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Development and Molecular Characterization of Microbial Inocula for Initiation of Graywater Waste Processing Systems on Long-Term Space Flights

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ABSTRACT

Microorganisms will be an integral part of biologically based waste processing systems used for water purification or nutrient recycling on space flights. Establishment of these systems with a defined group of microorganisms will provide a standardized means for conferring specific properties to the system. Phylogenetic analysis of 16S rDNA sequences from a clonal library of organisms from a graywater-degrading rhizosphere community suggested that members of the Cytophagales and Proteobacter phylogenetic groups dominated. A clonal library of organisms from an industrial wastewater treatment plant (WWTP) was more diverse and consisted of organisms from more phylogenetic groups. This analysis provided the basis for selection of organisms for use in a defined, constructed community and for selection of a source of an undefined, complex microbial inoculum. The constructed community and the sludge inoculum were inoculated into a model plant rhizosphere waste processing system to evaluate survival. Based on plant, microbiological and molecular biological measures, it appeared that both inoculated communities were able to become established and persist in this model wastewater processing system.

INTRODUCTION

The National Aeronautics and Space Administration (NASA) has long-term plans to undertake a manned mission to Mars. The mission would likely consist of a crew of 6 and would include a transit time of 360 days total and a surface stay of 600 days (15). Biological life support systems (BLSS) can be used on missions of this duration for food, water, and oxygen production and for waste processing (6, 41). BLSS, in contrast to physiochemical systems, are conducive to a sustainable environment, providing a measure of self-sufficiency, lowering the cost of re-supply and reducing dependence on technology. In a closed environment, plant-based BLSS would provide food and oxygen, and remove carbon dioxide. The same system can be used for remediation of graywater (non-toilet wastewater). It is estimated that less than 10% of plants grown for food would be needed for processing 100% of crew wastewater (27). It should, therefore, be possible to incorporate wastewater processing into hydroponic plant production to recycle water for non-hygienic uses on long-term space missions.

Microorganisms will be an integral component of any BLSS used for plant growth or waste processing. Microorganisms thrive in association with hydroponically grown plant roots and are found in concentrations as high as 10^{11} cells g^{-1} dry wt (17). Microorganisms in a closed system can have positive and potentially negative implications. The microorganisms in the plant root zone (rhizosphere) transform, mineralize or sequester organic or inorganic compounds while the plants purify the water through plant transpiration and provide exudates which supply nutrients to the rhizosphere population (7, 19). Plant-microbe interactions can be used for processing wastes, for recycling nutrients, and for inhibiting plant and human pathogens. However, these systems may also serve as a reservoir for pathogenic microbes (33). This is problematic because both the US and Russian space programs have found that astronauts exhibit changes in the immune system with long-duration space travel (42, 43). Therefore, common human associated organisms, not problematic under normal circumstances, may become potential human pathogens for immuno-compromised crewmembers on long-term missions. Furthermore, the turbulence and rapid flow of liquid through a recirculating hydroponic system could result in the spread of plant or human pathogens very rapidly (i.e., contamination of a 200 L system would occur less than 13 minutes) (44). For these reasons, it will be important to characterize and manage the microbial populations within these systems to obviate any negative interactions and to optimize the positive functions of microbial communities in BLSS.

16S rDNA sequence analysis has provided unique new insight into the breadth of microbial diversity in complex environmental ecosystems. This sequence is widely used for bacterial characterization because of the presence of highly conserved regions interspersed with variable regions that

facilitate the design of primers and probes for analysis of bacterial phylogeny at different levels of specificity (i.e. family, genus or species level) (2, 21). Bacterial identification is achieved by comparison of unknown clonal 16S rDNA sequences to sequences from known organisms deposited into public databases. In contrast to the 16S rRNA genes, the intergenic spacer region (ISR) located between the 16S and 23S rRNA genes exhibits much greater sequence diversity and length variation (21). Size and sequence divergence within the ISR has been used to characterize complex bacterial communities from soil and water samples through community profiling (9, 16). Using rDNA intergenic spacer analysis (RISA), the 16S-23S rDNA ISR of a broad range of organisms can be amplified from a mixed community sample in one reaction. Community profiles are obtained by gel electrophoresis of PCR products from mixed community DNA samples. The result is a banding pattern that corresponds to organisms within the sample that exhibit ISR sequence heterogeneities (1, 9). Community analysis in this manner provides a means for evaluating community shifts through temporal or environmental changes.

Defined microbial inocula have been used extensively to improve plant growth through enhanced nutrient uptake, production of plant growth promoting hormones or control of plant pathogens (24, 47). However, a lack of consistency and efficiency of results in field application has limited the widespread use of such organisms. A major goal in rhizosphere studies, therefore, has been to evaluate strategies to introduce or manipulate rhizosphere flora to improve survival of beneficial organisms or suppress deleterious ones. This is of direct importance for application to closed environmental systems, since microbial inocula will likely be used to establish any biological waste processing system used on long-term space missions. To assure safe, effective, reliable functioning of inoculated systems, it will be necessary to gain better understanding and control of the complex microbial interactions occurring within these systems. The aim of this study was to determine the capacity of constructed and undefined inocula to establish stable, functional rhizosphere communities for graywater treatment. Using molecular approaches including 16S rDNA sequence analysis, population composition of microbial communities from both an existing rhizosphere graywater treatment system and from an industrial wastewater treatment plant (WWTP) were determined and used to develop inocula to establish a new wheat rhizosphere-based treatment system. The results of these studies demonstrated that complex microbial inocula of differing diversity could successfully be maintained and demonstrated functional efficacy in the rhizosphere.

MATERIALS AND METHODS

EXTRACTION OF DNA FROM GRAYWATER RHIZOSPHERE COMMUNITY

Samples for molecular analysis were taken from an on-going plant growth study at Kennedy Space Center, FL (KSC). Briefly, wheat (*Triticum aestivum* L. cv. Apogee) was grown for 70 days in a 1.8 X 2.4 m, walk-in growth chamber using a recirculating nutrient film technique (19). Nutrient solution and surfactant (Igepon TC-42) were supplied to the plants as previously described for pulse surfactant addition (19). Suspensions of rhizosphere organisms were made by shaking rhizosphere sections (~ 1 cm x 1 cm) for 2 min in filter sterilized nutrient solution and glass beads (3 mm dia, Fisher Scientific, St. Louis MO). The resulting microbial suspension (15 ml) was centrifuged at 14,000 x G for 20 min, the supernatant decanted, and genomic DNA extracted using the FastDNA kit (Bio 101, Vista, CA). This DNA was used to construct a clonal library of 16S rDNA sequences from the graywater rhizosphere community.

ISOLATION OF CULTURABLE ORGANISMS FROM GRAYWATER RHIZOSPHERE COMMUNITY

A separate portion of the rhizosphere suspension was serially diluted and plated onto R2A agar (Difco, Detroit, MI). Surfactant degrading organisms were selected for by serial dilution and spread plating onto minimal salts media containing Igepon (750mg/l) as the sole carbon source prepared as described by Garland et al. (19). Cytophaga-Flavobacterium-Bacteroides (CFB) group isolates were obtained by plating on Dubos media (1.0g K_2HPO4, 0.5g KCl, 0.5g $MgSO_4 \cdot 7H_20$, 0.5g $NaNO_3$, and 0.01g $FeSO_4 \cdot 7H_20$, pH 7.2) or Dubos media with 1.0g casein or with ground Whatman #1 filter paper. Less dominant organisms were isolated using enrichment cultures in rhizosphere media (minimial salts media with rhizosphere solution extracted from 24 plants and having an electrical conductivity of 186 µS/cm and a pH of 7.0). Isolates were frozen at -80ºC in TSB with 15% glycerol. Organisms from the constructed community were tested for growth on surfactant using PAS media (8) with 0.2% polyoxyethylene 10 lauryl ether (Pol; Sigma, St. Louis MO) added as a carbon source.

EXTRACTION OF DNA FROM AN INDUSTRIAL WWTP COMMUNITY

Samples were collected from an industrial WWTP in July 2001. Genomic DNA was extracted from 2ml of WWTP sludge using the FastDNA™ Kit (BIO 101, Vista, CA) as previously described (14).

PREPARATION OF CLONAL LIBRARIES OF WASTE TREATMENT SYSTEM ORGANISMS

Clonal libraries were created by PCR amplification of mixed-community genomic DNA extract using primers specific for conserved sequences within the bacterial 16S rDNA and 23S rDNA. 16S-23S rDNA ISR sequences, including 500bp of the 16S rDNA sequence, from the mixed community were amplified using Ready-To-Go-PCR Beads (Amersham Pharmacia, Piscataway, NJ), with 800 nM primers, and 5-10 ng template DNA. The PCR primers were 1055f (5'ATG GCT GTC GTC AGC T 3'; (2) and 23Sr (5'GGG TTB CCC CAT TCR G 3'; (9). The touchdown PCR program used for 16S-23S rDNA ISR analysis was as follows: 5 min at 94ºC, then 10 cycles consisting of 15 s at 94ºC, 45 s at 65ºC, followed by a 1ºC decrease for each of the 10 cycles, and 60 s at 72ºC, followed by an additional 20 cycles of 15 s at 94ºC, 45 s at 55ºC, and 60 s at 72ºC and a final cycle of 60 s at 72ºC. PCR

products were cloned into the pCR®2.1-topo vector according to manufacturers instructions (TA vector; Invitrogen, Carlsbad, California). Alkaline lysis plasmid preparations were made from cultures of randomly selected clones from the industrial WWTP library that were grown in 50 ml of LB broth (Difco) with kanamycin (50µg/ml). Clones from the industrial WWTP were designated TED701 followed by a clone number (01 to 150).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) SCREENING OF CLONES AND ISOLATES FROM THE GRAYWATER RHIZOSPHERE LIBARARY

Plasmid DNA from randomly selected 16S-23S rDNA ISR clones from the graywater rhizosphere community and from cloned 16S-23S rDNA sequences from isolates was obtained using the RPM AFS kit (Bio 101). To discriminate between similar groups of clones or isolates, 16S-23S rDNA PCR products were subjected to RFLP analysis using two different four-base cutting restriction enzymes, *Hha*I and *Rsa*I (0.5U/µl; Promega, Madison, WI) and 1X compatible buffer (Buffer C). Digests were incubated overnight at 37ºC, products were electrophoresed in 4% agarose gels in 0.5X TBE, and visualized by UV transillumination after staining with ethidium bromide. Based on RFLP pattern analysis, similar isolates from the graywater rhizosphere sample and similar clones from the GWR999 clonal library were grouped and selected representatives from each RFLP-ISR group (from 1 to 5 depending on the number of similar genotypes in the group) were sequenced. Clones from the graywater rhizosphere library were designated GWR999 followed by a clone number (01 to 40).

SEQUENCE ANALYSIS

Randomly selected clones from the WWTP library were characterized using phylogenetic information obtained from sequencing 500 bp of the 16S rRNA using the 1055f primer. Selected clones from the graywater rhizosphere clonal library and isolates from the same system were sequenced using the 1492r primer (5'TAC GGY TAC CTT GTT ACG ACT T3'; (25). All sequencing was performed at the University of Tennessee Molecular Biology Resource Facility using an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, CA). Sequences of known phylogeny were obtained from GenBank. A phylogenetic tree was constructed using Clustal W (45) by distance matrix analysis and the neighbor-joining method (40). Bootstrap analysis was used to provide statistical confidence for the tree branch points. Phylogenetic trees were displayed using TREEVIEW (35).

PLANT CULTURAL TECHNIQUES

Wheat (*Triticum aestivum* L. cv. Apogee) was grown in a reach-in growth chamber (EGC, Inc. Chargin Falls, OH). Lighting was provided by very high output (VHO) daylight fluorescent lamps (Lucolux; General Electric Co., Cleveland, OH) set for a 20-h light/4-h dark photoperiod, and a constant temperature of 22ºC. Relative humidity (RH) was maintained at 70% for the duration of the study. Prior to sterilization, wheat seeds were wrapped in moist paper towels and refrigerated at 4ºC for 24 h. The imbibed wheat seeds were surface sterilized using a combination of mercuric chloride and hydroxylamine hydrochloride as previously described (4). Surface sterilized seeds were placed on filter paper (Whatman qualitative, 10cm) moistened with sterile distilled water in 100 mm glass petri dishes. The seeds were incubated in the reach-in chamber for 6 days in conditions described above. After 6 days, seedlings were randomly selected and placed into slits in autoclaved foam plugs which were then added to fitted holes in the lids of four liter wide-mouth plastic containers. The containers had filter sterilized, modified Hoaglands nutrient solution (19) with 5mM MES (Sigma, St. Louis MO) (32) added to maintain pH at or around 5.5 for the duration of the experiment. Nylon wicks were used to conduct nutrient solution to the seedlings until the roots reached the solution. Plants for the surfactant gradient study were harvested on day 29. Two separate plant growth studies were performed to evaluate treatment effects. Plants for the first study were harvested on day 28. Plants for the second study were harvested on day 21.

ANALYSIS OF SURFACTANT EFFECTS ON PLANT GROWTH

The nonionic surfactant polyoxyethylene 10 lauryl ether (Pol), an alkylethoxylate, was added to plant nutrient solution, to evaluate the effect of surfactant concentration on plant and microbial populations. Surfactant was added to four replicate containers at a final concentration of 0 ppm, 2 ppm, 10 ppm, 20 ppm, 100 ppm or 200 ppm. For each treatment, surfactant was added 2 days after the beginning of the experiment and every other day until the end of the experiment. Industrial WWTP inoculum (1 ml) was added to four replicate containers 7 days after planting. The inoculum for the undefined, industrial WWTP community was prepared by freezing 0.5 ml aliquots of the sludge with 1 ml of 40% glycerol at –80ºC.

EVALUATION OF INOCULUM SURVIVAL

The ability of the inocula to establish and persist in the root zone was evaluated in two separate studies. For the first inoculum study, Pol was added to each plant growth vessel at a final concentration of 1000 ppm. For the second inoculum analysis study, Pol was added to each plant growth vessel at a final concentration of 20 ppm. For each experiment, surfactant was added 2 days after the beginning of the experiment and every other day until the end of the experiment. Two different inocula (constructed community and sludge) and an uninoculated control were used to initiate the model plant-based graywater processing system.

Isolates from the constructed community were grown up separately on rhizosphere media to the same OD_{600} (approximately 0.75), each culture was frozen in 20% glycerol at -80ºC. Each member of the constructed community (0.5ml) was added separately to the nutrient solution of four replicate containers on day 7 of the experiment. Industrial WWTP sludge (1.0 mL) treatments were inoculated at the same time.

The plants for the uninoculated treatment received no inoculum and contained only contaminant species.

COMMUNITY-LEVEL MOLECULAR PROFILING OF INOCULA ESTABLISHMENT

Suspensions of rhizosphere organisms were prepared from sections of root mat taken from each treatment on days 0, 14 and 28 of the first inoculum study. Bacterial cells were pelleted (from 10-15mL suspensions) by centrifugation (4,500 x g for 15 min), mixed-community DNA was extracted using the FastDNA™ kit (Bio 101), and 16S-23S rDNA ISR sequences were PCR amplified as described above. Community profiles were obtained by gel electrophoresis of 16S-23S rDNA PCR product mixtures in 2% agarose gels.

PLANT AND MICROBIOLOGICAL ANALYSIS

Suspensions of rhizosphere organisms were prepared from sections of root mat from each treatment in the second inoculum study. The rhizosphere samples were shaken for 2 min in filter sterilized nutrient solution and glass beads (3 mm dia, Fisher Scientific, St. Louis MO). The resulting solution was serially diluted and plated onto R2A and CFU were enumerated after incubation for 72 h at 28ºC. Wheat root and shoot dry weight was determined after plant material was dried at 70ºC for 72 h.

RESULTS

ANALYSIS OF 16S rDNA SEQUENCES

Graywater rhizosphere clonal library

A clonal library of 16S rDNA sequences from a graywater rhizosphere population was aligned and placed into a phylogenetic tree along with sequences obtained from GenBank (Fig. 1). The graywater rhizosphere 16S-23S rDNA clonal library was dominated by members of the *Cytophaga-Flavobacter* cluster (CF) of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) division (60%). These clones fell into two large groups containing 10 and 9 clones, respectively, one more closely related to *Chryseobacterium* sp. and the second to *Cytophaga* sp. The second largest group of sequenced clones came from the gamma subdivision of proteobacteria (26%) followed by the beta and alpha subdivisions of proteobacteria (Fig. 1).

Graywater rhizosphere isolates

16S rDNA sequences from graywater rhizosphere isolates (45 total) revealed significant differences from sequences from 16S rDNA clones from the graywater rhizosphere library. In fact, only 5 rhizosphere isolates had 16S rDNA sequences that matched closely to those from the graywater rhizosphere clonal library (Fig. 1). Two of the isolates were from the beta subdivison of proteobacteria (*Ralstonia* and *Burkholderia*) and three pure cultures were from the gamma subdivison of proteobacteria (*Xanthamonas*, *Pseudomonas* and *Frateuria*). These isolates were used in the constructed community (designated Constructed 2, 3, 7,10, and 5, respectively in Fig. 1). Of these isolates, only *Ralstonia* and *Frateuria* were 100% matches to the respective clone. None of the sequenced isolates matched closely to the CF group clones from the graywater rhizosphere library. However, one isolate matching most closely to the *Chryseobacterium* sp of the CF group was isolated on selective media (Dubos media with casein) This isolate is designated Constructed 8 in Fig. 1. *Cytophaga hutchinsonii* **(**ATCC 33406**;** designated Constructed 9**)** was obtained from the American Type Culture Collection and grouped with the large *Cytophaga* sp. portion of the CF group. Eleven isolates were used in the final constructed community (Fig. 1 and Table 1).

Industrial WWTP clonal library

A total of 65 16S rDNA clonal sequences from the industrial WWTP library were classified into subdivisions by BLAST analysis against GenBank sequences. The library was diversewith representatives from the *Sphingobacteria*, CFB and *Nitrospira* groups and from the alpha, beta and delta subdivisions of proteobacteria (Fig. 2). No clones from the gamma proteobacteria were identified in this library. Not surprisingly, over 35% of the clones had greater than 95%

Table1. Isolates Included in the Constructed Community, Their Phylogenetic Placement and Ability to Degrade Surfactant

Designation	Identification[a]	Phylogenetic Placement	Surfactant Degradation
1	*Rhizobium leguminosarum* (U29388)(99%)	Proteobacteria, Alpha subdivision	-
2	*Ralstonia eutropha* (AB015605)(99%)	Proteobacteria, Beta subdivision	+
3	*Burkholderia cepacia* (U96927)(98%)	Proteobacteria, Beta subdivision	+
4	*Aquaspirillum metamorphum* (Y18618)(98%)	Proteobacteria, Beta subdivision	-
5	*Frateuria aerantia* (AJ010481)(95%)	Proteobacteria, Gamma subdivision	+
7	*Xanthomonas axonopodis* (AF123090)(99%)	Proteobacteria, Gamma subdivision	+
10	*Pseudomonas putida* (AE016774)(99%)	Proteobacteria, Gamma subdivision	+
8	*Chryseobacterium joosteii* (AJ271010)(97%)	Flavobacteriaceae, Chryseobacterium	-
9	*Flexibacter sp* (AF361187)(94%)	Flexibacteraceae, Flexibacter	+
11	*Cytophaga hutchinsonii* (M58768)(98%)	Flexibacteraceae, Cytophaga	ND
6	*Paenibacillus amylolyticus* (D85396)(100%)	Firmicutes, Bacillales	ND

[a]Closest match from GenBank (Accession number)(%16S rDNA sequence similarity)

ND, not determined

matches to previously sequenced clones from the same industrial WWTP system (26), (A. Layton unpublished data).

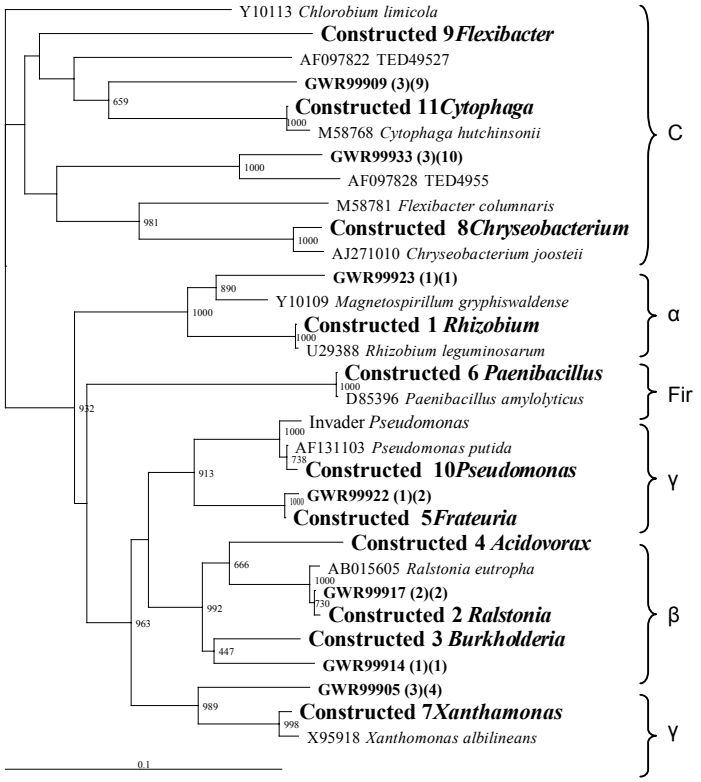

Figure 1. Phylogenetic tree showing the relationship between graywater rhizosphere clone library sequences (designated GWR999) and isolates chosen for use in the constructed community (designated Constructed 1-11). Clones are shown in boldface. Isolates used in the constructed community are shown enlarged and in boldface. For Graywater clones, numbers in the first parentheses represent of the number sequenced clones, while numbers in the second parentheses represent the number of clones in ISR-RFLP group. The GenBank accession number of reference strains used for alignment are given before the strain name. Bootstrap values per 1,000 replicates of bootstrap analyses are presented for values greater than 445. CF= Cytophaga-Flavobacter group; Fir = Firmicutes

PLANT GROWTH EXPERIMENTS

Surfactant gradient study

The nonionic surfactant Pol, an alkylethoxylate, was added to four replicate plant containers in 6 different concentrations (0ppm-200ppm). The effect of the surfactant on plant root dry weight and culturable cell numbers was evaluated 29 days after planting (Fig. 3). ANOVA indicated a signification effect of surfactant concentration on root dry weight only at 100ppm or 200 ppm surfactant ($P < 0.05$). However, culturable cells significantly increased at surfactant concentrations of 10 ppm or greater ($P < 0.05$). Based on this analysis, 20 ppm Pol was used in studies to evaluate survival of different inocula in the root zone.

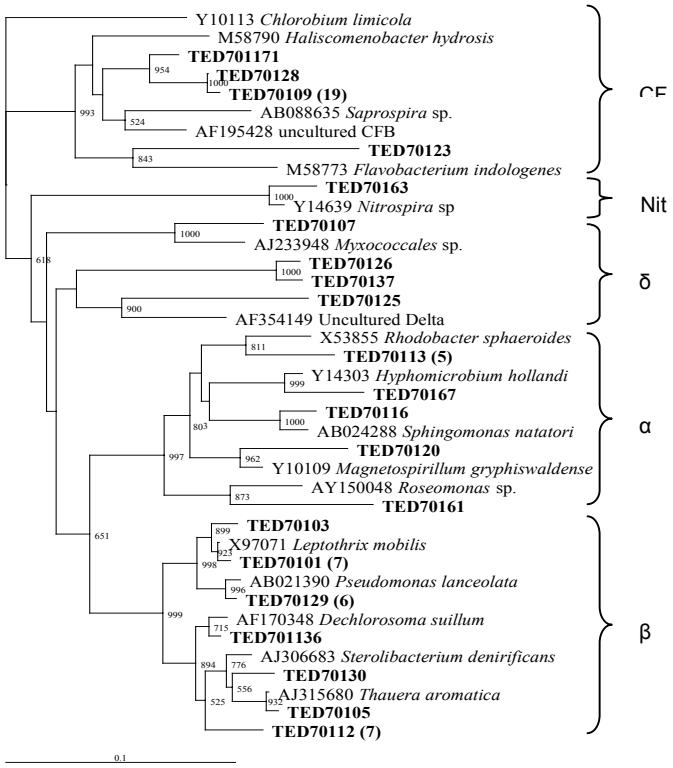

Figure 2. Phylogenetic tree showing the relationship of 65 clones from the industrial WWTP sludge library, aligned with reference strains from the domain Bacteria based on 16S rDNA sequences. Clones from the TED701 library are shown in boldface. The GenBank accession number of reference strains used for alignment are given before the strain name. Bootstrap values per 1,000 replicates of bootstrap analyses are presented for values greater than 500. CF= Cytophaga-Flavobacter group; N = Nitrospira

Inocula Analysis

Three different inocula were used to establish model plant-based graywater processing systems in two separate studies. The lowest complexity treatment (designated as Uninoculated) received no inoculum and therefore consisted of contaminant species. However, culturable cell density in the Uninoculated treatment was approximately the same as that of other treatments (Fig. 4B). The defined community (designated as Constructed) consisted of the eleven isolates, similar in structure and function to clones from the graywater rhizosphere library (Table 1). The industrial WWTP inoculum (designated as Sludge) was considered to be the most diverse based on 16S rDNA analysis. In one study, the plants received 20 ppm pol and plant root dry weight and culturable cells

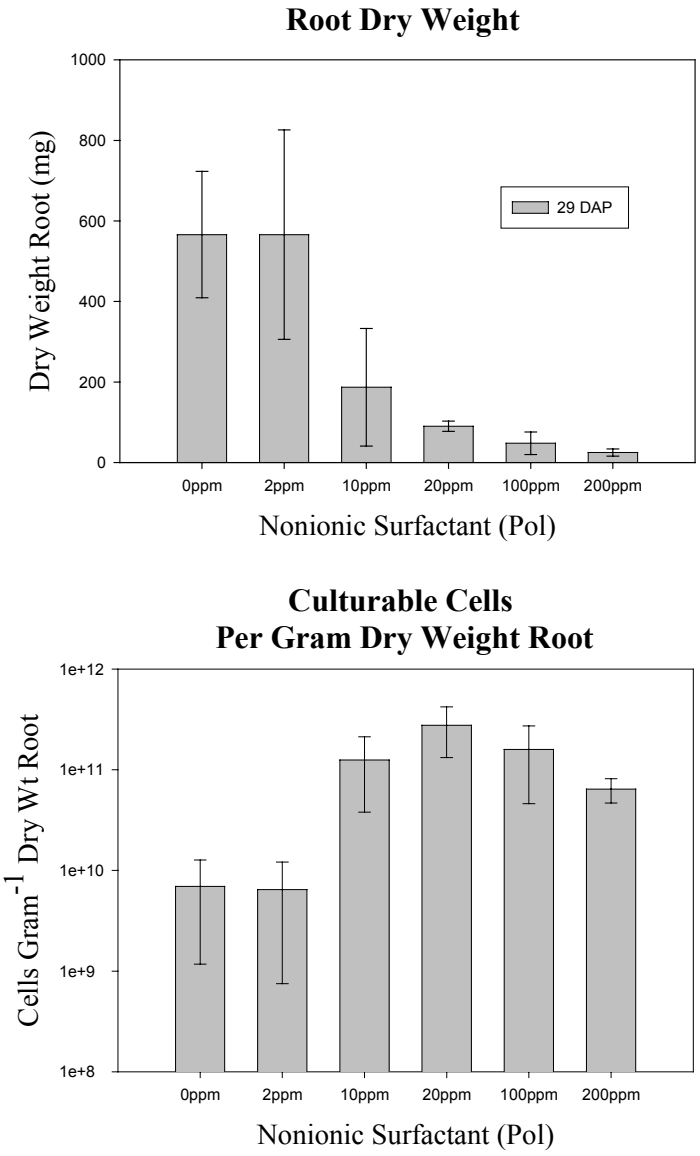

Figure 3. 3A, mg dry weight or 3B, culturable cell numbers for wheat plants grown in different concentrations of surfactant (Pol) from 0 ppm to 200 ppm. DAP, Days after planting. Error bars represent the standard deviation of four replicates.

numbers were measured from each of the plant treatments 21 days after planting (Fig. 4A and Fig. 4B). ANOVA indicates that root dry weight in treatments that did not receive Pol were significantly higher than the Sludge treatment that did receive Pol ($P < 0.05$), and root dry weight in the Constructed treatment with no added surfactant was significantly higher than in any of the surfactant treatments ($P < 0.05$). Although not significant at the $P = 0.05$ level, in general the root dry weight of plants that did not receive surfactant was higher than that of plants that did receive Pol ($P < 0.1$). There were no significant differences in culturable cell numbers in any of the treatments ($P < 0.05$).

In the other study, 1000 ppm Pol was used to simulate graywater addition. This concentration of Pol was toxic to the plant roots (root dry weight < 40mg g^{-1}), however molecular analysis using the RISA approach showed that microorganisms in the root zone persisted throughout the experiment. The RISA approach was used to evaluate differences between communities before and 14, and 28 days after inoculation into the model plant-based wastewater processing system (Fig. 5). Comparison of community profiles for the constructed community and the sludge community from before inoculation to the end of the study at 28 days shows that organisms from both communities persisted for the duration of the study. Surfactant addition had a more apparent affect on the constructed community structure, based on the community profile in Lanes 5 and 6, than on the sludge community, Lanes 9 and 10.

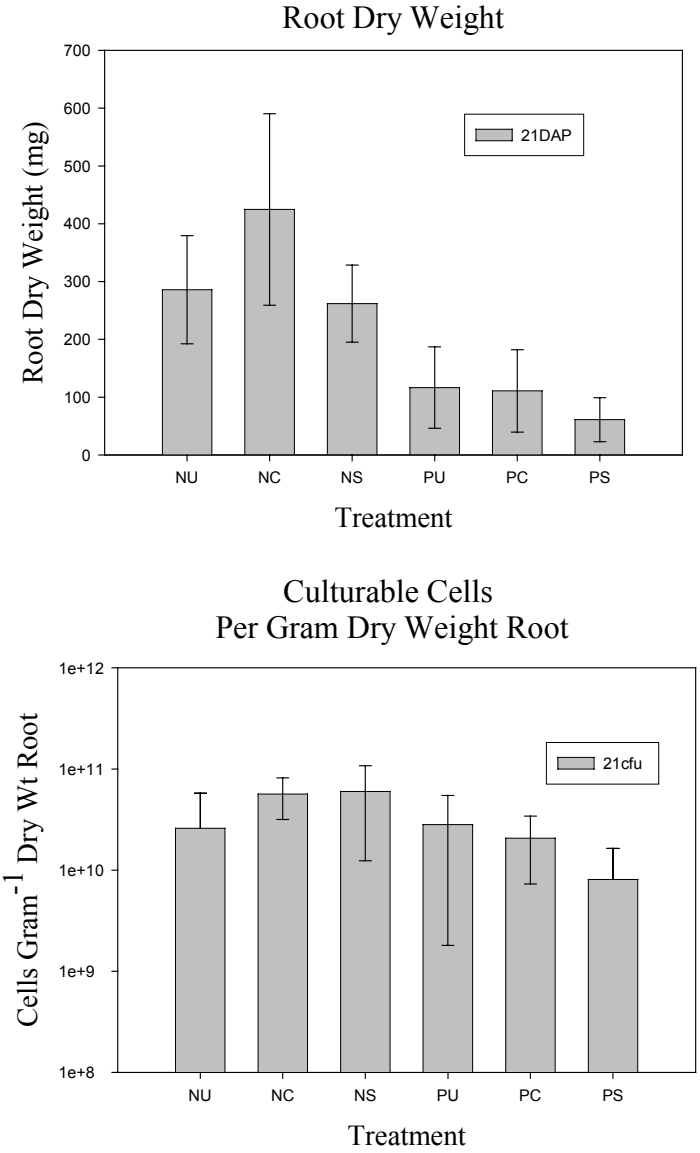

Figure 4. 4A, mg root dry weight or 4B, culturable cell numbers for wheat plants grown with 20 ppm Pol and different iocula where N, No Pol; P, Pol; C, Constructed; S, Sludge; U, Uninoculated; DAP, Days After Planting. Error bars represent the standard deviation of four replicates.

DISCUSSION

16S rDNA sequence analysis was used to describe the composition of a graywater rhizosphere community and an industrial wastewater community. The graywater rhizosphere library was dominated by members of the *Cytophaga-*

Flavobacter (CF) cluster of the CFB division (Fig.1). The rest of the clones from the graywater rhizosphere library were distributed among the common groups of rhizosphere organisms included in the alpha, beta and gamma subdivisions of the proteobacteria. Evaluation of the composition of the graywater rhizosphere library was used as the basis for selection of the eleven isolates included in the constructed community. Isolates used in the constructed community were, for the most part, not identical to clones from the library, however, selected organisms did fill functional niches associated with the major clonal groups from the graywater rhizosphere population. Characterization of 16S rDNA clones from the industrial WWTP library showed that, as expected, this community was more diverse, but consisted of fewer rhizosphere-specific species (Fig. 2). Alpha and beta proteobacteria were abundant in the TED701 sludge library. The sludge library also contained a large number of clones from the CF cluster within the CFB division. No clones from the gamma subdivision of proteobacteria were identified in the industrial WWTP library. The industrial WWTP sludge was used as a source of inoculum because of the extensive diversity and expected functional versatility of species from the community. This population contains species similar to clones present in the graywater rhizosphere library and should be pathogen-free since no human wastes pass through the industrial wastewater.

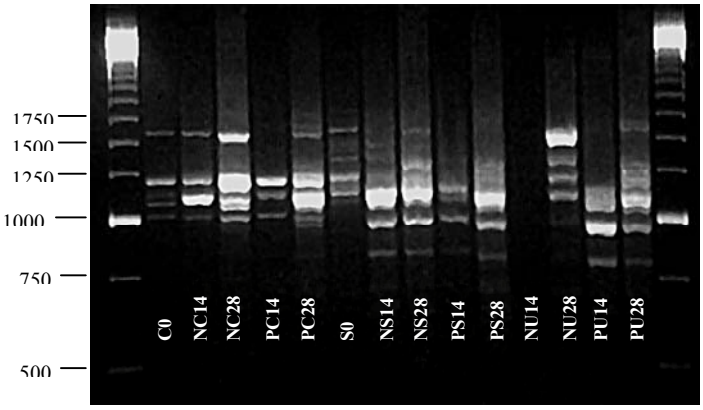

Figure 5. 16S-23S rDNA ISR community profiles for the graywater rhizosphere inoculum treatments. N, No Pol; P, Pol; C, Constructed; S, Sludge; U, Uninoculated; 0, Before Addition; 14 Days After Planting; 28 Days After Planting

The graywater rhizosphere library was dominated (60%) by members of the *Cytophaga-Flavobacter* cluster of the CFB division (Fig. 2). The CFB are a large and diverse group of free-living bacteria commonly found in terrestrial environments, including rhizosphere soils (23, 28, 38). Members of the CFB are often found in association with particulate matter and degrade diverse macromolecules including cellulose, chitin, N-acetylglucosamine, protein and starch (13, 22, 38). This evidence suggests that the CF group from the graywater population may be living on plant debris and algal or bacterial biomass present in the rhizosphere system. Despite attempts to select or enrich for corresponding CF group isolates from the graywater rhizosphere system, no isolate was recovered which closely matched clones from the dominant groups. Chelius et al. (12) also found that isolates from the CF group were significantly different than uncultured clones. Therefore, to approximate the CF group from the library, two isolates were obtained that were similar to species within these groups. One member of the constructed community (*Cytophaga hutchinsonii*) was obtained from ATCC and another, a *Chryseobacterium* isolate, was isolated on selective media. *Chryseobacterium* is a common rhizosphere isolate and has been shown to dominate early in plant growth (28). A large number of clones from the sludge library also fell into the CF cluster within the CFB division. Clones from the CFB division are common in this sludge. In fact, the GenBank sequence matches with the highest similarity to the two large CF-group clones from the graywater rhizosphere library, unidentified clone 4955 (Accession AF097828) and unidentified clone 49527 (Accession AF097822) came from the industrial WWTP sludge used in these studies (Fig.1). Although, these two clones were not part of the TED701 library, other CF division clones were identified, suggesting that organisms from this group may potentially function in similar roles (degradation of plant debris and algal or bacterial biomass) upon inoculation into the graywater rhizosphere system.

The remainder of clones from the graywater rhizosphere library are distributed among the common groups of rhizosphere organisms included in the alpha (3%), beta (9%) and gamma (26%) subdivisions of the proteobacteria including members of the *Pseudomonas*, *Rhizobium*, *Xanthamonas*, and *Burkholderia* genera (3, 28, 31). In the graywater rhizosphere library, gamma proteobacteria were the second most dominant members of the graywater rhizosphere library (Fig. 1). *Pseudomonas* sp., in particular frequently dominate in hydroponic systems, especially in the case of hydroponic systems using recycled nutrient solutions (5, 44). Pseudomonads are used as inocula in rhizosphere systems to control bacterial and fungal plant pathogens through competitive mechanisms including antiobiosis and siderophore production (36, 47). These species are competitive colonizers and have been shown to survive for long periods of time following inoculation in competition with indigenous species (31, 39). Three gamma proteobacteria were included in the constructed community, including a *Pseudomonas* sp., a *Xanthanomas* sp., and a *Frateuria* sp. (Table 1). Although alpha proteobacteria often dominate rhizosphere soils as measured by 16S rDNA sequence analysis and common cultural methods, our library had only one clone in this subdivision (Fig. 1). We were able to isolate a *Rhizobium* sp and this organism was included in the constructed community. Three beta proteobacteria were included in the consortium, an *Acidovorax* sp., a *Ralstonia* sp., and a *Burkholderia* sp. These are ubiquitous rhizosphere organisms that most likely thrive on amino aids and organic acids in rhizosphere exudates (3, 13, 47). Alpha and beta proteobacteria were abundant in the TED701 sludge library. The alpha proteobacteria, in particular have been shown to dominate in this sludge (26). The alpha proteobacterial cluster had one clone, *Magnetospirillum* sp., which was also found in the graywater rhizosphere library. The beta proteobacterial cluster consisted of clones related to common sludge and soil organisms (i.e., *Thauera* sp., *Dechlorosoma* sp. and *Leptothrix* sp.). One group of clones clustered with the delta

proteobacterium in the *Mycoccales* genus. The lack of clones from the gamma subdivision was not surprising since the occurrence of gamma proteobacteria varies in this industrial WWTP sludge depending on plant conditions and season (26). Given the diversity and versatility of the sludge population it is unlikely that the lack of gamma proteobacteria will significantly impact the stability of this community in the plant rhizosphere. This is supported by the molecular community analysis and culturable plate counts which suggest that high concentrations of organisms from the sludge community were able to become established and persist in the rhizosphere of these plants (Fig. 4B and Fig. 5).

Two groups of common rhizosphere organisms were not represented in the rhizosphere library, the *Bacillus* sp. and the *Actinomycetes* sp. (28, 31). These species are most common in soil systems and in the soil around the rhizosphere, presumably because of the general continuum of microbial species from soil to rhizosphere (28). In hydroponically grown plants, these species are not as common (5, 18, 44) and this may be due to the lack of contact with soil-based organisms. This difference suggests that the *Bacillus* and *Actinomycetes* represent "seeded" soil-based organisms found in association with rhizosphere due to the fastidious and competitive nature of the species. A *Paenibacillus* sp. was isolated from a rhizosphere sample and was included in the constructed community because of the ubiquity of *Bacillus* species in rhizosphere and because *Paenibacillus* sp. have been shown to have plant-growth promoting properties (47).

Results from the surfactant study suggest that surfactant concentration can have a potentially negative impact on the root mass (Fig. 3A). This is due to the fact that surfactants are surface-active agents that may increase the permeability of the plant cell wall causing root leakage. Exudate leakage may have occurred in these experiments since culturable cell numbers increased significantly when the surfactant concentration was greater than 10 ppm, presumably in response to increased nutrient availability from rhizosphere exudates (Fig. 3B). To optimize the amount of surfactant added to the system (to more closely approximate the amount of surfactant that might be added with graywater treament; (19), but avoid potentially detrimental effects to the plant root zone (Fig 4A), 20ppm Pol was added to the rhizosphere of plants used in the inocula analysis studies. Many of the isolates in the constructed community are anticipated to degrade surfactant (Table 1). Diverse surfactant-degrading isolates were included in the constructed community for two reasons 1) the surfactant is anticipated to be the major carbon input from graywater addition and 2) research suggests that microbial consortia are often required to effectively and completely degrade surfactants (19, 46). The inclusion of at least 6 isolates capable of growth on surfactant media should increase the likelihood of achieving complete degradation of the surfactant in situ thereby reducing the potential for negative side-effects from surfactant accumulation (10, 20, 27). However, results from these preliminary experiments suggest that surfactant was able to accumulate and to affect both root dry weight and the concentration and structure of the constructed community inoculum (Fig. 4A and 4B and Fig. 5). Nonionic surfactants such as Pol are used in a variety of industrial processes (including pulp and paper manufacturing, textiles, rubber, and plastics), and surfactant degrading organisms (including *P. fluorescens*, *P. aeruginosa*) have been isolated from wastewater treatment systems (29, 46). Therefore, it is possible that some surfactant degraders are present in the industrial sludge inoculum. However, the roots of plants inoculated with the sludge community were also impacted by the surfactant addition (Fig. 4A), suggesting that the sludge community was either not enriched for surfactant degraders and/or did not have enough time in the 21 day study to completely degrade the surfactant.

In the inoculum analysis studies, the uninoculated treatment received no inoculum but was apparently colonized by contaminant species (Fig. 4B). Morales et al. (33) also found that contaminant species readily colonized the root of axenic plants. They speculate that the contamination occurs either during planting of seedlings in the plant growth vessels or through humidifier water which pools on the surface of the plant and the plant growth vessel. A limited analysis of organisms present in uninoulated treatments from other studies has shown a predominance of *Enterobacter* and *Pseudomonas* species (K. Cook, unpublished data). Other researchers have found that both species are early rhizosphere colonizers with *Enterobacter* sp. often dominant in the endorhiza, suggesting that some portion of the contaminant may be present inside the seed cover and unaffected by seed sterilization (28). Results from this treatment were highly variable with plate counts and molecular analysis showing unpredictable colonization patterns (Figs 5B and 6).

Although the constructed community and the sludge community are of different overall complexity in terms of the numbers and kinds of different organisms, both of these populations consist of diverse species from multiple bacterial genera and preliminary data from these studies suggest that the inocula were able to become established and persist in the rhizosphere for up to 28 days (Fig. 5). This approach was used to select inocula, based on results that suggest that inoculant mixtures (rather than single species) added to the rhizosphere for purposes of biocontrol have been the most successful in terms of long-term survival and overall ability to effect changes in rhizosphere structure and plant growth (11, 37). The use of complex mixtures of organisms is also supported by general ecological theory which suggests that more diverse populations are also more stable (30, 34). Therefore, the most prudent means of achieving the ultimate goals of reliable, effective and productive functioning of inoculants may be to approximate the structure of indigenious populations to obtain benefits from naturally acquired traits of the organism and to permit species interactions as well as functional redundancy.

CONCLUSION

Molecular techniques, including 16S rDNA sequence analysis enabled characaterization of two wastewater treatment communities. Clones from 16S rDNA libraries from a graywater rhizosphere community and an industrial WWTP community were diverse with species from 4 or more genera illustrating functional and structural diversity of such systems. The eleven isolates included in the constructed community

were not identical to clones from the graywater rhizosphere library, but did fill functional niches associated with the major clonal groups from the graywater rhizosphere population. Establishment of graywater processing rhizosphere systems using inocula from the constructed community and the industrial WWTP demonstrated that complex microbial inocula of differing diversity could successfully be maintained and demonstrated functional efficacy in the rhizosphere. However, more work is need to understand the differential ability of inocula to adjust to and function in situ. In future experiments, an integrated approach, incorporating physiological and molecular biological measures, will be used to evaluate the effects of temporal and environmental perturbations on the dynamics of these inocula.

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