DETECTION OF AN INTRODUCED BACTERIAL CULTURE IN GREYWATER TREATMENT REACTORS

THESIS

Presented to the Graduate Council
of Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Master of SCIENCE

by

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San Marcos, Texas
May 2005

ACKNOWLEDGEMENTS

This work was supported by a grant from Sam Houston State University, Texas Research Institute for Environmental Studies, and the U.S. Air Force.

I need to thank my committee members for their support. Dr. Bob McLean, my committee chair, gave me incredible opportunities to excel both in my thesis work and in my role as his research assistant. Dr. Gary Aron and Dr. Patricia Richardson gave me helpful encouragement with the design and writing of this work. Dr. Dittmar Hahn gave me invaluable advice and expertise, particularly near the end of this project.

Several others in Dr. McLean's lab helped with this work either directly or indirectly. Mary Barnes, Dr. McLean's former research assistant, trained me in molecular techniques. Sue Reilly completed much of the bacterial sequencing and conceptual design of the project. Chris McGowin, an undergraduate summer worker, assisted Sue and me in technique development. Mubina Merchant served as a respected peer and helped in the overall presentation of my work.

Additionally, I need to thank my family, specifically: Elisabeth Welsh, Kevin Anderson, Melanie Welsh, Tom Welsh, Wilma Seuser and Stephanie Seuser. Without their support none of my work would have been possible.

This manuscript was submitted on April 6, 2005.

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ABSTRACT

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This research describes overall bacterial community structure within a greywater wastewater treatment reactor, and identifies one specific bacterial inoculum. Biological samples were removed from both the unattached (planktonic) and surface-adherent (biofilm) populations in several inoculated reactors. Nucleic acids were isolated and community structure investigated using molecular tools. Analysis of the 16S rRNA gene by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) revealed community profiles of the dominant bacterial species. Species and strain-specific primers targeting either the 16S rRNA gene or the intergenic spacer (ITS) between the 16S and 23S rRNA genes were developed to detect the inoculum strain and

to distinguish it from closely related organisms, present in the reactors. PCR-DGGE analyses indicated differences between planktonic and biofilm populations in the reactors. Sequence-based analyses from the DGGE gel identified seven community members, which may not have been detected by conventional culturing approaches. However, neither DGGE nor PCR with specific primers were able to detect the original inoculum strain in the reactors suggesting that it did not survive or was a minor, undetectable community member following inoculation.

INTRODUCTION

Wastewater reclamation is a serious concern worldwide, with increasing human populations and reduction in supplies of fresh water. From 1990 to 1995, global freshwater demand rose six fold which was more than twice the human population increase (6). The UN states that half of the human population worldwide lacks adequate sanitation (6). Bacterium caused diseases like shigellosis, cholera and typhoid have been linked directly to inadequate drinking water sources and poor wastewater sanitation (77). Inadequate sanitation and unsafe water sources are the chief cause of disease in developing countries and primarily affect children (6, 11). Diarrheal diseases attributable to contaminated water are estimated to cause the death of 2 million children worldwide each year (77). Additionally, children who were infected and suffered from diarrheal disease with *Giardia lambila* in infancy showed severe stunting and reduced cognitive function later in childhood (11). In order to prevent these tragedies, access to safe sources of drinking water and practical wastewater treatment options are a growing necessity as the world population grows.

Wastewater treatment is one key to addressing the crisis of our dwindling global fresh water resources (6). Households are the primary generators of wastewater in most communities (12). Household wastewater is generally separated into two categories: blackwater originating from the toilets and greywater from all other sources (12). Household greywater kept separate from blackwater can be re-used for irrigation or

shunted into blackwater streams and used for toilet flushing (29). However, the daily pollutant loads in greywater can be dangerously high in bacterial biomass and chemical oxygen demand (COD), ammonia, phosphate, anionic detergents, chlorides, boron and fecal coliforms (29). Greywater must be treated to reduce the bacterial biomass and total suspended solids (TSS) and thus the carbonaceous biochemical oxygen demand (CBOD) if it is to be re-claimed as drinking water (29).

Currently there are several accepted methods for treating greywater. Greywater can be treated by vertical flow through a constructed wetland, but requires additional treatment to remove enteric pathogens, and thus may not be practical for large scale use (52). Small scale, single household wastewater treatment typically uses an anaerobic holding tank for storage, followed by release into wetlands which depends on nutrient uptake by aquatic plants (34). These small scale reactors are expensive to install and maintain for individual households and its treatment efficiency varies seasonally (34). These traditional modes of wastewater treatment are not practical for smaller communities, which lack the infrastructure to transport wastewater to a large centralized location, but have enough community members to prevent the practical treatment of each household's wastewater in individual septic tanks and wetlands. The technology to create a treatment facility for smaller communities that is both cost-effective and consistent in effluent quality is in development.

Collaborators at Sam Houston State University (SHSU), Texas Research Institute for Environmental Studies (TRIES), and the US Air Force are developing a low-cost, portable greywater treatment reactor to allow an Air Force regiment to treat their greywater on site. Additionally, the reactor will be used in unincorporated, low-income

communities referred to as "*Colonias*" in South Texas counties bordering Mexico (8). Half of those living in *Colonias* do not have wastewater treatment facilities of any kind and these communities have a high prevalence of water-borne disease (8). Briefly, the greywater reactor is a rotating biological contactor (RBC) consisting of four aerated tanks (65). The tanks have a plastic mesh material suspended on a rotating, vertical shaft completely submerged in greywater for biofilm community development (12). Sample is retained in the reactor for about 10.5 hours. The mechanisms of RBCs are not fully understood, but good effluent quality depends on aeration, biofilm maintenance, nutrient load, and participation of planktonic biomass (65). The bioreactor developed by SHSU shows a consistent reduction in TSS and BOD and seems to be operating well.

Performance of the greywater reactor may be directed by the bioaugmentation of the indigenous bacterial community. The first in the series of four tanks in the SHSU greywater bioreactor is bioaugmented with an inoculum of seven specific organisms (Table 1). Bioaugmentation involves the addition of known or unknown consortia of bacteria to a wastewater treatment reactor to aid in reactor performance and pollutant degradation (12). Addition of bacterial consortia to the complex microbial community within a wastewater treatment reactor has had mixed success in terms of consistent pollutant degradation and inoculum survivability (36, 75). It is unknown whether these seven inoculum species survive and directly contribute to the overall reactor performance. Boon *et al.*, 2000, reported that inoculum organisms present in an activated sludge reactor changed the microbial community structure and resulted in reduced pollutants, but the effect was short lived and the inoculum died off (13). However, Van der Gast *et al.*, 2004, showed that inoculum organisms can survive and thrive in waste

metal-working fluid degradation without the need for repeated inoculations (80). Bioaugmentation with bacteria well adapted to wastewater reactor dynamics is crucial for their survivability and reduction of their target pollutant *in situ* (14, 54). Additionally, bioaugmentation with specific organisms known to be beneficial in treatment reactors results in better reactor performance than bioaugmentation with an unknown, uncharacterized consortia of bacteria like those present in activated sludge (80). However, reports of inoculum species survival or demise in wastewater reactors have been solely based on planktonic bacterial community assessments. Chao and Ramsdell, 1985, showed that biofilm community structure can impact the planktonic bacterial community and act as "seed" for the planktonic community members in a laboratory chemostat (16). Therefore, inoculum organisms in a wastewater reactor may survive in biofilm bacterial communities and be undetectable in planktonic communities.

Detection of inoculum species within a wastewater reactor implies that it is a functional member of the community and contributes to the performance of the reactor overall (1, 80). The primary modes of detection of specific bacteria in a complex environment are traditional culturing techniques and more recently developed molecular-based techniques. Van der Gast *et al.*, 2004, have focused on following four inoculum organisms through a bioreactor treating metal working fluid (MWF). The greatest reduction in MWF toxins occurred when the bacterial consortium populations were most active and most culturable (80). However, these four organisms were easily culturable on antibiotic media and this type of culture-based identification cannot be applied to inoculum organisms that do not have genetically altered antibiotic resistance or some other phenotypic characteristic (e.g. pigmentation). Also, environmental bacteria have

been known to enter a viable, but non-culturable state in which they are active, but not detectable using traditional culture based techniques (56). Molecular-based detection methods also have limitations, but are the primary tools currently used to detect bacteria in complex environments since they are unaffected by the limitations of culturability. Specifically, molecular tools have been used to monitor wastewater treatment reactor microbial community dynamics to better understand biological treatment processes (14, 74, 83).

Denaturing gradient gel electrophoresis (DGGE) is an excellent molecular tool for examining bacteria in mixed communities particularly where traditional culturing techniques are not possible (1, 59). DGGE has been used to examine bacterial communities in a variety of samples: rhizosphere, lakes, and biofilms in water reclamation (22, 23, 42). Total bacterial DNA from an environmental sample is extracted and fragments amplified using PCR typically targeting the 16S small ribosomal subunit rRNA gene using specially modified primers (57). This mixed template PCR product is electrophoresed through an acrylamide gel with an increasing gradient of formamide and urea. The mixed template PCR product is separated into individual bands in the DGGE gel by guanine and cytosine (GC) content and results in a distinct banding pattern for each sample. Excising and sequencing of bands from DGGE gels can identify prominent bacteria in the sample (14, 21). However, DGGE, like other PCR-based molecular tools, is not quantitative and can not be directly related to population size of specific bacteria in the environment (22, 26, 50). Also, sensitivity has been a drawback in interpreting the data generated from a community profile. DGGE has been shown to only detect bacteria that are at least 1% of the population and can not detect minor populations of bacteria

(58, 59, 84). To detect these minor populations, other molecular tools would need to be used.

One way to target one specific organism in a complex environment that may be a minor component of the bacterial population is the use of PCR with species-specific primers. Typically species-specific primers target a hypervariable region of the 16S rRNA gene, as this gene is found in all free-living prokaryotes. However, 16S rRNA gene segments usually lack specificity to distinguish between closely related species and strains of bacteria (17, 42). An alternative is to create a specific primer to target the intergenic spacer region (ITS) between the 16S and 23S rRNA genes on the bacterial chromosome (43). The ITS regions vary in length and sequence at the level of genus and species and is typically non-coding DNA, but may contain various transfer RNA (tRNA) sequences (43). The ITS region has been used with other molecular tools like restriction fragment length polymorphisms (RFLP) and DGGE to distinguish between strains of bacteria with good reproducibility (35, 42). Species-specific primers can detect their target even in very low numbers in the sample, however, the actual numbers of organisms in the environment can not be quantitated (81, 82).

Quantification can only be achieved with advanced PCR applications like quantitative PCR (qPCR) or with fluorescent *in situ* hybridization (FISH) (4). The later technique allows bacteria to labeled with a fluorescent probe and visualized directly *in situ*. Typically, the probe targets the 16S small subunit of the bacterial ribosome. Ribosomal RNA (rRNA) is ubiquitous in all bacteria and most active bacteria have an estimated 10,000 functioning ribosomes (3). Regions of high and low variability on the ribosome allow for the design of oligonucleotide probes that can target a range of bacteria

of interest from the subspecies to the phylum (4, 20). Cells are visualized using epi-fluorescent microscopy. FISH can be the culmination of DGGE, sequencing, and species-specific primer techniques to elucidate the presence and abundance of a target organism in the environment (79, 87).

This research will use DGGE, sequencing, species-specific primers and FISH to detect the inoculum species in the greywater treatment reactor at various sampling points. Detection of the inoculum species within the reactor implies that it is a functional member of the community and directly contributes to the overall performance of the reactor. Thus, detection of augmented organisms will demonstrate that bioaugmentation of the reactor with these organisms is important to the consistent effluent quality of the reactor.

MATERIALS AND METHODS

Overall Strategy

This research described overall bacterial community structure within the reactor and attempted to detect one specific inoculum species in a functional greywater treatment reactor with species and strain level specificity for both biofilm and planktonic bacterial communities. DGGE was used to assess overall bacterial populations with in the reactor with the emphasis on detection of any of the seven inoculum strains (Table 1). Inoculum species detection techniques will be developed for one organism with the potential to apply the detection strategy to the six other inoculum organisms in the SHSU greywater treatment reactors. The organism that was chosen for this research is *Agrobacterium sp.* ATCC 31529 (Strain 31529). The presence of Strain 31529 in the greywater samples was assessed using published primers and primers created for this assay. FISH was utilized to visualize Strain 31529 in the samples.

Sampling

The greywater samples with planktonic bacteria used in this assay were frozen immediately following sampling by collaborators at SHSU and stored at –20 ^{o}C. One-liter plastic carboys of planktonic samples were shipped frozen to Texas State University and remained at –20 ^{o}C. Collaborators at SHSU also obtained pieces of plastic mesh suspended in the reactor tanks harboring the biofilm community. We are unsure of sterility during collection and storage. The plastic mesh was placed in plastic bags and stored at –20 ^{o}C. Bags that contained biofilm organisms on plastic mesh were also

shipped to Texas State University and stored at –20 °C. Table 2 describes planktonic and biofilm samples used in this analysis.

Six greywater samples were thawed at 4 °C over a 1-2 day period to assess the planktonic bacterial community. Samples in one-liter carboys were shaken for 30 sec. Then 50 mL of greywater was aseptically moved to a 50 mL conical centrifuge tube. The effect of sample size on Strain 31529 detection, described below, was investigated before deciding on 50mL sample sizes. Aliquots were removed from this 50 mL sample for a dilution series in sterile water for heterotrophic plate counts and into sterile microfuge tubes for fixation for FISH. Heterotrophic plates counts were performed as described (49) and traditionally provide a indicator test for the number of bacteria present in wastewater samples (12).

The six corresponding plastic mesh fragments harboring biofilm communities were thawed at 4 °C, cut into 1.2-gram sizes and placed in a 250 mL beaker with 50 mL sterile water. Next, the glass beakers were subjected to sonication for 10 min to disrupt the biofilm and suspend the organisms in the water surrounding the mesh (55). The 50 mL of biofilm suspension was moved to a 50 mL conical centrifuge tube. Aliquots were removed from this 50 mL sample into sterile microfuge tubes for fixation for FISH.

The 50 mL conical tubes containing biofilm and planktonic samples were centrifuged in a fixed angle rotor at 8000 rpm for 10 min, the supernatant decanted and the pellet dissolved in 1 mL sterile TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.2). DNA extractions were performed immediately on 1 mL of concentrated planktonic samples and 0.25 mL of concentrated biofilm samples using the DNeasy ®Tissue Kit Gram positive bacterial DNA protocol (Qiagen Sciences, Valencia, CA).

Overall Community Analysis

DGGE conditions were optimized for these greywater samples to generate the best community profiles possible. PCR for the overall community DGGE analysis using universal bacterial primers GC357 F and 907 R targeted the V3-V6 regions of the 16S rRNA gene (85). Table 3 provides a list of primers used in this assay. All the primers used in this assay were purchased from IDT (Chicago, IL). The addition of a 40 base pair GC clamp to the 357F primer prevents the duplex PCR product from completely denaturing in the DGGE gel (59). To reduce PCR biases from varying matches with these universal primers, a low annealing temperature of 46° C was used in PCR (39). To reduce biases in multi-template PCR caused by G-C rich templates amplifying with higher efficiency, the number of thermocyler cycles was reduced to 30 (63). A final extension at 72° C for 30 minutes was added to the thermocycler program for PCR to reduce artifactual double bands in the DGGE gel (41). Table 4 lists all of the thermocycler parameters used in this assay. The PCR reaction was completed using 2.5 units of Ampli-Taq Gold polymerase (Applied Biosystems, Foster City, CA), 1 X PCR Buffer, 3.0 mM $MgCl_2$, 200μM each dNTP blend, 0.5μM primer concentrations and 2.5% template concentration. PCR product was concentrated in an isopropanol precipitation reaction to reduce the volume of product loaded into each well of the DGGE gel (70). Presence of PCR product was confirmed using an ethidium bromide stained agarose gel. The amount of PCR product was estimated by comparison with the Low Mass DNA Ladder (Invitrogen, Carlsbad, CA).

DGGE was performed on a DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA). Gels contained 6% polyacrylamide (ratio of acrylamide to

bisacrylamide, 37.5:1). To determine the ideal denaturing gradient for our samples a perpendicular gel was run with a 0-100% denaturing gradient perpendicular to the electrophoretic current according to the manufacturers specifications (Bio-Rad) in 0.5 X TAE buffer (Tris Base 0.02M, glacial acetic acid 0.01M, EDTA 0.5mM, pH 8.0) where 100% denaturant contains 7M urea and 40% formamide (59). The time and voltage for the parallel DGGE gels needed to be optimized for these samples. Longer electrophoresis times have been shown to result in lower number of bands and loss of the denaturant into the buffer (72). To determine the overall effect of longer electrophoresis times and lower voltage, gels with similar samples were run at 70 volts for 18 hrs or at 200 volts for 12 hrs (22, 44). To determine the ideal electrophoresis time for these greywater samples a DGGE gel with 40-60% denaturant was loaded with a mixture of inoculum species or greywater samples every hour for 8 hrs (21, 59, 72). Fine tuning of the denaturant concentration was necessary to determine the best gradient for the greywater samples (21). Gels of 20-40%, 30-50% and 40-60% denaturant were loaded with similar greywater samples and compared to determine the best denaturant concentration.

Following these optimization steps, the ideal DGGE parameters were utilized for all the planktonic and biofilm greywater samples. Approximately 500-800 ng of concentrated planktonic or biofilm PCR product was loaded into each well (9). Gels electrophoresed at 60 ^{o} C and 180 volts for 7 hrs with a denaturant concentration of 30-50% gradient. Gels were stained with ethidium bromide (0.5μg/ml) in 0.5 X TAE for 30 min, destained in water, and visualized on a UV transilluminator. Final images were adjusted in Adobe Photoshop 7.0.1 ®.

What appeared to be individual bands in this community analysis DGGE gel were poked with a sterile pipette tips and soaked in 10µl sterile water for 2 hrs at 4 °C (42). This was used as template in a PCR reaction utilizing the 357 F primer with no GC clamp and the same 907R primer. This reaction was used for sequence analysis using the Big Dye Terminator v. 3.1 Kit (Applied Biosystems) and an ABI 377 gene sequencer according to the manufactures specifications. All sequences from the DGGE were analyzed both through CHIMERA_CHECK version 2.7 from the RDP-II v. 8.1 (19) and Bellerophon (38) to detect possible chimeric sequences.

To determine if the inoculum species listed in Table 1 were dominant in the greywater samples, DGGE banding patterns of each pure culture was compared to that of the community analysis gel. Pure cultures of each of the inoculum species were grown per ATCC recommendations. DNA extraction, PCR with DGGE primers and isopropanol precipitation was performed as stated above. These samples were run on the DGGE gel with identical parameters as those optimized for the greywater samples. DGGE melting profiles of each individual inoculum strain were compared with the DGGE community profile to look for similar band migrations and possible matches (57, 84).

Characterization of Strain 31529

Shimiza *et al.* patented *Agrobacterium sp.* ATCC 31529 on October 26, 1982, as a microorganism for treating wastewater, US Patent # 4,356,268. The function of Strain 31529 in our greywater reactors is unknown. To better characterize Strain 31529 and begin to describe its possible role in the greywater reactor, we chose to sequence its 16S small ribosomal subunit rRNA gene. Pure cultures of Strain 31529 were grown in nutrient broth (NB) (Difco, Sparks, MD) at 30 °C and DNA extracted using the DNeasy

®Tissue Kit as stated above. Four primers were used to obtain 16S rRNA gene sequence for Strain 31529: 27F, 355F, 1096R, and 1492H2R (Table 3). 16S rRNA gene sequences were also obtained for the six other inoculum species to aid in subsequent characterization. Pure cultures of each of the six other inoculum species were grown per ATCC recommendations and partial 16S rRNA gene sequence obtained as stated for Strain 31529.

In order to distinguish Strain 31529 from those in the wastewater that may be closely related and share similar 16S sequences, we also chose to sequence the ITS region. Strain 31529 DNA could not be amplified in PCR using the published universal ITS region primers G1 and L1 (43). Based on the presumptive identification of Strain 31529 from the 16S rRNA gene sequence, an alignment of 23S rRNA gene sequences of related organisms available in Genbank (2) was created using Sequencher version 4.2.2 (7, 70). Based on regions of homology in these sequences, new primers, ITS2F and ITS2R, were designed as described (70). These primers were used to amplify the ITS region and a large portion of the 23S rRNA gene of Strain 31529. Approximately 500 base pairs of sequence was obtained as stated above for Strain 31529 including the ITS region and beginning of the 23S rRNA gene.

Basic biochemical utilization and microscopic morphology were determined for Strain 31529 (47, 51). Thirteen biochemical tests were performed on Strain 31529 and compared with 3 other closely related species to attempt to confirm the presumptive identification made by 16S rRNA gene sequencing. Additionally, phase contrast microscopy helped to identify Strain 31529. Based on the presumptive 16S rRNA gene identification, several medias were tried to better cultivate this organism: proteose

peptone yeast extract, tryptone glucose extract agar and R_2A agar (Difco, Sparks, MD) (30, 47). The best growth, however, was obtained in nutrient broth (NB) at 30 °C as per ATCC recommendations for this organism, *Agrobacterium sp.* ATCC 31529.

Strain 31529 PCR Detection

Presence of Strain 31529 in greywater was assessed using two different sets of species specific primers. The first primer set was selected from the literature and based on the presumptive identification made following sequencing the 16S rRNA gene. The forward primer ZAL F was designed for detection of a specific wastewater bacterium and shared 100% homology to our organism (67). It targeted a portion of the 16S rRNA gene corresponding to sequence position 647 to 664 on the *Escherichia coli* 16S rRNA gene (67). ZAL F and 907R generated PCR product of about 250 base pairs (Table 3). Presence or absence of product was confirmed by electrophoresis in a 1.25% agarose gel.

The second primer set served as a confirmatory identification of Strain 31529. A new reverse primer was generated based on the sequence generated from the forward ITS region, DGGE Agro ITSR primer (Table 3). The DGGE Agro ITS R is located on positions 199-214 of the ITS sequence obtained. The primer set, ITS2F and DGGE Agro ITS R, was designed to yield a PCR product of about 500 base pairs, the limit of PCR fragment resolution on the DGGE (60). A GC clamp was added to the 5' end of this primer with the intension of running this ITS PCR product on a DGGE to confirm that Strain 31529 was amplified (61). Presence or absence of PCR product with these primers was confirmed by electrophoresis in a 1.25% agarose gel.

FISH

Fluorescent *in situ* hybridization (FISH) was attempted to visualize and quantitate the presence of Strain 31529 in the samples that were positive in the species-specific PCR

assay. Aliquots of thawed samples for FISH were immediately fixed in a 4% paraformaldehyde solution as described (3). Each sample was prepared in eight well glass microscope slides as described (3). 10µl of the fixed cell suspension was added to a gelatin coated slide and spread around gently to fill the 5mm diameter well. The oligonucleotide probes used in this assay were purchased from IDT (Chicago, IL). The *Zoogloea ramigera* probe, ZAL, targets a portion of the 16S rRNA corresponding to sequence position 647 to 664 on the *E. coli* 16S rRNA gene and was labeled on its 5' end with the indocarbocyanine fluorochrome, Cy3 (67). Table 3 lists probe sequence details. The hybridization was performed as described (3) with a 25ng/µl concentration of probe, 35% formamide concentration, and 1 hr hybridization incubation at 46° C (67). To ensure that the signal received from the probe was due to probe binding and not autofluorescence of the sample, the cells were also stained with the DNA intercalating dye, 4', 6-diamidino-2 phenylindole (DAPI). To ensure that the cells were permeable and could be probed the samples were hybridized to the eubacterial probe EUB 338 with a Cy-3 label in duplicate wells (3). Severe autofluorescence in the sample necessitated pretreatment with a 1 X Blocking reagent (Roche, Indianapolis, IN) for 30 min followed by hybridization as described (3). To determine the ideal formamide concentration and hybridization temperature, the hybridization procedure was optimized for these samples. Optimization of the hybridization reaction included altering the published hybridization conditions for the ZAL probe (67). The hybridization temperature was reduced from 46° C to 37° C. The formamide concentration within the hybridization buffer was decreased from 35% to 10%, 15%, or 20% (53). Slides were viewed with an epi-fluorescent microscope Olympus CKX41 Inverted Microscope with MagnaFire SP digital camera

and filter sets UV-3A (EX330-380, DM400, BA435-485 for DAPI) and HQ-Cy3 (G535/50, FT565, BP610/75 for Cy3).

Phylogenetic Analysis

The 16S rRNA gene sequences of Strain 31529 were complied in Sequencher 4.2.2 into one consensus sequence (7). This sequence was compiled into one 'contig' with sequences of close relatives downloaded from Genbank also using Sequencher (2). *E. coli* was set as the reference sequence in Sequencher and the outgroup for all analyses in PAUP*4.0b10 (78).

Bacterial community sequence information that was retrieved from the DGGE gel was analyzed with 16S rRNA gene information generated for all of the inoculum organisms. All sequence data was aligned using Sequencher with *E. coli* as the reference sequence. No outgroup was specified for this data set in PAUP* because the sequences were so diverse. To clarify the possible relatedness of inoculum and community members phylogenetic data were compared in an analysis solely focusing on those relationships, separate from the overall community/inoculum phylogenetic analysis.

All data sets were analyzed with heuristic, neighbor joining (NJ) and maximum likelihood (ML) methods in PAUP* (78). Modeltest 3.01 and PAUP*was used to determine the best model of sequence evolution that fit the dataset (64, 78). Modeltest recommendations from both the Akaike Information Criterion (AIC) and the high frequency Likelihood Ratio Test (hfLRT) were used to determine the best model of sequence evolution that fit each dataset. NJ settings revealed the minimum evolution tree topology using the maximum likelihood assumptions for distance correction specified by Modeltest (68). All other settings within the Distance windows were set at factory settings and not changed. The heuristic parsimony settings included: 10000 random

addition sequence reps, start from random trees, gaps treated as "missing", accelerated transformation character-state optimization, tree-bisection-reconnection (TBR) branch swapping algorithm, original 'MaxTrees' setting= 100 (will be auto increased by 100), branches collapsed if MPR-sets of incident nodes are identical, 'MulTrees' not in effect, and topology constraints are not enforced. Equally parsimonious trees were printed with branch lengths from the 'Describe trees' menu in PAUP*. The initial tree that served as the basis for the ML tree in PAUP* was the parsimony heuristic tree or consensus tree. The recommendations from Modeltest as stated above were used as the parameters to generate a ML tree topology. All settings in PAUP* were similar to those of the heuristic search stated above except no addition sequence replicates were used. Instead the ML search was based on the parsimony tree in memory.

Confidence in topologies created in these analyses was gauged using bootstrap re-sampling methods (27). The bootstrap test in PAUP* included 10000 replications and a full heuristic search. The analysis included the following settings: starting trees obtained via stepwise addition, 10 random sequence addition replicates, TBR, swap on best trees only and 'MulTrees' not in effect. The bootstrap replicates for the ML tree varied: 100 replications and a full heuristic search, 1 random sequence addition replicate, and TBR swapping.

RESULTS

Sampling

DNA extraction from 50ml of sample yielded the greatest amount of PCR product. Figure 1 shows the effect of sample size on bacterial detection in wastewater. Two planktonic greywater samples were chosen. Duplicate samples were removed from the 1 liter carboys in 1.5ml, 15ml, and 50ml increments. Figure 1 clearly shows that for one of the two samples an increase in sample size showed an increase in PCR product generated. Table 5 lists heterotrophic plate counts of the planktonic samples used.

Overall Community Analysis

DGGE analysis of these samples required a great deal of optimization. A perpendicular DGGE gel was loaded with a mixture of the 7-inoculum organisms and 2 planktonic greywater samples (Fig. 2). The point in the gel where the bands seem to rise asymptotically indicates the ideal melting range for that sample (60). The melting pattern in this gel reveals that a denaturant concentration of 40-75% should be adequate to resolve the organisms present. However, when applied to the greywater samples without the addition of the inoculum species the 40-75% gradient did not result in any clear banding, so the gradient was reduced to 40-60%.

The effect of low electrophoresis times versus high times was clearly illustrated in Figure 3. Longer electrophoresis time resulted in fewer bands and more smearing of the bands present. Figure 4 illustrates the melting properties of a greywater samples and a mix of inoculum species with time. Samples were loaded each hour for 8 hrs. This gel

shows that the inoculum species and greywater samples stop migrating after 7 hrs. Additionally, this gel illustrates that the denaturing gradient for these samples is not correct. The samples should melt further down the gel, not in the middle of the gel. Also, there is little resolution of individual bands.

Next, the denaturing gradient concentration was optimized. Similar biofilm and planktonic samples were prepared as stated above and were loaded and run on gels with differing gradients: 20-40%, 30-50%, and 40-60% at 180 volts for 7 hrs (Fig. 5). The 30-50% gradient clearly is the best for these samples. All subsequent gels were run with this gradient.

All 12 biofilm and planktonic samples were loaded and run using a 30-50% gradient at 180 volts for 7 hrs. Figure 6 shows these greywater community profiles. Table 3 lists details on the biofilm and planktonic samples used. Qualitatively, planktonic samples 52 and 62 show very similar banding patterns and thus community profiles. Biofilm samples 52B and 62B also show banding similarity. The planktonic samples 52 and 62 and biofilm samples 52B and 62B are clearly distinct from one another. Samples 12 and 12B do not seem to have enough PCR product to form good banding patterns. Samples 53 and 63 display some similar banding patterns, though they are not as well matched as 52 and 62. Also, 53B and 63B are similar, but not as much as 52B and 62B. The banding patterns in 13 and 13B are very different from any of the other samples. There are faint double bands labeled A and B that appear in most of the samples. Band C also appears to be in more than one kind of sample.

DGGE bands were poked immediately to prepare for sequencing. Twenty four bands were poked. Chromatograms viewed as output from the ABI sequencer in

Sequencher revealed messy, low peaks for many samples or revealed multiple overlapping high peaks. Good quality sequence data was only obtained from 7 bands (Fig. 7).

Sequence data obtained from the DGGE gel was analyzed to provide presumptive identification of bacterial community members in the greywater samples. Table 6 lists the 16S rRNA gene sequences obtained for each of the poked DGGE bands that yielded sequence information. Both CHIMERA_CHECK and Bellerophon suggest that DGGE band # 1 is a chimeric sequence, composed of sequence data for more than one organism (19, 38). Table 7 lists the comparative identification results from Genbank of each of the 6 DGGE bands that yielded bacterial sequences (2). Sequence data obtained from the DGGE gel was also complied with all the inoculum species 16S rRNA gene sequences into one phylogenetic tree. The parsimony heuristic analysis revealed the best-supported tree topology for this dataset. The parsimony analysis resulted in three consensus trees with identical topology, but slightly varying steps between the #2 and #5 clade. Figure 8 is one of these parsimony trees. This tree reveals that none of the inoculum strains are closely related to the sequences from the DGGE bands with the exception of the *P. stutzeri-P.putida*-#2-#5 clade. The close relationship of these sequences warrants a more specific analysis.

The closely related inoculum and DGGE organisms were compared using similar analyses as the other datasets. A 'contig' was created in Sequencher with the 4 sequences of our target organisms. The top 2 or 3 sequence results for each of these 4 were downloaded from Genbank and added to the 'contig' (2). #2 and #5 resulted in the exact same sequence identification using Genbank (Table 7). The ML heuristic analysis

revealed the best supported tree topology for this dataset (Fig. 9). *Pseudomonas aeruginosa* was used as the outgroup in PAUP*. #2 and #5 are clearly in their own clade with 92% bootstrap support to indicate that delineation. The inoculum species *P. stutzeri* and *P. putida* form their own monophyletic groups. #2 and #5 are not inoculum species.

To determine whether the inoculum species are dominant in the reactor, DGGE with the inoculum species was run concurrently with the community profile DGGE samples to ensure identical electrophoresis conditions (Fig. 10). Comparing DGGE profiles with the community profile can presumptively identify no inoculum species in the greywater reactor samples.

Characterization of Strain 31529

Most of the 16S rRNA gene sequence of Strain 31529 can be found in Table 8. Phylogenetic analysis has revealed that this organism is most closely related to *Zoogloea ramigera,* a Gram-negative rod commonly found in wastewater (67). Figure 11 displays a phylogenetic tree of Strain 31529 and its close relatives and other proteobacteria. ML heuristic analyses revealed the best supported tree topology. Also, 353 base pairs of sequence data was obtained for the ITS region of Strain 31529 and used for primer design (Table 9).

Basic biochemical tests performed on Strain 31529 and the reported results of *Z. ramigera* ATCC 19623, *A. tumefaciens* Biovar 1, and *Z. ramigera* T ATCC 19544 type strain are listed in Table 10 (47). Of the thirteen biochemical tests performed, Strain 31529 results varied from each of the other bacteria listed by 3 or 4 tests (47). These biochemical tests are not useful in determining or confirming the identity of Strain 31529. One defining characteristic of *Z. ramigera* is flocculent growth patterns in broth media

(30, 47). Figure 12 is a picture of flocs of Strain 31529 in nutrient broth culture. The most recognized feature of *Z. ramigera* in wastewater is the formation of zoogloea, thick masses of cells with finger-like projections emanating from the floc (25, 30). Figure 13 features a phase contrast micrograph of finger-like projections from Strain 31529 flocs.

Strain 31529 PCR Detection

The 2 primer sets to detect Strain 31529 yielded contrasting results. The primer ZAL F designed to target *Z. ramigera* in wastewater yielded PCR product in all greywater samples (Fig. 14). Lane 14-16 yielded good product, indicating that the Strain 31529 does generate PCR product with these samples and if it were in the greywater samples it would also generate product. Lane 17 did not generate product because *E. coli* does not share homology with the ZAL F primer.

The ITS region primer set did not yield product for the greywater samples (Fig. 15). The Strain 31529 and the greywater samples spiked with Strain 31529 lanes did yield product (Lanes 14-16) indicating that Strain 31529 does share homology with the ITSR primer and could be detected if present in the greywater samples.

FISH

No data could be generated from FISH that would allow quantitation of the Strain 31529 if it had been detected in the greywater samples. Pretreatment of the samples with 1 X Blocking buffer effectively reduced the severe autofluorescence of the greywater samples. DAPI staining revealed many cells and worked well in all the samples. Hybridization with EUB 338 yielded good fluorescence in both greywater samples and in pure cultures of Strain 31529. Successful EUB hybridization indicated that the Strain 31529 cells in the pure culture samples were permeable and could be hybridized.

However, no hybridization signal was received from pure culture preparations of Strain 31529 with the *Z. ramigera* probe, ZAL.

DISCUSSION

Overall Community Analysis

The community profile illustrated by DGGE and its subsequent analysis illustrates both beneficial data that can be generated using DGGE and the serious drawbacks associated with this technique (Fig. 6). Inferences about overall community structure in the greywater reactor can be gauged based on the types of samples in the DGGE gel (Table 2). Samples 52-12B are from tank 2 in the greywater reactor series. Samples 53-13B are from tank 3 in the reactor. Samples 52, 52B, 53, and 53B were all collected at the same time. Eight hours later the samples designated 6 were taken. Samples 12, 12B, 13, and 13B were taken about one week after the 6 samples were taken. Samples 52 and 62 and 53 and 63 show similar banding patterns indicating that the bacterial community structure is similar for samples collected within hours of one another in the same tank. Stamper *et al.*, 2003, also reported similar DGGE profiles in samples taken in daily intervals from a membrane bioreactor for greywater treatment (74). However sample 52 is very different from 53 indicating that each tank has it's own bacterial community perhaps caused by a reduction in TSS in the greywater from tank 1 to tank 4 (65). Also biofilm and planktonic samples seem to be very different despite the time of collection or the series tank. Sample 13 is different from 53 and 63 suggesting that the reactor tank community changes over time, however, there are no other data for these samples to support this inference. Stamper *et al.*, 2003, showed that DGGE profiles of wastewater treatment reactor organisms varied greatly over 100 days of reactor function even though

there appeared to be not loss in effluent quality (74). Additionally Fernandez *et al.*, 1999, reported that the community dynamics of a methanogenic reactor analyzed using amplified ribosomal DNA restriction analysis (ARDRA) revealed high fluctuations in community structure and stable ecosystem function (28). However Smith *et al.*, 2003, reported stable wastewater reactor DGGE community profiles throughout the treatment process (73). Fluctuations in community structure over time could not be determined for our greywater samples. DGGE profiles describe overall community dynamics and structure, however the subsequent molecular analysis did not adequately describe individuals in the wastewater reactor represented by bands in the DGGE.

Several notable difficulties in interpreting the identity of bacterial community members using DGGE bands have been described. Bano *et al.*, 2002 noted 4 different bacterial clones migrated to exactly the same spot in the DGGE gel (9). Those bacteria that may be distinctly different, but have similar DNA G+C content may migrate to the same place in the gel (40, 46). The chromatogram output from the sequencer seemed to show at least two overlapping sequences resulting from PCR amplification of one DGGE band. Perhaps, what appeared to be one band and thus one organism in Figure 6 was actually multiple organisms migrating to the same band. The chimeric origin of DGGE band # 1 also confirms the difficulty in getting sequence data for one organism from one DGGE band.

Additional difficulties arise in interpreting DGGE data and identifying organisms from the gel because bacteria often have more than one copy of their 16S rRNA gene operon. Several known organisms have more than one copy of the 16S rRNA gene operon: *E.coli* has 7 copies, *P. aeruginosa* has 4 copies and *Bacillus subtilis* has 10

copies (45). Nubel *et al.*, 1996, first reported sequence heterogeneities within the various copies of the 16S rRNA gene interfering with DGGE and interpretation DGGE banding patterns (62). They discovered that *Paenibacillus polymyxa* has at least 12 copies of its 16S rRNA operon and thus one bacteria made 12 bands in the DGGE (62). Figure 7 illustrates band #2 clearly migrating to a different position than band #5, however both have almost the same sequence and group very closely together in phylogenetic analysis (Fig. 9). #2 and #5 may represent different copies of the 16S rRNA gene from the same species of bacteria. One organism may be represented by more than one band in the DGGE gel.

Finally, the primary difficulty in identifying bacterial community members using general bacterial primers and DGGE is the insensitivities inherent in the technique. DGGE has been reported to only be able to detect bacteria that are 1% or greater in the total population (58, 59). Most of the bacteria present in the complex community with in the greywater reactor will not be detected using DGGE. Additionally, the intensities of the bands in DGGE gels cannot be interpreted as higher or lower abundance of that particular organism in the environment (9, 50). DNA extraction biases, template DNA target ratios to genome size, primer specificity, other PCR biases, and inherent gel to gel differences affect band intensities (32, 88). Therefore, Figure 6 is by no means a total look at all the bacteria present in the greywater reactors, but rather is a glimpse of some dominant bacterial community members.

To determine the presence of our inoculum species in the reactor, melting profiles of the 7 inocula were compared to the community profile generated (Fig 10). Several lanes lack sufficient DNA to generate good bands. Additionally, the smearing present

prevents good band isolation and comparison. The perpendicular gel (Fig. 2) was loaded with all the inoculum strains and 1 greywater sample. It seems clear that the 40-75% denaturant concentration would be better for resolution of the inoculum species than for the greywater samples. To assess whether bands in the community may match bands generated by the inoculum several gels with only narrower denaturing gradients may be necessary (44). Kisand and Wikner, 2003, used an overall denaturing gradient of 20-70% to assess all clusters of bands and gradients varying only by 10% to resolve subsets of those bands (44). Variations in gel denaturant in 5 or 10% increments may be necessary to definitively gauge the presence or absence of our inoculum organisms as dominant community members in the reactor or not. However, the inoculum does not appear to be present in the community profile in Figure 10.

Characterization of Strain 31529

Bacterial systematics is in a constant state of flux as new organisms are being discovered and traditional culture-based identifications are replaced with DNA sequence analysis. Currently, *Z. ramigera* and *Z. resiniphila* are both assigned to the beta proteobacterium subclass (47). However, *Z. ramigera* has been shown to be a very diverse species (66). Traditionally, organisms had been classified as *Z. ramigera* by the formation of zoogloea, thick masses of cells with finger-like projections emanating from the floc (25, 30). Organisms that may be distantly related were grouped into the *Z. ramigera* species based on this morphology in activated sludge and wastewater (25). *Z. ramigera* strains IAM 12136, *Zoogloea sp.* ATCC 19324, *Z. ramigera* IAM 12670, *Z ramigera* ATCC 19544 (neotype strain) and ATCC 25935 all group in the beta subclass according to 16S rRNA gene sequence analysis (66, 71). However, 16S rRNA gene analysis placed *Z ramigera* ATCC 19623 (former type strain) and *Z. ramigera* IAM

12669 in the alpha proteobacterium subclass (66, 71). *Z. ramigera* ATCC 19623 and IAM 12669 need to be assigned to a different genus. Analysis of 16S rRNA gene sequences may not be sufficient to describe bacterial species due to high levels of intraspecific variation and multiple 16S operons for one organism (17). However, chemotaxonomic data based on rhodoquinone analysis of various *Z. ramigera* species has not clarified the genus well either (37). Figure 11 illustrates this divergence of *Zoogloea sp.* into alpha and beta proteobacteria lineages. Bacterial taxonomists have long grouped species by morphological or phenotypic similarities, now molecular analysis is being used in a number of taxa to resolve similar taxonomic incongruities as *Z. ramigera*. The *Caedibacter* genus is also poorly delineated, primarily because they all are endosymbionts of *Paramecium* spp., some species belong to the alpha and some to the gamma proteobacteria subclasses (10).

Figure 1 illustrates that Strain 31529 groups well with the alpha subclass *Z. ramigera* including *Agrobacterium tumefaciens*. The closest isolated species related to Strain 31529 is *Granuella daejeonensis*. It was isolated from activated sludge, but it has no other information available about it. *Z. ramigera* is the next closest described species to Strain 31529. Based on 16S rRNA gene analysis (Figure 11) and zoogloea formation (Figure 13), Strain 31529 is closely related to *Z. ramigera*, belonging to the alpha subclass of the proteobacteria.

Z. ramigera have been detected in a wide range of environments: membrane-aerated biofilms in wastewater reactors (18), uranium-contaminated sediments (76), subterranean oil-storage cavities (86), ammonia biofilters (69) and polluted waters (30). In activated sludge, *Z. ramigera* is purported to form activated sludge flocs (67). These

flocs can be regarded as small microbial communities that stimulate pollutant degradation (36). There have been no other reports of the function of *Z. ramigera* in wastewater. The primary role of Strain 31529 in the greywater reactor may be to act as a floc former for other organisms that reduce the pollutant load.

Strain 31529 PCR Detection

Species-targeted primer specificity for only the target organism is a chronic problem in molecular microbial ecology. The primer ZAL F (67) in the Ribosomal Database Project II (RDP-II), matches with 31 other organisms, mostly *Z. ramigera* and *Rhizobium* sp. (19). Some of these environmental organisms may be present in the greywater samples and responsible for eliciting PCR product in Figure 14. The primer DGGE Agro ITSR does not show any matches in the RDP-II (19). The ITS region has been shown to be highly variable and good for distinguishing closely related species and even strains (42). Janse *et al.*, 2003, designed species-targeted primers to amplify the ITS region of cyanobacteria and was able to distinguish different strains of *Microcystis* in environmental samples by running samples on the DGGE (42). If Figure 15 had yielded product for the greywater samples, they would have been run on the DGGE to ensure Strain 31529 was present.

Strain 31529 was not detected in the greywater reactor in planktonic or biofilm samples. Reasons for failure of inoculum species in wastewater reactors may include: predation by protozoa within the reactor (15), poor growth *in situ* (33), and inoculum species interacting with indigenous bacteria can have unforeseen competition and toxic effects on one another (36, 54). Van der Gast *et al.*, 2004, showed that their inoculum was shown to persist throughout the treatment process and reduce toxic load regardless of the presence or absence of indigenous microbial populations (80). However, Hamer,

1997, stresses the importance of viewing microbes in treatment reactors as a consortia with unforeseen interactions and complex homeostasis (36). McClure *et al.*, 1991, emphasizes the importance of well-acclimated organisms being introduced into a treatment reactor, preferably those organisms naturally isolated in similar treatment reactors (54). Strain 31529 was isolated from wastewater. It is possible that the Strain 31529 is present in the reactor tanks and simply beneath the detection range.

FISH

The initial FISH protocols were completed as described by Rosello-Mora *et al.*, 1995 (67). They experimentally determined the melting temperature and ideal formamide concentration for the hybridization buffer of the probe ZAL in pure cultures of the alpha proteobacteria *Z. ramigera* ATCC 19623 (67). Upon further reflection on the sequence for the probe (Table 2), the calculated T_m should be 44° C (70). A hybridization temperature of 46° C and formamide concentration of 35% is entirely too high. At 46° C, there may be no need for formamide in the hybridization reaction at all. Additionally, Fuchs *et al.*, 1998, indicate that position 647-664 on the *E. coli* 16S rRNA gene corresponding to the ZAL probe has the lowest fluorescent intensity brightness class, meaning that an oligonucleotide probe for that region of the *E. coli* 16S rRNA did not bind well (31). Binding of a rRNA probe can be blocked by secondary and tertiary structure of the ribosome and by proteins in association with the ribosome (31). Despite the results obtained from Rosello-Mora discovering a small number of cells binding ZAL in wastewater (67), the probe and hybridization conditions are poorly designed. The best solution would be to re-design a probe for Strain 31529.

Concluding Remarks

DGGE community profiles reveal similarities in the bacterial community structure of samples taken from the same tanks and similar times. Profiles also reveal distinct differences in community structure between different tanks in the reactor and biofilm and planktonic communities. DGGE profiles did not show that any of the inoculum species are dominant community members in the reactors. *Agrobacterium sp.* ATCC 31529 could not be detected in either the biofilm or planktonic greywater samples tested. Future work can entail expanding the search for *Agrobacterium sp.* to tanks 1 and 4 in the reactor and re-design of a FISH probe. Additionally, the protocols developed here can be used to create ITS species-specific primers for the other 6-inoculum species as well. Development of FISH probes for each of the inoculum species will also be critical.

This assay has focused on developing a polyphasic approach to the study of very specific bacteria in a complex environment. Multiple overlapping techniques are vital to the generation of data that is reflective of bacteria in their natural environment. No one molecular technique can be used to infer bacterial community dynamics in the environment. Ahn *et al.*, 2002, detected *Dechlorimonas* sp. in most DGGE gels using universal primers from a biological treatment reactor and inferred that it was a dominant community members, however FISH data with a *Dechlorimonas* sp. probe did not reveal many cells at all (1). LaPara, *et al.*, 2000, used both DGGE profiles and sequencing of 16S rRNA gene clonal libraries to assess bacterial community diversity in pharmaceutical wastewater and each technique revealed different degrees of diversity and different organisms (50). Eschenhagen *et al.*, 2003, detected only minor fluctuations in bacterial community structure in activated sludge treatment reactors using group-specific FISH

probes, but generated wildly differing community fingerprints from terminal restriction-fragment length polymorphism (T-RFLP) analysis (24). No one molecular tool is sufficient to describe microbial community diversity or abundance in the environment. Polyphasic approaches to the study of environmental bacteria are vital to understanding bacterial community structure in complex environments.

APPENDIX

Table 1: Organisms used as inoculum species in greywater treatment reactor.

Organism	ATCC Strain number
Unidentified sp.	21160
Gordonia sp.	BAA-558
Agrobacterium sp.	31529
Pseudomonas putida	700412
Pseudomonas stutzeri	BAA-172
Enterobacter cloaocae	700411
Caulobacter vibrioides	15252

Table 2: List of biofilm and planktonic greywater samples used in this assay.

Sample	Series	Tank	Sample Date	Collection Time	Planktonic or Biofilm
52	5	2	11/06/03	0:00	P
52B	5	2	11/05/03	0:00	B
53	5	3	11/06/03	0:00	P
53B	5	3	11/06/03	0:00	B
62	6	2	11/06/03	8:00	P
62B	6	2	11/06/03	8:00	B
63	6	3	11/06/03	8:00	P
63B	6	3	11/06/03	8:00	B
12	11	2	12/05/03	4:00	P
12B	11	2	12/05/03	4:00	B
13	11	3	12/05/03	4:00	P
13B	11	3	12/05/03	4:00	B

Table 3: Primers and probes used in this study.

Primer	Sequence 5' to 3'	Purpose	Reference
GC 357 F	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	Eubacterial DGGE forward primer	(59)
907 R	CCG TCA ATT CMT TTG AGT TT	Eubacterial DGGE reverse primer	(5)
ITS2 F (1492 H2 R Reverse and Complement)	AAG TCG TAA CAA GGT AG	ITS region forward primer	(49)
ITS2 R	TAC CGA ACT GTC TCA CG	ITS region reverse primer	This study
ZAL F primer	GTA CCT AGA GTA TGG AAG	*Z.ramigera* PCR detection	(67)
DGGE Agro ITS R	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGT CAW GCA CG	Strain 31529 specific DGGE for PCR detection	This study
27 F	AGA GTT TGA TCA TGG CTC AG	Sequencing 16S rRNA gene	(49)
355 F	CCA GAC TCC TAC GGG AGG CAG C	Sequencing 16S rRNA gene	(4)
1096 R	AGG GTT GCG CTC GTT GC	Sequencing 16S rRNA gene	(48)
1492 H2 R	TAC GGC TAC CTT GTT ACG ACT T	Sequencing 16S rRNA gene	(49)
Probe	Sequence 5' to 3'	Purpose	Reference
ZAL probe	Cy3-CTT CCA TAC TCT AGG TAC	FISH probe	(67)
EUB 338 probe	Cy3-GCT GCC TCC CGT AGG AGT	FISH probe	(4)

Table 4: List of specific thermocycler parameters used with each primer set and the purpose of each reaction performed.

Primer pair	Program	Purpose
ITS2F & ITS2R	96° C 10 min; 96° C 45 sec, 45° C 45 sec, 72° C 3 min X 30 cycles; 72° C 5 min; 4° C infinity	ITS sequencing
GC357F & 907R	96° C 10 min; 96° C 45 sec, 46° C 45 sec, 72° C 1 min X 30 cycles; 72° C 30 min; 4° C infinity.	DGGE analysis
ZALF & 907R	96° C 5 min; 96° C 30 sec, 45° C 30 sec, 72° C 30 sec X 30 cycles; 72° C 5min; 4° C infinity	Published primers for *Z. ramigera* detection
ITS2F & DGGE Agro ITSR	96° C 10 min; 96° C 45 sec, 45° C 45 sec, 72° C 1 min X 30 cycles; 72° C 5 min; 4° C infinity	New primer pair designed for Strain 31529 ITS region and Strain 31529 detection

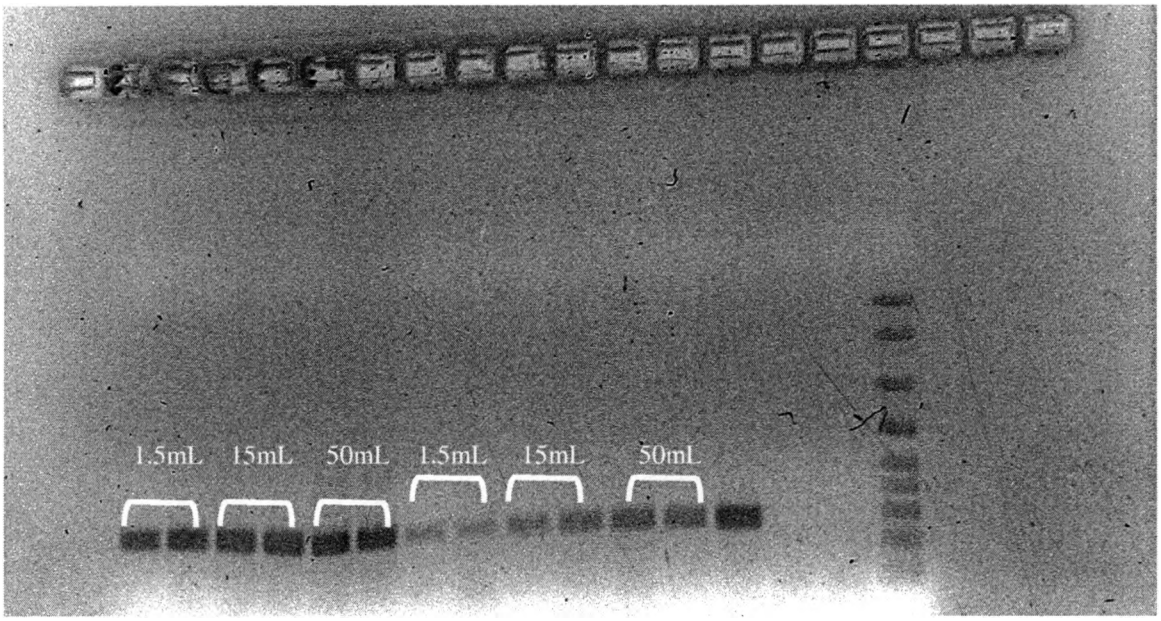

Figure 1: 1.25% agarose gel with PCR product from primers ZAL F and 907R. Two greywater samples were chosen at random. Samples were taken in duplicate from each 1-liter carboy of greywater in 1.5 ml, 15 ml, and 50 ml aliquots. Sample 1 is in lanes 1-6. Sample 2 is in lanes 7-12. Lane 13 is *Agrobacterium sp* ATCC 31529 positive control. Lane 14 is *Escherichia coli* MG1655 negative control. Lane 15 is a PCR no template control. Lane 16 is HI LO DNA ladder (Minnesota Molecular Inc., Minnesota, MN). PCR product was electrophoresed on a 1.25% agarose gel and visualized by ethidium bromide staining under UV light.

Table 5: Heterotrophic plate count agar (Difco) colony counts for 6 planktonic samples. Sample descriptions are listed in Table 2. All plates incubated at 30^{o} C for 72hrs.

Sample #	Plate count (CFU/ml)
52	2.5×10^{5}
53	9.3×10^{3}
62	9.0×10^{4}
63	3.8×10^{3}
12	3.8×10^{3}
13	6.4×10^{4}

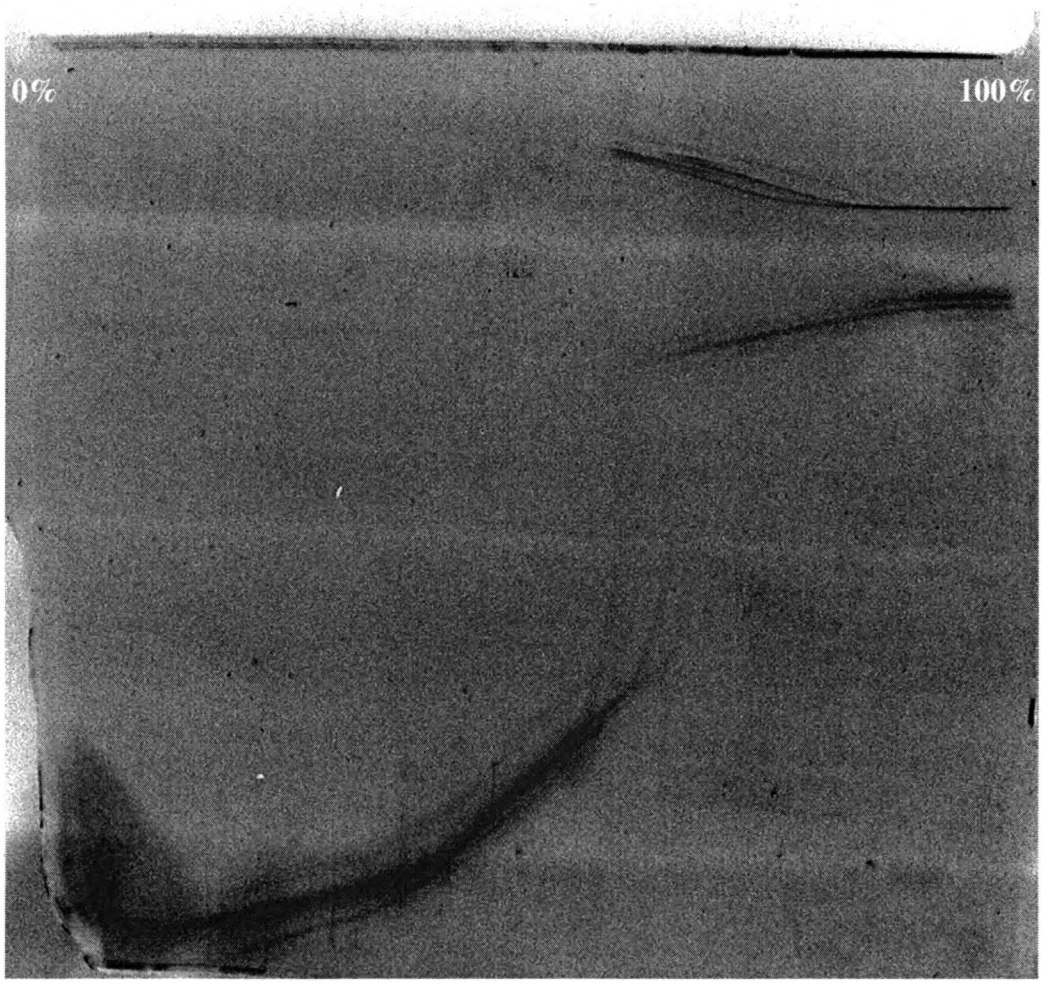

Figure 2: Perpendicular DGGE gel with all 7 inoculum organisms (Table 1) and 1 greywater sample 52 (Table 2). PCR product was generated using universal primers GC357F and 907R. Gel ran for 18 hrs at 80volts and 60° C.

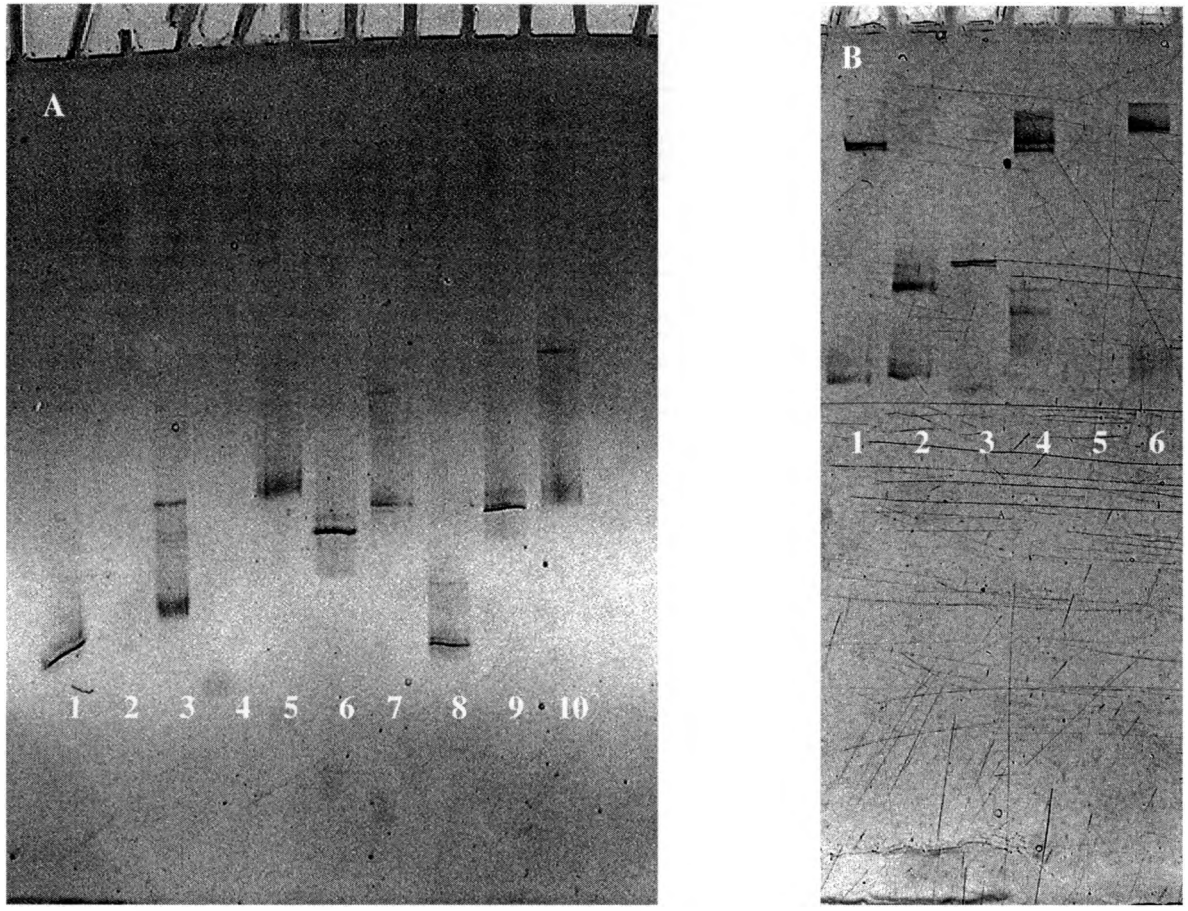

Figure 3: DGGE gels illustrating the affect of variations in electrophoresis time and voltage. Gel A is a 50-75% denaturing gradient and ran 16 hrs at 80 volts and 60° C. Table 1 lists all the inoculum organisms. Lanes: 1 *Gordonia* sp, 2 empty, 3 *Escherichia coli* MG1655, 4 empty, 5 *Agrobacterium* sp., 6 *C. vibriodes*, 7 *P. putida*, 8 *E. cloacae*, 9 Unidentified sp., and 10 *P. stutzeri*. Gel B is a 40-75% denaturing gradient and ran 5.4 hrs at 180 volts. Lanes: 1 *C. vibriodes*, 2 *E. cloacae*, 3 *Gordonia* sp, 5 *P stutzeri*, 6 *P putida*, 7 *Agrobacterium* sp.

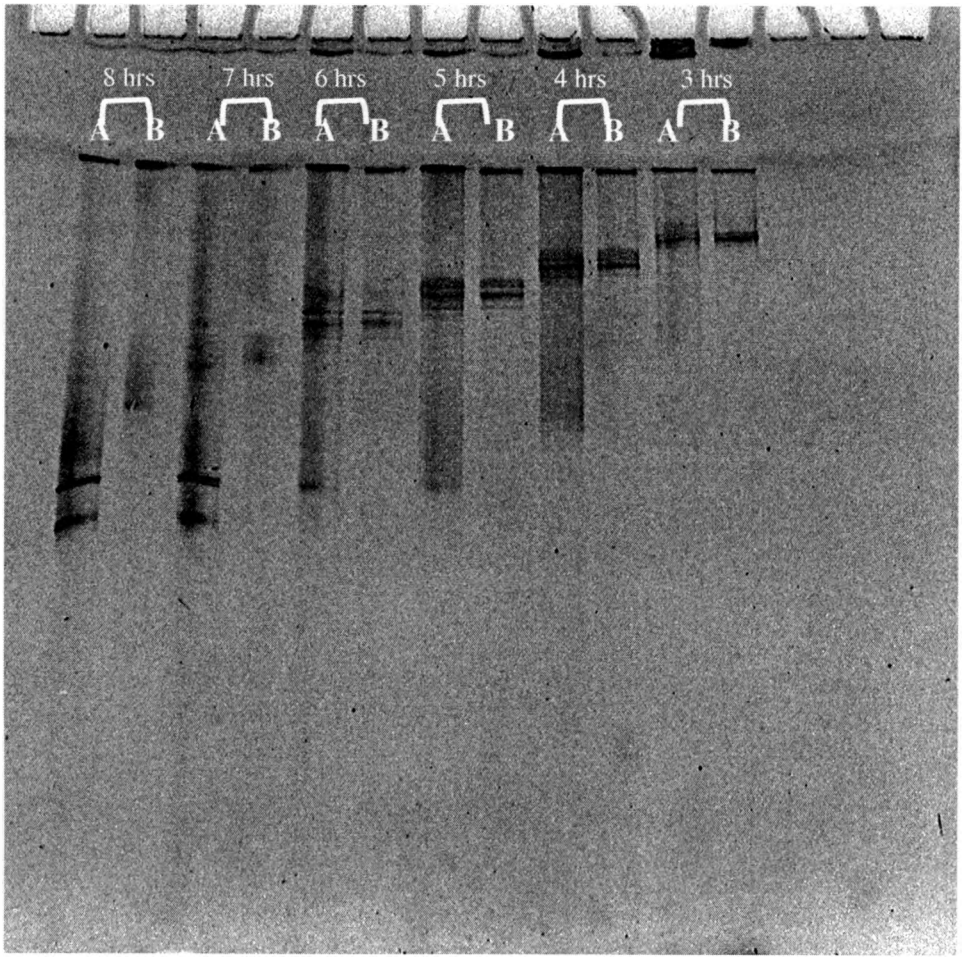

Figure 4: Time trial DGGE gel in which samples were loaded every hour for 8 hrs. A is a mixture of inoculum species. B is a planktonic greywater sample. PCR product was generated using universal primers GC357F and 907R.Gel is 40-60% denaturant concentration and ran at 180 volts and 60° C.

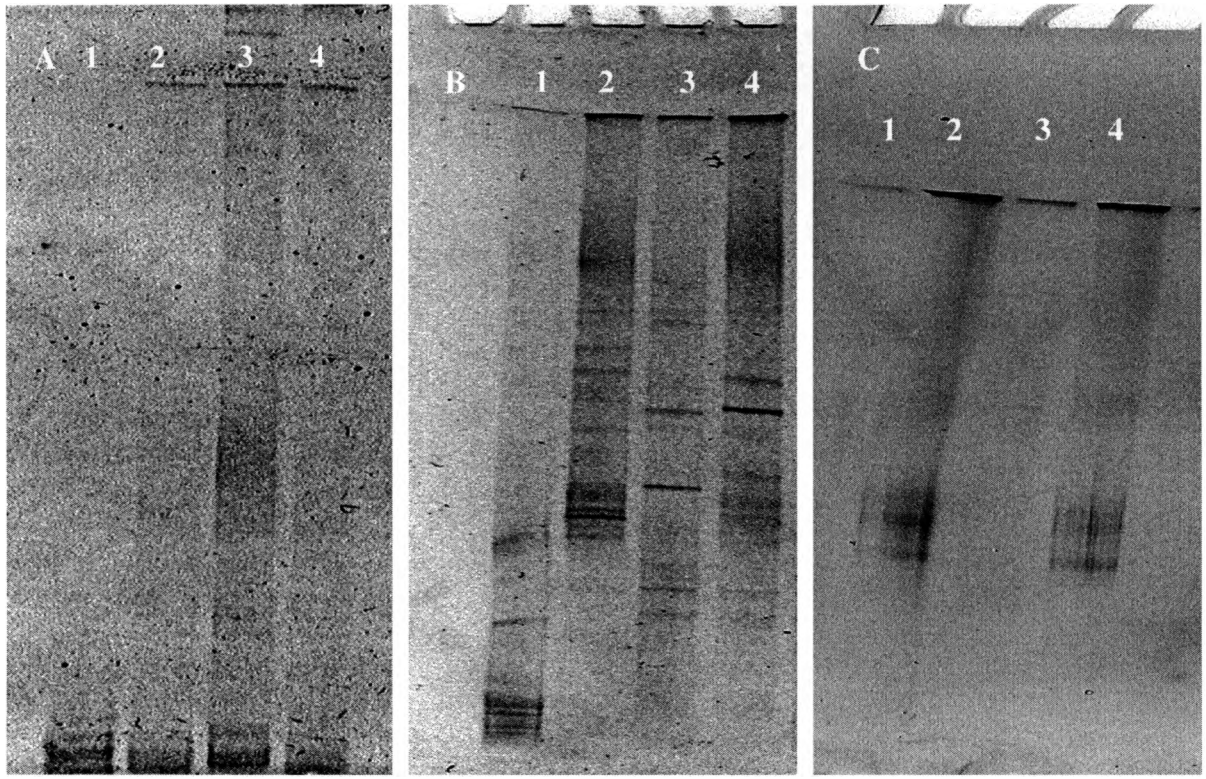

Figure 5: Optimization of DGGE formamide and urea denaturing gradients. Gel A is 20-40%. Gel B is 30-50%. Gel C is 40-60%. Lane 1 is a mix of inoculum organisms. Lane 2 and 4 are planktonic samples. Lane 3 is a biofilm sample. Gels ran for 8 to 12 hrs at 180 volts and 60° C.

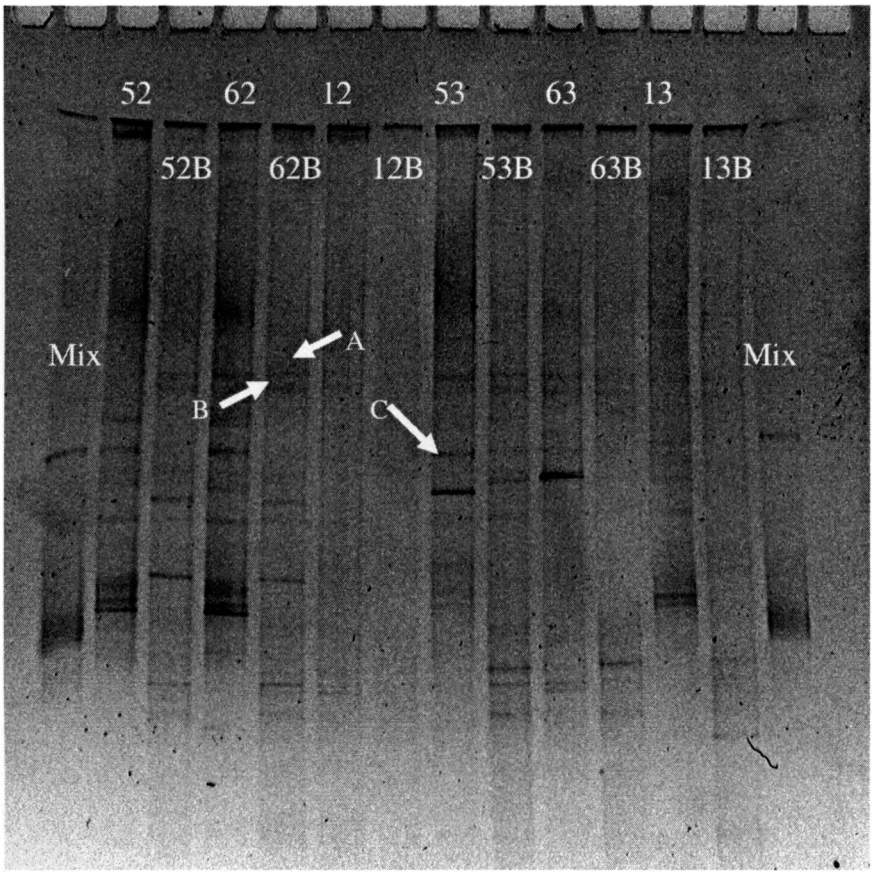

Figure 6: Overall community profile of greywater samples generated using DGGE and universal bacterial primers. Details on samples 52-13B are listed in Table 2. A, B, and C represent organisms that may be present in multiple tanks in the reactor. The Mix is a 1:1 mixture of *Agrobacterium* sp ATCC 31529 and *Enterobacter cloacae* ATCC 700411. The gel ran at 60 ^{o}C and 180 volts for 7 hours with a denaturant concentration of 30-50% linear gradient.

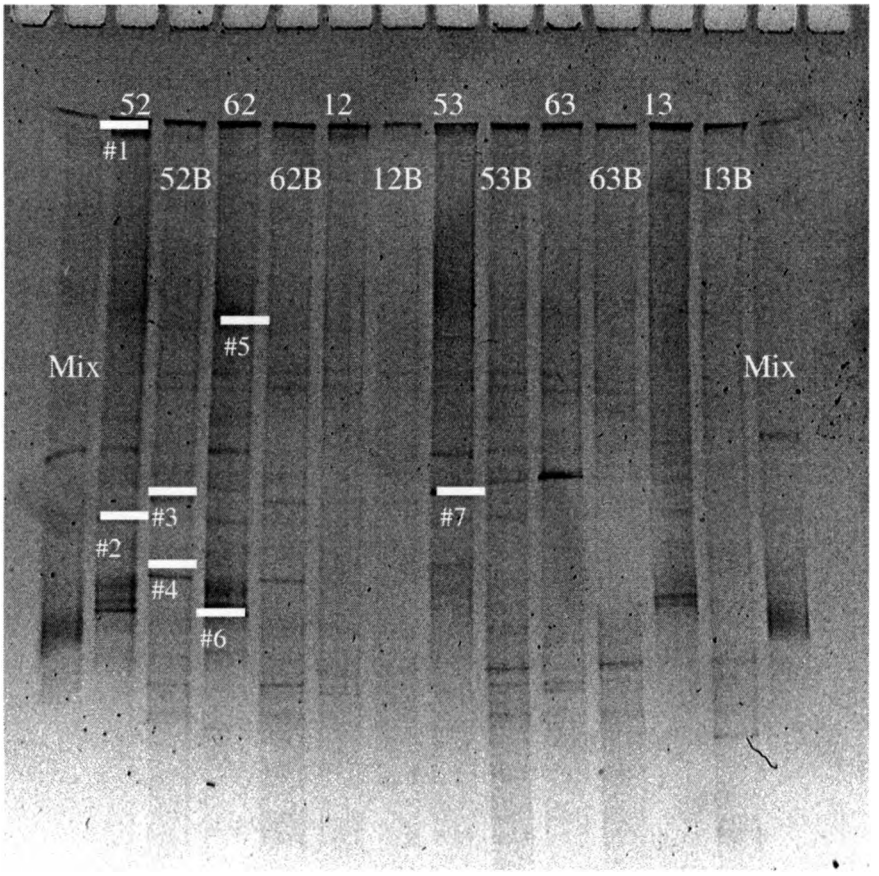

Figure 7: Excised bands from overall community profile of greywater samples generated using DGGE and universal bacterial primers. Details on samples 52-13B are listed in Table 2. Lines and numbers represent DGGE bands that were excised and yielded good sequence information.

Table 6: 16S rRNA gene sequence data for each of the 7 bands from community profile DGGE gel (Fig. 7).

DGGE band number	Sequence 5'-3'
1	ACCAGCCATGCCGCGTGCAGGAAGAAGCATCTATGGTGTGTAAACT GCTTTTATACAGGAAGAAACNCCCCCACGTGTGGGGGCTTGACGGT ACTGTAGGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTA ATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGT CCGTAGGCGGTCTTATAAGTCAGTGGTGAAAGCCCATCGCTCAACG ATGGAACTGCCATTGATACTGTAAGACTTGAATTTTTGTGAAGTAAC TAGAATATGTAGTGTAGCGGTGAAATGCTTAGATATTACATGGAAT ACCCATTGCGAAGGCAGGGTACTAACAAACGATTGACGCTGATGGA CGAAAGCGTGG
2	CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAA AGCACTTTAAGTTGGGAGGAAGGGCATTAACCTAATACGTTAGTGT NTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCA GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGC GTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGAATGTGAAATCCCCG GGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGG TAGAGGGTNGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATAT AGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACTGATACTGA CACTGAGGTGC
3	AGCCTGAACCAGCCAAGTCGCGTGAAGGAAGAAGGATCTATGGTTC GTAAACTTCTTTTGCAGGGGAATAAAGTGCAGGACGTGTCCTGTTTT GTATGTACCCTGAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCC GCGGTAATACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAA AGGGTGCGTAGGTGGTTTGATAAGTCAGCGGTGAAAGTTTGCAGCT TAACTGTAAAAATGCCGTTGAAACTGTCGGACTTGAGTGTAAATGA GGTAGGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATATCACG CAGAACTCCGATTGCGAAGGCAGCTTACTAAGCTACAACTGACACT GAAGCACGAA
4	GAACCAGCCATCCCGCGTGCAGGAAGACGGCCCTATGGGTTGTAAA CTGCTTTTGCGCATGAAGAATGTTACCGACGTGTCGGTAAGTGACGG TAATGCGTGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGT AATACGGAGGATGCAAGCGTTATCCGGATTTATTGGGTTTAAAGGG TGCGTAGGCGGCCTTGTAAGTCAGTGGTGAAAACCTGCAGCTCAAC TGTAGGCGTGCCAATGAAACTGTGAGGCTTGAGTGACGTTGAGGCA GGCGGAATGTGTAGTGTAGCGGTGAAATGCTTAGATATTACACAGA ACACCGATTGCGTAGGCAGCTTGCTAAAGGTTAACTGACGCTGATG CACGAAAGCGT

Table 6: Continued 16S rRNA gene sequence data for each of the 7 bands from community profile DGGE gel (Fig. 7).

5	AGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG CNCTTTNAGTTGGGAGGAANGGCATTNACCTAATACGTTANTGTCTTGACG NTACCGACAGAATAAGCACCGGNTAACTCTGTGCCAGCAGCCGCGGTAAT ACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG GTGGTTTGTTAAGTTGNATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA TCCAAAACTGGCNNGCTAGAGTATGGTAGAGGGNNGTGGAATTTCCTGTGT AGCGGTGAAATGCGTAGATATAGGAAGGAACACCNGTGGCGAAGGCGACN ACCTGGACTGATACTGACACTGAGG
6	CCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGNTTGTAAAGCTCTTTTG TCAGGGAAGAAACGGNGGGAGCTAATATCTCCTGNTAATGACGGTACCTG AAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACNTAGG GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGNGCGCAGGCGGTTT NGTAAGTCTGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTGGAGA CTGNAAGGCTAGAATCTGGCAGAGGGGGGTNGAATTCCNCGTGTAGCAGT GAAATGCGTAGATATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTGG GTCAAGATTGACGCTCATGCACGAAAGC
7	AACCAGCCACGCCGCGTGCAGGAAGAAGGCCCTACGGGTTGTAAACTGCT TTTATACGGGAAGAACCGCCCTCCTGCGGGGGGGTATGACGGTACCGTAGG AATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATG CAAGCGTTATCCGGATTCATTGGGTTTAAAGGGTGCGTAGGTGGGTCCATA AGTCAGTGGTGAAATCCGGCAGCTCAACTGTCGCACTGCCTCTGATACTGT GGGTCTTGAGTTAAGTTGAAGCAGGCGGAATATGACATGTAGCGGTGAAA TGCATAGATATGTCATAGAACACCGATAGCGAAGGCAGCCTGCTAAACTTA CACTGACACTGATGCACGAAAGCGTG

Table 7: Sequence comparison results from similar sequences downloaded from Genbank of the 6 DGGE bacterial sequences (Table 6). Sequences are presented with their most similar results from Genbank and or the closest described species (2).

DGGE band	% match and Highest blast result	Closest related known organism
2	99% #AY450556.1 *Pseudomonas putida* strain L	
3	99% #AY570639 Uncultured bacterium clone PL-7B7	92% #AY1691116.1 *Bacteriodes merdae*
4	93% #AY168724 Uncultured bacterium clone	87% #AB015525.1 *Cytophaga* sp.
5	96% #AY450556.1 *Pseudomonas putida* strain L	
6	97% AF488664 Beta proteobacterium Z 29	96% #AJ551147 *Janthinobacterium* sp. An8
7	91% #AF469375 Uncultured clone 33-PA63B98	88% #AY711048 Uncultured *Bacteriodetes* bacterium clone SIMO-1682

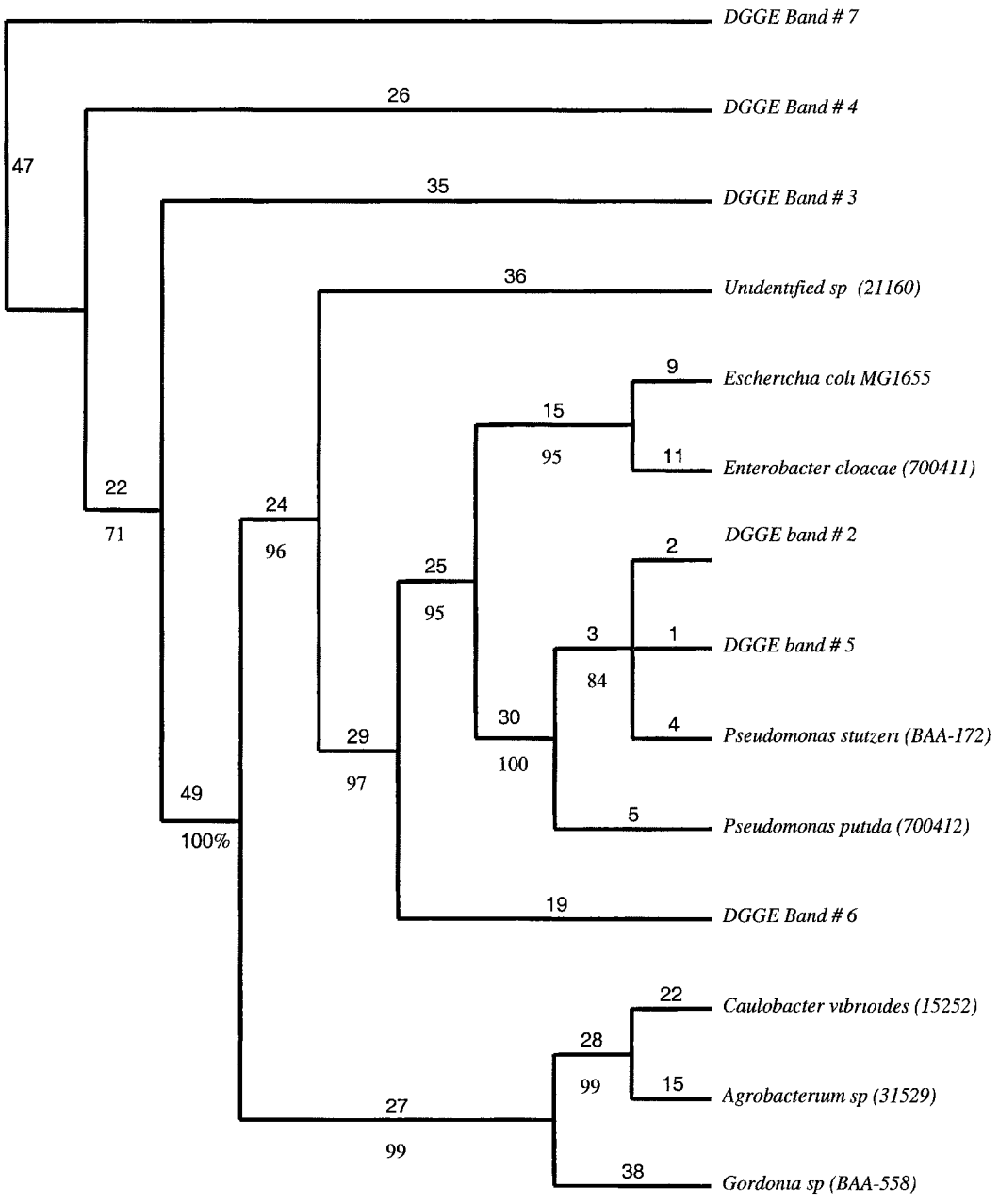

Figure 8: Heuristic parsimony phylogenetic tree based on 16S rRNA gene created using PAUP* (78) for the inoculum organisms (Table 1) and the DGGE band sequences (Table 6 and 7). Numbers in parentheses following the organism reflect ATCC culture collection strain numbers. This tree is unrooted. Numbers represent the numbers of changes between sequences. Percentages reflect bootstrap support measures for this topology generated using PAUP*.

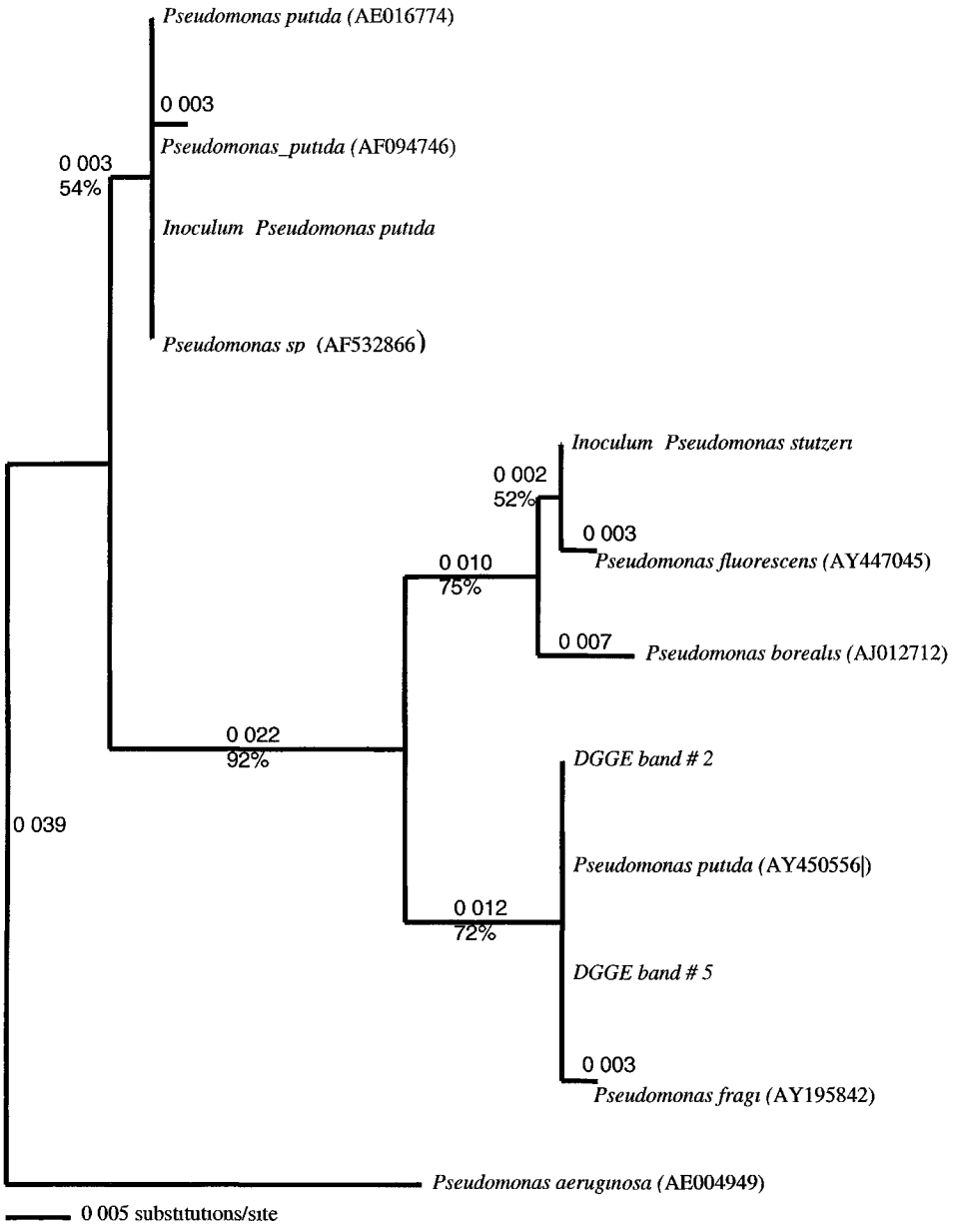

Figure 9: Maximum likelihood heuristic phylogenetic phylogram created using PAUP* to distinguish the inoculum strains *P. putida* and *P. stutzeri* (Table 2) from the DGGE bands #2 and #5 (Tables 6 and 7). Numbers in parentheses following the organism reflect Genbank accession numbers (2). Numbers reflect the likely number of base substitutions for this topology. Percentages reflect bootstrap support measures for this topology generated using PAUP*.

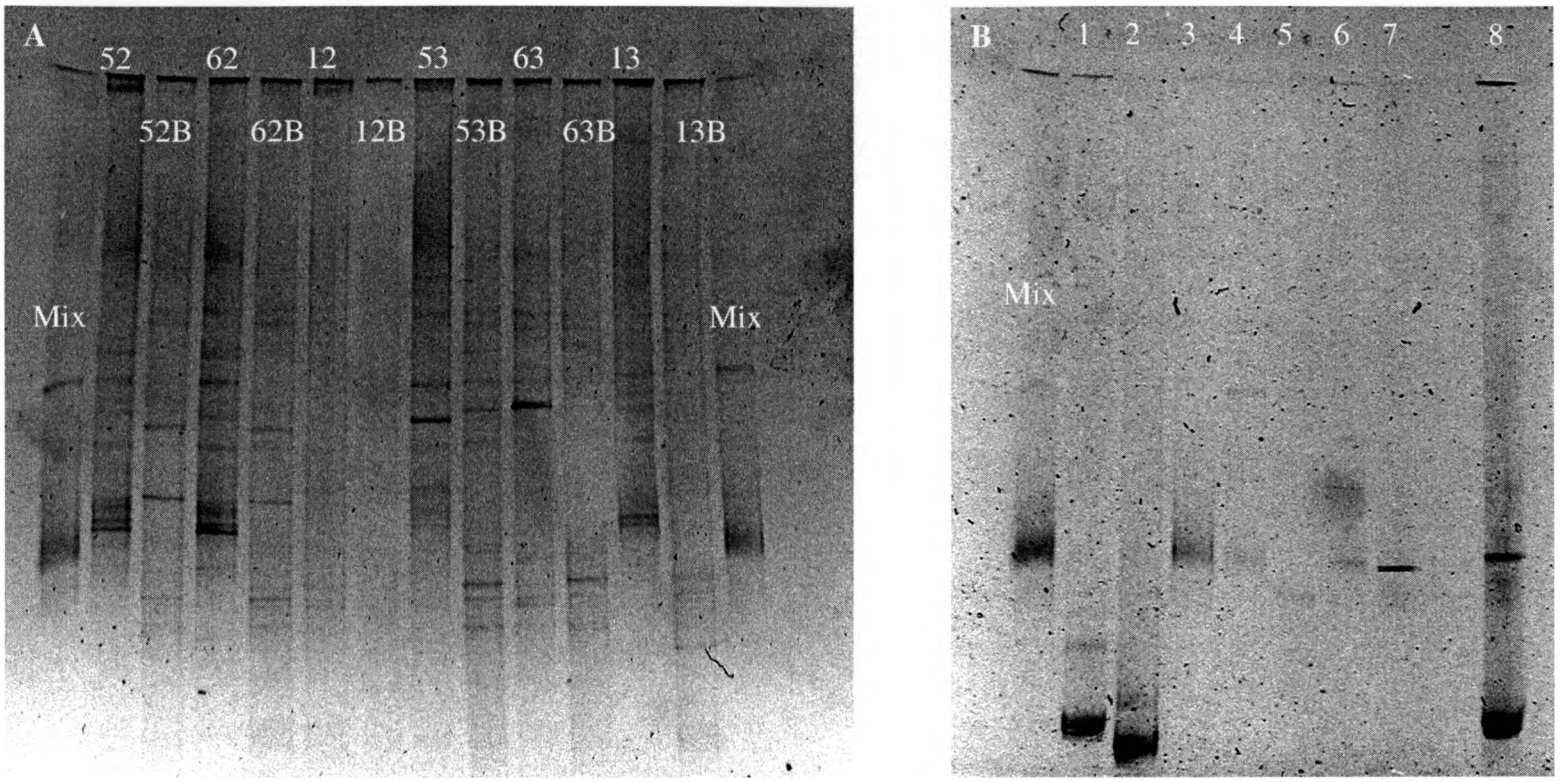

Figure 10: DGGE gels comparing the overall community profile of greywater samples and the profile of each inoculum strain. Gel A:Community profile DGGE gel(Fig. 6) next to Gel B:7 inoculum organisms DGGE gel. Gel A is labeled with the sample numbers (Table 2). Gel B is labeled with inoculum numbers: 1 *E. cloacae*, 2 *Gordonia*, 3 *Agrobacterium sp.*, 4 *P. stutzeri*, 5 *C. vibriodes*, 6 *P. putida*, 7 Unidentified sp. and 8 *Escherichia coli* MG1655 (Table 1). The Mix is a 1:1 mixture of *Agrobacterium sp.* and *E. cloaocae*. The gels ran concurrently at 60 ^{o}C and 180 volts for 7 hours with a denaturant concentration of 30-50% gradient.

Table 8: Partial 16S rRNA gene sequence generated for *Agrobacterium* sp. ATCC 31529. The sequence corresponds to positions 119-1472 on the *E. coli* 16S rRNA gene.

Sequence 5'-3'
GTGATACGCGTGGACGTACCTTTCTACGGATACTCAGGGAACTTGTGCTAATACCGTATGTGCCCTTCGGGG
GAAAGATTTTCGGTAAAGGATCGGCCCGCGTTGGATAGCTAGTTGGTGGGGTAAAGCCTACCAGGCGACGAT
CCATAGCTGGTCTGAGAGGATGACAGCCACATTGGGACTGAGACACGGCCCAACTCCTACGGGAGGCAGCAG
TGGGGAATATTGGACAATGGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTG
TAAAGCTCTTTCACCGGTGAAGATAATTGACCGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCA
GCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTARGCGGGTATTTA
AGTCAGGGGTGAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATACTGGGTACCTARAGTATGGAAGAGG
TAAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTAC
TGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGAATGTTAGCCGTCGGCATGCATGCATGTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCT
GGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGCAGAACCYTACCAGCCCTTGACATGTCGGTCGCGGATTACAGAGATGTTTTCCTT
CAGTTAGGCTGGACCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTGGTTAATCCCG
CAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGC
CGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTG
GTGACAGTGGGCAGCGAGACAGCGATGTCGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGC
AACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCN
TTGTACACACCGCCCGTCACACCATGGAGTTGTTTTACCCGAAGGCGTGCGCCAACCCGCAGGAAGCAGCG

Figure 11: Maximum likelihood heuristic phylogram based on 16S rRNA gene for *Agrobacterium sp.* ATCC 31529 and sequences of its close relatives downloaded from Genbank (2) created using PAUP* (78). Numbers in parentheses following the organism reflect Genbank accession numbers (2). *E. coli* MG1655 was set as the outgroup. Percentages reflect bootstrap support measures for this topology generated using PAUP*.

Table 9: ITS sequence data retrieved for *Agrobacterium* sp. ATCC 31529 (Strain 35129). 353 base pairs of sequence including the ITS region and beginning of the 23S rRNA gene were obtained for Strain 35129.

Sequence 5'-3'
GAAGCTGTGGAATTGGTAAGACGCCTAACTTGATTAGGATGAACCTTCCCGTG CTTTTTAGAACATAGATGGCGNCAGTCAGGCGACCATCGAAACGCAATACGCT GCGGAAATGCTTTGGCATTCGGACAGTATGGCGATTATCGCCGACCACGTTTC TCTTTCTTCACNAGGATATACNAACCACGCCCGCGTCAGCGTGCTTGACCGTA ATGGGCCCGNANCTCAGGNGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCG GCAGTTCGAGTCTGCCCGGGCCCACCATCCCAACGATTGAGTGGTCAGCCGGT CAGGTTATCNAAACCTGAATGGGGCTGTAGCTCA

Table 10: Results of basic biochemical tests performed on *Agrobacterium sp.* ATCC 31529 listed with test data reported from *Zoogloea ramigera* ATCC 19544 the beta proteobacteria type strain, *Zoogloea ramigera* ATCC 19623 the alpha proteobacteria former type stain, and *Agrobacterium tumefaciens* Biovar 1 (47). All tests performed as described (51) and incubated at 30° C.

Test	*Agrobacterium sp.* ATCC 31529	*Z. Ramigera* ATCC 19623	*A. tumefaciens* Biovar *1*	*Z. Ramigera* T ATCC 19544
Urease	-	-	+	+
Oxidase	+	+	+	+
Glucose fermentation	-	+	+	-
Gelatinase	-	-	-	+
Starch hydrolysis	-	-	-	-
Sucrose fermentation	-	+	+	-
Lactose fermentation	-	-	+	-
Citrate utilization	-	-	-	+
Nitrate to N_2	+	-	ND	+
SIM (H_2S production)	-	-	-	-
SIM (indole)	-	-	-	-
Motility Agar	+	+	+	+
Catalase	+	ND	+	+

ND=Not determined

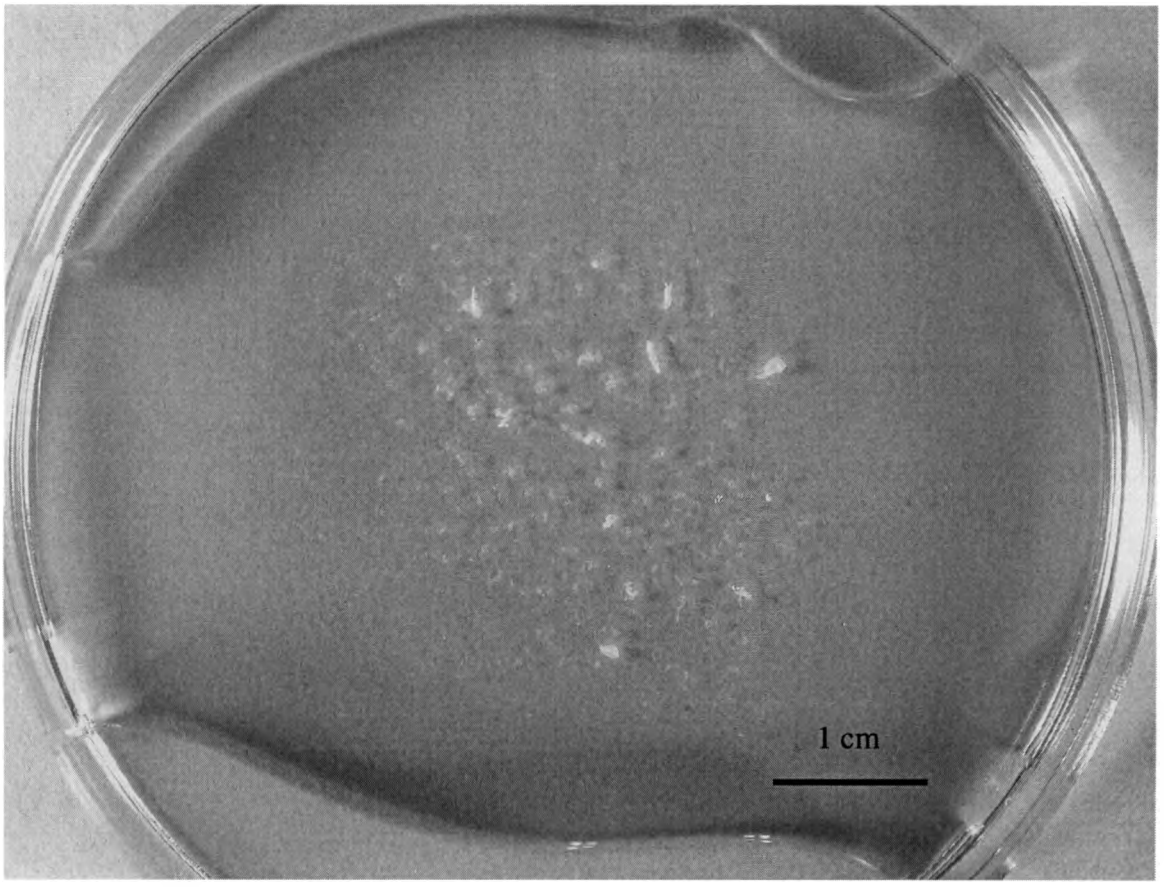

Figure 12: Broth culture of *Agrobacterium sp* ATTC 31529 exhibiting flocculent growth. Culture was grown in nutrient broth (Difco, Sparks, MD) at 30 ^{o}C and 150 rpm for 3 days. Scale bar represents 1 cm.

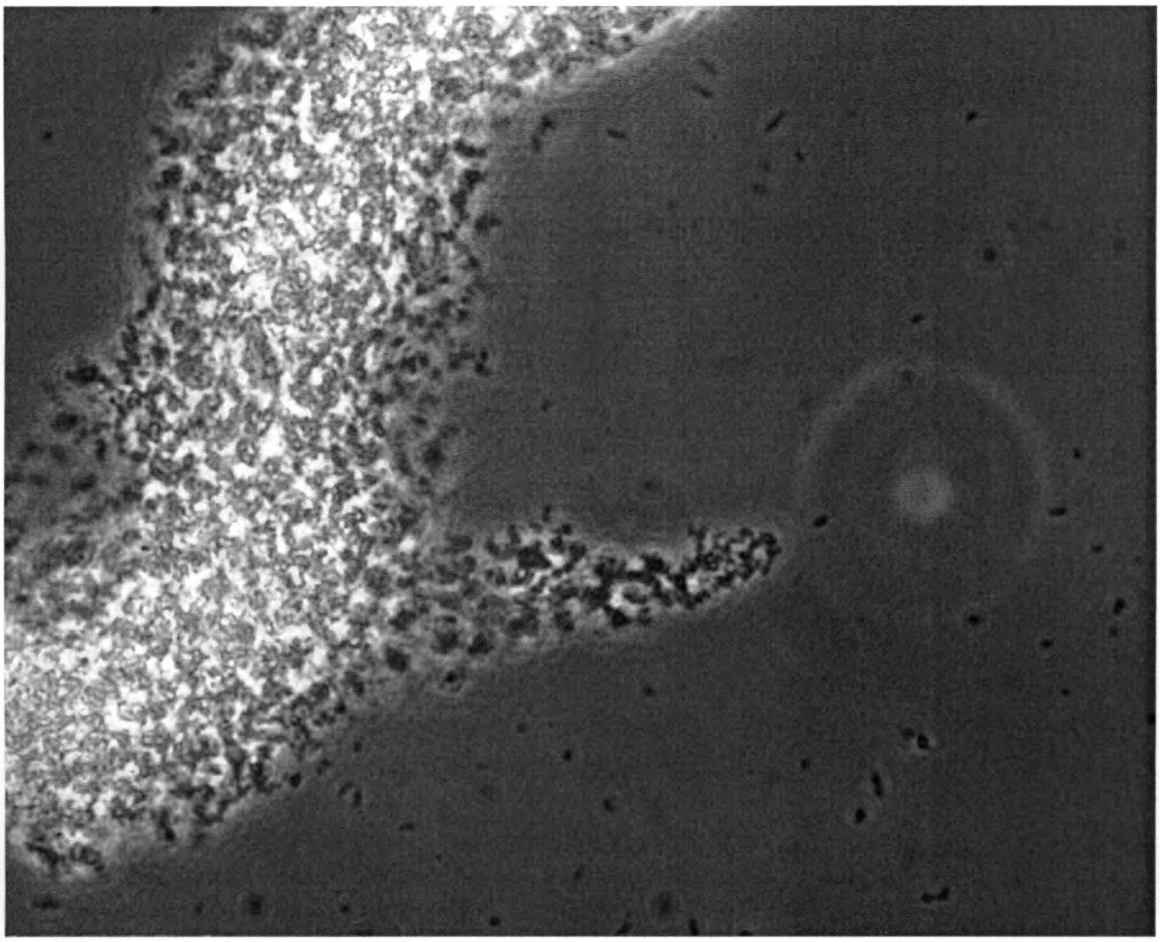

Figure 13: Phase contrast picture of *Agrobacterium sp* ATCC 31529 flocs exhibiting characteristic finger-like projection indicative of *Zoogloea ramigera*. Floc viewed at 200X magnification.

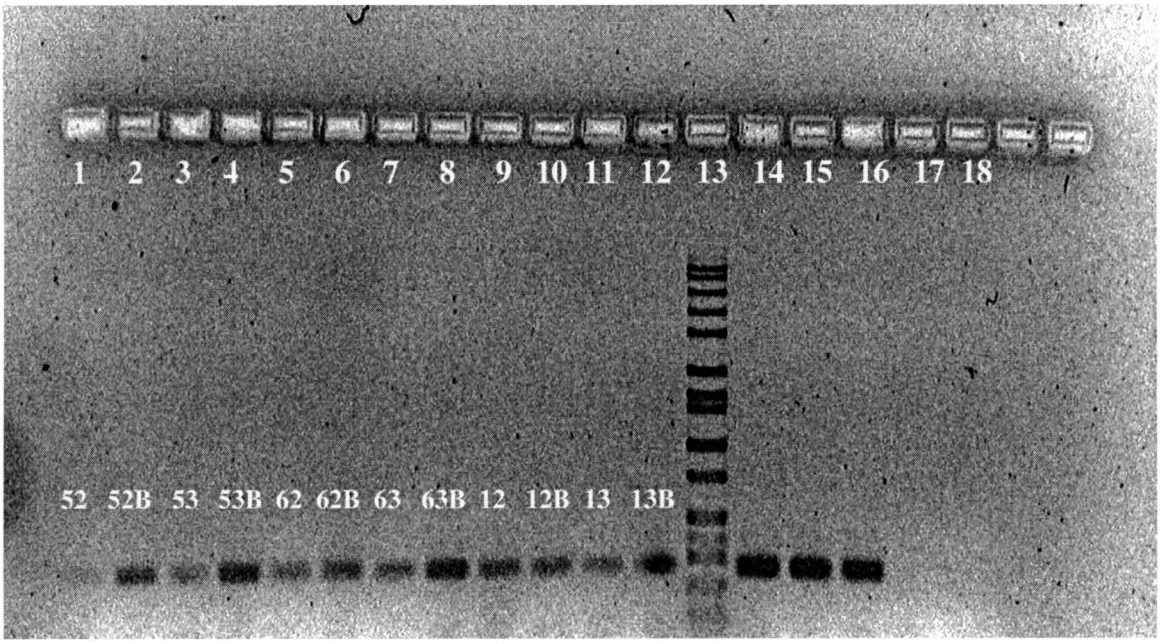

Figure 14: PCR product from the ZAL F and 907R primer set from a published species-specific primer for *Zoogloea ramigera*, ZAL F (Table 3). Lanes 1-12 are labeled with the sample number (Table 2). Lane 13 is HI LO DNA ladder (Minnesota Molecular Inc., Minnesota, MN). Lane 14 is a pure culture of *Agrobacterium* sp. ATCC 31529. Lane 15 and 16 are samples 52 and 52B with *Agrobacterium* sp tDNA added. Lane 17 is *E. coli* and 18 is a PCR no template control. PCR product was electrophoresed on a 1.25% agarose gel and visualized by ethidium bromide staining under UV light.

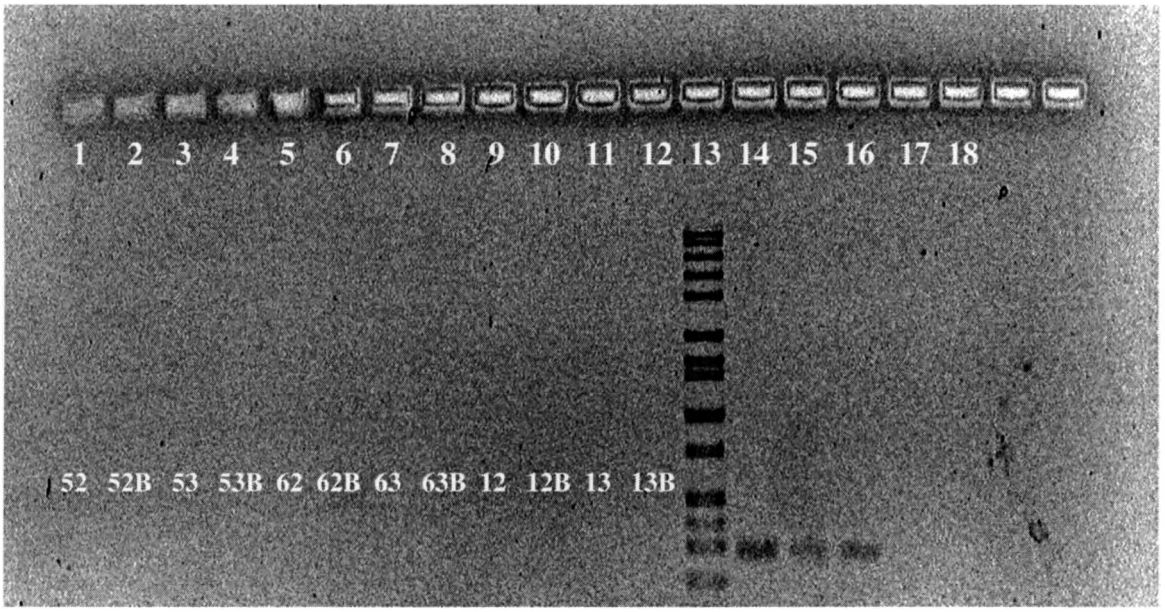

Figure 15: PCR product from the ITS2F and DGGE Agro ITSR primer set created from ITS sequence data for *Agrobacterium* sp. ATCC 31529 (Table 3). Lanes 1-12 are labeled with the sample number (Table 2). Lane 13 is HI LO DNA ladder (Minnesota Molecular Inc., Minnesota, MN). Lane 14 is a pure culture of *Agrobacterium* sp. Lane 15 and 16 are samples 52 and 52B with *Agrobacterium* sp tDNA added. Lane 17 is *E. coli* and 18 is a PCR no template control. PCR product was electrophoresed on a 1.25% agarose gel and visualized by ethidium bromide staining under UV light.

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