are 5 conserved products across the DGGE profiles and a total of approximately 15 distinct products (Figure 5C). This lack of diversity is backed up by two previous studies which showed only 3 distinct products per individual [3] and 8-10 products per individual [4]. In these studies, Scanlan *et al.* reported some similarity (43%) between the fungal profiles of different donors, while Nam *et al.*, reported 31% similarity to the fungal communities from different donors. Here we report that fungal flora between our 3 healthy donors is fairly similar, and major species seem stable over time in the chemostat. This indicated that the chemostat can retain fungal communities seen in fecal matter (inoculums sample). Cluster analysis reports an average of 75% similarity between all fungal profiles from different donors and time points and 73% similarity of the fungal profile from the same donor but across different time points (Figure 5A and B). This is much higher than previously reported. Fungal diversity seems to be as dependent on time point in the chemostat run, as much as which donor they we isolated from, although it is hard to make conclusions with a donor sample size of 3. Interestingly, fungal DGGE profiles from chemostat profiles in steady state (day 36) and after norepinephrine was added (day 48) were clustered together (Figure 5A). There are also visible changes in the banding patterns. While the dominant, constant species are still detectable, products appear and disappear indicating a possible shift in the fungal population. Most of the inoculums samples are clustered together and these samples seem to have less diversity of products compared to DGGE profiles from later in the run. More studies are needed on different healthy donors and possibly from donors with an IBD. Three DGGE products were cloned and sequenced successfully from donor 2 at day 36 (2 bands) and donor 3 at day 23 (Table 1). Two of the sequences turned out to be from an uncultured fungal species, while the other amplified the carbamoyl-phosphate synthase gene from *Bacillus fragilis* a common prokaryotic inhabitant of the GI tract. More cloning and sequence analysis is needed but this indicated that the primers may be binding non-specifically in some cases.

Table 1 Sequence data collected from DGGE Bands

Clone	Nearest Sequence	Identity	E-Value	Score	Kingdom
D2B	Uncultured fungus clone WD4-32 18S ribosomal RNA gene	100%	1e-178	634	Fungi
D3B	Carbamoyl-phosphate synthase from <i>Bacteroides fragilis</i>	98%	1e-110	410	Bacteria
D2C	Uncultured fungus isolate DGGE gel band 2 18S ribosomal RNA gene	100%	1e-139	510	Fungi
P4E1	Uncultured T7-like podovirus clone 49 DNA polymerase gene	100%	0.70	41	Bacterio phage
P4E2	Uncultured T7-like podovirus clone 41 DNA polymerase gene	100%	0.81	40	Bacterio phage
P4E3	Uncultured T7-like podovirus clone 36 DNA polymerase gene	100%	1.3	38	Bacterio phage
P61	Uncultured T7-like podovirus clone 44 DNA polymerase gene	100%	0.84	40	Bacterio phage

Archaeal Diversity in the Human Distal Gut

Previous studies indicate that each individual possesses a distinct population of archaea in their gut and with methanogens being the predominate archaeal species found in the human distal gut [4]. Our DGGE profiles show 6 distinct products conserved across 2 donors at different chemostat time points (Figure 6). Overall the archaeal community is stable over time, with greater than 88% similarity between the 3 DGGE profiles of each donor over time in the chemostat (Figure 6). The 3 DGGE profiles from donor 3 were clustered together and subsequently the DGGE profiles of donor 4 were all clustered together with an average similarity value of 91%. This supports the fact that each individual has its own unique set of archaeal flora, but with high similarity in species content. The chemostat vessels seem to retain the archaeal populations well from the original inoculums sample. Given this, the two post-inoculum chemostat samples (day 12 and 23 for donor 3 and day 15 and 24 for donor 4) were each clustered together (Figure 5). The inoculums samples were outliers, indicating slight some change over time. More DGGE analysis needs to be carried out to confirm this.

Podophage in the Human Distal Gut

Based on the sequencing data, the PARIS primers successfully amplified Podophage DNA. Also, they amplified similar sequences from an isolated phage DNA sample (P61) as well as from a mixed sample of bacterial, fungal and archaeal DNA. This is a good sign, however the length of the amplicons are much shorter than predicted by the primers and in the paper Breitbart *et al.*, 2004. This could be because the primers amplified many parts of this DNA polymerase gene sequence, yet only the shortest bands ligated into the vector since shorter DNA bands tend to ligate at higher efficiencies. Also the e-values are low, yet this also may be because of the short sequence relative to the hit found on BLAST, and shows the novelty of the sequence. Future studies will be directed towards separating the bacteriophage DNA sequences using DGGE to get an overall community profile. Subsequent products could then be sent for sequencing.

Conclusions and Future Directions

In summary, in this study, we began to characterize the overall archaeal, fungal and bacteriophage diversity in the human distal gut using a molecular PCR-DGGE method. Clustering analysis of the DGGE band patterns revealed that the archaeal communities among individuals are much more similar than the fungal community. Also, fungal diversity is low and seemingly more variable between hosts and depending on the time point of sample collection. There were definite changes in the population of fungal species in chemostat samples observed over time, and after addition of norepinephrine. Yet, there is some stability in our fungal diversity over time indicated by a presence of six identical products in each profile. Therefore there seems to be a core

set of archaeal and fungal species living as residents in our gut. It is also clear that there are bacteriophages who reside in our gut, and their effect on the global population of microbes there is relatively unknown. There are Podophage that can be found in our gut, and how many of them are there as well as the significance of their functioning role in the ecosystem still needs to be answered.

This study is still in its infancy, and more DGGE analysis needs to be done to confirm findings. All visible products will be sequenced to provide a better understanding of which species of fungus and archaea reside in our distal gut. The gut flora of IBD patients can also be studied to come to grips with how not only the bacteria are altered during these diseases but also our fungal, and archaeal flora along with bacteriophage.

METHODS**Chemostat Sample Collection and Extraction of DNA**

The DNA used in this study was isolated from different time points in chemostat runs with fecal samples from healthy donors in each chemostat vessel (Figure 1). All DNA samples were extracted by Julie McDonald using a bead-beating method, removing the inhibitors using a kit (Omega E.Z.N.A. stool kit), and adding the resulting supernatant to a Maxwell kit.

Phage DNA Isolation

Approximately 2 mL of a chemostat sample was collected, containing fecal matter from a healthy donor, and mixed with a 4X volume of Phage Buffer (100 mM MgCl₂, 50 mM NaCl, 50 mM Tris-HCl pH 7.5). The mixture was shaken vigorously to dislodge phage particles from particulate matter and filtered with a 0.2 µm Nitex filter. The filtrate was treated with 1 µL of 10U/µL DNase for 30 min at room temperature. The reaction mixture was heated for 20 min at 60°C to inhibit the enzyme. The phage particles were lysed and the DNA was extracted following a previously published protocol [10].

PCR Amplification

The extracted DNA from each chemostat sample was amplified by PCR with different primer sets for each target gene (see Table 2), followed by separation of the PCR products using Denaturing Gel Gradient Electrophoresis (DGGE). Nested PCR amplification was used to analyze the fungal and archaeal diversity. PCR amplification of archaeal 16S rRNA genes was performed using the arch21F-958R primer set [11] for the first amplification step, followed by the Parch519-915 [12] for the second amplification step, as described previously [13]. PCR amplification of fungal 18S rRNA genes was performed using the fungalaNSO-EF3 primer set [14] for the first amplification step and the fungalbNS1-FR1 primer set [15] for the second amplification step as described previously [16]. Each primer used in the second amplification step had a 40 bp GC clamp for DGGE

Table 2 PCR primers used in this study

	Name	Sequence (5'-3')
Fungal-specific primers 18S rRNA gene amplification	fungalaNSO fungalaEF3	TACCTGGTTGATCCTGCC TCCTCTAAATGACCAAGTTTG
	fungalbNS1 fungalbFR1	GTAGTCATATGCTTGTCTC *AICCATTCAATCGGTAIT
Archaeal-specific primers 16S rRNA gene amplification	21FArch 958RArch	TTCCGGTTGATCCYGCCGGA YCCGGCGTTGAMTCCAATT
	Parch519 Arch915	CAGCCGCCGCGGTAA **GTGCTCCCCGCCAATTCCT
Phage-specific primers DNA polymerase amplification	HECTORf HECTORr	GCAAGCAAGTTTACTGTGG CGAGAGATACACCAACGAA
	PARISf PARISr	ATACTACACGCTACTCTGG CACTGGCAAGAGGAGTTAT

*A 40bp long GC-clamp (5'-CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC G-3') was attached to the end of the primer fungalbFR1 to prevent the complete melting of the PCR product during DGGE.

**A 40bp long GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3') was attached to the end of the primer Arch915 to prevent the complete melting of the PCR product

analysis. For amplification of phage DNA, primers PARIS and HECTOR were designed to amplify a specific region of the phage DNA polymerase gene [9] from the polymerases of phage from the family of Podophage (see Table 2). The reaction mixtures for this amplification consisted of ~25 ng DNA template, DNA Pol. Buffer (NEB), 0.2 mM dNTPs, 1 mM MgCl₂, 0.2 µM of each primer and 5U *Taq* DNA polymerase. The reaction mixtures for the 18S and 16S rRNA PCR consisted of ~25 ng DNA template, DNA Pol. Buffer (NEB), 0.2 mM dNTPs, 0.2 µM of each primer and 5U *Taq* DNA polymerase. Fungal PCRs were cycled with 8 min of denaturation at 94°C, 30 cycles of 30 sec at 94°C, 45 sec at 48°C and 3 min at 72°C followed by a 10 min extension step at 72°C. Archaeal PCRs were cycled with 2 min of denaturation at 94°C, 30 cycles of 1 min at 94°C, 30 sec at 57°C and 1 min at 72°C, followed by a 5 min extension step at 72°C. Bacteriophage PCRs were cycled with 5 min of denaturation at 94°C, 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C, followed by a 10 min extension step at 72°C.

DGGE Analysis

Resulting PCR products were separated by DGGE using electrophoresis apparatus from Bio-Rad Laboratories, USA. A volume of 30 µL of each PCR product was loaded on a 6% acrylamide gel with a denaturing gradient of 18 to 38% for fungal DNA and 30 to 55% for archaeal DNA in a running

buffer of 1X Tris-acetate-EDTA. The denaturing agents used in the gel were urea and formamide. The DGGE was electrophoresed for 18 hours at 58°C, 180 V for fungal DNA and 5 hours at 60°C, 120 V for archaeal DNA. After electrophoresis, the gels were stained using ethidium bromide and photographed under UV transillumination. The DGGE profiles were analyzed using cluster analysis (Gene Tools by SynGene) and the lanes were normalized in order to compensate for differences in the migration distance of the DNA in each lane. Dendograms were computed (Gene Tools by SynGene) using the DICE similarity coefficient and unweighted pair group method and arithmetic averages clustering algorithm (UPGMA). The average similarity value was calculated by adding up all similarities between gels and dividing by the total.

DGGE Band Excision, Cloning and Sequence analysis of Fungal and Bacteriophage species

Sterile blades were used to excise products from the DGGE gels, which were then mixed with 50 µL of TE buffer and incubated overnight at 4°C. Resulting DNA was cloned into the pCR2.1 TOPO cloning vector using a TOPO cloning kit (Invitrogen, USA). PCR product for phage DNA was also cloned this way. The ligation reaction was transformed into *E. coli* DH5α cells and plated on LB/kanamycin/X-gal/IPTG plates. White colonies were selected and grown in 5 mL of LB broth. The plasmid was mini-prepped from *E. coli* using a plasmid mini-prep kit (Qiagen, USA) and eluted in 50 µL distilled water. Concentration of the plasmid DNA was checked using a nanospectrometer and sent to Bio-Basic for sequencing. The sequence was then analyzed using BLAST, on the NCBI database.

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