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EVALUATION OF MICROBIAL INOCULA FOR INITIATION OF BIOLOGICAL LIFE SUPPORT SYSTEMS FOR WASTEWATER PROCESSING ON LONG TERM AND DEEP SPACE MISSIONS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Kimberly L. Cook

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DEDICATION

This work is dedicated to my family. They are the heart and soul of who I am and I owe them everything.

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This work is the culmination of research that I have done for many years and for that reason I have more people to thank than can be mentioned by name, but I will mention a few. Gary Sayler who has made all of this possible and who shown me how to succeed in this business. Jay Garland who has been my mentor from the moment I started doing real science. Alice Layton who has been a role model as a scientist, a mother and a human being. Hebe Dionisi who has been my friend and mentor and has taught me the true meaning of dedication. Stacey Patterson my friend through it all and the one who has convinced me that perseverance is the key to success. Thanks to the rest of my friends at CEB for taking the time to laugh with me and at me. Thanks to my friends at KSC, especially Lisa, Cheryl F. and Cheryl M., for being there for the duration and giving me a place to go, a shoulder to cry on, and many good pep talks. Finally, to my family who have been loving, patient, tolerant and always supportive.

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ABSTRACT

A fundamental goal of ecological research is to better understand the relationship between structural diversity, functional diversity and ecosystem stability. Insight into the mechanisms that regulate microbial ecosystem function should provide important new information to improve system control for ecological, agricultural and biotechnological applications that depend on microbial processes. For example, defined microbial consortia have been employed to improve plant growth through enhanced nutrient uptake, production of plant growth promoting hormones or control of pathogens. However, a lack of consistency of results in field application has limited the widespread use of microbial inoculants. 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 employed by the National Aeronautics and Space Administration (NASA), since microbial inocula will likely be used to initiate any biological waste processing system used on long-term and deep space missions. To assure safe, effective, reliable functioning of inoculated systems, it will be important to manage and potentially manipulate microbial populations within these systems to obviate any negative interactions and to optimize the positive functions of plant associated communities.

This research focuses on the use of a model plant-based graywater waste processing system to evaluate the effect of species richness on community phenotype and community invasibility. The principal hypothesis of this research is that a community of rhizosphere organisms, constructed using parameters described in this research, will be as effective as an undefined industrial activated sludge community in terms of its ability to:

establish in the rhizosphere, persist over time, resist invasion, and degrade organic compounds. To address this hypothesis, the microbial inocula of different structure were evaluated based on the ability of the populations to establish and persist *in situ*, resist invasion by outside species, and withstand changes in nutritional or environmental conditions. A combination of PCR-based phylogenetic analysis and quantification, community level physiological profiling (CLPP) and microarray analysis was employed to evaluate the effect of microbial community structure on community dynamics, community stability and ecosystem processes.

Differences in species richness corresponded to differences in phenotypic potential (surfactant degradation and CLPP) and differences in resistance to invasion. All of the communities were phenotypically diverse, however, only communities with higher species richness were able to degrade surfactant and to resist invasion by a competitor. The constructed community was phenotypically diverse, and even degraded surfactant to a greater extent than any of other the other treatments. However, this community was invaded to a greater extent than any of the other communities, the invader persisted in the population, and one member of the community was completely displaced upon invasion. These results suggest that species richness improved system performance by increasing community persistence, resistance to invasion and ability to degrade surfactant.

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PART I

INTRODUCTION

BIOLOGICAL LIFE SUPPORT SYSTEMS (BLSS) FOR USE ON LONG-TERM AND DEEP SPACE MISSIONS

The National Aeronautics and Space Administration (NASA) is exploring model systems for wastewater processing and food production as part of a Biological Life Support System (BLSS) for long term and deep space missions. One of NASA's interests is the use of hydroponic plant growth systems for food production and for treatment, processing, and polishing of wastewater for recycle applications. A significant concern is the establishment of robust and reliable microbial communities in the rhizosphere to accomplish wastewater treatment as well as to ward off invasive species and microbial pathogens. Consequently, the dynamic nature and stability of inoculated communities needs to be evaluated. The ability of inoculants to survive and form a cohesive community in the system will be vital to ensure reproducible, reliable and resilient system function.

NASA has long-term plans to undertake a manned mission to Mars. The mission will likely consist of a crew of 6 and will include a transit time of 360 days total and a surface stay of 600 days (45). A mission of this duration will require bioregenerative life support systems (BLSS) for food, water, and oxygen production and waste processing (20, 183). Re-supply of materials on long-term space missions will be extremely costly (as much as $22,000/kg) (33). Therefore, NASA has undertaken an agency-wide initiative to evaluate self-sustaining life support systems to reduce these costs. Although both biological and physiochemical systems have been evaluated, a combination of processes is likely to be used to meet crew requirements (20, 45). BLSS have the advantage of being self-sustaining and less dependent on technology and re-supply (45).

Plants are the primary component of BLSS (33). Plants will be used for food production and will supply 100% of crew water and oxygen needs. Researchers at Kennedy Space Center, FL (KSC) have demonstrated the long-term production capabilities of BLSS for plant production. The biomass production chamber and other environmental growth chambers located at KSC provide a closed environment for large-scale baseline testing of candidate crops for use on long-term space missions (209). Plants are grown hydroponically using a recirculating nutrient film technique (209). Nutrients were supplied from prepared nutrient solution or from nutrients recovered from inedible plant biomass processed through aerobic bioreactors and recycled for plant growth. Using recycled nutrients, 50% of plant nutrient requirements were meet with no phytotoxic effects and no effect on microbial communities (70, 127). BLSS have also been used for long-term growth of plants, demonstrating that these BLSS can operate on a continuous basis with no effect on the plant and no build-up of the microbial community (70). These data suggest that use of BLSS on long-duration space missions will provide a reliable, cost-effective, sustainable means to provide crew food, oxygen and water requirements.

Microorganisms will be an integral component of any BLSS used for plant growth or waste processing. Microorganisms thrive in association with plant roots and are found in high concentrations (as high as 10^{11} cells g^{-1} dry wt) within the in root zone of hydroponically grown plants (69). High concentrations of organisms in a closed system can have positive and potentially negative implications. Plant-microbe interactions can be used for processing wastes, for recycling nutrients, and for inhibiting plant and human pathogens. However, the potential exists for the BLSS to serve as a reservoir for

pathogenic microbes (142). This is problematic as both the US and Russian space programs have found that astronauts exhibit changes in the immune system with long-duration space travel. Recently, Russian scientists found that following 3 months in a closed BLSS, microflora of the large intestines of the crew began to shift to include some pathogenic strains of common intestinal flora and some organisms not normally found in the intestine (188). This shift in microflora is common in closed systems due to limited mobility, drastic decreases or changes in the presence of incoming flora, and the psychological effects of prolonged isolation (188). As a consequence, 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 through an entire 200 L system in less than 13 minutes (191). For these reasons, it will be important to manage and manipulate microbial communities within these systems to obviate any potentially negative interactions and to optimize the positive functions of plant associated communities.

The high cost of re-supplying food and water, coupled with the need for self-sufficiency for astronauts on long-term space missions necessitates the development of safe, effective methods for initiation of food production and waste processing systems. Recycling wastewater through BLSS designed for plant production represents an ideal method for re-supplying water at substantial cost savings (83). Graywater, the waste from showers, sinks, laundry, and dishwashing will make up the single largest waste stream on long-term space missions (up to 25 L person^{-1} day^{-1}) (33, 189). In a closed

system, it is estimated that plant production for one person would produce 40 L of atmospheric condensate day^{-1} (74). It should, therefore, be possible to incorporate wastewater processing into hydroponic plant production to recycle water for non-hygenic uses. Implementation of an effective plant-based waste processing system requires careful consideration of plant-microbe and microbe-microbe interactions in the root zone. In the next sections, aspects of plant-microbe and microbe-microbe interactions important to establish effective, stable root-based microbial communities and methods for evaluating these processes will be discussed.

THE PLANT RHIZOSPHERE AND RHIZOSPHERE EXUDATES

The area surrounding plant roots has been recognized for many years as a unique environment that contains a large and diverse microbial community. The rhizoplane and rhizosphere are the location of the bulk of microbial biomass. The rhizoplane refers to the surface of the plant root, while the rhizosphere refers to both the root surface and the layer of material surrounding the root and driven by physical, chemical and biochemical parameters resulting from the plant's physiology (9). Rhizobacteria are beneficial, deleterious, or neutral root colonizing microorganisms that exhibit plant-microbe interactions within the root zone (173). Rhizosphere or rhizoplane organisms are bacteria present in the root zone but these organisms may only be transient members of the rhizosphere community rather than root colonizers. There is a significant difference in the number of microorganisms found in the rhizosphere versus the number of microorganisms found in soil remote from roots (normally 5 to 20 times greater) (175, 204). This phenomenon is known as the rhizosphere effect and it is a result of

rhizodeposition, the loss of carbon compounds from plant roots into the surrounding soil (125).

Plant species and plant physiology influence the rhizosphere bacterial community composition (130, 177, 207) (Table 1). The type and concentration of organic compounds released by roots vary along the root and in the soil (26). The root structure and the amount of root branching influence root exudation, which in turn influence rhizosphere colonization. Root depth, diameter, surface-to-volume ratio, and total biomass vary between and within plant species (204). Monocotyledons such as wheat have roots that branch extensively and penetrate an area in excess of 6 m^2 (9). Plant secretions also directly influence microbial growth and composition in the rhizosphere. Root exudates are estimated to be between 5% and 20% of total fixed carbon (57). Between 40% and 90% of the carbon transferred to the root is lost to soil, and respiration from the roots and rhizosphere organisms (125). Lynch and Whips (125) divide root exudates into four groups: water soluble exudates (sugars, organic acids, hormones and vitamins), secretions (polymeric carbohydrates and enzymes), lysates released when cells autolyse, and gases such as ethylene and CO_2. Low molecular weight root exudates (amino acids, organic acids, sugars, phenolics, and secondary metabolites) represent the bulk of root exudates (57).

Root exudation is tightly linked to microbial processes in the rhizosphere. Different plant lines have been shown to support different rhizosphere communities (184, 187, 208). For example, enhanced exudation of organic acids such as citrate and malate contribute to the mobilization of phosphorus iron in the rhizosphere (57). This increase in organic acid production in limiting conditions correlates with selection for

Table 1. Microbial interactions in the rhizosphere

Interaction	Property	Process	Reference(s)
Plant-Microbe	Plant Species	Exudate Production, Mucigel Formation	26, 57, 125
	Rhizosphere Structure	Root Surface Area	204
	Plant Metabolism	Secondary Metabolites	100
Microbe-Plant	Beneficial	Provide Nutrients, Biocontrol	202
	Deleterious	Disease, Compete for Nutrients	
Environment-Microbe	Aeration, Moisture,	Nutrient Availability, Exudation, and	165, 202
	Temperature, and pH	Community Structure (Bacterial vs Fungal)	148, 191
Microbe-Microbe	Intrinsic Species Competence	Motility, Adaptation, Growth Rate,	97, 123
		Adhesion	52
	Cooperation	Community/Species Interaction	26, 157,163
	Competition	Grazing, Physiology, Antibiosis	21, 94, 109, 136

rhizosphere communities that increase the availability of limiting nutrients (i.e., phosphorous) (25, 100). Secondary metabolites can serve as a signal to beneficial microorganisms. For example, flavonoids stimulate the growth rate of *Rhizobium meliloti* and induce the *nod* gene in this and other *Rhizobium* species (57). Plant exudates such as flavonoids can be simultaneously stimulatory for one organism, neutral for second organism and inhibitory for a third (26). Both root exudation and mucilage formation are increased in the presence of microorganisms (10, 138). Aseptically grown roots respire about 1% of total carbon, while over 7% is released into unsterilized soil as root exudate. Increased respiration of root cells in unsterilized soil may be the result of microbial stimulation in the root zone (10). Mucigel is a high molecular weight root exudate produced by senescing root cap cells, but also contains bacterial cells and their metabolic products (26, 181). It serves as a lubricant for root movement through soil and may provide contact between soil and roots for diffusion of nutrients (26). Mucigel is produced under axenic conditions, but it is thicker in the presence of bacteria. It is part of the root-microbe complex and provides protection and support for the growth of rhizosphere communities (26, 57).

COLONIZATION AND SUCCESSION IN THE RHIZOSPHERE

Just as the plant influences the dynamics of the rhizosphere community, the microorganisms influence the health of the plant (Table 1). Rhizobacteria enhance plant health by providing nutrients to the plant, competing against plant pathogens, or producing compounds that promote plant growth. Alternatively, microorganisms may be detrimental to plant health by causing disease or competing for nutrients. Inoculation of

plant rhizosphere with beneficial organisms has been evaluated extensively, but has provided mixed results. Inoculated organisms often fail to successfully colonize the root zone. The inability to successfully establish and persist in the rhizosphere is a major cause for inconsistent results in field trials with plant growth promoting organisms (105, 161). A better understanding of issues related to rhizosphere colonization should result in enhanced inoculum success.

Successful colonization of the rhizosphere is affected by characteristics of the microorganism, the plant and the abiotic environment (161) (Table 1). In the rhizosphere, nutrient availability, temperature, moisture and pH affect colonization (202). Abiotic factors influence inoculum survival either directly (i.e., by causing physiological stress) or indirectly (i.e., by affecting the activity of the indigenous flora) (202). Moisture is a major determinant in microbial survival in the root zone. Rattray et al. (165) used a *Escherichia coli lux* bioreporter to evaluate the effect of water stress on viable cell concentrations and microbial activity. They found that light output and CO_2 production per cell decreased with increased water stress (165). This agrees with other work that showed GFP-marked *Pseudomonas fluorescens* viability was not dependent on root decay or location on the rhizosphere, but was reduced in dry soil (152). Root exudation is affected by moisture. Bowen and Rovira (25) found that exudation of organic materials from cereal roots in hydroponic culture was 5-20 times lower than in soil. Soil pH can select for tolerant species (beneficial or deleterious) and influences nutrient availability (202). Changes in soil pH affect the activity of biocontrol agents and thereby affect the invasibility of plant pathogens such as *Fusarium* and *Rhizoctonia solani* (173). Decreases in soil pH lead to higher fungal and yeast communities, while bacterial communities

increase at pHs closer to neutral (i.e., pH 6.0 to pH 7.0) (148). Oxygen is important for root and microbial respiration and it affects the rhizosphere redox potential (191, 204). In oxygen depleted microhabitats within the root zone, denitrification, sulfate reduction, and organic acid production may influence nutrient availability (191). Temperature has been shown to influence the survival of rhizosphere bacteria (161). This may be related to the physiology of the organism or to the effect of temperature on the amount and nature of exudates produced by the plant (161).

Properties intrinsic to the inoculated organisms also affect its ability to establish and persist in the rhizosphere including its concentration in the root zone, its competitive attributes, and its ability to adapt and cope with adverse and fluctuating environmental conditions (Table 1). In a study to evaluate essential genetic characteristics important for rhizosphere colonization, motility, growth rate, and growth on organic acids were found to be essential for rhizosphere colonization (123). Lutenberg et al. (123) identified the major sugars and organic acid root exudates produced by tomato plants and found that mutants defective in utilization of the sugars colonized rhizosphere in a manner similar to the parent strain. However, mutants defective in utilization of organic acids were not recovered from the rhizosphere. These results suggest that rapid growth and perhaps exudate-induced motility towards organic acids produced in the root zone are important for colonization success. Adherence to seeds and root has been proposed as a factor in colonization since adhering cells are transported along with the root and therefore remain intimately attached to the nutrient source (26, 97). Chao et al. (37) showed that bacteria that could be agglutinated by root extract followed the rhizosphere growth pattern and were able to colonize to a greater extent. Research suggests that mechanisms for adhesion

by rhizobacteria may be similar to those used by pathogenic organisms in colonization of host tissues (52). This suggests that attachment is a non-specific event that does not involve specific recognition sites.

Protozoa are known to be important predators of soil microorganisms. Although protozoa generally cause a decrease in microbial biomass, protozoan grazing also stimulates metabolic activity in bacterial communities (109, 215). Protozoan grazing improves carbon and nitrogen mineralization by stimulating microbial turnover (109). Slow-growing or senescent organisms are more susceptible to grazing, therefore more active, rapidly growing cells predominate in heavily grazed communities (6, 97). This increase in active cells tends to stimulate overall activity in the rhizosphere and correlates to greater mineral uptake by plants (109). Alternatively, protozoan grazing may alter rhizosphere microbial community structure, contributing to plant growth by decreasing growth of plant pathogens or enhancing plant growth promoting communities (95, 98).

Mycorrhizal fungi also influence rhizosphere community structure (95). Mycorrhizal fungi are found inside roots, in the rhizosphere and in the bulk soil (56). Mycorrhiza may reduce the incidence of some plant pathogens through physical and chemical interactions and increase plant interactions with nitrogen-fixing bacteria by increasing plant nutrient uptake thereby improving conditions for the symbiotic organisms (56). Other research suggests that the same mycorrhiza may stimulate bacterial growth in some conditions and inhibit it in others, depending on nutrient limitations (157).

One factor that is essential for survival of inoculated organisms in the rhizosphere is the ability to compete effectively with indigenous rhizosphere communities (202).

There is a substantial influence of the resident rhizosphere community on inoculated species. Inoculants often survive and grow in sterilized soils, but then decline in competition with the indigenous rhizosphere flora (21, 136). Successful inoculants are able to aggressively colonize the rhizosphere and displace niches filled by indigenious rhizosphere organisms (173). Adaptation and colonization in competition with indigenous flora is correlated with inoculum success (140). Competition in the rhizosphere generally occurs through resource competition, which occurs when one community affects another community by utilizing a resource that is limited in availability, whether it be space or a limiting nutrient (9, 22, 107). Competition for resources is mediated through either direct or indirect interactions. Direct or interference competition occurs when one organism inhibits another by production of chemicals that have toxic effects on other microbial communities (61). Indirect, exploitive, or resource competition occurs when one organism utilizes resources that reduce the net growth rates of communities requiring them (61). Resource and interference competition often occur simultaneously and cannot easily be divorced (22).

MICROBIAL PLANT GROWTH PROMOTION

Biocontrol is the use of beneficial microorganisms to suppress diseases caused by plant pathogens. If effective, biological control agents (BCAs) would reduce the need for and accumulation of environmentally damaging pesticides (15). However, the commercial application of BCAs has been limited due to a lack of consistency in field application. A major reason for this failure is postulated to be the inability of the BCA to compete effectively with indigenous flora and become established in the rhizosphere (15,

160). A major goal in rhizosphere studies has been to evaluate strategies to introduce or manipulate rhizosphere flora to improve survival of beneficial organisms or eliminate deleterious ones. *Arthrobacter*, *Bacillus*, *Paenibacillus*, *Azotobacter*, *Rhizobium*, and *Pseudomonas* species are rhizosphere inoculants introduced to improve plant growth (100, 202). Understanding the mode of action of these organisms in the rhizosphere will facilitate the use of inoculants for remediation of chemically contaminated soils (204) and for biocontrol and plant growth promotion (97).

Rhizosphere inocula improve plant growth by enhancing nutrient uptake, through production of plant growth promoting hormones, or control of plant pathogens (100, 202). Rhizosphere communities may increase nutrient availability through mineralization or solubilization of limiting nutrients. The soil and rhizosphere are oligotrophic environments (163). Competition from plant roots, macro- and microorganisms for limited resources in the densely populated root zone is intense (47). Additionally, many inorganic plant nutrients may be complexed to soil or only sparingly soluble. Plant growth promoting bacteria provide inorganic nutrients to plants by mineralizing organic forms of nitrogen, phosphorous, and sulfur that plants cannot use as nutrients (136). For example, less than 5% of total phosphate is available to plants (30). However, phosphate availability is enhanced in the presence of phosphate-dissolving microbes present in the root zone (predominantly *Pseudomonas* sp. and *Bacillus* sp.) (30, 100). In an early attempt to evaluate the role of rhizosphere organisms and their benefit to plant communities, Katznelson and Bose (101) characterized carbon source utilization and phosphate-solubilization from rhizosphere isolates. They found that more than one third of wheat rhizosphere isolates were capable of dissolving insoluble phosphorous and

suggested that this may contribute significantly to the plant nutritional status (101). Later work showed that phosphate solubilization from calcium phosphates was due to 2-ketogluconic acid, a product of microbial oxidation of carbohydrate exudates from wheat plants (141). Although these results suggest that rhizosphere bacteria are actively involved in increasing nutrient availability, alternative explanations have been suggested. For example, increases in plant growth may also be the result of enhanced nutrient uptake through promotion of root hair development or root elongation. Promotion of root growth would increase root surface area thereby improving plant nutrient uptake (100).

Many beneficial rhizosphere bacteria produce hormones that may be partially responsible for their plant growth promoting properties. Auxins, gibberellins, and cytokinins are some plant growth promoting substances produced by rhizosphere bacteria (30). One species can produce several different growth regulators and many growth regulators can interact with each other (124). Evidence for plant-growth promotion through hormone production results from findings that plants in sterile growth chambers inoculated with plant growth-promoting organisms exhibited enhanced growth (118). Additionally, plants in the presence of rhizosphere bacteria have higher concentrations of auxins than those grown in sterile conditions (30). Many soil organisms produce the auxin indolyl-3-acetic acid (IAA), which stimulates root growth (124). Microbial production of IAA in the rhizosphere may result from root exudation of tryptophan, a precursor of IAA (30). Ethylene is another plant growth promoting hormone produced by many microorganisms, however, ethylene is also readily metabolized by many rhizosphere bacteria (124). The production and utilization of most plant growth-promoting hormones is likely balanced in the soil (124). Therefore, it is questionable

whether concentrations of plant hormones produced by rhizosphere bacteria are sufficient to have any effect on plants (100, 124).

Many biocontrol organisms inoculated into the root zone inhibit deleterious or pathogenic organisms through antibiosis. Antibiosis involves production of substances that kill or inhibit the growth of competing bacteria of different genotypes through allelopathy (48). Inhibition of plant pathogens through antibiosis has been studied most extensively in the fluorescent pseudomonads. This group of organisms produces antibiotics including 2,4-diacetylphoroglucinol, phenazines, and pyoluteorin (111, 182). Antibiotic producing strains are genotypically and phenotypically diverse and vary in ability to suppress root diseases (111, 134). The ecological significance of antibiosis in the soil has not been well characterized, and it is possible that the energetic cost of these compounds may be too great for bacteria to be energetically favorable in most soil habitats (11, 211). Less than 5 percent of bacteria that produce antibiotics *in vitro* have any affect on plant growth (173). However, recent evidence suggests that some organisms cannot compete effectively in the rhizosphere when they loose their ability to produce antibiotics (11, 132). These results suggest that antibiosis may be ecologically important in microsites where there is sufficient substrate to support their production, especially in the face of intense competition (30, 132).

Siderophore production represents another form of antibiosis and is used by rhizosphere bacteria to compete for limiting nutrients (173). Siderphores are low molecular weight compounds with a high affinity for specific compounds (i.e., iron). The production of siderophores that chelate ferric iron in the rhizosphere is suggested as a means for inhibition of plant pathogens that have a lower affinity for iron (100). The

addition of a purified siderophore from *Pseudomonas*, pseudobactin, to soil caused increases in plant growth and reductions in fungal colonization in rhizosphere (173). Benizri *et. al* (14) demonstrated that a plant growth promoting *Pseudomonas* sp. inhibited *Fusarium*, a fungal maize pathogen, in part due to production of siderophores with a higher affinity for iron. However, the organism only produced siderophores in iron-limiting conditions (14). In the rhizosphere, iron concentration is related to soil pH. At lower pH (below 6), iron availability increases and, consequently, siderophore production does not provide a selective advantage for biocontrol organisms in such cases (100).

MICROBIAL DEGRADATION OF NONIONIC SURFACTANTS

Phytoremediation is the use of plants to remove pollutants through the combined effect of the plant and its rhizosphere community (23). Plants are commonly used to treat water and soil systems contaminated with organic pollutants (83, 180). The plants absorb and metabolize pollutants and provide an active rhizosphere community that can transform, mineralize or complex recalcitrant organic or inorganic compounds (23). Efficient degradation of complex waste streams through plant-microbe interactions suggests that the rhizosphere of plants grown for food on long-term space missions may serve a dual purpose: food production and recycling of wastewater. It is estimated that less than 10% of plants grown for food would be needed for processing 100% of crew wastewater (120).

The primary contaminant in used hygiene water will be surfactants (the cleansing agents or soaps) (83). Surfactants are synthetic surface-active substances that consist of a polar head group, which is water-soluble and a nonpolar hydrocarbon tail, which is

hydrophobic (196, 201). The hydrocarbon tail normally consists of a C_8 to C_{22} alkyl chain (201). Surfactants are classified according to the charge of the polar head group. Igeopon TC-42 is an anionic surfactant used on the International Space Station. Igeopon degradation has been evaluated in hydroponically grown wheat (120) and lettuce (33, 83) rhizosphere. Concerns regarding the direct incorporation of graywater into crop production systems include: plant toxicity, sodium build-up from the polar group of the anionic surfactant, and build up of microbial biomass due to carbon addition (33, 83, 120). Results suggest that there are phytotoxic effects at high concentrations of surfactant (1000 ppm) (83). In another study, Garland et al. (73) found that phytotoxicity of Igeopon depended on the mode of addition (continuous or pulse) and the plant type (wheat vs. lettuce). When actual graywater was added to hydroponically grown plants, overall plant growth was not affected, surfactant did not accumulate, and soap-degrading organisms were isolated in high concentrations (120).

To eliminate potential phytotoxic effects due to sodium buildup from the anionic moiety of the Igeopon polar head group, it may be prudent to consider alternative surfactants for use on long-term space missions. Nonionic surfactants contain no ionic constituents and are usually polyethoxylated. Nonionic surfactants are used in a variety of industrial processes (including pulp and paper manufacturing, textiles, rubber, and plastics), in household detergents, and for bioremediation of hydrophobic organic compounds (5, 90, 110, 112). In bioremediation, nonionic surfactants are used for solubilization of hydrophobic organic compounds (110, 119). Hydrophobic compounds are solubilized by incorporation into micellar aggregates formed by surfactant molecules (119). This process increases the concentration of the hydrophobic compound in the

aqueous phase. Liu et al. (119) found that naphthalene was solubilized by micelles of alkylethoxylates and made bioavailable and degradable by mixed microbial cultures.

The method of microbial degradation of nonionic surfactants has not been fully elucidated (128), however, several things are known about the general pathway. Surfactants are degraded starting at the hydrophilic part of their molecules (5, 128, 158). Branching in the alkyl chain leads to preferential degradation of the ethyoxylate (EO) chain by microorganisms (4, 201). This mode of degradation (from the hydrophilic portion of the molecule) leads to the formation of more lipophilic intermediates, including nonylphenol, short-chain nonylphenol polyethoxylates and nonylphenoxycarboxylic acids (4).

Complete degradation of polyethoxylated (PEO) surfactants may require complex consortia of microorganisms (128). The requirement for microbial consortia for complete degradation of most surfactants is suggested by the amphiphilic nature of these molecules with mixed communities of hydrophobic and hydrophilic moiety-degrading species (201). Nguyen and Sigoillot (150) isolated a bacterial community from coastal seawater that degraded nonionic surfactants. They found that only four of 25 isolates could degrade the initial substrate, while two other organisms could only degrade the intermediate products. Additionally, nonionic surfactants with less than three ethoxylate units are degraded by mixed cultures to alkylphenoyl carboxylic acids, while the intermediates must be degraded by another group of microorganisms (62). It has been suggested that the limited level of degradation exhibited by pure cultures of microorganisms growing on surfactants also points to the requirement of consortia for complete degradation (201). This is supported by the findings of Maki et al. (128), who

isolated an alkylphenol ethoxylate-degrading *Pseudomonas* sp. from activated sludge. They found that the bacterium could degrade the polyether moiety of Triton N-101 (a nonylphenol ethoxylate) but could not mineralize the Triton N-101 itself, suggesting the requirement for a microbial consortium. The results of these studies suggest that microbial transformation of nonionic surfactant waste is through microbial consortia capable of degrading different moieties within the surfactant molecule.

CONSIDERATIONS FOR THE USE OF MICROBIAL INOCULA

To provide consistent protection against pathogens and plant growth promotion using biocontrol agents, it may be necessary to move away from the commonly used single inoculum approach. Inoculants are often chosen for biocontrol properties in controlled situations that do not reflect *in situ* environmental conditions. Furthermore, the rhizosphere community has emergent properties that are not taken into consideration and are difficult to simulate *in vitro*. Bowen and Rovira (26) suggest that plant bioassays are the best means for evaluating biocontrol activity, since *in vitro* assay results do not correlate well with biocontrol success. Plant assays integrate plant-microbe and microbe-microbe interactions that are not considered using *in vitro* assays. In general, biocontrol is likely the result of the combined activity of an assortment of rhizosphere bacteria acting in concert in favorable biotic and abiotic conditions (173). A single biocontrol agent is not likely to be active against a broad range of pathogens in a broad range of environmental conditions (166). Selection of organisms for their ability to establish and persist *in situ* and adapt to constantly change biotic and abiotic conditions is key to inoculum success.

Inoculation of a single biocontrol agent to control a single pathogen may be responsible, at least in part, for inconsistent performance in biological control. Antagonism of one organism for another can be nullified by the presence of an outside organism that has little interaction with the other two alone (25). The outside organism may produce or degrade compounds important to the competitive interaction of the antagonistic organism. Therefore, to control a deleterious organism by competitive means it may be necessary to inoculate with a wide range of fast-growing organisms to fill niches the deleterious organisms normally occupy (25). Raupach and Kloepper (166) found that consistent protection against pathogens occurred in the presence of mixtures of plant growth promoting rhizobacteria. Wild type antibiotic producing strains of *Pseudomonas aureofaciens* were found to have enhanced fitness in the presence of the same strain with mutations for production of secondary metabolites (GacS/GacA mutants) (36). The authors suggest that enhanced siderophore production by mutants may improve the fitness of the mixed community by enhancing the ability of both strains to acquire and sequester iron (36). This may serve to limit growth of competing organisms or to make antibiotic production energetically and evolutionarily more favorable for the wild type strain. These results suggest that co-inoculation with a diverse group of organism provides the most feasible means for achieving biological control.

Successful application of microorganisms to improve plant growth requires careful consideration of interactions between the plant, the biocontrol agent and the native rhizosphere flora (184). Manipulation of rhizosphere conditions may provide a significant advantage to growth of inoculants (173). Inoculant patterns of nutrient utilization, nutrient production, and antibiosis may be used to an advantage to alter the

makeup of the rhizosphere community and thereby control the pathogen fitness (78). Creation of temporary ecological "niches" by altering abiotic factors can be used to favor growth of the biocontrol agent (21, 173). For example, Lajoie et al. (110) used surfactants that were not readily degradable by indigenous communities to enhance growth of a polychlorinated biphenyl-degrading inoculant that could also degrade the surfactant.

The rhizosphere is never static; root exudates and microbial communities within the root zone are constantly shifting (26). The timing of colonization and the variance or magnitude of fluctuation of species affects the species-specific biology of the inoculant within the dynamic environment of the rhizosphere community (172). Therefore, an effective biocontrol strategy must include an evaluation of the mechanisms and processes that affect the system and how these processes are influenced by spatial and temporal dynamics (172). *Pythium* is a common fungal pathogen of wheat (140) and the most common and destructive root-infective pathogen in hydroponic systems (190). *Pythium* may infect the root system at a low level until the plant is stressed. Pathogenesis is then rapid, and can decimate the root system with 3-9 days (164). Therefore, it is important for the biocontrol agent to be present and active at the spatial/temporal peak for activity of the pathogen (202). Otherwise concentrations or activity of the biocontrol agent may be too low to be effective at the onset or peak of pathogen infection (139). Therefore, if a pathogen infects seeds or young plants, seed bacterization may be the most effective means to control the pathogen. Alteration of the plant through selection or genetic manipulation may aid in the success of biocontrol agents (143). Plant nutrition can be managed to adjust root exudate composition, crop rotation can be used to influence

indigenous flora, and alteration of carbon economy within the plant-microbe interaction have all been suggested as means to improve microbial biocontrol (26, 138). Greater understanding of plant-microbe and microbe-microbe interactions in the rhizosphere will permit biocontrol for plant growth promotion by enabling better assessment of ecological and environmental parameters for selection of organisms best suited to the task.

MONITORING MICROBIAL COMMUNITIES IN ENVIRONMENTAL SYSTEMS

In recent years, evaluation of microbial communities in environmental systems has been aided by the use of molecular methods that are less selective than traditional cultural techniques and provide a more complete view of the microbial complexity (7, 102, 154). Sequences obtained from the environment are seldom identical to sequences from cultured organisms and even cloned sequences often fail to match any other known group of organisms (92). This suggests that culture-based methods provided a limited view of the phylogenetic makeup of the sampled ecosystem. Molecular methods based on the 16S rRNA gene (16S rDNA) have provided unique insight into the breadth of microbial diversity (92). This sequence is universally present in all microorganisms and contains highly conserved regions interspersed with variable regions, thereby facilitating the design of primers and probes for analysis of bacterial phylogeny at different levels of specificity (i.e. family, genus or species level)(7, 40). 16S rRNA gene sequences from environmental samples are extracted and analyzed using a combination of PCR amplification, cloning and sequence analysis (92). Although comparative analysis of 16S rDNA sequences provides valuable information about the composition of the community,

little real data about the function or dynamics of the community is obtained. Methods are needed to provide a link between phylogenetic groups and their functional role in the ecosystem (197). An integrated approach incorporating phylogenetic analysis, functional evaluation, and assessment of community dynamics should provide a more complete view of microbial community processes *in situ*. In this research, a combination of PCR-based phylogenetic analysis and quantification, community level physiological profiling (CLPP) and microarray analysis was used to evaluate the effect of species richness on community phenotype and community invasibility.

QUANTITATIVE REAL-TIME PCR

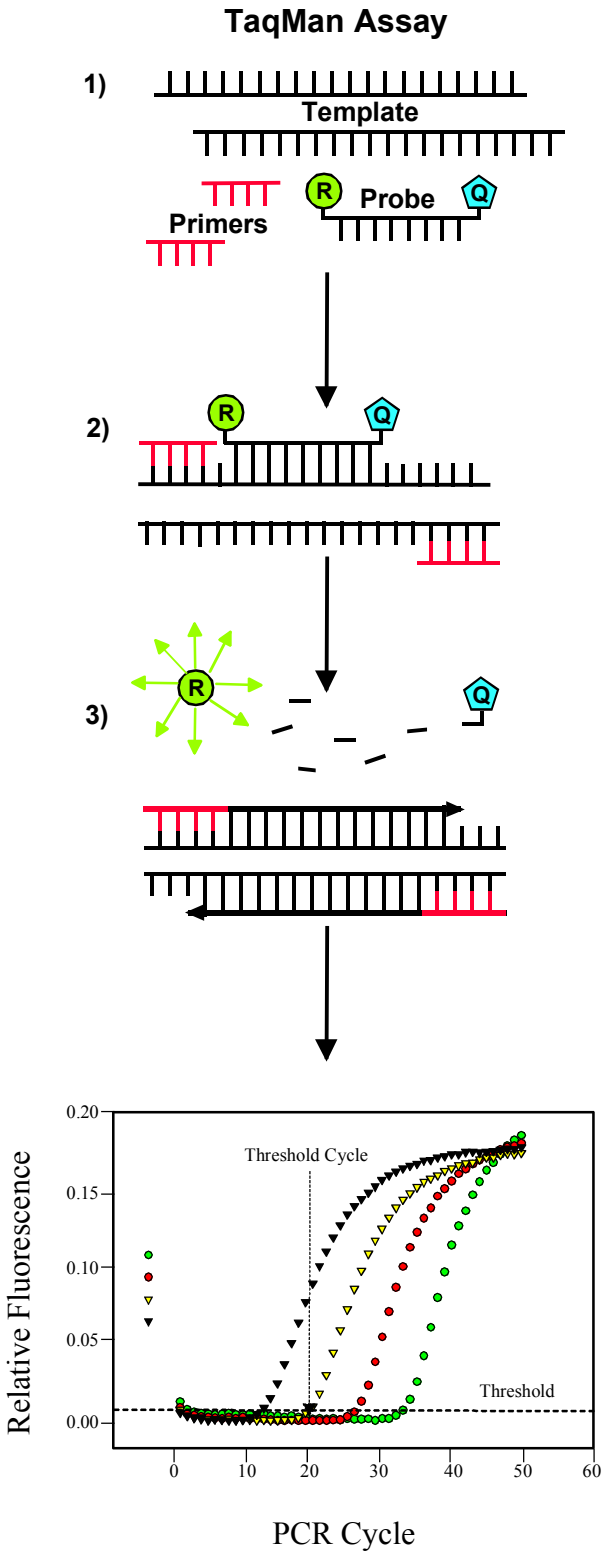
The Polymerase chain reaction (PCR) is a simple but elegant process that enables sensitive, specific, timely molecular analysis of genetic sequences obtained from minute quantities of template. PCR-based techniques using primers specific for a particular genotype provide a high level of resolution for microbial identification and characterization (12). Comparative analysis of 16S rDNA sequences provides valuable information about microbial composition; however, PCR analysis of genetic diversity in environmental systems is not limited to 16S rDNA sequence analysis. The sensitivity (amplification of a single target molecule 10^5 to 10^6-fold) and specificity of PCR permits analysis of any gene sequence given proper primer selection and optimization of amplification conditions (32). This technique has revolutionized studies in most areas of the life sciences, including the field of molecular microbial ecology.

Evaluation of mixed microbial communities or specific genetic sequences using PCR is subject to biases encountered at each step in the process including DNA

extraction, PCR amplification, hybridization and analysis (151, 155, 192, 213). DNA extraction efficiency, and the low concentration of biomass and high concentrations of PCR inhibitors affect the template material extracted from environmental samples (151, 155). Lack of primer specificity, formation of secondary structures in the template, misincorporation by *Taq* polymerase and the formation of chimeric compounds all influence the amplification process (12, 213). PCR reactions are often considered to be semi-quantitative with the relative abundance of amplicons produced by amplification reflecting the initial template concentration (12). However, reagent limitation, PCR inhibitors and the tendency for some reactions to generate more product than others make end-point quantification unreliable (79).

The most effective means of determining the abundance of a target sequence is to measure PCR products as they are accumulating and to quantify the amount of target while the PCR reaction is still in the exponential range (32, 79). Quantification of PCR products is made possible by real-time quantitative PCR analysis (Fig. 1). This process permits detection and measurement of products as they are generated during each PCR cycle (79). In this method, laser power is used to generate a fluorescent signal emitted from a hybridization probe. Fluorescence emission data are collected every cycle as the PCR process continues. One of the most commonly used detection methods is the Taq nuclease assay, which uses the 5' to 3' exonuclease activity of *Taq* DNA polymerase to cleave a fluorescently labeled oligonucleotide, the TaqMan probe, that hybridizes to the PCR template but is 3' phosphorylated to prevent polymerazation by *Taq* polymerase (12). The TaqMan probe is an oligonucleotide labeled at the 5' end with a fluorescein derivative (usually 6-carboxyfluorescein (6-FAM)) and at the 3' end with a quencher dye

Figure 1. A typical fluorogenic 5’ nuclease assay using a TaqMan probe with a reporter and quencher. 1) Two fluorescent dyes a reporter (R) and a quencher (Q), are attached to the 5’ and 3’ ends of a TaqMan probe, 2) The TaqMan probe hybridizes to the template between the two PCR primers. The reporter dye emission is quenched as long as the probe is intact, 3) Once cleaved from the rest of the probe, the 5’ dye molecule is freed from the quenching effect and fluorescence increases in proportion to the amount of amplification.


TaqMan Assay
1)
Template
Primers
R
Probe
Q
2)
R
Q
3)
R
Q
Relative Fluorescence
0.20
0.15
0.10
0.05
0.00
Threshold Cycle
Threshold
0
10
20
30
40
50
60
PCR Cycle

(a rhodamine derivative) (12). The intact probe anneals to the target between the forward and reverse primers sites. The 5'fluor is quenched by the quencher molecule at the 3' end of the intact probe through fluorescence resonance energy transfer (FRET). The intact probe emits light in a wavelength characteristic of the 3' quencher molecule, shifting the energy released by the fluorophore to the quencher molecule, reducing the amount of emitted fluorescence (79). During the PCR extension, the probe is digested by the 5' nuclease activity of the DNA polymerase. This separates the reporter dye from the quencher, and the probe is displaced from the target sequence without disrupting polymerase activity. Once cleaved from the rest of the probe, the reporter dye is freed from the quenching effect and the fluorescence signal increases in proportion to the amount of amplicon produced (12).

The advantage of the fluorogenic probes is that specific hybridization between probe and target is required to generate fluorescent signal. The polymerase will cleave the probe only while it remains hybridized to its complementary strand. Because the probe remain bound to the complementary template strand, proper optimization of probe and primer binding conditions must be established to allow the probe to anneal to all the targets and saturate the sample without the primers annealing at the same time (35). The probe must have a melting temperature (Tm) greater than that of the primer pair by 8-10°C so annealing of the probe occurs at a temperature too high for the primes to anneal, but low enough so the probe will bind (Fig. 1). At the primer annealing temperature, below the Tm of the primers, the primers will extend and cleave off the probe. Ideally the probe design will be less than 100 bp since shorter amplicons amplify more

efficiently than longer ones and extension times can be shortened thereby reducing the likelihood of amplifying DNA contaminants (35).

The reaction is calibrated by amplification of known amounts of the target sequence and monitoring fluorescence cycle by cycle. A standard curve of the known sample from each gene is added to each plate spanning the range above and below the amount of the gene in the unknown samples (79). Usually the known sample is from a plasmid for the gene of interest and the standard curve is generated based on serial dilution of a quantified amount of starting material (79). Known standards and unknown samples are irradiated from a laser source and the resulting fluorescence is detected using a CCD array. In a real-time assay, reactions are measured at the point in time during cycling when amplification of PCR product is first detected above the background threshold. The higher the starting copy number of the target material, the sooner a significant increase in fluorescence is observed. An amplification plot of fluorescence signal versus cycle number is used to quantify the amplicon (Figure 1). In the initial cycles of PCR, a baseline value is established which indicates the detection of fluorescence signal that is fairly stable. An increase in fluorescence above the baseline signifies the detection of accumulated PCR product. The threshold cycle (C_T) is defined as the PCR cycle at which a fluorescence of the reporter dye passes the fixed threshold. The log of initial target copy number for the standard versus C_T produces a straight line. The amount of target in the unknown sample is quantified by measuring the C_T and using the standard curve to determine starting copy number (12).

16S-23S rDNA INTERGENIC SPACER ANALYSIS

Evaluation of microbial community structure through identification of target organisms and/or characterization of whole community changes occurring throughout temporal, spatial or environmental gradients has been greatly aided by the use of the bacterial rRNA operon (*rrn*) as a genetic marker. The 16S rRNA gene in particular has proven a useful target for evaluating bacterial phylogeny (probing, sequence analysis) and bacterial community structure (ribotyping) (7, 87, 131). 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) (7, 40, 64, 87). Bacterial identification is aided by the availability of a vast database of 16S rRNA gene sequences. However, there are several disadvantages to the use of the 16S rRNA for characterization of bacterial isolates or bacterial communities. The size of the 16S rRNA gene is extremely constant (1.5 kb), thereby prohibiting differentiation of organisms based on size (64). Additionally, 16S rRNA gene sequences may be too highly conserved to permit analysis of phylogenetic relationships between closely related species (1, 40, 64, 137). The 23S rRNA is more useful for resolving closely related species than the 16S rRNA because of its larger size (about 3kb) and because it contains domains of rapid expansion and higher mutation rates (40). However, use of the 23S rRNA gene sequence has been limited due to the paucity of sequences available in gene databases (87).

Compared to rRNA genes, the intergenic spacer region (ISR) located between the 16S and 23S rRNA genes exhibits much greater sequence diversity and length variation

(8, 87, 94) (Fig. 2). Size and sequence divergence within the ISR has been used to confirm 16S rRNA gene-based phylogeny and provides better discrimination of closely related species than 16S rRNA characterization (1, 18, 94, 137, 149). More recently, ISR size differences have been used to characterize complex bacterial communities from soil and water samples (24, 55).

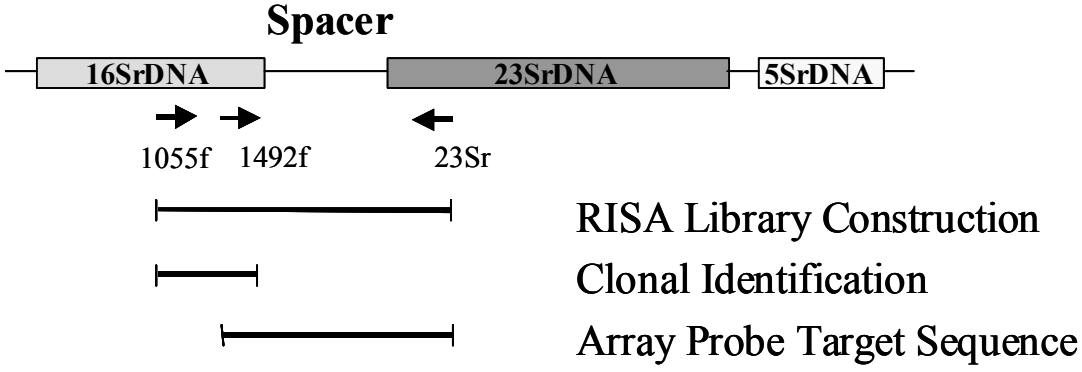
Ribosomal RNA operon (*rrn*) organization

Ribosomal RNA (*rrn*) operons are highly expressed genes accounting for more than half of *E. coli* transcriptional activity at high growth rates (8). The bacterial *rrn* operon is a multigene family that contains the genes that code for the three rRNA genes (16S, 23S, and 5S) and the intergenic spacer region (ISR). The components of the operon normally follow the order 16S, spacer, 23S, spacer, 5S rRNA (87, 116). The *rrn* operon is transcribed into a single RNA precursor that is later cleaved into separate rRNA and tRNA molecules. The 16S and 23 srDNA sequences are highly conserved and are commonly used for analysis of microbial phylogeny. The ISR is more variable and is believed to be involved in the formation of double-stranded processing stems involved in the maturation of rRNA genes, it also contains coding sequences for tRNA genes as well as several different regulatory sequences (8, 29, 34, 85). Regulatory and tRNA sequences in the ISR make up less than 50% of the total intergenic sequence.

The 16S rRNA gene sequence is highly conserved among bacterial genera and even for closely related species. For example, the similarity between *Bacillus anthracis* and *Bacillus cereus* is 99.9%-100%. The more distantly related *Bacillus subtilis* is still 92%-94% similar to *B. anthracis* and *B. cereus* (147). The number of rRNA operons varies greatly depending on the growth rate of the species. For example, in the slow-

Figure 2. The ribosomal RNA (*rrn*) operon. The intergenic spacer region (ISR) is a region situated between the small (16S rDNA) and large (23S rDNA) ribosomal subunit genes within the *rrn* operon. Conserved primers in the 16S (1055 Forward) and 23S (23S reverse) regions are used to amplify the ISR region from a mixed community sample. Amplicons are used for community analysis (RISA) or for construction of clonal libraries. Phylogenetic information is obtained by sequencing the 16S portion of clones. Species to strain level resolution of unknown organisms or closely related organisms is possible using only the ISR product for sequence analysis, as probe target for hybridization studies or for restriction digestion.

Ribosomal RNA (*rrn*) Operon



growing bacterium *Mycoplasma pneumoniae* there is only one *rrn* operon, while the faster growing organisms *E. coli* and *B. subtilis*, have 7 and 10 *rrn* operons per genome, respectively (27, 64, 87).

In contrast to the 16S rRNA and 23S rRNA genes, the ISR is under minimal selective pressure (34). In fact, the evolutionary rate of the ISR is assumed to be 10 times greater than that of the 16S rRNA (113). As a result, the ISR of bacterial species is much more variable in sequence and in length than the adjacent 16S rRNA and 23S rRNA genes (65). ISR variability in size and sequence exists between strains, species and genera, and even between distinct *rrn* operons within one bacterium (1, 87). In general, the pattern of variation within the ISR is very similar to that found in the 16S rRNA gene, with stretches of DNA sequences that are conserved interspersed with variable segments (8, 85). However, in the case of the ISR, sequences are conserved within a single species, but rarely beyond the genus or family level, while variable segments such as block substitutions and insertions/deletions result in species or strain specific differences that often result in ISR variation (65).

Much of the variation in the ISR is due to the presence or absence of tRNA sequences (73-76 nt in length). The tRNA sequences also represent the most highly conserved regions within the ISR. However, tRNAs are not present in all copies of the *rrn* operon of a single organism and their presence is variable between species. In general, there are three patterns of tRNA sequence composition: the absence of tRNA sequences, the presence of two tRNA sequences ($tRNA^{Ile}$ and $tRNA^{Ala}$), or the presence of a lone tRNA which may be $tRNA^{Ile}$, $tRNA^{Ala}$, or $tRNA^{Glu}$ (8, 27, 85). Most of the spacers in Gram-negative bacteria contain either $tRNA^{Ile}$ and $tRNA^{Ala}$, or only a $tRNA^{glu}$

gene, while the majority of Gram-positive species have no tRNA genes at all (87). Within the seven *rrn* operons of *E. coli*, four contain genes for $tRNA^{Glu}$ and three contain genes for the $tRNA^{Ile}$ and $tRNA^{Ala}$ (8). Comparison of the ten *rrn* operons of *B. subtilis* 168 showed that two of the ten 16S-23S ISRs contain the sequences for $tRNA^{Ile}$, $tRNA^{Ala}$ and are identical in sequence. The remaining 8 ISRs lack tRNA sequences (147).

Despite significant intercistronic sequence differences, studies have found a high level of conservation in the kind of ISR (i.e., number and kind of tRNA containing ISR blocks). For example, Nagpal et al. (147) found that equivalent ISRs within *B. subtilis* strains W23 and 168 were 99.9-100% similar. Variability between ISRs of one strain of *B. subtilis* was found to be greater than variability between equivalent ISRs from different strains of *B. subtilis* (147). Anton et al. (8) found that variation between equivalent ISRs in *E. coli* was in the range found for housekeeping genes. It is hypothesized that ISR type is highly conserved in order to maintain equilibrium in the number of tRNA genes within the cell (8).

Other functional units that are conserved within the ISR include the RNAase III recognition site, several nucleotides surrounding the tRNA and the antitermination sequence, *boxA*. Every 16S-23S rDNA ISR contains RNAse III recognition sites. These regions include conserved nucleotide sequences that are involved in the formation of the 16S rRNA and 23S rRNA processing stems that are cleaved by RNAase III (29). RNAase III is involved in the process of splicing to yield the mature ribosomal RNA (64, 116). Nucleotides surrounding the tRNA are conserved due to their importance for processing the transcript (8). The ISR of the *rrn* operon also contains a *boxA* sequence that is required for binding bacterial proteins necessary for anti-termination during transcription

(16, 116). These are the only functional units within the ISR and they do not comprise more than 50% of the size (64).

Substitutions, insertions and deletions represent another major source of heterogeneity within the ISR. Frequent insertions and deletions occur. Other changes involve rearrangement of 20-50 nt sequence blocks some of which may alter secondary structure, but do not affect growth of mutant strains (8) (64, 85). Surprisingly few single nucleotide substitutions occur in the ISR. Sites prone to nucleotide substitution are located at the beginning and end of the ISR and are found in nucleotides that do not affect the secondary structure of tRNAs or the stem-structure required for production of mature rRNA genes (64). Therefore these sites have low functional restrictions. Even so, the presence of a substitution in one position is often conserved throughout all ISRs of a single strain (8, 86).

Concerted evolution of multigene families and homologous recombination between similar ISRs have been suggested as mechanisms by which the ISR maintains a high degree of sequence conservation despite limited evolutionary constraints (8, 85). This tendency towards homogenization is common in multigene families. The location of the ISR between the highly conserved 16S rRNA and 23 rRNA genes and the fact that the *rrn* operon is transcribed as a single pre-RNA transcript, contribute to the high level of conservation of ISR sequences among closely related strains (64). Evidence also suggests that recombination may be involved in this process (85). A higher incidence of ISR recombination leads to greater sequence conservation, while lower levels of ISR recombination result in greater variability. Conserved sequence blocks may allow homologous recombination between ISRs (85). This could explain why insertions,

deletions, and nucleotide substitutions within one ISR are often conserved throughout the species or strain (8, 86). Anton et al. (8) found that ISRs of most strains of *E. coli* showed a strong tendency toward homogenization and suggested that intercistronic ISR heterogeneity may reduce the incidence of recombination thereby preventing large genomic rearrangements.

Characterization of bacterial isolates and communities using the 16S-23S rDNA spacer

In recent years, the 16S-23S rDNA spacer has been used for characterization of relationships between closely related bacterial species (1, 87) and as a community-fingerprinting technique (2, 55). Conserved sequences within the 16S rRNA and 23S rRNA are used as primer sequences for amplifying the spacer region from a broad range of organisms (94). Jensen et al. (94) were the first to use a universal set of primers anchored in the 16S rRNA and 23S rRNA for amplification of the ISR of diverse bacterial species (over 300 species or strains). They found that these sequences were conserved enough among bacterial genera and species to permit amplification of the ISR without modification of PCR conditions. Subsequent analysis of length and sequence variability within the 16S-23S rDNA ISR permits characterization of bacterial strain, species, or community-level differences.

The most widespread use of the method has been to distinguish closely related bacterial species and strains by taking advantage of length and/or sequence differences within the 16S-23S rDNA ISR. The RISSC (Ribosomal Intergenic Spacer Sequence Collection) database (http://ulises.umh.es/RISSC) was established to compile 16S-23S ISR sequences (65). Amplification of the 16S-23S rDNA ISR or amplification of the

16S-23S rDNA ISR coupled with restriction enzyme digestion has been used to differentiate closely related species or strains of *E. coli* (34), *Staphylococcus* sp. (137), *Bacillus* sp. (178), *Bifidobacterium* sp. (113), *Nitrobacter* sp. (149) and ammonia-oxidizing bacteria (1), among others. Primers directed to species- or strain-specific sequences within the 16S-23S rDNA ISR have been used differentiate closely related lactobacilli (18) and *Streptococcus* sp. (17) and to monitor *Vibrio cholerae* concentrations in water samples (96).

In environmental samples, the 16S-23S rDNA ISR from a broad range of organisms can be amplified in one reaction. These products may also include DNA sequences from within the 16S rRNA that can be used for phylogenetic analysis (2). Target organisms have been detected using PCR amplification of 16S-23S rDNA ISR sequences from water (34, 96) and soil (193) samples. In 1997, Borneman and Tripplett (24) introduced RISA (rDNA intergenic spacer analysis) as a community-level fingerprinting technique. Using RISA, size differences within a mixed community DNA sample can be differentiated by either gel electrophoresis or automated analysis using a fluorescently labeled forward primer (ARISA)(55). The result is a banding pattern or electropherogram pattern corresponding to organisms within the sample that exhibit ISR sequence size heterogeneity. The RISA technique has been used to characterize community samples from Western Amazonian soils (24), freshwater bacterial communities (55), plant rhizosphere (169), and Mediterranean offshore waters (2). The use of RISA reduces the need for costly sequence analysis, but permits simultaneous phylogenetic analysis through sequencing of the 16S rRNA portion of the RISA-amplified product if desired. Borneman and Triplett (24) were able to detect an effect of

human activities on soil microbial diversity in Western Amazonia using RISA and without the need to extensively evaluate the high diversity soil using costly 16S rRNA sequence analysis. Acinas et al. (2) found that the sequence diversity obtained using RISA was higher than that obtained by sequencing random clones from a 16S rRNA library.

There are several potential issues related to the use of the ISR for bacterial or community characterization. Extensive evidence indicates significant variation in the number of *rrn* operons within a single organism and significant variation in ISR sequence between multiple *rrn* operons within a single organism (8, 27, 29, 137, 147). The fact that the technique requires PCR-based amplification of the ISR sequence introduces several inherent biases related to PCR technology. For example, Boyer (27) found that in the case of cyanobacteria ISRs lacking tRNA sequences were preferentially amplified. Biases may also result from preferential amplification of shorter templates and from lack of amplification of templates with secondary structure (55). Additionally, characterization of isolates and communities may be affected by the fact that multiple organisms (either closely related strains or completely unrelated species) may possess ISR regions of identical length (64). Alternatively, one organism may possess multiple ISRs with significant length differences so that a single organism may contribute multiple bands to a community profile (55).

THE USE OF MICROARRAYS FOR ENVIRONMENTAL APPLICATIONS

Widely used and reviewed (82, 108, 117, 162, 179) in the biomedical, pharmaceutical, and clinical research fields, array technology has only recently been used in

environmental analysis. While there appears to be unlimited opportunity to use array technology to address important questions in microbial ecology and environmental microbiology, there are also limitations that may slow implementation of the technology. The term array technology describes any high-throughput methodology that permits analysis of hundreds or thousands of genetic sequences in parallel. However, array technology is most commonly associated with genome-wide or system-wide analysis of gene expression. Arrays containing most or all of the open reading frames (ORFs) of an organism are used to measure expression of thousands of genes simultaneously. More recently, gene expression analysis has been used to characterize important biochemical functions in environmental organisms (53, 174, 194, 217). Use of array technology in environmental studies should facilitate a greater fundamental understanding of the ecology, physiology, structure and function of complex environmental systems. However, application of array technology to environmental samples presents unique challenges. Issues relating to the extent of genetic diversity in the environment, low concentration of biomass and high concentration of contaminants must be addressed for this technology to realize its full potential in environmental studies.

The array platform

Arrays have been fabricated on a variety of different materials, but the most common are oligonucleotide or DNA-based arrays fabricated on glass slides or nylon membranes. In the field of array technology, the probe or known sequence is the arrayed material, while the unknown or target sequence is labeled and hybridized to the array. Oligonucleotide arrays are synthesized on a glass slide using photolithographic techniques (159, 212), and contain perfect match probes and mismatch probes arranged

into probe sets (114, 205). Probe sets are used for quantification, subtraction of non-specific signals, and for determination of transcript abundance. Affymetrix now carries GeneChips® with probe sets representing as many as 20,000 transcripts (3, 179). DNA arrays, on the other hand, consist of larger DNA fragments spotted onto glass slides or nylon membranes. The spotted probe material generally consists of PCR product. DNA arrays of 10,000 to 15,000 genetic elements are common (19, 99). A comparison of general parameters for synthesized oligonucleotide arrays and spotted DNA arrays is given in Table 2. In an array experiment, target sequences from environmental samples are labeled and hybridized to the arrayed probes (Fig. 3). When using a fluorescent label, the intensity of hybridized sequences is then quantified and the resulting data are subjected to a detailed statistical or quantitative analysis.

Application of array technology to environmental samples: gene expression analysis

Gene expression studies have provided insight into unique aspects of an organisms' physiology and have demonstrated the feasibility of applying array technology to microbial systems to obtain a broader, more complete view of the cellular response (121). Gene expression analysis has been used to evaluate transcriptional profiles for sporulation (53), biosynthesis (114) and anaerobic growth (217) in *Bacillus subtilis*. More recently, however, gene expression technology has been applied in more novel ways to address questions in environmental studies. Delisa et al. (43) used an *E. coli* genome-based array to evaluate transcriptional changes occurring in response to the alternative autoinducer (AI-2) quorum-sensing compound. They found that 242 genes, including 20 sensors, transcriptional regulators, and signal transduction genes responded to the signal molecule. Whitley et al. (210) investigated gene expression in *Pseudomonas*

Table 2. Comparison of typical parameters for synthesized versus spotted DNA arrays

	Synthesized Oligonucleotide Arrays	Spotted DNA Arrays
Deposited Material[a]	50-75ng/spot	1-10ng/spot
Specificity[b]	90-95%	75-80%
Sensitivity[c]	$>10^9$ copies	$>10^6$ copies
Probe Density[d]	$10^6/cm^2$	$10^4/cm^2$
Probe Sets[e]	22,000	Not Applicable
Represented Genes[f]	20,000 (typically 2,000-5,000)	60,000 (typically 5,000-15,000)
Qualtity Control	High	Medium
Probe Size	Short oligos - 15-25 nt Long oligos - 50-80 nt	>200 base pairs
Sequence Information	Required	Not Required
Probe Material	Oligonucleotides synthesized *in situ*	DNA from clonal libraries cDNAs, SAGE, PCR product, etc.
Printing Method	Photolithography[g]	Robotic Printing, Inkjet

[a]Amout of probe material deposited, although the maximum loading capacity for glass slides may be as low as 10-20 pg/spot (see reference 39)

[b]Discrimination of related genes

[c]Reported values for detection limits in enviornmental samples (see references 39,185)

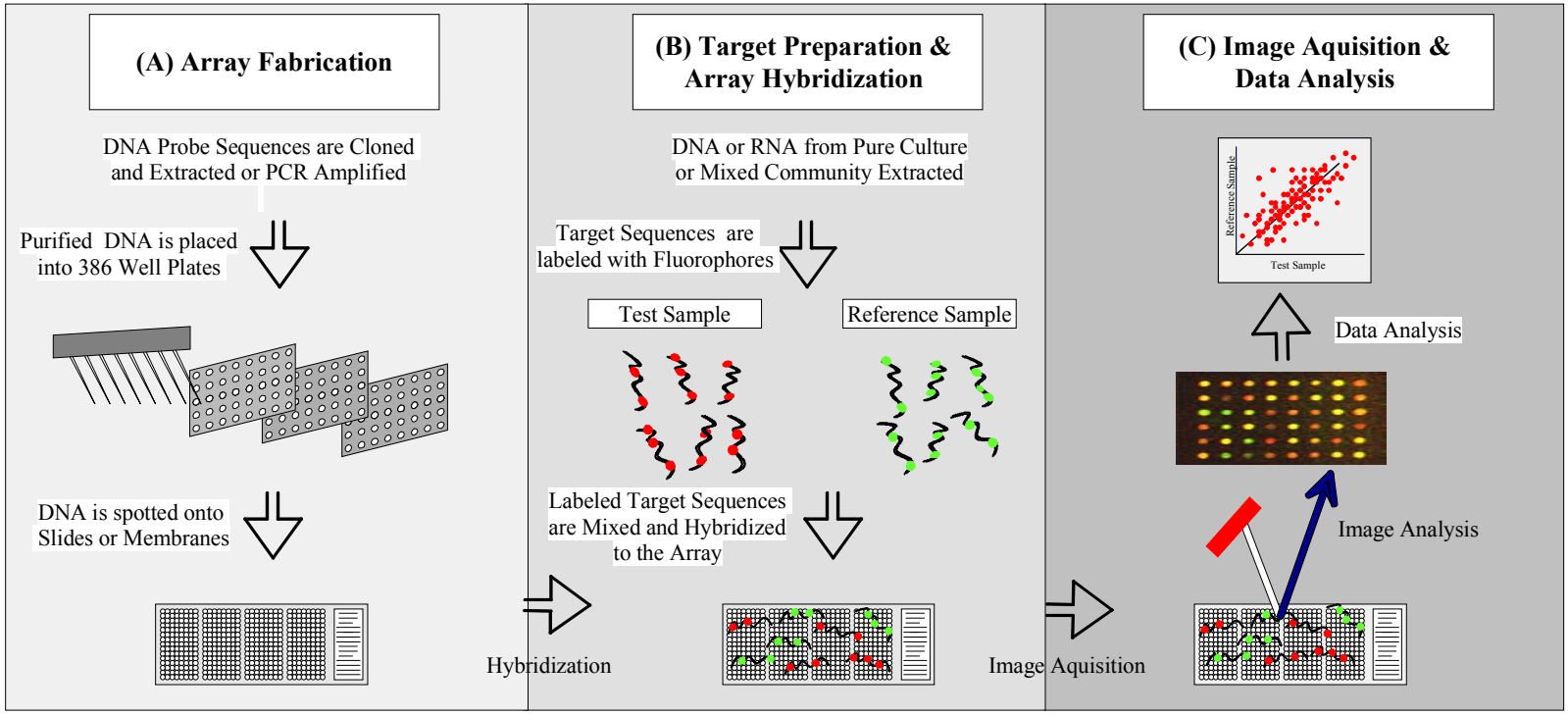
[d]Maximum attainable density of arrayed probes

[e]Each probe set contains 10-20 probes in perfect match/mismatch pairs that represent a single transcript

[f]Genes represented on the array using either oligonucleotide probe sets or arrayed DNA

[g]Light-directed combinatorial synthesis (see reference 159)

Figure 3. Schematic showing the steps involved in fabrication, hybridization, and analysis of a spotted DNA microarray. Target sequences are from a test sample and a reference sample that have been fluorescently labeled with different fluorophores, mixed and hybridized to the same array. The probe sequence is DNA from gene sequences, expressed sequence tags (ESTs), genome fragments, oligonucleotides, or similar. The reference sample is from an untreated control, a time course reference point, a known standard, or similar.


(A) Array Fabrication
DNA Probe Sequences are Cloned and Extracted or PCR Amplified
Purified DNA is placed into 386 Well Plates
DNA is spotted onto Slides or Membranes
(B) Target Preparation & Array Hybridization
DNA or RNA from Pure Culture or Mixed Community Extracted
Target Sequences are labeled with Fluorophores
Test Sample
Reference Sample
Labeled Target Sequences are Mixed and Hybridized to the Array
Hybridization
(C) Image Aquisition & Data Analysis
Reference Sample
Test Sample
Data Analysis
Image Analysis
Image Aquisition

aeruginoa biofilm formation by comparing expression profiles of free-living and attached cells. They found only 1% of genes were differentially expressed between the two different growth forms. These results lead to identification of genes important in biofilm formation and to others possibly responsible for resistance of biofilms to antibiotics.

An alternative approach to gene expression analysis involves the use of partial arrays of a specified subset of genes. For example, Schut et al. (174) characterized sulfur-mediated gene regulation using an array from a hyperthermophilic archaeon, *Pyrococcus furiosus*, which consisted of 271 ORFs encoding proteins proposed to be involved in metabolic pathways, energy conservation, and metal metabolism. Cytoplasmic hydrogenases, thought to function in sulfur reduction, were actually repressed by sulfur. Two uncharacterized ORFs were unregulated more than 25-fold, and were proposed to be a novel sulfur-reducing enzyme complex. These results illustrate the breadth of information that can be obtained using a more directed methodology. Evaluation of gene expression using a defined group of genes may provide as much information as genome-wide analysis, but in a more practical and manageable format (91).

Application of array technology to environmental samples: comparative genomics

Comparative genomics is used for analysis of genetic similarities and differences between species or strains of closely related organisms. Known or fractioned genomic sequences from one bacterial species are arrayed and genomic DNA from closely related species or strains is labeled and hybridized to the array. This technique permits evaluation of genetic elements that might be responsible for unique functions of an organism. For example, Dziejman et al. (49) used an array of 93% of the predicted genes from the etiologic agent of the 7th pandemic *V. cholerae* (El Tor strain) outbreak to evaluate

similarities and differences between nine isolates from different geographical locations. Despite 99% similarity between the arrayed and the test strains, the 7th pandemic isolates of *V. cholerae* contained two chromosomal gene clusters which were hypothesized to impart adaptive properties that enhance the organisms fitness in the human host or in environmental systems. A similar approach was used to identify genetic differences between a pathogenic and a nonpathogenic species of *Listeria* (80).

Comparative hybridization has also been used for phylogenetic analysis among related species to gain insight into the importance of horizontal gene transfer as a mechanism of bacterial speciation (129, 144, 153). A partial array containing 192 ORFs from *Shewanella oneidenis* was used to compare the genome of that organism to nine other *Shewanella* species and *E. coli* (144). Hybridization profiles for some genes, including *gyr*B (encoding DNA topoisomerase subunit B) and *arc*A (encoding a key gene in the two-component system responsible for repressing transcription of aerobic genes under low oxygen conditions), were highly conserved between *Shewanella* sp. suggesting that these universally conserved genes may provide new phylogenetic markers to complement 16S rRNA sequence analysis (144). In fact, sequence divergence patterns of closely related species were better predicted by *gyr*B hybridization profiles than hybridization profiles of 16S rRNA gene sequences, suggesting an evolutionary lineage based more on functional rather than structural characteristics (144). Using another variation of the comparative genomics approach, Cho and Tiedje (38) arrayed 338 total genome fragments from four fluorescent *Pseudomonas* strains. Cluster analysis of hybridization profiles for twelve test strains resulted in phylogenetic dendograms providing species to strain level resolution. The results of these studies suggest that a

comparative genomics approach can be used to obtain information about the identity and relatedness of isolated organisms without the need for whole-genome sequence data. Comparative genomics may represent the most useful array-based approach for determination of important discriminating characteristics of bacterial species or strains isolated from environmental systems or utilized in environmental processes.

Application of array technology to environmental samples: mixed community analysis

The major goal of community-based array analysis is characterization of community structure and function. In some instances identification of all or target organisms is desired while in others characterization of specific functional aspects of the community is the goal. Oligonucleotide-based arrays have been used to target specific diagnostic sequences of pathogens identified as candidates for biological terrorism (212), for detection of 16S rRNA from *Geobacter* and *Desulfovibrio* sequences in unpurified soil extracts (185) and for evaluation of cyanobacterial abundance in mesotrophic and eutrophic lakes (170). Most recently, Loy et al. (122) developed an oligonucleotide based-array targeting 16S rRNA of sulfate-reducing prokaryotes (SRP). The authors used a PCR-based technique to label environmental samples from lake and periodontal samples to obtain a fingerprint of SRP diversity.

Modifications of the conventional oligonucleotide and DNA-based arrays have also been used to characterize target sequences in environmental samples. For example, Valinsky et al. (199) arrayed 16S rRNA sequences from soil clonal libraries and hybridized using 27, 10-mer discriminating oligonucleotide probes. Cluster analysis from "fingerprint" profiles of 1,536 rDNA clones from two agricultural soils revealed

compositional differences which may make it possible to correlate specific bacterial communities to soil disease-suppressive qualities. Guschin et al. (88) modified a polyacrylamide gel pad, oligonucleotide-based technique used previously for genome sequence analysis, for characterization of nitrifying bacteria. This technique was recently applied for characterization of anaerobic toluene and ethylbenzene degrading consortia (106). Results suggest that detection sensitivity in environmental samples is limited. On-going work to optimize probe design and hybridization conditions may ultimately alleviate some issues related to the sensitivity and specificity of this array format (198).

Wu et al. (216) developed and evaluated the specificity, sensitivity and quantitative characteristics of a DNA-based array containing genes important in biogeochemical cycles. These authors were able to qualitatively detect hybridization to functional genes encoding key denitrification, nitrification and methane oxidation enzymes using 25 to 50 ng of fluorescently labeled soil community DNA. However, these and other authors suggest that the quantitative aspects of community-based arrays have not been well established and issues related to many aspects of array fabrication, hybridization, and analysis remain to be addressed (39, 216). Techniques for identification, classification and expression analysis of pure or mixed microbial samples show great promise and provide environmental and ecological data on a high-throughput, short time-scale not possible even in the recent past. However, to date quantitative analysis of specific genetic sequences, especially in mixed community environmental samples remains an elusive goal.

Array Issues: specificity, sensitivity and analysis

Researchers have begun to more rigorously evaluate experimental and analytical aspects of array experiments. In the past, the high cost and overwhelming magnitude of data accrued from one array experiment has limited replication and validation of results. This practice has resulted in an unprecedented lack of scientific stringency when reporting array data. However, the trend is abating as scientific groups begin to organize and send out guidelines for publication (28). To best interpret array results, careful consideration must be given to potential sources of error. In general, sources of error in array experiments can be placed into two categories, experimental error and analytical error. Experimental issues in array experiments arise from sample variability, array design and fabrication as well as from variation in sample preparation, processing, labeling and hybridization (117). Analytical error occurs at the level of data analysis and quantitation. Potential sources of experimental and analytical error must be pinpointed and proper standards and controls established to avoid misinterpretation of results. This is perhaps more important for environmental array applications where there is less control over the target community than in traditional reductionistic laboratory studies.

The breadth of genetic diversity in the environment influences both array fabrication and data interpretation. Structural gene sequence diversity in functionally related communities (i.e., nitrifying bacteria, SRP, nitrogen fixers) is large and makes up-front bioinformatics work cumbersome, but essential especially when short oligonucleotide probes are used. Specificity is critical since parallel hybridization of hundreds or thousands of arrayed probes increases the potential for cross-hybridization between unrelated genetic sequences (218). Interpretation of data is equally vexing.

Changes in hybridization patterns between samples may likely represent genuine differences between samples; however, a lack of differences in hybridization patterns does not necessarily mean that the samples are similar. It may instead reflect the possibility that dissimilarity exists in sequences that are not represented in the arrayed probes. Some researchers have made progress toward determination of confidence limits for evaluation of array data. For example, Wu et al. (216) reported that their functional gene arrays could accurately discriminate probe sequences of 80-100% identity. Oligonucleotide-based arrays are more sensitive to base-pair mismatches and therefore exhibit greater hybridization specificity (198). However, recent research has shown that cross-hybridization with oligonucleotide-based arrays is also an issue (122). More detailed studies of array specificity are needed to permit proper analysis of array data. As pointed out by Zhou (218), one of the most difficult issues in terms of application of array technology to environmental samples is differentiating changes in hybridization signal resulting from community differences and those resulting from sequence divergence.

Sensitivity may be the most important parameter to consider in environmental array-based studies. In environmental studies, low biomass or low target sequence concentration and high concentrations of contaminants often reduce detection limits (117, 216). Therefore, many researchers have resorted to enrichment culture or PCR amplification to obtain enough labeled target for array analysis (122, 167). This has the obvious limitation of altering *in situ* environmental conditions, which can have a profound effect on gene expression and on community composition. Target labeling can also influence sensitivity depending on the type and efficiency of incorporation of the

fluorophore (117). The length of the arrayed probe, and the location, type and number of base pair mismatches between probe and target affect oligonucleotide-based array signal intensity (198). These issues should also be explored for DNA-based arrays.

Specificity and sensitivity are both dependent on experimental factors relating to array fabrication and hybridization. Slide surface chemistry, spot morphology, probe binding capacity and hybridization and wash conditions each affect specificity and sensitivity in array experiments (176). These factors are all related to the material used for adherence of arrayed probe sequences. Glass slides and nylon membrane are the most common array surfaces. Glass slides are smaller, have lower background and require reduced hybridization volumes. However, glass slides cannot be re-used while membrane arrays can be stripped and re-used several times. Nylon membranes are hybridized with radiolabeled probes, while glass slides are hybridized with fluorescently labeled probes. Fluorescent signals are advantageous because the signal does not disperse as readily as radioactive signals, thereby permitting denser array spacing. Additionally, hybridization signals from multiple probes, differentially labeled and hybridized to the same slide can be detected separately (176). However, the sensitivity of detection is significantly less using glass-slide based techniques due to lower dynamic range as a result of the limited binding capacity of the glass. It has been suggested that there is up to 10^5-fold difference in the amount of DNA probe that can be immobilized on a glass slide versus a nylon hybridization membrane, resulting in an equivalent difference in detection sensitivity (39). The efficiency of extracting and labeling target material from low-copy probe sequences must be improved to eliminate the need for enrichment or PCR amplification.

Until these problems are addressed, quantitative analysis of array data from environmental samples will be primarily hindered by the lack of sensitivity.

Analytical issues related to extraction and interpretation of relevant data from array experiments are currently the focus of intensive investigation. Interpretation of data is made difficult by inherent variability in array experiments due, in part, to experimental factors discussed above. Additionally, the lack of replication and the magnitude of simultaneously derived data points make extraction of relevant information cumbersome. Currently, a movement is underway to describe methods for standardizing and normalizing array experiments to accommodate better interpretation of array data (41, 135, 156).

Since many experimental factors in an array experiment are difficult to calibrate (i.e., efficiency of incorporation of fluorophores, concentration of spotted target material, or the amount of target available for hybridization) it is important to incorporate proper controls and methods for normalizing to account for slide-to-slide variability (117, 122). These sources of experimental error are controlled, to some extent, by pre-processing of array data through background subtraction and data normalization. Background subtraction is the simple process of subtracting local and nonspecific background from spot intensity values. This step controls for nonspecific hybridization, differential blocking, and some mechanical spotting errors. The most simple and most common method for data normalization involves normalizing against the total image intensity or average intensity of all spots on the array (117, 145). More recently, methods have been developed to incorporate arrayed control spots (206), internal standards (39) or calibrated

standards (46) to correct for some experimental variability and to make slide-to-slide comparison easier.

Following pre-processing of array data, the actual analysis presents an enormous obstacle. The challenge has been to arrange data in a meaningful context without losing important information or overstating the statistical significance of the data. Most commonly, array data is analyzed by comparing ratios of gene expression from control and treatment samples. Confounding experimental factors are eliminated when control and treatment probes are hybridized simultaneously because they affect both probes equivalently (176). However, these values cannot be related to absolute expression levels and samples without similar controls cannot be compared. Additionally, this represents a reductionistic approach that does not consider the breadth of information available from an array experiment. To glean more information from array data, Eisen et al. (51) introduced a hierarchical clustering technique, which has been used extensively and modified by others (104, 135, 156). Hierarchical clustering organizes large sets of data based on similarities in patterns of gene expression. The resulting groups of co-expressed genes have been shown to have similar properties reflecting gene function, genetic regulation or physical location (31, 50). Importantly, genes of unknown function often cluster with genes with defined function, making these genes obvious choices for further analysis (99). With a few notable exceptions (38, 39, 198), even the most cursory attention to analytical standards has been ignored. This is due in large part to the fact that most environmental array technology is currently in the developmental stage. As the technology becomes more established, methods for normalization and standardization of data should be incorporated into the experimental design. Until these techniques have

been established for environmental studies, however, array data should be held to high standards and rigorously validated.

Future applications of array technology to environmental studies

Integration of disparate technologies and disciplinary fields is required to incorporate array technology into a holistic approach to analysis of complex environmental systems. For example, Ideker et al. (93) used a combination of deletion mutants, microarray analysis and proteomics to evaluate the yeast galactose utilization pathway. Similarly, Fouts et al. (59) used an iterative approach to identify genes involved in *Pseudomonas syringae* pathogenicity. Using a combination of miniTN5gus mutagenesis, gene expression and computational analysis, these researchers identified gene promoters, which control virulence factors that activate the hypersensitive response and pathogenicity (Hrp) system. These studies illustrate how directed research using array-technology in conjunction with other proven methodologies can produce new fundamental knowledge about important concepts in environmental science.

Issues relating to sensitivity and specificity, as well as data analysis and interpretation make it important to proceed with caution when applying array technology to studies in the environment. Recognizing the fact that mRNA expression does not necessarily reflect the cellular response (more closely approximated by protein expression) researchers are developing new techniques to evaluate whole cell response. For example, the Lux array developed by DuPont provides a cellular array of gene reporter fusions in *E. coli*. This array is composed of *E. coli* cells with defined reporter gene fusions in a 96-well array format. The Lux array is used in the same fashion as a traditional array, but enables analysis of the whole cell response and can be used to

collect kinetic data (by measuring bioluminescence for a single array over time)(200). Other researchers are arraying plasmid DNAs onto glass slides and spreading cells in a monolayer over the slide surface. Cells coming into contact with the expression vector divide and form clusters of transfected cells, forming living cell arrays (219). Proteomics represents the newest challenge in array research. To evaluate protein function, robotic technologies are being used to spot proteins at high density. Covalently attached proteins have been shown to interact with other proteins or molecules revealing new information about protein-protein interactions, the substrates of protein kinases, and the protein targets of small molecules (126). Regardless of the form, array technology promises to revolutionize environmental studies as researchers apply array technology to the environment in ways that suggests that many new aspects of ecology, physiology, systematics, structure and function can be explored in a more compete and probing manner than ever before.

BIOLOG AND BD OXYGEN BIOSENSOR PLATE COMMUNITY LEVEL PHYSIOLOGICAL PROFILING (CLPP)

Evaluation of microbial community structure over temporal or spatial gradients provides a more complete view of community dynamics than the more popular phylogenetic methods based on 16S rDNA sequence analysis. Molecular methods involving sequence analysis give more detailed information about community composition but take a more reductionistic approach to characterization of microbial communities. Often, the result is a “point in time” measure of community structure, without any linkage to community dynamics or ecosystem function. Community

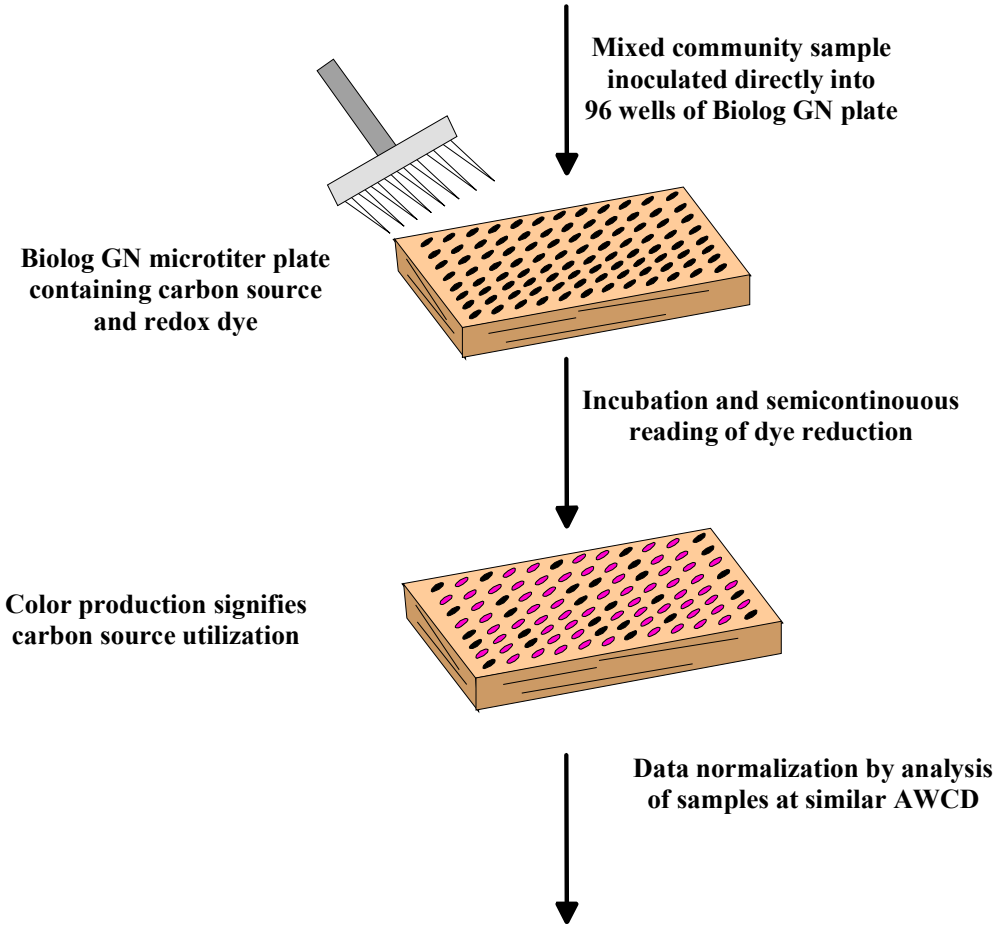
fingerprinting techniques based on the 16S rDNA sequence (i.e. TGGE, DGGE, ARDRA) permit analysis of differences in microbial composition associated with environmental, spatial or temporal changes. However, these analyses are based on genetic differences that cannot be directly related to community ecology and community function.

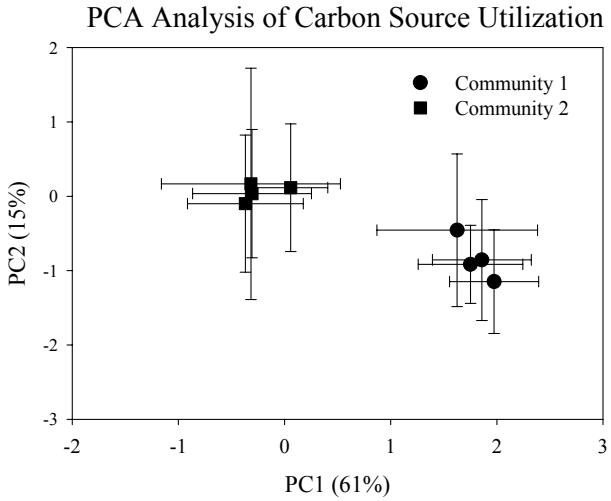
Community level physiological profiling (CLPP) using Biolog was developed by Garland and Mills (75) as a means to characterize differences among microbial communities on the basis of substrate utilization. In this method, commercially available microtiter plates (Biolog GN; Biolog, Inc. Hayward, CA) containing 95 different carbon substrates are directly inoculated with environmental samples (Fig. 4). The functional potential of microbial mixtures is quantified by measuring the reduction of tetrazolium violet to colored formazan (71). Reduced tetrazolium violet produces a colored product (the formazan) that serves as an indicator of respiration of the carbon source (75). Patterns of carbon source utilization by mixed communities are analyzed using multivariate statistical methods. Community samples are thereby differentiated based on differences in carbon metabolism (89).

CLPP through analysis of carbon source utilization patterns is a rapid, high-throughput method with significant resolving power (66). Haack et al. (89) found that replicate microplates inoculated with the same microbial communities (either bacterial isolates, model bacterial communities composed of combinations of strains or soil microbial communities) exhibited reproducible substrate utilization profiles. Furthermore, different communities were clearly distinguished following principal component analysis (PCA) suggesting that differences in patterns of carbon source

Figure 4. Schematic showing community level profiling procedure for mixed community analysis of carbon source utilization.

Community-level Physiological Profiling of Mixed Microbial Communities





utilization reflect real differences in community composition. Garland et al. (68) found that subtle shifts in microbial communities in response to plant type and plant age were discernible. This is supported by data from other researchers using Biolog CLPP to distinguish communities from plant, soil and water ecosystems (63, 71, 89, 103, 203).

Variation in the rate of color development in the Biolog CLPP assay can result from differences in diversity or distribution of organisms in the sample (true differences between communities) or from differences in inoculum density. Correlations between inoculum cell density and the rate of color development occur since more dilute samples will require longer incubation times to develop while the opposite is true for more dense inocula. To use Biolog CLPP to analyze for differences in composition (as opposed to differences in density) of communities, steps must be taken to normalize for inoculum density (66). Garland and Mills (75) adjusted for inoculum density by calculating an average well color development (AWCD) value for each sample and dividing each sample by this value. AWCD is calculated as the mean of the blanked absorbance values for all 95 responses per reading time (67). Subsequent research has shown that the most effective means of minimizing inoculum effects is to monitor color development in a semi-continuous manner, and evaluate samples at equivalent points of ACWD, but not necessarily incubation times (66). The relationship between samples is evaluated using multivariate statistical analysis; most often PCA is used. PCA is applicable when the number of variables per observation is higher than the number of observations (high multivariate dimensions but few observations) (81). PCA can often summarize, in a few dimensions, most of the variability of a dispersion matrix of a large number of descriptors (115). The first principal component is the line that goes through (or explains) the

greatest amount of the variance in the original data (115). Successive principal components correspond to progressively smaller fractions of the total variance. Relationships among samples are discerned by plotting on the basis of their scores for the first two principal components (75). Each of the principal components can be correlated to the original variables so that axes descriptors can be obtained. The significance of the variance associated with the principal components may be obtained through subsequent statistical analysis (81).

Interpretation of the ecological meaning of Biolog CLPPs has been difficult. The degree to which growth of cells in the microtiter plate affects the overall response and the ultimate meaning of a positive response have been the subject of much debate. Haack et al. (89) found that growth does occur during incubation of Biolog plates, and that the plates are selective for a subset of the inoculated community. This is supported from TGGE and DGGE analysis that suggests that organisms utilizing sole carbon sources are not necessarily the dominant members of the original community (186). Furthermore, utilization of a carbon source by a combination of isolates was not a summation of the individual carbon source utilization profiles suggesting that the degree of oxidation of a particular substrate could not be correlated with the number of utilizers or with individual community members (89). Garland et al. (71) found that addition of amended substrate (up to 50% of readily available carbon) to microbial communities in continuously stirred tank bioreactors resulted in weak to no additional response in the Biolog plate well containing the corresponding carbon substrate. These results suggest that the method has limited application as a measure of *in situ* utilization of specific carbon sources by the community, but rather serves as tool to discriminate between communities based on

functional potential (71, 81). Color development in the wells of Biolog plates depends on the inoculum density and the physiology and interactions between inoculated organisms (81). However, if communities of similar overall cell density and of sufficient complexity are compared, patterns of carbon source utilization provide a rapid, reproducible method for differentiating those communities based on metabolic profiles.

More recently, a CLPP method has been developed which may provide more information concerning *in situ* community carbon source utilization (77). The improved system provides substrate at lower concentrations and results in detectable signal over shorter incubation periods, thereby minimizing biases associated with cell growth and selective enrichment. The BD Oxygen Biosensor System (BD Biosciences, Bedford, MA) is used to detect shifts in substrate utilization by mixed microbial communities in either a 96 or 384-well microplate format. This technology, developed for use in the medical and pharmaceutical fields, is based on an O_2-sensitive fluorophore, 4, 7-diphenyl-1,10-phenathroline ruthenium (II) chloride (214). The Oxygen BioSensor contains the ruthenium dye that is immobilized within an oxygen-permeable silicon matrix (214). The dye is quenched in the presence of oxygen, but increases in response to oxygen depletion. The response to oxygen depletion (i.e., respiration of the substrate) is measured with an automated fluorescence plate reader. Preliminary studies suggest that the rate of the fluorescent response is related to *in situ* rates of degradation and is less influenced by selective enrichment (77).

THE DIVERSITY-STABILITY QUESTION

A fundamental goal of ecological research is to better understand the relationship between biological diversity, functional diversity and ecosystem stability. It has been argued that diversity increases ecosystem function by ensuring that more productive species are present to provide more complete resource utilization and better coverage in heterogeneous habitats (146, 195). Other research suggests that functional diversity is a more reliable indicator of ecosystem stability and that overall diversity is not directly related to ecosystem function (13, 84). A 605-day study of the community dynamics within a functionally stable methanogenic reactor found that no climax community was established (54). Instead, the bioreactor functioned effectively for the duration of the study, but structural diversity was constantly changing from one sample to the next. These results suggest that genetic changes may be more dramatic than functional changes, supporting the theory that in functionally redundant communities, shifts in community structure at the individual level may not be reflected at the community level (13).

A related philosophy holds that ecosystem stability results from functional diversity relative to a few key processes that vary with time and space (58). These niches are filled by a "keystone process species" that drive critical processes required for system stability (58). Distinct environmental conditions correspond to unique community assemblages that may be correlated to a few keystone processes and resultant, distinctive keystone process species. These species create niches that are occupied by the remainder of the community. The loss of keystone species results in a loss of plasticity and capacity for self-organization and minimizes the systems' stability by reducing the ability of the

community to provide a buffer to environmental perturbations (58). Alternatively, fundamental aspects of the stability-diversity relationship may be a function of initial assembly mechanics (44, 168). Drake (44) found that differences in invasion order and species persistence led to alternative community states. This suggests that events occurring during early community assembly may be more important to understanding community structure and function than extant community structure (171). The resultant communities studied in soil, bioreactor, and rhizosphere systems may reflect one community state or "climax community" of many possible alternative community states.

Microbial communities are highly versatile and dynamic and therefore, provide a good model system for evaluation of diversity-stability hypotheses (66). However, methodological limitations have made the study of details concerning community dynamics difficult. Recent advances in molecular techniques and high throughput methods for analyzing specific structural and functional aspects of microbial diversity coupled with new tools for analysis of multidimensional data have permitted more detailed studies of interactions in environmental systems (197). As a result several recent studies have used microbial communities for analysis of the relationship between community structure and ecosystem stability. McGrady-Steed et al. (133) used constructed communities to create a diversity gradient in several different combinations. Using these artificial communities of aquatic eukaryotic species, they found that biodiversity enhanced system predictability (133). In separate studies, researchers used fumigation to evaluate the effect of decreases in species richness on the number and type of processes carried out in two different soil systems (42, 84). These researchers found the decrease in species richness correlated to the loss of function in specific groups

(nitrifiers, methane oxiders) and not to a loss in overall functional capacity (42, 84). Dilution to extinction has also been used to create diversity gradients. In this approach, dilutions of a diverse community are used to initiate new systems and re-growth of the community produces communities of progressively lower diversity, but similar cell density. This approach has been used in rhizosphere and bioreactor studies and has been shown to produce communities of different structural and functional potential (60, 72, 76).

RESEARCH GOALS AND OBJECTIVES

The goal of this research was to contribute new fundamental understanding of the effect of structural diversity on community stability and function. Insight into the interaction between these factors may make it possible to improve process control for ecological and biotechnological applications (13). However, these issues are also relevant in closed systems such as those employed by NASA for long term and deep space missions. Closed systems are self-sustained ecosystems, reliant on biological and physio-chemical processes for production and processing of important nutrients including food and water. Microbial inoculants will likely be used to initiate biological waste processing systems employed on these missions. The need for system inoculation is driven by the lack of suitable indigenous "inoculant" species in closed systems, and the need for a taxonomically, physiologically, and genetically well-characterized group of organisms to avoid potential health hazards (76). Historically, inoculants have had limited success in environmental applications due to the lack of consistency of results in field application (202). Inoculants either fail to become established and persist in the inoculated system or

they survive and grow in sterilized systems, but then decline in competition with more competent species (21, 136).

Microorganisms metabolize recalcitrant organic and inorganic compounds and prevent invasion by plant and human pathogens through competitive interactions. Furthermore, emergent properties resulting from complex interactions among members of a stable microbial community provide a resilient populace suited to survival in dynamic environments. The ability of inoculants to survive and form a cohesive community in closed systems will, therefore, be vital to ensure reproducible, reliable and resilient system function. Therefore, this research focused on the use of a model plant-based graywater waste processing system to evaluate the effect of microbial community structure on community processes (i.e., degradation of organics, susceptibility to invasion). An integrated approach incorporating phylogenetic analysis, phenotypic analysis, and assessment of community dynamics should provide a more complete view of microbial community processes, and provide specific information regarding the suitability of microbial inocula for initiation of BLSS for use on long term and deep space missions. In this research, an integrated approach using PCR-based phylogenetic analysis and quantification, CLPP and microarray analysis was employed to evaluate the effect of structural diversity on community phenotype and community invasibility.

The *principal hypothesis* of this research is that:

A community of rhizosphere organisms, constructed using parameters described in this research, will be as effective as an undefined industrial activated sludge community in terms of its ability to:

- Establish in the rhizosphere
- Persist over time
- Resist invasion
- Degrade organic compounds

In Part II of this dissertation, the composition of microbial communities within a rhizosphere system used for graywater waste processing and an industrial wastewater treatment plant (WWTP) activated sludge system were described. The results of this analysis were used as the basis for selection of organisms for use in a constructed community and for selection of a source for an undefined inoculum. To assess community structure and dynamics, it was necessary to establish new methods for monitoring inoculated communities. To this end, a novel 16S-23S rDNA intergenic spacer microarray was developed and is described in Part III. In Part IV, a model plant-based graywater waste processing system was initiated with six different microbial inocula to evaluate the effect of species richness on community phenotype and community invasibility.

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PART II

DEVELOPMENT AND MOLECULAR CHARACTERIZATION OF MICROBIAL INOCULA FOR INITIATION OF GRAYWATER WASTE PROCESSING SYSTEMS ON LONG-TERM SPACE FLIGHTS

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. The purpose of this study was to develop microbial inocula (a defined, constructed community and an undefined community) for initiation of plant-based graywater waste processing systems. To this end, small-subunit 16S rDNA sequence analysis was used to describe the population composition of microbial communities from a plant-based graywater waste treatment system and from an industrial wastewater treatment plant (WWTP). The clonal library of organisms from the graywater-degrading rhizosphere community suggested that members of the *Cytophagales* and *Proteobacteria* phylogenetic groups dominated. The clonal library of organisms from the industrial WWTP was taxonomically more diverse. The phylogenetic analysis provided the basis for the selection of organisms for use in the constructed community and for the selection of a source of an undefined, complex microbial inoculum. The constructed and the industrial WWTP sludge communities were inoculated into a model plant-based graywater waste processing system to evaluate their survival. Based on plant, microbiological and molecular biological measures, it appeared that both inoculated communities were able to become established and persist in the 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) may be used for 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 microbial inocula of differing diversity could successfully be maintained in a BLSS.

MATERIALS AND METHODS

Extraction of DNA from graywater rhizosphere community

Samples for molecular analysis were 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 (1.0 g K_2HPO_4, 0.5g KCl, 0.5g $MgSO_4 \cdot 7H_20$, 0.5g $NaNO_3$, and 0.01g $FeSO_4 \cdot 7H_20$,

pH7.2) or Dubos media with 1.0 g casein or with ground Whatman #1 filter paper. Additional rhizosphere organisms were isolated using enrichment cultures in rhizosphere media (minimal salts media with rhizosphere solution extracted from 24 plants and having an electrical conductivity of 186 µS/cm and a pH of 7.0). Phylogenetic information was obtained from 45 rhizosphere isolates by sequencing 500bp of the 16S rDNA. Each isolate was frozen at –80 ºC in TSB with 15% glycerol. Phylogenetic information from these isolates was compared to clone sequences from the graywater rhizosphere clonal library. 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^{TM} 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. In this way, a variety of 16S-23S rDNA ISR sequences from a mixed-community sample could be amplified using a single set of PCR primers and PCR conditions. 16S-23S rDNA ISR sequences 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 94 ºC, then 10 cycles consisting of 15 s at 94 ºC, 45 s at 65 ºC, followed by a 1 ºC decrease for each of the 10 cycles, and 60 s at 72 ºC, followed by an additional 20 cycles of 15 s at 94 ºC, 45 s at 55 ºC, and 60 s at 72 ºC and a final cycle of 60 s at 72 ºC. 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 library

Plasmid DNA from randomly selected 16S-23S rDNA ISR clones from the graywater rhizosphere community and 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 analyzed by RFLP 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 37 ºC, products were electrophoresed in 4% agarose gels in 0.5X TBE, and visualized by UV transillumination after staining with ethidium bromide. GWR999 clones with similar RFLP-ISR patterns 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. Similar isolates were also grouped, selected and sequenced. Clones from the graywater rhizosphere library were designated GWR999 followed by a clone number (01 to 40) (Fig. 1).

Figure 1. Schematic showing the procedure used to distinguish unique clone groups. Plasmid was extracted from randomly selected clones from a 16S-23S rDNA library. The ISR region of plasmid inserts digested with two restriction enzymes. Following digestion samples were subjected to gel electrophoresis. Clones exhibiting similar restriction digestion patterns were grouped. Selected clones from each group were sequenced.

DNA from Clones and Isolates Extracted & Amplified by PCR

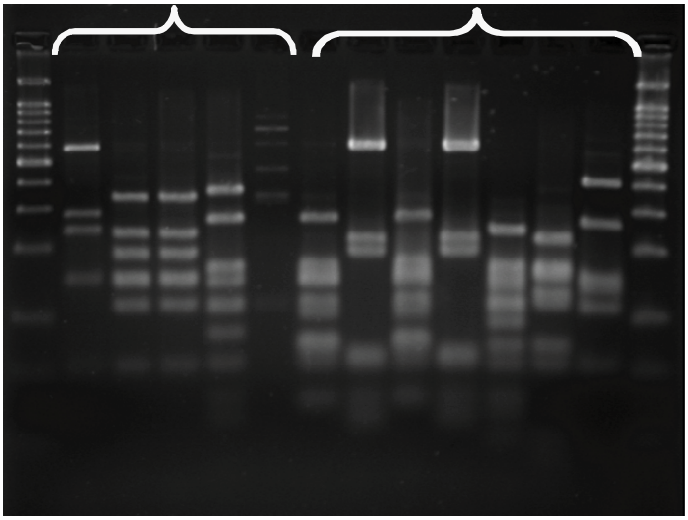
PCR using primers 1055f and 23Sr

Restriction Digestion of PCR Products with *RsaI* & *HhaI*

RISA-RFLP

RISA-RFLP

Isolates Lanes 2-6 Clones Lanes 7-13



Phylogenetic analysis

Randomly selected clones from the WWTP library were characterized based on sequencing 500 bp of the 16S rRNA using the 1055f primer. Selected clones from the graywater rhizosphere clonal library and isolates were sequenced using the 1492r primer (5'TAC GGY TAC CTT GTT ACG ACT T3'; (25). Sequencing was performed at the University of Tennessee Molecular Biology Resource Facility using an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, CA). DNA sequences were aligned using the Clustal W program (45). Sequences of known phylogeny were obtained from GenBank. A phylogenetic tree was constructed using Clustal W 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 4 ºC 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 experiment, 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.

Preparation of inocula

The inoculum for the undefined, industrial WWTP community was prepared by freezing 0.5ml aliquots of the sludge with 1 ml of 40% glycerol at –80 ºC. Isolates from the constructed community were grown separately on rhizosphere media to approximately the same cell density as determined by turbidity measured at 600 nm. Each culture was frozen in 20% glycerol at –80 ºC.

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, surfactant was added to each plant growth vessel at a final concentration of 1000 ppm. For the second inoculum analysis study, surfactant 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 to simulate graywater addition. The constructed community and the industrial WWTP sludge community were inoculated into the rhizosphere of four replicate plants. 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.

Plant and microbiological analysis

Suspensions of rhizosphere organisms were prepared by removing a single plant from each treatment on days 0, 14 and 28 of the first inoculum study, placing the rhizosphere into 50 mL tubes with filter sterilized nutrient solution and glass beads and shaking for two minutes. Rhizosphere suspensions were serially diluted and plated onto R2A. CFU were enumerated after incubation for 72 h at 28 ºC. Wheat root and shoot dry weights were determined after plant material was dried at 70 ºC for 72 h.

Community-level molecular profiling of inocula establishment

Bacterial cells were pelleted (from 10-15 mL of rhizosphere suspension) 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.

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 known sequences obtained from GenBank (Fig. 2). 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 clones came from the gamma subdivision of proteobacteria (26%) followed by the beta and alpha subdivisions of proteobacteria (Fig. 2).

Selection of consortium isolates. The process used to select isolates for inclusion in the constructed community is shown in Fig. 3. 16S rDNA sequences from 45 graywater rhizosphere isolates were significantly different from corresponding sequences of 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. 2). Two of the isolates were from the beta subdivision of proteobacteria (*Ralstonia* and *Burkholderia*) and three isolates were from the gamma subdivision of proteobacteria (*Xanthamonas*, *Pseudomonas* and *Frateuria*). These

Figure 2. Phylogenetic tree showing the relationship between graywater rhizosphere clone library sequences and isolates chosen for use in the constructed community. Clones are shown in boldface and are designated GWR999. Isolates used in the constructed community are enlarged and in boldface and are designated Constructed 1-11. For graywater rhizosphere clones, numbers in the first parentheses represent the number of sequenced clones, while numbers in the second parentheses represent the number of clones in the 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.

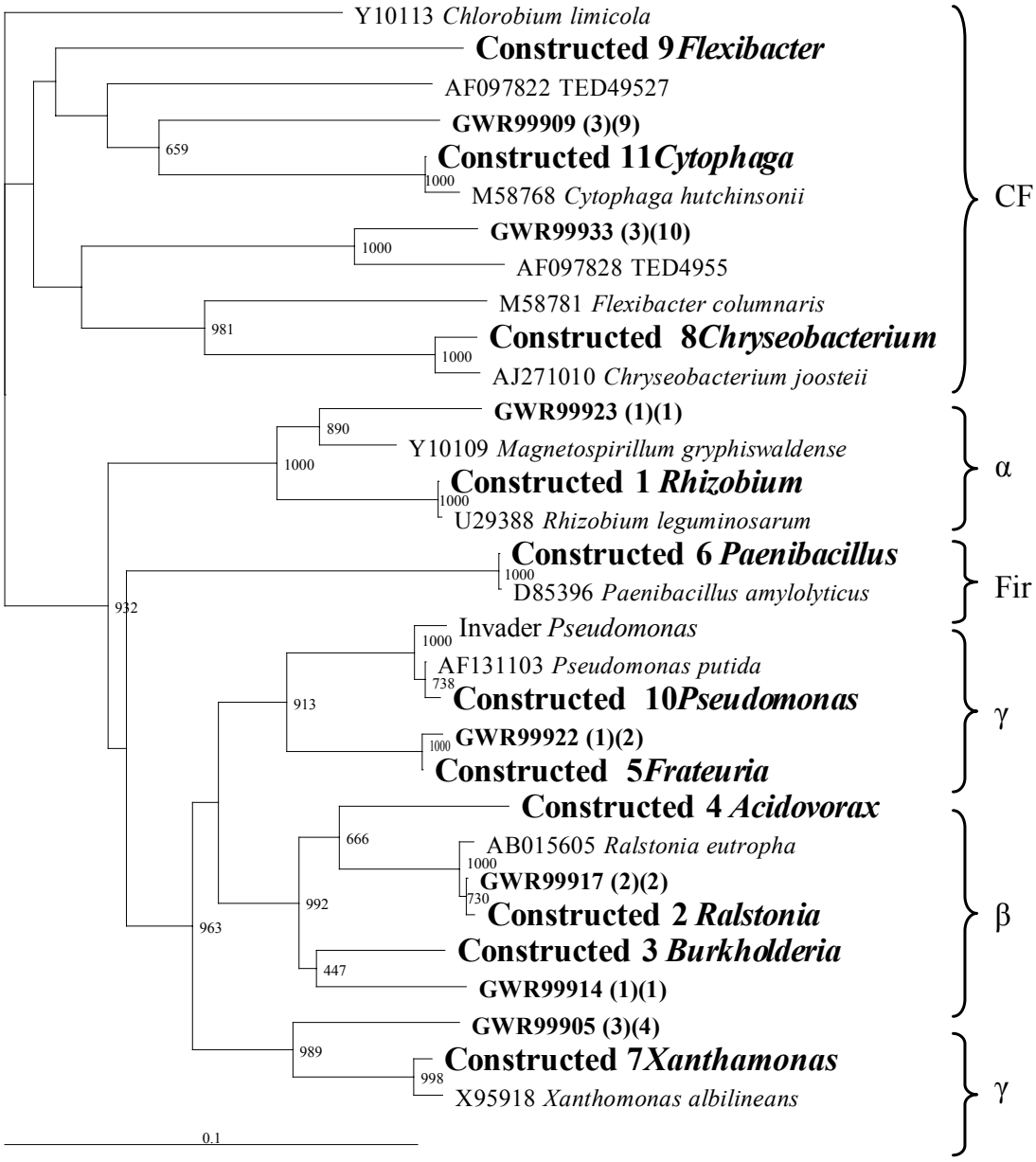
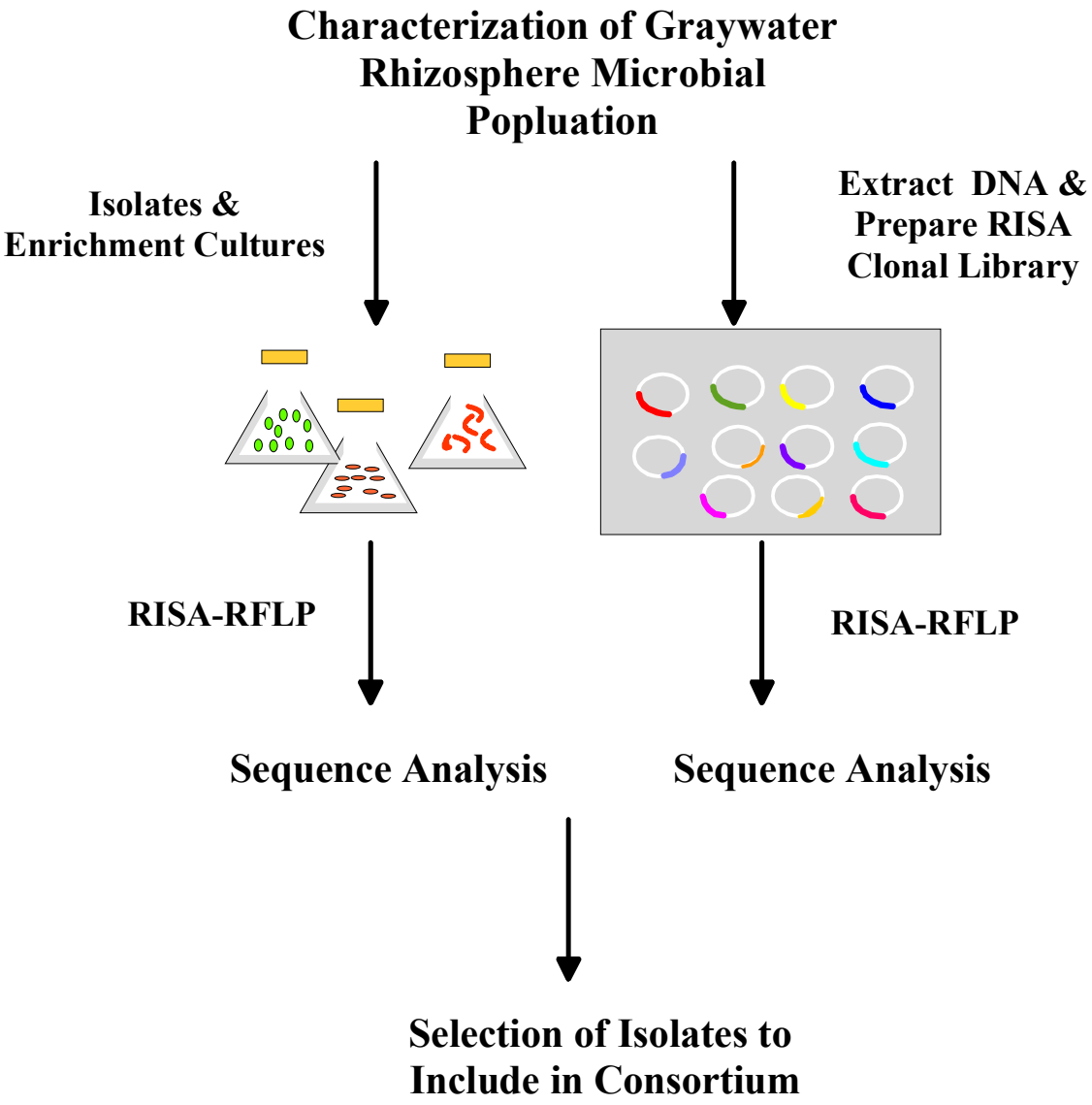

Y10113 Chlorobium limicola
Constructed 9 Flexibacter
AF097822 TED49527
GWR99909 (3)(9)
659
Constructed 11 Cytophaga
1000
M58768 Cytophaga hutchinsonii
GWR99933 (3)(10)
1000
AF097828 TED4955
M58781 Flexibacter columnaris
981
Constructed 8 Chryseobacterium
1000
AJ271010 Chryseobacterium joosteii
CF
GWR99923 (1)(1)
890
Y10109 Magnetospirillum gryphiswaldense
1000
Constructed 1 Rhizobium
1000
U29388 Rhizobium leguminosarum
α
Constructed 6 Paenibacillus
1000
D85396 Paenibacillus amylolyticus
Fir
932
Invader Pseudomonas
1000
AF131103 Pseudomonas putida
738
913
Constructed 10 Pseudomonas
GWR99922 (1)(2)
1000
Constructed 5 Frateuria
γ
Constructed 4 Acidovorax
666
AB015605 Ralstonia eutropha
1000
GWR99917 (2)(2)
730
992
Constructed 2 Ralstonia
963
Constructed 3 Burkholderia
447
GWR99914 (1)(1)
β
GWR99905 (3)(4)
989
Constructed 7 Xanthamonas
998
X95918 Xanthomonas albilineans
γ
0.1

Figure 3. Schematic showing the procedure used to select isolates to be included in the constructed community. Isolates from the rhizosphere of a graywater processing rhizosphere community were compared to 16S-23S rDNA clones from the same system. Isolates included in the constructed community were similar to clones from the graywater rhizosphere clonal library, commonly found in the rhizosphere and/or degraded surfactant.


Characterization of Graywater Rhizosphere Microbial Popluation
Isolates & Enrichment Cultures
Extract DNA & Prepare RISA Clonal Library
RISA-RFLP
RISA-RFLP
Sequence Analysis
Sequence Analysis
Selection of Isolates to Include in Consortium

isolates were used in the constructed community (designated Constructed 2, 3, 7,10, and 5, respectively in Fig. 2). Of these isolates, only *Ralstonia* and *Frateuria* had a 100% match to a graywater rhizosphere clone sequence. None of the sequenced isolates closely matched 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 was designated Constructed 8 in Fig. 2. *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. Isolates chosen for use in the constructed community are shown in Fig. 2. Table 1 shows the phylogenetic placement, source of isolation and indicates the ability (+/-) of the organism to degrade surfactant (Pol).

Industrial WWTP clonal library. A total of 65 16S rDNA clonal sequences from the industrial WWTP library were placed into subdivisions based on comparison of GenBank sequences by BLAST analysis. Based on these identifications, the cloned 16S rDNAs were assigned to *Eubacteria* groups and *Proteobacteria* subdivisions. The library was diverse with representatives from the *Sphingobacteria*, CFB and *Nitrospira* groups and the alpha, beta and delta subdivisions of proteobacteria (Fig. 4). No clones from the gamma proteobacteria were identified in this library. Not surprisingly, over 35% of the clones had greater than 95% matches to previously sequenced clones from the same industrial WWTP system (26) (A. Layton unpublished data).

Plant growth experiments

Surfactant gradient study. The nonionic surfactant Pol, an alkylethoxylate, was added to four replicate plant containers in 6 different concentrations (0 ppm to 200 pp

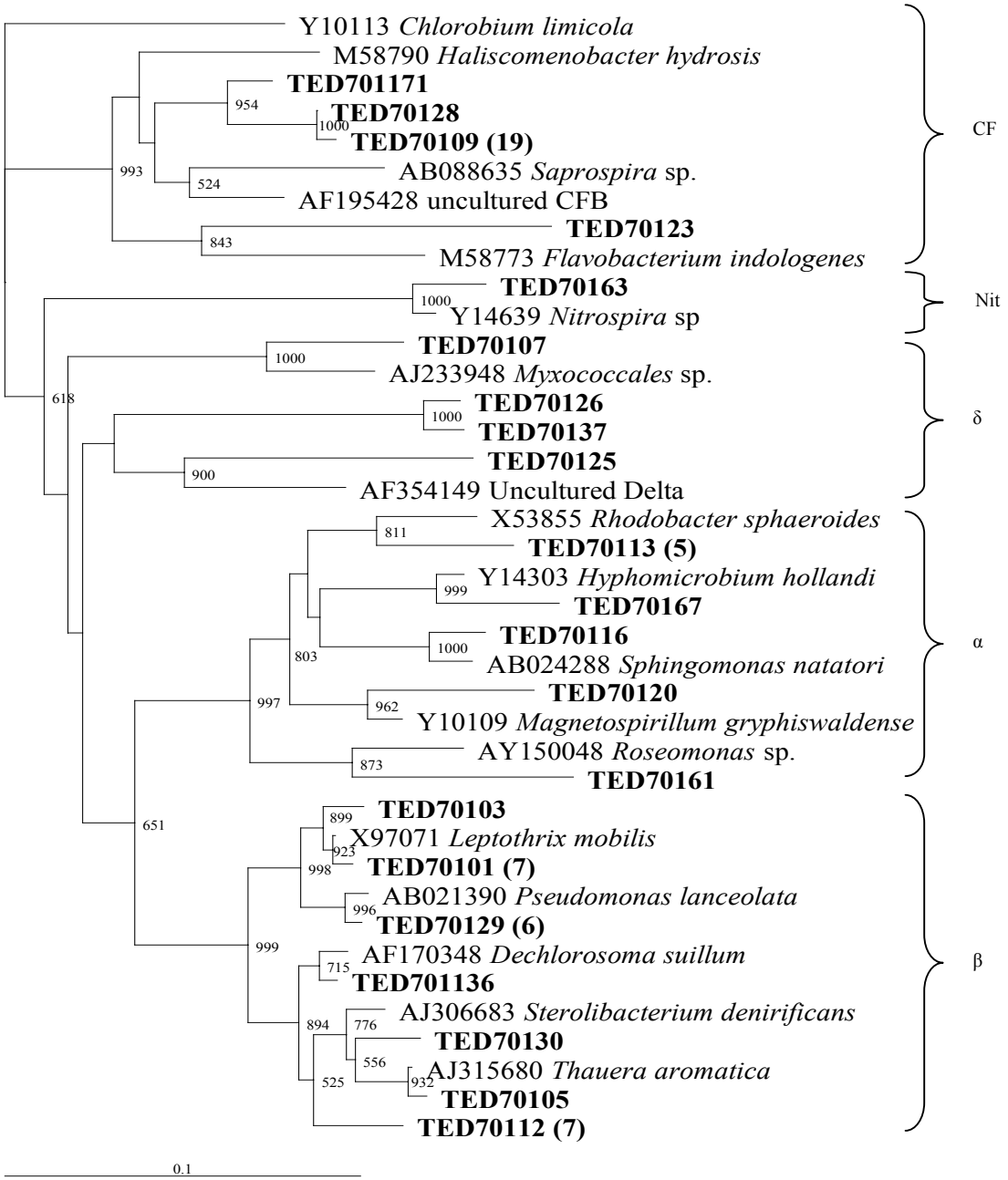
Table1. Isolates included in the constructed community, their phylogenetic placement and ability to degrade surfactant

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

Figure 4. Phylogenetic tree showing the relationship between clones from the industrial WWTP sludge library. The 65 clonal sequences were aligned with reference strains from the domain Bacteria based on 16S rDNA sequence analysis. 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


Y10113 *Chlorobium limicola*
M58790 *Haliscomenobacter hydrosis*
TED701171
954
TED70128
1000
TED70109 (19)
993
AB088635 *Saprospira* sp.
524
AF195428 uncultured CFB
TED70123
843
M58773 *Flavobacterium indologenes*
CF
TED70163
1000
Y14639 *Nitrospira* sp
Nit
TED70107
1000
AJ233948 *Myxococcales* sp.
618
TED70126
1000
TED70137
TED70125
900
AF354149 Uncultured Delta
δ
X53855 *Rhodobacter sphaeroides*
811
TED70113 (5)
Y14303 *Hyphomicrobium hollandi*
999
TED70167
TED70116
1000
803
AB024288 *Sphingomonas natatori*
TED70120
997
962
Y10109 *Magnetospirillum gryphiswaldense*
AY150048 *Roseomonas* sp.
873
TED70161
α
TED70103
899
X97071 *Leptothrix mobilis*
923
998
TED70101 (7)
AB021390 *Pseudomonas lanceolata*
996
TED70129 (6)
651
999
AF170348 *Dechlorosoma suillum*
715
TED701136
AJ306683 *Sterolibacterium denirificans*
894
776
TED70130
556
AJ315680 *Thauera aromatica*
525
932
TED70105
TED70112 (7)
β
0.1

The effect of the surfactant on plant root dry weight and culturable cell numbers was evaluated 29 days after planting (Fig. 5). ANOVA indicated a significant effect of surfactant concentration on root dry weight at concentrations higher than 2 ppm (P = 0.05 at 10 or 20 ppm) with the most significant effects at the higher concentrations (100 ppm or 200 ppm surfactant; $P < 0.05$). Culturable cell counts 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.

Inocula Analysis. Three different inocula were used to establish model plant-based graywater processing systems in two separate studies. The uninoculated treatment 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. 6B). The defined community (designated as constructed) consisted of the eleven isolates, similar in phylogenetic placement 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 sequence analysis. In one study, the plants received 20 ppm surfactant and plant root dry weight and culturable cells numbers were measured from each of the plant treatments 21 days after planting (Fig. 6A and Fig. 6B). ANOVA indicated that root dry weights in treatments not receiving Pol were significantly higher than the Sludge treatment that received Pol ($P <$ 0.05). 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, the root dry weight of plants that did not receive

Figure 5. Plant and microbiological analyses for surfactant study. Root dry weight (A) and culturable cell numbers (B) for wheat plants grown in different concentrations of surfactant (Pol) from 0 ppm to 200 ppm. Plants were inoculated with industrial WWTP sludge community. DAP, Days after planting. Error bars represent the standard deviation of four replicates.

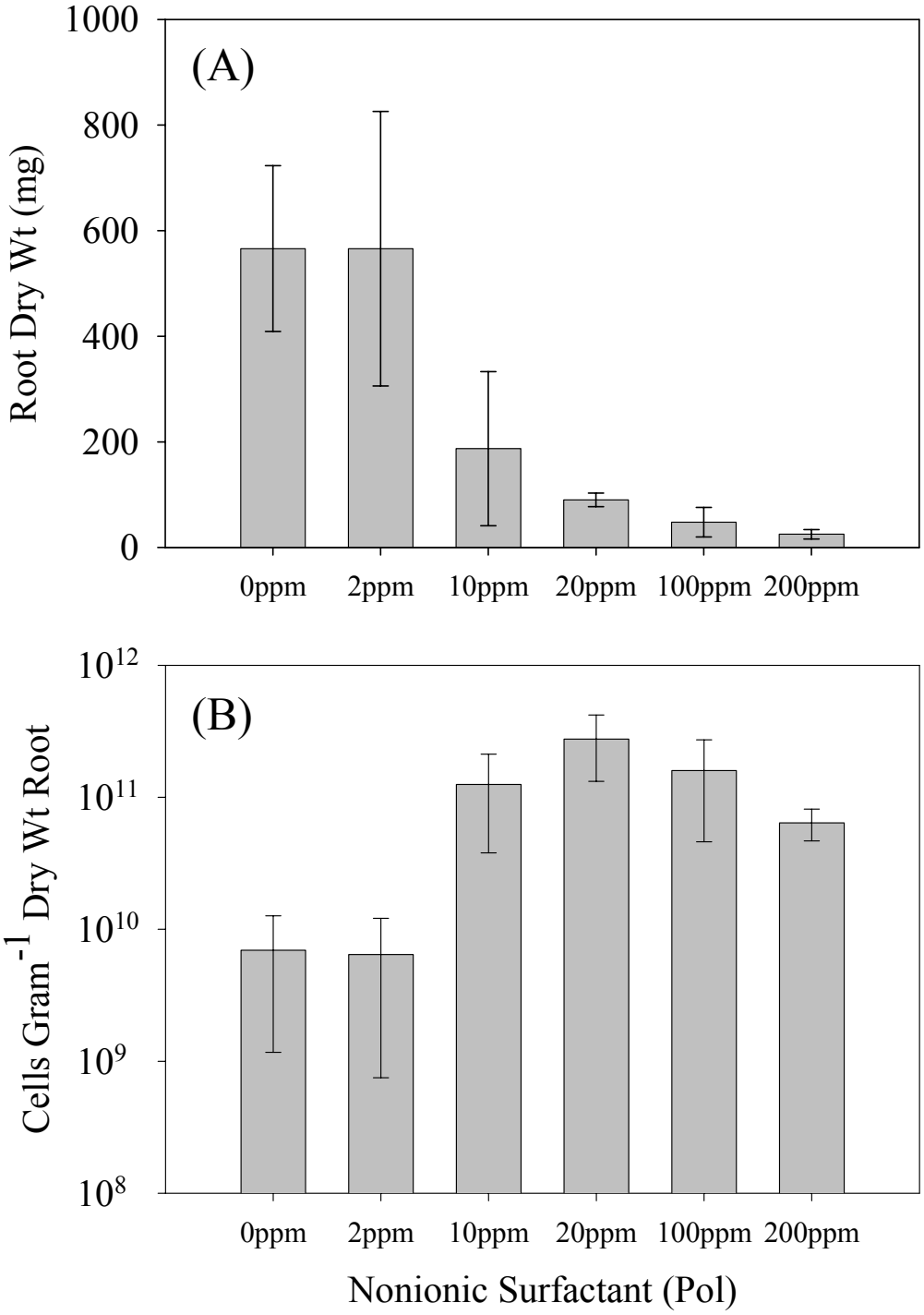
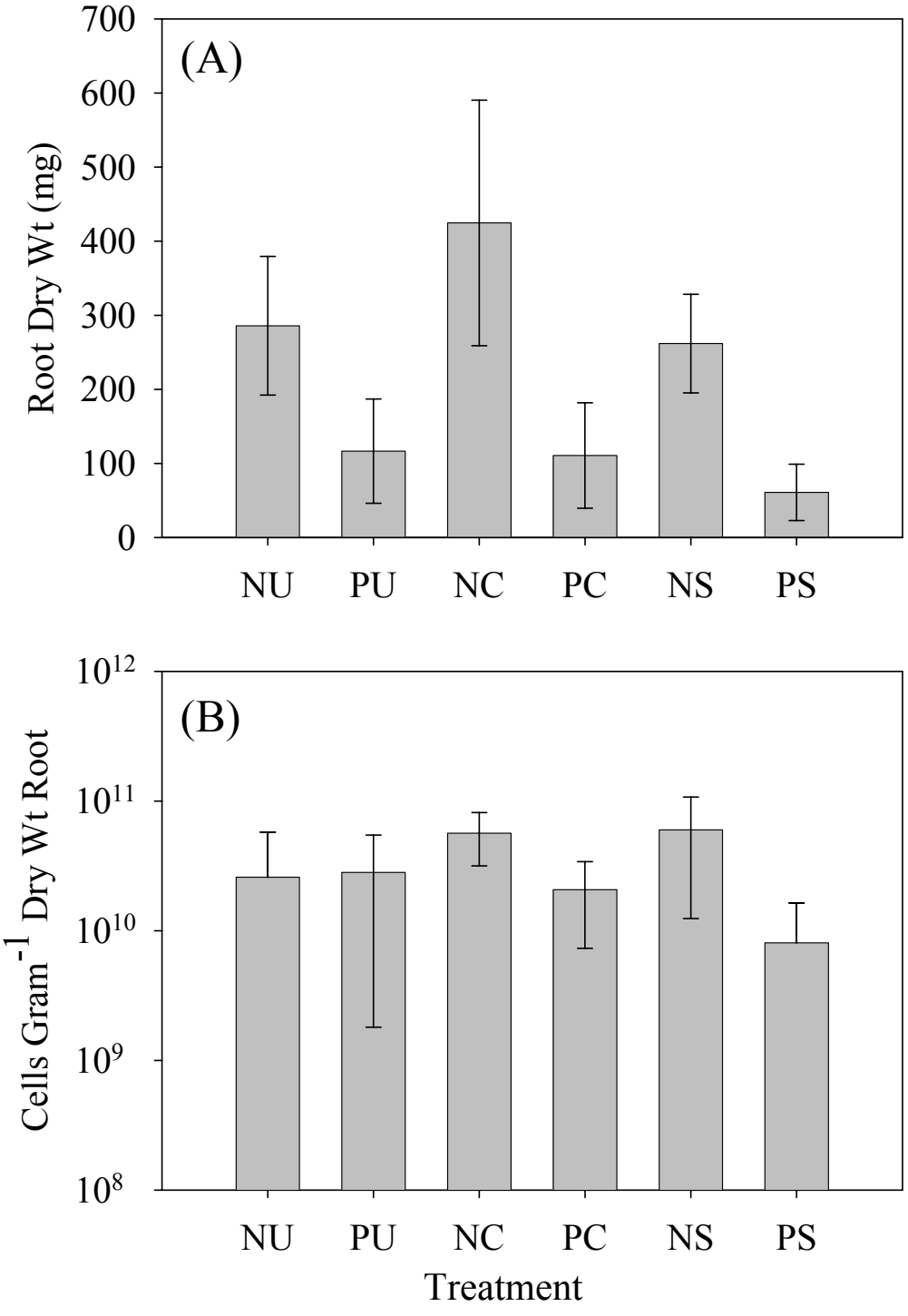

(A)
Root Dry Wt (mg)
1000
800
600
400
200
0
0ppm
2ppm
10ppm
20ppm
100ppm
200ppm
(B)
Cells Gram-1 Dry Wt Root
10^12
10^11
10^10
10^9
10^8
0ppm
2ppm
10ppm
20ppm
100ppm
200ppm
Nonionic Surfactant (Pol)

Figure 6. Plant and microbiological analyses for inoculum study. Root dry weight (A) and culturable cell numbers (B) for wheat plants grown with 20 ppm Pol and different inocula where N, No Pol; P, Pol; C, Constructed; S, Sludge; U, Uninoculated. Error bars represent the standard deviation of four replicates.


(A)
Root Dry Wt (mg)
700
600
500
400
300
200
100
0
NU
PU
NC
PC
NS
PS
(B)
Cells Gram-1 Dry Wt Root
10^12
10^11
10^10
10^9
10^8
NU
PU
NC
PC
NS
PS
Treatment

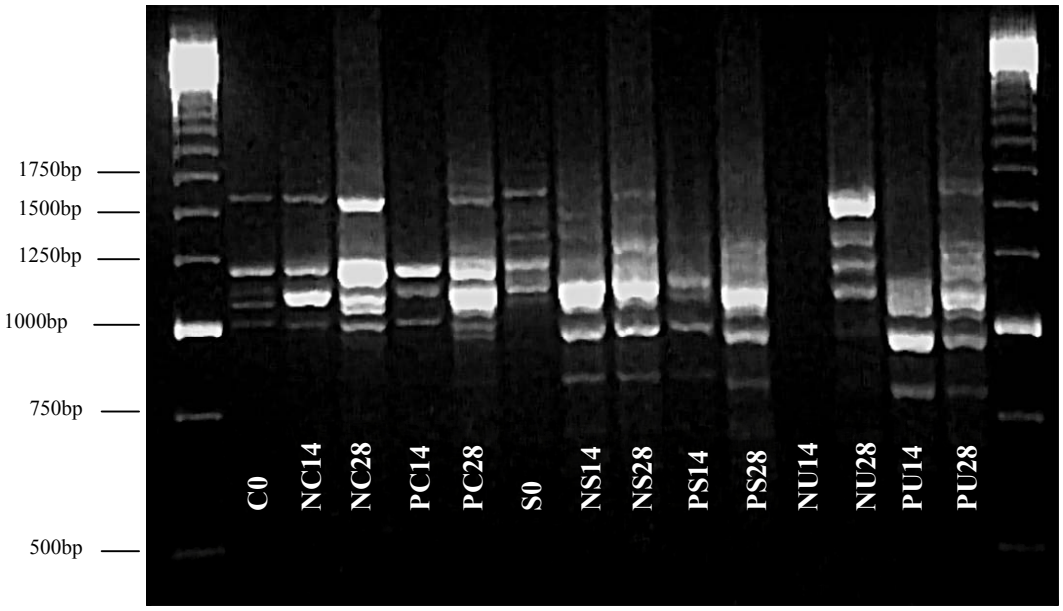
surfactant was higher than that of plants receiving Pol ($P < 0.1$). There were no significant differences in culturable cell numbers in any of the treatments ($P < 0.05$).

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 dry weight root), however molecular analysis by the RISA 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. 7). Comparison of community profiles for the constructed community and the sludge community from before inoculation to the end of the study at 28 days showed 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 (Fig. 7).

DISCUSSION

Using molecular approaches, including 16S rDNA sequence analysis, the population composition of microbial communities from both an existing rhizosphere graywater treatment system and from an industrial WWTP was determined and used to develop inocula to establish a new wheat rhizosphere based treatment system. The graywater rhizosphere library was dominated by members of the *Cytophaga-Flavobacter* (CF) cluster of the CFB division (Fig.2). The rest of the clones from the graywater rhizosphere library were distributed among the common groups of rhizosphere organisms including the alpha, beta and gamma subdivisions of the proteobacteria. The composition

Figure 7. 16S-23S rDNA ISR community profiles for 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.


1750bp
1500bp
1250bp
1000bp
750bp
500bp
C0
NC14
NC28
PC14
PC28
S0
NS14
NS28
PS14
PS28
NU14
NU28
PU14
PU28

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 were phylogenetically similar to clones 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 associated species (Fig.4). Alpha and beta proteobacteria were abundant in the TED701 sludge library. However, 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.

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 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 (35%) from the sludge library were in the CF cluster within the CFB division. The best GenBank sequence matches for the two large CF-group clones from the graywater rhizosphere library, unidentified clone 4955 (Accession AF097828) and unidentified clone 49527 (Accession AF097822) were previously identified in the industrial WWTP sludge used in these studies (Fig.2). 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 were distributed among the common groups of rhizosphere organisms included in the alpha (3%), beta (9%) and gamma (26%) subdivisions of the proteobacteria (3, 28, 31). The gamma proteobacteria were the second most dominant members of the graywater rhizosphere library (Fig. 2). *Pseudomonas* sp., in particular frequently dominate in hydroponic systems, especially those 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 siderophore production and other forms of antibiosis and (36, 47). These species are competitive colonizers and 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. 2). 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 acids 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. 6B and Fig. 7).

Two groups of common rhizosphere organisms not represented in the rhizosphere library, were the *Bacillus* sp. and the *Actinomycetes* sp. (28, 31). These species are 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 plant-growth promoting properties (47).

Results from the surfactant study (Fig. 5A) suggest that surfactant concentration can negatively impact the root mass, because 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 because culturable cell numbers increased significantly when the surfactant concentration was greater than 10 ppm, in response to increased nutrient availability from rhizosphere exudates (Fig. 5B). To more closely approximate the amount of surfactant that might be added with graywater treatment; (19), but avoid potentially detrimental effects to the plant root zone (Fig 4A),

20 ppm Pol was added to the rhizosphere of plants used in the inocula analysis studies. Many of the isolates in the constructed community degrade surfactant (Table 1). Diverse surfactant-degrading isolates were included in the constructed community for two reasons (1) the surfactant is a 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 5 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 suggested that surfactant accumulated and to affected both root dry weight and the concentration and structure of the constructed community inoculum (Fig. 6A and 6B and Fig. 7). 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 negatively impacted by the surfactant addition (Fig. 6A), 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 study, the uninoculated treatment received no inoculum but was apparently colonized by contaminant species (Fig. 6B). Morales et al. (33) found that contaminant species readily colonized the root of axenic plants. They speculated that

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 6B and Fig. 7).

Although the constructed community and the sludge community were of different species richness preliminary data from these studies suggest that organisms from both sources of inocula were able to become established and persist in the rhizosphere for up to 28 days (Fig.7). A constructed community was used, based on results that suggest that inoculant mixtures (rather than single species) added to the rhizosphere for purposes of biocontrol (as opposed to single isolates) have been the most successful in terms of long-term survival of inoculants 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 that more diverse populations are also more stable (30, 34). Therefore, the most prudent means of achieving the ultimate goals of survival and reliability 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 characterization of a microbial community from a plant-based graywater waste processing system was used as the basis for selection of isolates to be included in a constructed community. Organisms included in the constructed community were not all identical to clones from the graywater rhizosphere library, but these organisms are similar genetically to identified clones. The eleven isolates included in the constructed community are all competitive root colonizers, many were closely related to organisms that have plant growth promoting properties, and six of the isolates grew well on surfactant. The industrial WWTP sludge 16S rDNA library was made up of clones from many different species suggesting that the community should be structurally and functionally diverse. 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. Preliminary studies to evaluate the survival of the constructed community and the industrial WWTP sludge community in a plant-based graywater waste processing system suggest that organisms from the inocula were able to become established and persist. However, more work is needed 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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PART III

EVALUATION OF A PLASMID-BASED 16S-23S rDNA INTERGENIC SPACER REGION (ISR) ARRAY FOR ANALYSIS OF MICROBIAL DIVERSITY IN INDUSTRIAL WASTEWATER

ABSTRACT

A plasmid-based 16S-23S rDNA intergenic spacer (ISR) array was evaluated for analysis of microbial diversity within complex environmental samples. Plasmid probes with 16S-23S rDNA ISR inserts (800 to 1,500 bp) from industrial wastewater treatment plant (WWTP) organisms were arrayed onto glass slides. Hybridization was found to be highly specific (average background from arrayed probes with less than 80% similarity in ISR sequence was less than 7%). Hybridization of fluorescently labeled target sequences from two clones from the ISR WWTP library showed that there was a good linear relationship between hybridization intensity and ISR similarity (r^2=0.82). Strong fluorescence intensity corresponded to near perfect match clones (99% or greater match in ISR sequence). A majority of probes (79%) showed no background hybridization. However, weak background (less than 50% for arrayed probes matching between 90% and 95% in 16S rDNA) was observed from closely related organisms. Background fluorescence from the negative control (plasmid vector with no insert) was similar to water and DMSO negative control spots. Hybridization using fluorescently labeled ISR from a mixed community sample produced strong fluorescent signal with no background from negative controls. A Cy-5 labeled reference standard, part of the vector and present in every spotted probe, was used to normalize hybridization values. These results indicate that arrayed plasmid containing ISR probe insert sequences provides specificity and sensitivity for microbial community analysis in a high throughput array format.

INTRODUCTION

In recent years, evaluation of microbial diversity in environmental systems has been aided by molecular techniques that are less selective than traditional cultural techniques and provide a more complete view of the complexity within environmental systems (4, 26, 31, 35). In particular, molecular methods based on the 16S rRNA gene (16S rDNA) have provided unique insight into the breadth of microbial diversity (22). This sequence is universally present in all microorganisms and contains highly conserved regions interspersed with variable regions, thereby facilitating the design of primers and probes for analysis of bacterial phylogeny at different levels of specificity (i.e. family, genus or species level)(4, 13). Many 16S rDNA-based molecular techniques (DGGE, tRFLP, and ARDRA) have been developed for community-level analysis of differences in microbial composition associated with environmental, spatial or temporal changes. However, these methods yield complex community profiles that provide only superficial information about community ecology (26, 40). Additionally, the size of the 16S rRNA gene is extremely constant limiting its use in gel-based community-fingerprinting techniques and 16S rRNA gene sequences may be too highly conserved to permit analysis of phylogenetic relationships between closely related species (1, 13, 32).

In contrast to the rRNA genes, the intergenic spacer region (ISR) located between the 16S rRNA and 23S rRNA genes is under less selective pressure (10). Therefore, the 16S-23S rDNA ISR exhibits greater sequence diversity and length variation than 16S rRNA genes (19, 24, 34). This variability is due primarily to insertion and deletion events and to the presence or absence of tRNA sequences. Divergence within the ISR has been

used to confirm 16S rRNA gene-based phylogeny and has been shown to provide better discrimination of closely related species than 16S rRNA characterization (1, 3, 13, 32). However, ISR sequences among closely related organisms (species level) are conserved due to the location of the ISR between the highly conserved 16S rRNA and 23 rRNA genes and transcription of the *rrn* operon as a single pre-RNA transcript (17). Concerted evolution of multigene families and homologous recombination between similar ISR's also contribute to sequence conservation within the ISR despite limited evolutionary constraints (5, 18). Therefore, ISR sequences are often conserved within a species, but rarely beyond the genus or family level.

The 16S-23S rDNA ISR has been used to evaluate mixed microbial communities within environmental samples via clonal library construction and community-level fingerprinting (2, 16, 36). Conserved sequences within the 16S rRNA and 23S rRNA genes are used as primer targets for amplifying the spacer region of a broad range of organisms in a single amplification reaction (23). PCR products, including a portion of the 16S rRNA gene, can be used for phylogenetic analysis (2). ISR community level-profiling was used to characterize community samples from Amazonian soils (7), freshwater bacterial communities (16), plant rhizosphere (37), and Mediterranean offshore waters (2). Microbial community profiling based on ISR size differences is limited by the fact that multiple organisms (either closely related strains or completely unrelated species) may possess ISR regions of identical length (17). Alternatively, one organism may possess multiple ISR's with significant length differences so that a single organism may contribute multiple bands to a community profile (16).

Array technology is a high-throughput methodology that permits parallel analysis of hundreds or thousands of genetic sequences. Recently, alternative array methodologies have been applied for analysis of environmental samples through gene expression analysis (33, 38), bacterial identification (11, 30, 41), and evaluation of functional genes important in environmental ecosystems (42). However, these arrays rely on highly conserved 16S rRNA gene sequences and/or require costly and time-consuming selection of oligonucleotide probe sequences (20, 30, 40, 41).

This report describes the first use of a plasmid-based 16S-23S rDNA ISR array for characterization of microbial diversity within an industrial wastewater treatment plant (WWTP). Conserved sequences in the 16S rRNA and 23S rRNA genes were used as primer targets to amplify ISR sequences from a mixed microbial community. Phylogenetic information was obtained through analysis of 500bp of 16S rRNA gene sequence. The PCR products were cloned into plasmid vector and extracted plasmid was arrayed onto glass slides. Hybridization of fluorescently labeled samples against arrayed plasmid with 16S-23S rDNA ISR insert sequences provided a strong, specific fluorescent signal. This plasmid-based ISR array represents a new application of array technology in which community analysis can be achieved with a high level of specificity without the need for culture-based approaches or sequence analysis. Coupling of the array format with ISR community-level profiling will permit parallel analysis of hundreds to thousands of clone sequences from complex environmental samples with greater specificity than 16S rDNA-based methods.

MATERIALS AND METHODS

A) Evaluation of the ISR as a probe target for characterization of mixed microbial communities

DNA extraction and preparation of clonal library. DNA was extracted from industrial WWTP activated sludge as described by Layton et al. (28). 16S-23S rDNA ISR sequences from industrial WWTP microbial communities 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') (4) and 23Sr (5'GGG TTB CCC CAT TCR G 3') (7). The touchdown PCR program used for 16S-23S rDNA ISR analysis was as follows: 5 min at 94 ºC, then 10 cycles consisting of 15 s at 94 ºC, 45 s at 65 ºC, followed by a 1 ºC decrease for each of the 10 cycles, and 60 s at 72 ºC, followed by an additional 20 cycles of 15 s at 94 ºC, 45 s at 55 ºC, and 60 s at 72 ºC and a final cycle of 60 s at 72 ºC. Aliquots of 20 µl of the PCR product were electrophoresed in 2% agarose gels in 0.5X Tris-Borate (TBE) solution (0.045M Tris-borate, 0.001M EDTA, pH 8.0).

PCR products were cloned into the pCR®2.1-topo vector (TA vector; Invitrogen, Carlsbad, California). Large-scale plasmid DNA extractions were performed by using an alkaline lysis procedure from Promega Tech Bulletin .009 (Promega, Madison, WI) and then degrading the RNA with 10 µg of DNase-free RNAase per ml. Clones were characterized using phylogenetic information obtained from sequencing approximately 450bp of the 16S rRNA using the 1492r primer (5'TAC GGY TAC CTT GTT ACG ACT T3') (27). 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).

Construction of a prototype nylon-membrane ISR array. The 16S-23S rDNA ISR inserts were amplified from purified plasmid from eleven WWTP clones as described above except 0.8 ng to 1.1 ng of plasmid template DNA was used along with the primers 1492f (5'AAG TCG TAA CAA GGT ARC CGT A 3') and 23Sr. The amplified fragments were cleaned using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA). Purified PCR products (probes) were quantified using Hoechst Dye (Biorad, Hercules, CA) measured at an excitation of 346 nm and emission of 460 nm with a DyNA Quant 200 fluorometer (Amersham Biosciences, Piscataway, NJ). The PCR product was diluted to produce probes at final concentrations of 0.4 pmol, 0.2 pmol, and 0.04 pmol. The probes were blotted using the dot blot hybridization protocol described by Dionisi et al. (14).

Cross-hybridization analysis. Arrays were prehybridized in solution containing 0.5 M NaH_2PO_4, 1mM EDTA, and 7% (wt/vol) sodium dodecyl sulfate (SDS) for 1 hour at 65 ºC in a shaking water bath. Hybridizations were carried out overnight following addition of ^{32}P- labeled target ISR DNA (1.0×10^6 to 3.0×10^6 cpm). Eleven separate arrays were hybridized using labeled target ISR sequences from each of the different clones. Hybridization was carried out at 65 ºC and membranes were successively washed in high stringency wash buffer (10mM NaCl, 10mM Tris base, 1mM EDTA, and 0.5% SDS) at 65 ºC, 70 ºC, and 75 ºC. Following each wash, the arrays were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale CA) and the exposed screen was scanned on a STORM 840 PhosphorImager (Molecular Dynamics). The resulting images

were analyzed by determining pixel density for each spot using ImageQuaNT™ 5.0 analysis software (Molecular Dynamics).

B) Evaluation of Glass-slide Plasmid-based ISR array

Construction of plasmid-based 16S-23S rDNA ISR array. Samples from the industrial WWTP were collected in July 2001 and genomic DNA was extracted using the FastDNA kit (Bio 101, Vista, Calif.) as previously described (14). 16S-23S rDNA ISR's of microorganisms from the industrial WWTP were amplified, PCR products were cloned into the pCR®2.1-topo vector and plasmid DNA was extracted.

The ISR array was composed of 160 plasmids from the industrial WWTP 16S-23S rDNA ISR library. Each glass slide contained triplicate ISR arrays. TA vector with no insert, dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and water blanks served as negative controls. Probes consisting of plasmid containing an 16S-23S rDNA ISR insert were spotted in 50% DMSO at a final concentration between 400 ng/μl and 550 ng/μl. Ten μl of plasmid probe in an equal volume of DMSO was placed into 384-well microtiter plates. Probes were arrayed at a spacing of 350 μm onto Cel Associates (Houston, TX) poly-L-Lysine coated slides in a grid of 8 rows by 6 columns. Arrays were printed at 57% relative humidity and 22°C using a Virtek robotic printer (Ontario, Canada) with 8 pins (TeleChem International, Sunnyvale, CA). Following printing, ISR arrays were re-hydrated in a glass petri dish containing moist filter paper for 5 min and snap dried at 80 °C for 5 s. ISR arrays were then UV crosslinked at 150 mJ in a UV Spectrolinker XL1500 (Spectronics Corporation, Westbury, NY). Arrays were blocked in a solution of 1.5% succinic anhydride (Sigma, St. Louis, M), 90% 1-methyl-2-pyrrolidinone (Sigma, St. Louis, Mo) and 43mM boric acid (pH8.0) (Sigma, St. Louis,

MO) for 15 min at room temperature. Following blocking, DNA on the arrays was denatured by boiling at 95°C for 2 min. Slides were dried by centrifugation using a microarray centrifuge (Telechem, Sunnyvale, CA) at 2,000 x g for 20 s. Two slides from each printed batch were evaluated for quality of printing by staining with Sybr Green II (Molecular Probes, Eugene, OR) diluted 1:10,000 in 0.5X TBE. Arrays were stained for 1 minute, and then washed in 0.5X TBE and sterile deionized water for 30 s each prior to scanning using the Scan Array 5000 MicroArray Analysis System (GSI Lumonics, Watertown, MA) at an emission wavelength of 522 nm.

A sub-array containing probes from six 16S-23S rDNA ISR clones from the WWTP library was used for analysis of cross-hybridization and for optimization of hybridization conditions for the ISR array (Table 1). The probes as well as negative controls (TA vector with no insert and DMSO) were spotted under the conditions described above. Five replicates of the sub-array were printed on each slide.

Preparation of fluorescently labeled target DNA. 16S-23S rDNA ISR sequences from industrial WWTP samples (2-5 ng) were amplified using the PCR conditions described above to increase overall target signal intensity. 16S-23S rDNA ISR from plasmid insert sequences were selectively amplified using PCR to minimize background from vector. The resultant PCR products from the first reactions were purified using the Zymo DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, CA). Cleaned PCR product was diluted 1:1000, and 1ul was used as template for an incorporation reaction using the PCR bead, primers (1492f and 23Sr), and 4μM Cy3 labeled dCTP (Amersham Pharmacia, Peapack, NJ). The labeled target DNA was

purified by precipitation with 2.5 volumes of ethanol and 0.1 volume of sodium acetate (Sigma, St. Louis, MO) at –20°C overnight.

Optimization of hybridization specificity and intensity using a formamide gradient. To optimize hybridization specificity and signal intensity, sub-arrays were hybridized in hybridization solution containing 30%, 40%, 50%, 60%, or 70% formamide. Labeled target DNA was resuspended in hybridization solution containing different concentrations of formamide (Ambion, Austin, TX), 5X SSC, 0.1% SDS and 0.5μg unlabeled herring sperm DNA (Sigma, St. Louis, MO) in a total volume of 60 μl. Fluorescently labeled target was denatured at 95°C for 2 min and cooled to ambient temperature by centrifugation for 2 min. Denatured probe was deposited into an adhesive-backed Frame Seal (MJ Research, Waltham, MA) chamber which was placed around arrayed DNA on the glass slide sub-array, the chamber was then sealed with the adhesive plastic coverslip. All hybridizations were performed in a MJ Research Twin Tower Sample Block (MJ Research, Waltham, MA) at 45 °C. Following hybridization, the arrays were washed in separate chambers with 2X SSC and 0.1% SDS for 5 min at the hybridization temperature and then with 0.1X SSC and 0.1% SDS for 5 min at room temperature, followed by 0.1X SSC for 1 min four times at room temperature. Residual salts were removed by centrifugation. Slides were scanned at a resolution of 5 μM with the ScanArray 3000 confocal laser scanner. Signal intensities for each spot were determined using QuantArray 3.0 image analysis software (Perkin Elmer, Boston, MA). ANOVA was carried out using SPSS version 11.0 (SPSS, Chicago, IL).

Analysis of industrial WWTP sludge library using the 16S-23S rDNA ISR array. ISR array library analysis was performed by hybridization of labeled target

sequences from clones TED70103 or TED70109 (Table 1) to the arrayed plasmid probes on the ISR array. A formamide concentration of 50% was used in the hybridization solution and all hybridizations were performed at 45°C. Following hybridization, arrays were scanned and analyzed as described above. Plasmids corresponding to selected spots were sequenced for phylogenetic analysis, using the 16S rDNA portion of the insert (1055f primer), and for ISR similarity, using the 1492f primer. Spearman correlation coefficients (*r*) were calculated to find relationships between hybridization ratio and ISR or 16S rRNA gene sequences.

Hybridization of plasmid-based ISR array with DNA from Mixed Community. An environmental sample from plant rhizosphere inoculated with industrial WWTP organisms was extracted using the MoBio Soil Extraction Kit (Solana Beach, CA) and genomic extract was labeled with Cy3-dCTP as described above. The kanamycin gene from the pCR®2.1-topo vector was used as reference DNA by incorporation of Cy5-dCTP (Amersham Pharmacia, Peapack, NJ) using primers KanF (5'TCA GGG CGC AAG GGC3'; corresponding to sequence positions 963-977 of the TA vector) and KanR (5'ACT CTT CCT TTT TCA ATT CAG AAG AAC3'; corresponding to sequence positions 2107-2133 of the TA vector). The Cy3 labeled target and the Cy5 labeled reference standard were mixed in equal portions and hybridized to the array simultaneously. The arrays were hybridized, washed and analyzed as described above.

RESULTS

Specificity of ISR in DNA hybridization

16S-23S rDNA ISR probes from a phylogenetically diverse group of WWTP clones were used in a prototype array to evaluate the feasibility of using the 16S-23S rDNA ISR as a probe target for characterization of mixed microbial communities in an array format. The 1492f primer was used to produce labeled target sequences to avoid amplification of conserved sequences present in the 16S rDNA portion of the ISR insert. The 23Sr primer used in these studies anneals 115 bp into the 23S rRNA. This primer was chosen because it is more highly conserved than previously used 23S rRNA-targeted primers that are specific for sequences closer to the ISR region (19). Clones were sequenced from the 16S rDNA end of the PCR product using the 1055f primer. The approximately 500 bp of partial 16S rRNA gene sequence was used to obtain phylogenetic information for selected clones. The names and accession numbers of organisms from GenBank that most closely matched each of the 11 clones used for cross-hybridization analysis, as well as the tentative phylogenetic placement, clone number, %GC of the ISR, ISR size and Tm are given in Table 1A. The 16S-23S rDNA ISR library produced from WWTP mixed communities was diverse with representatives from the alpha, beta, and gamma proteobacteria as well as *Nitrospira*, *Verrucomicrobia*, and *Sphingobacteria*. ISR size varied between 440 bp and 978 bp. Tm for the 11 clones varied between 96°C and 103°C, with an average of 99°C (±2 °C).

Cross-hybridization analysis using selected clones revealed that at low stringency, (65°C wash) some cross-hybridization between related groups of bacteria occurred, i.e., a labeled target using clone TED9317 (*Asticcacaulis* sp.), an alpha proteobacterium,

Table 1. Industrial WWTP 16S-23S rDNA ISR clones used for optimization of ISR array hybridization

Clone Name	Closest Match (%similarity)	Phylogenetic Placement	%GC	ISR Size (bp)	Tm[a]
A. Prototype ISR Array Clones[b]					
TED9317	*Hyphomicrobium hollandicum* Y14303 (95%)	Alpha Subdivision, Hyphomicrobium	56	728	101
TEDB3119	*Magnetospirillum gryphiswaldense* Y10109 (94%)	Alpha Subdivision, Rhodospirillaceae	57	829	101
TED937	*Asticcacaulis excentricus* AJ247194 (93%)	Alpha Subdivision, Caulobacter group	61	643	103
TED9315	*Leptothrix discophora* L33974 (98%)	Beta Subdivision, Comamonadaceae	56	558	100
TED933	*Thauera aromatica* AJ315680 (99%)	Beta Subdivision, Rhodocyclus Branch	56	537	100
TED9328	*Pseudomonas lanceolata* AB021390 (99%)	Beta Subdivision, Comamonadaceae	49	616	98
TED9310	*Methylococcus* sp. X72769 (93%)	Gamma Subdivision, Methylococcaceae	54	548	100
TED932	*Lewinella persicus* AF039295 (90%)	Sphingobacteria, Sphingobacteriales	48	876	98
TED936	*Flexibacter tractuosus* AB078076 (91%)	Sphingobacteria, Sphingobacteriales	44	978	96
TED931	*Nitrospira* sp. Y14638 (98%)	Nitrospirales, Nitrospiraceae	53	511	99
TED9326	*Verrucomicrobium* sp. AF027005 (97%)	Verrucomicrobia	54	440	99
B. ISR Sub-Array Clones[c]					
TED70103	*Leptothrix mobilis* X97071 (98%)	Beta Subdivision, Comamonadaceae	55	577	100
TED70101	*Leptothrix mobilis* X97071 (99%)	Beta Subdivision, Comamonadaceae	53	692	99
TED70129	*Pseudomonas lanceolata* AB021390 (99%)	Beta Subdivision, Comamonadaceae	54	521	100
TED70105	*Thauera aromatica* AJ315680 (99%)	Beta Subdivision, Rhodocyclus Branch	56	511	100
TED70113	*Rhodobacter sphaeroides* X53855 (94%)	Alpha Subdivision, Rhodobacter group	54	621	100
TED70109	*Haliscomenobacter hydrossis* M58790 (90%)	Sphingobacteria, Sphingobacteriales	42	305	94

[a]Melting temperature of ISR sequence

[b]Clones used on proto-type ISR array for cross-hybridization analysis

[c]Clones used on ISR sub-array for formamide optimization experiments

produced 56% background signal from spotted probe sequence TEDB3119 (*Magnetospirillum* sp.), another alpha proteobacterium (Table 2). This background was reduced as wash stringency increased. At a wash temperature of 75 °C, the same labeled target (TED9317) showed only 4.5% background from the alpha proteobacterium TEDB3119. At the lowest stringency wash temperature (65 °C) background averaged 10% (±20%) over the entire group. At the highest stringency wash temperature (75 °C) background averaged 1.5% (± 2%). Based on these results, it was determined that the arrayed ISR provides a useful target for hybridization analysis with minimum potential for cross-hybridization given sufficiently stringent hybridization conditions.

Arraying of 16S-23S rDNA ISR plasmids

Plasmid probes extracted from a clonal library of 16S-23S rDNA ISR sequences from industrial WWTP organisms were spotted onto glass slides at a concentration of 400-550 ng/µl. This concentration of starting material was necessary to achieve saturating concentrations of spotted probe since approximately ¼ of the plasmid is probe ISR sequence, the rest being vector sequence. No effect of this concentration of starting material was observed, i.e. there was no evidence of the "comet effect" typically seen when excessively high concentrations of probe are spotted (15). TA vector with no insert was spotted onto the array and its intensity following hybridization was subtracted from all other background-subtracted intensity values to eliminate the effect of non-specific hybridization to the plasmid. Initial background levels from TA vector were minimal (5% to 7%) and were only slightly higher than values for the DMSO control (2% to 3%).

Table 2. Characterization of percent cross-hybridization between ISRs of different phylogenetic groups

Wash	Clone	Phylogeny	Alpha Proteobacteria			Beta Proteobacteria			Gamma	CFB[a]		Nitrospira	Verruco[b]
Temp.	Name		TED9317	TEDB3119	TED937	TED9315	TED933	TED9328	TED9310	TED932	TED936	TED931	TED9326
65 ºC	TED9317	*Hyphomicrobium*	100.00	47.70	41.85	1.73	6.39	4.30	2.16	1.03	1.21	6.37	18.80
65 ºC	TEDB3119	*Magnetosprillum*	17.46	100.00	56.00	11.08	3.47	6.26	3.42	0.29	1.75	23.83	62.20
65 ºC	TED937	*Asticcacaulis*	3.16	35.76	100.00	0.74	15.25	4.80	1.05	3.38	3.67	6.19	22.91
65 ºC	TED9315	*Leptothrix*	1.44	5.40	18.18	100.00	73.20	86.77	4.13	0.13	6.69	5.82	5.19
65 ºC	TED933	*Thauera*	1.05	0.85	4.42	18.63	100.00	21.63	8.38	0.14	1.96	0.34	2.14
65 ºC	TED9328	*Pseudomonas*	0.13	0.66	1.52	0.51	14.14	100.00	0.20	0.11	1.26	-0.02	3.68
65 ºC	TED9310	*Methylococcus*	1.10	12.24	24.29	3.89	76.01	17.37	100.00	3.19	1.42	1.81	11.20
65 ºC	TED932	*Lewinella*	0.69	0.25	0.92	0.51	0.70	1.53	2.04	100.00	6.32	1.21	1.62
65 ºC	TED936	*Flexibacter*	0.86	0.67	1.54	2.60	0.85	2.27	2.29	21.33	100.00	5.79	1.52
65 ºC	TED931	*Nitrospira*	2.76	54.72	61.67	0.97	0.30	4.34	3.42	0.38	1.35	100.00	57.77
65 ºC	TED9326	*Verrucomicrobium*	1.42	38.68	40.26	0.18	8.52	1.46	1.34	0.30	0.21	15.14	100.00
70 ºC	TED9317	*Hyphomicrobium*	100.00	21.22	18.56	0.95	0.50	3.92	1.60	0.73	0.81	4.69	0.86
70 ºC	TEDB3119	*Magnetosprillum*	12.55	100.00	28.88	7.60	0.36	3.22	2.42	0.44	0.71	5.07	2.82
70 ºC	TED937	*Asticcacaulis*	2.02	15.17	100.00	3.53	0.47	2.62	1.14	3.06	3.08	4.76	1.24
70 ºC	TED9315	*Leptothrix*	1.43	1.17	9.35	100.00	2.76	66.37	0.78	0.26	4.29	3.99	0.34
70 ºC	TED933	*Thauera*	0.82	0.50	3.05	8.27	100.00	13.01	2.65	0.16	1.18	0.43	0.15
70 ºC	TED9328	*Pseudomonas*	0.02	0.38	1.24	1.31	1.20	100.00	0.22	0.25	0.34	0.21	0.05
70 ºC	TED9310	*Methylococcus*	0.79	6.58	10.93	1.89	0.32	9.73	100.00	4.41	0.58	0.82	0.64
70 ºC	TED932	*Lewinella*	0.73	0.13	0.50	0.34	0.15	0.85	0.46	100.00	1.61	0.71	0.17
70 ºC	TED936	*Flexibacter*	0.69	0.23	0.96	0.77	0.12	0.75	0.29	5.33	100.00	2.53	0.18
70 ºC	TED931	*Nitrospira*	1.17	26.16	31.41	0.58	0.10	2.08	0.42	0.24	0.80	100.00	1.51
70 ºC	TED9326	*Verrucomicrobium*	0.66	15.74	20.24	0.10	0.78	0.95	0.12	0.19	0.05	3.71	100.00
75 ºC	TED9317	*Hyphomicrobium*	100.00	2.68	2.65	1.73	0.28	2.24	1.60	0.34	0.47	5.96	2.06
75 ºC	TEDB3119	*Magnetosprillum*	3.97	100.00	4.47	2.83	0.16	1.50	2.42	0.88	0.19	1.03	3.49
75 ºC	TED937	*Asticcacaulis*	0.59	2.13	100.00	0.94	0.19	1.46	1.14	10.01	3.35	6.51	1.73
75 ºC	TED9315	*Leptothrix*	0.93	0.41	5.08	100.00	1.09	8.88	0.78	2.81	2.23	4.21	1.29
75 ºC	TED933	*Thauera*	0.61	0.23	1.73	3.18	100.00	2.53	2.65	1.81	0.73	0.85	0.88
75 ºC	TED9328	*Pseudomonas*	0.03	0.12	0.80	0.14	0.47	100.00	0.22	1.77	0.22	0.50	2.03
75 ºC	TED9310	*Methylococcus*	0.40	0.76	2.51	0.87	0.14	3.26	100.00	4.98	0.44	1.07	1.22
75 ºC	TED932	*Lewinella*	0.58	0.06	0.29	0.34	0.07	0.62	0.46	100.00	1.70	0.31	0.70
75 ºC	TED936	*Flexibacter*	0.60	0.17	0.65	1.03	0.06	0.58	0.29	4.51	100.00	1.76	0.95
75 ºC	TED931	*Nitrospira*	0.25	2.50	2.64	0.54	0.06	1.29	0.42	1.90	0.77	100.00	2.89
75 ºC	TED9326	*Verrucomicrobium*	0.15	1.69	1.32	0.11	0.32	0.58	0.12	0.67	0.10	1.67	100.00

[a]CFB = Cytophaga, Flavobacerium, Bacteroides Group

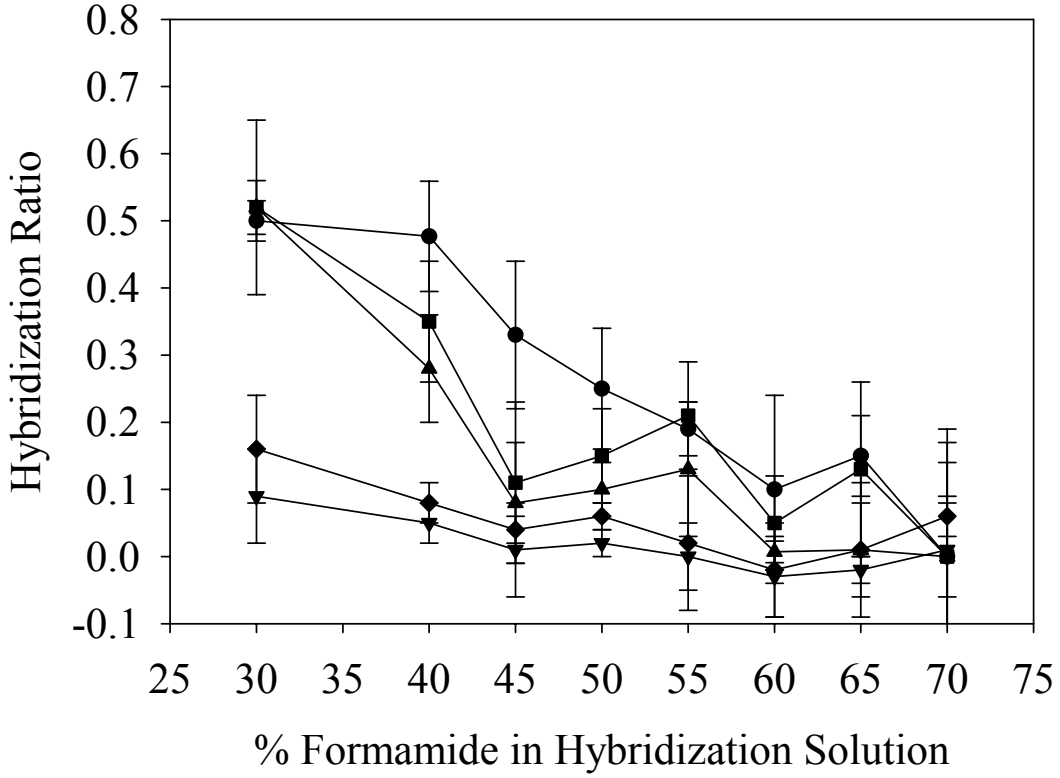
[b]Abrreviation for Verrucomicrobium

Plasmid-based ISR array hybridization specificity

Hybridization specificity was optimized using a sub-array of 6 probes from the WWTP ISR array. Selected probe 16S-23S rDNA ISR insert sequences matched the labeled target 16S-23S rDNA ISR sequence (TED70103) between 58% and 100% (Fig. 1). The names and accession numbers of organisms from GenBank that most closely matched each of the 6 plasmid probes spotted onto the sub-array, as well as their tentative phylogenetic placements, clone number, %GC of the ISR, ISR size and Tm are given in Table 1B. The sub-array also contained TA vector with no insert. The array was replicated 5 times on each slide and hybridization at each formamide concentration was replicated on 3 to 5 separate slides. Replicate spots from each array were averaged to obtain one value per probe per slide. Final fluorescence intensity (I') for each spot resulted from subtraction of local mean background and negative control (TA vector with no insert) values from the initial fluorescence intensity (I). The hybridization ratio for each array spot was measured as the ratio of I' for the test spot to I' for the control spot (the spot matching the labeled target 100%; hybridization ratio = I'-test/ I'-control).

ISR insert from the 100% matched clone (TED70103) was labeled with Cy3-dCTP and hybridized to the sub-array in hybridization solution with formamide concentrations ranging between 30% and 70%. Specificity was found to increase with formamide concentration (Fig. 1). ANOVA suggests that hybridization specificity significantly increased at a formamide concentration of 50% or greater ($P<0.05$ [Fig. 1]). At low hybridization stringency, the closest sequence matches exhibited some background hybridization. At a formamide concentration of 70%, there was no detectable background from other clones, however; overall intensity was also lower (30%

Figure 1. Effect of formamide concentration on hybridization specificity. The hybridization ratio is derived from the ratio of final fluorescence intensity (I') for each spot in the sub-array to the final fluorescence intensity of the 100% 16S rDNA and ISR matched probe (TED70103). TED70101, 98% 16S rDNA and 80% ISR match to target sequence (#), TED70129, 94% 16S rDNA and 74% ISR match to target sequence (!), TED70105, 90% 16S rDNA and 70% ISR match to target sequence (%), TED70113, 80% 16S rDNA and 64% ISR match to target sequence (&), TED70109, 80% 16S rDNA and 58% ISR match to target sequence (").


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
Hybridization Ratio
25
30
35
40
45
50
55
60
65
70
75
% Formamide in Hybridization Solution

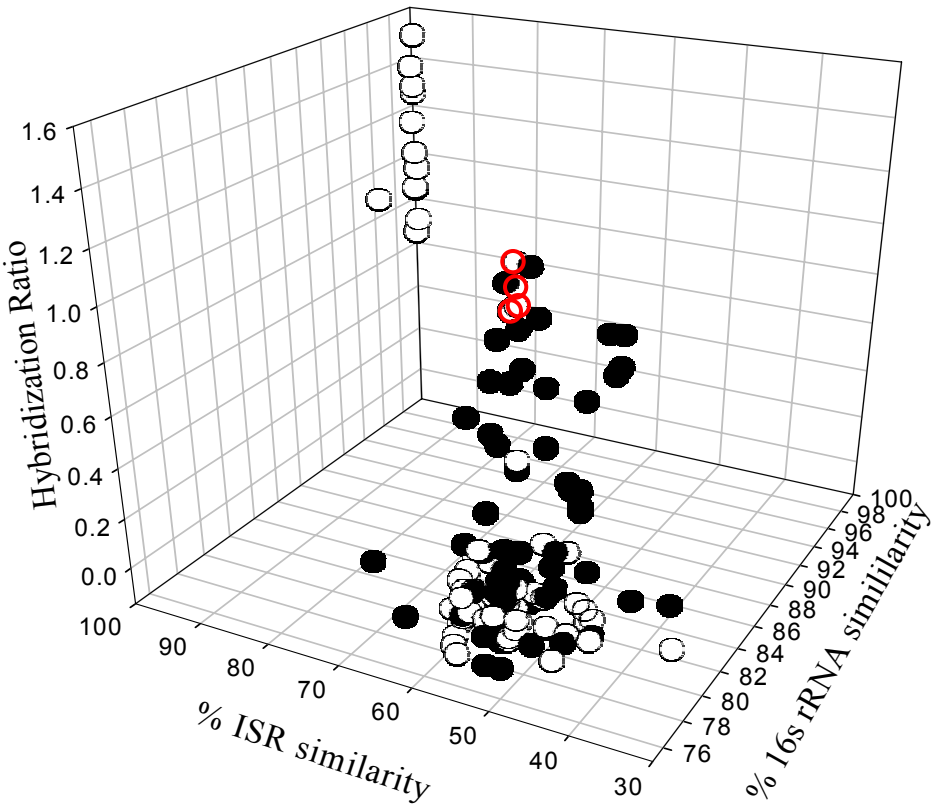
lower than the intensity at the lowest concentration of formamide). At a formamide concentration of 50%, background hybridization was less than 25% and overall intensity was 95% of the intensity at the lowest concentration of formamide. Based on the level of background hybridization and overall intensity, it was decided that all further experiments would be conducted at 45°C in hybridization solution containing 50% formamide.

Validation of plasmid-based ISR array WWTP community analysis

Based on phylogenetic analysis of data from sequenced clones from the July 2001 16S-23S rDNA ISR clonal library, two clones were chosen for WWTP community analysis. A common clone (TED70109; 16% of sequenced clones in the library matched 99% in ISR sequence) was chosen to evaluate hybridization specificity. A beta proteobacterium (TED70103) was less common in the sequenced portion of the WWTP library, but was chosen to evaluate the potential for cross-hybridization between closely related organisms. Figure 2A shows the relationship between hybridization ratio, 16S rDNA sequence similarity and ISR sequence similarity for 69 sequenced clones hybridized against labeled target sequences from clones TED70103 and TED70109. Spearman correlation analysis of this data showed a substantial positive correlation between hybridization ratio and 16S-23S rDNA ISR sequence ($r = 0.63$, $P < 0.0005$). Slide to slide variation in signal intensity for replicate spots averaged 8.0% with a standard deviation of 5.5%. Given that labeled targets are complementary to the ISR portion of arrayed probes, it was not surprising to find that the linear relationship between hybridization ratio and ISR sequence similarity ($r^2 = 0.82$) was better than that for hybridization ratio and 16S rDNA sequence similarity ($r^2=0.77$). Two of four ISR

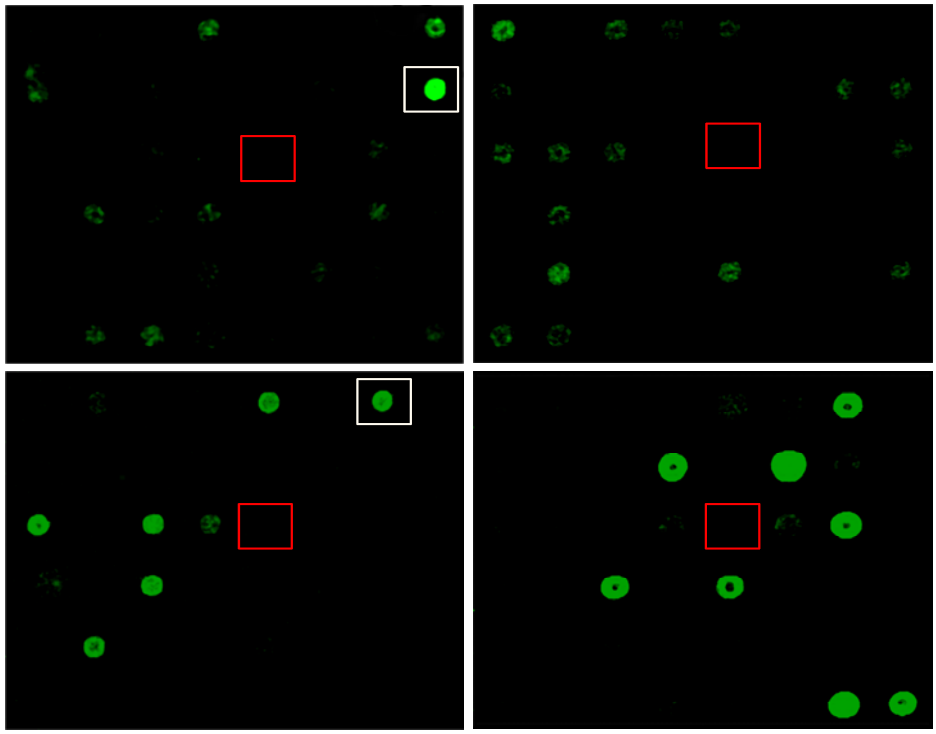
Figure 2. Hybridization of fluorescently labeled target sequences to the 16S-23S rDNA ISR array. (A) Relationship between hybridization ratio, ISR sequence similarity and 16S rDNA sequence similarity. ISR sequences from two clones, TED70103 (#), and TED70109 ( ) were labeled with Cy3 dCTP and hybridized to separate arrays in triplicate. Cluster of four clones with 99% 16S rDNA sequence similarity to clone TED70109, but low ISR similarity and low hybridization ratios ( ) (B) Two sub-arrays of the ISR array hybridized with Cy3-dCTP labeled ISR from clone TED70103. Red boxes indicate TA vector with no insert (negative control); white boxes indicate the 100% match to the labeled target sequence. (C) Two subarrays of the ISR array hybridized with Cy3-dCTP labeled ISR from clone TED70109. Red boxes indicate TA vector with no insert (negative control); white boxes indicate the 100% match to the labeled target sequence.

(A)


Hybridization Ratio
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
100
90
80
70
60
50
40
30
% ISR similarity
100
98
96
94
92
90
88
86
84
82
80
78
76
% 16s rRNA simililarity

(B)

(C)



subarrays from the ISR array, hybridized with labeled target from TED70103 and TED70109, are shown in figures 2B and 2C, respectively.

Hybridization of labeled target from clone TED70109 to the array resulted in 10 clones with a hybridization ratio greater than 0.85 (Fig. 2A). These clones were all greater than 99% matches to the labeled target sequence in terms of ISR and 16S rRNA gene sequence. A cluster of four clones had 99% 16S rRNA gene sequence similarity to clone TED70109, but had low hybridization ratios (between 0.60 and 0.38) (Fig. 2A). This discrepancy was due to the low level of ISR sequence similarity (84%) between the 100% match clone and this cluster of clones. Differences in the ISR sequence were due to insertions and single base-pair mismatches in comparison to the ISR sequence of the perfect match clone.

Hybridization of the less common clone (TED70103) to the array produced only two matches with a hybridization ratio greater than 0.55, both were 98% similar in 16S rRNA gene sequence, but only 78% to 80% similar in ISR sequence to the perfect match (Fig. 2A). Only one other 98% 16S rRNA match to TED70103 was identified in the sequenced portion of the library. The clone had a hybridization ratio of 0.36. This finding was consistent with a significantly longer ISR region (692 bp versus 577 bp for clone TED70103) containing several long insertions. Despite fewer matched clones, hybridization with TED70103, a beta proteobacterium, resulted in greater background hybridization by related beta proteobacteria than that observed for the *Sphingobacterium* clone TED70109, a more common clonal library sequence (Fig. 2B and 2C). Background hybridization ratio was less than 0.50 for arrayed probes matching between 90% and 95% in 16S rRNA gene sequence (less than 80% ISR match) and less than 0.10

for arrayed probes with less than 90% match in 16S rRNA sequence. More than 79% of the probes produced little or no signal (hybridization ratio less than 0.20) in comparison to the hybridization intensity of the perfect match probe for both of the labeled targets used for hybridization.

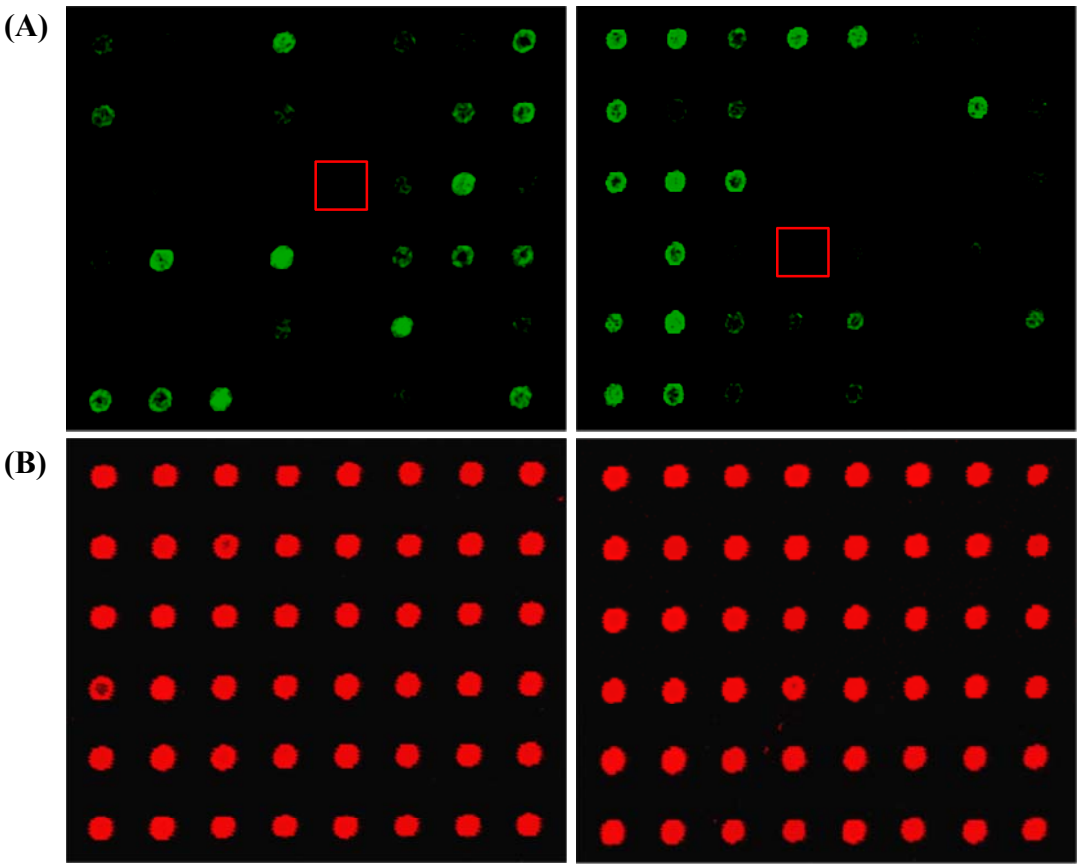
Hybridization of plasmid-based ISR array with DNA from Mixed Community

Hybridization using fluorescently labeled target DNA from a sludge inoculum resulted in a strong fluorescent signal from matching probe sequences. As expected, hybridization with labeled target from the environmental samples resulted in no background from plasmid vector sequences (vector control intensity less than or equal to DMSO and water blanks [Fig. 3A]). Simultaneous hybridization with Cy5-labeled standard, targeting the kanamycin gene on the TA vector, was used as a reference for hybridization signal ratio normalization (Fig. 3B). Normalization to the kanamycin reference resulted in 145 of 160 probes with hybridization ratios above threshold levels (higher than negative controls). Over half of these probes produced a hybridization ratio (where hybridization ratio = (I'-Cy-3 probe/ I'-Cy-5 reference)∗1000) of 76 while more than 75% produced a hybridization ratio over 116. Using this technique, diverse phylogenetic groups from a mixed community sample were probed in parallel with simultaneous hybridization to normalize for differences in probe concentrations and hybridization efficiency.

DISCUSSION

In this study, we report the first use of a plasmid-based 16S-23S rDNA ISR array for analysis of microbial diversity within complex environmental samples. The ISR array

Figure 3. Hybridization of industrial WWTP sample to the 16S-23S rDNA ISR array. (A) Two sub-arrays of the ISR array hybridized with Cy3-dCTP labeled ISR from mixed community sludge inoculum. (B) The same two sub-arrays of the same ISR array hybridized with Cy5-dCTP labeled kanamycin gene targeted to every probe on the array. Red boxes indicate TA vector with no insert (negative control).


(A)
(B)

utilizes the sequence diversity of the 16S-23S rDNA ISR to obtain greater specificity than traditional 16S rDNA-based methods. The array format eliminates the need for length-based evaluation of ISR differences and provides a high-throughput, rapid and cost-effective means for simultaneous analysis of hundreds or thousands of phlyotypes from clonal libraries or environmental samples.

Specificity is a critical aspect of array experiments since parallel hybridization of hundreds or thousands of arrayed probes increases the potential for cross-hybridization between unrelated genetic sequences. Probes must have sufficiently high specificity and uniformity for simultaneous hybridization in an array format. Cross-hybridization analysis using a prototype ISR array showed that the 16S-23S rDNA ISR used as probes provides specificity over a broad range of closely and distantly related organisms (Table 2). These results agree with those of other researchers that have found that the ISR provides species to strain level differentiation of bacteria (10, 29, 32). The arrayed probes must also exhibit uniformity in terms of optimal conditions for hybridization. The size range of ISR's of selected organisms resulted in probes with Tm values within a relatively narrow range (Table 1). Based on these data, 16S-23S rDNA ISR probes appear to provide specificity and uniformity for hybridization in a high-throughput array format.

Arraying plasmids eliminated the need for costly and time-consuming PCR amplification steps. Often, multiple PCR reactions must be combined and purified to provide product for arraying. Arrayed plasmid remained tightly bound to the slide (i.e., no comet effect) and produced strong hybridization signals with no need for modification (i.e., partial digestion, nicking, tailing or amination of nucleotide sequences). Plasmid

vector with no insert was spotted in each subarray to permit subtraction of fluorescence contributed by the local vector (Fig. 2 and Fig. 3). For most of these studies, ISR insert from plasmid was used as the labeled target. Even in this case, background from the vector was minimal and only slightly greater than that for other negative controls (DMSO and water). When hybridization was carried out with a labeled probe from a mixed community extract, background from the vector control was less than or equal to that from other negative controls. These results suggest that arrayed plasmid containing probe insert sequences provides the same specificity as the more laborious and costly arraying of PCR product.

Given adequate probe specificity, successful analysis of mixed communities using array technology depends on sufficiently stringent hybridization conditions. In this study, formamide concentration was adjusted to optimize hybridization specificity and sensitivity. Formamide is normally adjusted in conjunction with temperature. However, elevated hybridization temperatures resulted in probe removal from glass slides in a non-linear and unpredictable fashion (data not shown). As a result, formamide concentrations were adjusted and a constant hybridization temperature of 45°C was employed. As expected, hybridization specificity increased as a function of formamide concentration (Fig. 1). This was consistent with previous work that showed that formamide addition increases hybridization specificity and intensity thereby increasing the dynamic range of the array (39). Our final hybridization conditions, 50% formamide in the hybridization solution and 45°C hybridization temperature, were sufficiently stringent to permit high specificity without diminishing signal intensity.

There was a good linear relationship between hybridization intensity and ISR similarity (r^2=0.82) and results were highly reproducible. Strong hybridization intensity (hybridization ratio of 0.85 or greater than that of the perfect match probe) corresponded to near perfect match clones (99% or greater match in ISR sequence). Weak background was observed from closely related organisms (i.e., beta proteobacteria related greater than 95% in 16S rDNA to the labeled target). This background was due, primarily, to hybridization within conserved $tRNA^{ile}$ and $tRNA^{ala}$ sequences, which were the only regions of significant similarity between clones with 96% or less 16S rDNA sequence similarity to the perfect match sequence. The tRNA sequences represent the most highly conserved regions within the ISR, but generally make up less than 50% of the length of the ISR (17).

The linear relationship between hybridization intensity and 16S rDNA similarity was not as robust (r^2=0.77). This was expected because the ISR is known to be more variable than the 16S rDNA. For example, there were three sequences that matched the hybridized target by 98% in 16S rRNA gene sequence, but had very low hybridization ratio values. These sequences contained insertions or deletions resulting in an ISR match to the labeled target of less than 80%. Evidence suggests that there can be significant variation in the number of *rrn* operons within a single organism and variation in ISR sequence between multiple *rrn* operons within a single organism (5, 8, 9, 34). This may be an issue in array analysis, since only the arrayed 16S-23s rDNA ISR's would be represented. Additionally, there may be PCR-based biases affecting the number or kind of ISR's amplified. For example, Boyer et al. (8) found that ISR's lacking tRNA sequences were preferentially amplified. Shorter templates and those without secondary

structure also may be preferentially amplified (16). Gürtler and Stanisich (19) found that differences in primers specific for 23S rRNA gene sequences affected the detection of spacer variation. For these reasons, it may be prudent to improve the resolution of the array by including clonal libraries from diverse environments using primers specific for different regions in the bacterial 16S rRNA or 23S rRNA genes (17, 19), or primer sets targeting specific taxa (6, 25).

Hybridization using fluorescently labeled 16S-23S rDNA ISR from a mixed community sample produced strong fluorescent signal with no background from negative controls (Fig. 3). Simultaneous hybridization with a Cy5-labeled standard targeting the kanamycin gene served as a reference for normalizing hybridization signal. This is important as differences in signal intensity may result from overall differences in the amount of the organism in the sample population, as well as variations in the amount of DNA probe deposited during arraying (12, 42). Using the kanamycin gene, present in the TA vector and therefore part of every arrayed probe, it was possible to normalize for differences in the amount of arrayed material and to account for some differences in hybridization conditions.

The ISR array format described here permits the use of 16S-23S rDNA ISR sequences from mixed microbial communities as probe targets for characterization of community diversity. This method, based on PCR amplification for labeling of target sequences, can only be considered semi-quantitative and is prone to PCR biases. However, for detection of target organisms or for analysis of microbial community dynamics or community characterization, PCR amplification of target sequences diminishes issues relating to array sensitivity. Detection of target sequences in

environmental samples is limited by low concentrations of biomass and high concentrations of inhibitors and by the limited loading capacity of glass-slide arrays (12, 21, 43). Target labeling through PCR amplification, thereby enhances the ability to effectively characterize mixed populations of organisms by increasing sensitivity.

Many aspects of the ISR array make it a useful tool for analysis of mixed microbial communities. It was possible to obtain clone probes from a mixed population of organisms in one PCR reaction, to hybridize with a high level of specificity, and to have uniformity of hybridization due to the narrow Tm range of ISR sequences. The ISR array provides a sensitive, rapid, cost-effective method for analyzing mixed microbial communities. These arrays are now being used for analysis of microbial community dynamics within microbial populations exposed to temporal and environmental perturbations.

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PART IV

EVALUATION OF THE EFFECT OF SPECIES DIVERSITY ON MICROBIAL COMMUNITY PHENOTYPE AND INVASIBILITY IN THE RHIZOSPHERE OF A MODEL PLANT-BASED GRAYWATER WASTE PROCESSING SYSTEM

ABSTRACT

A model plant-based graywater processing system was initiated with six different microbial inocula to evaluate the effect of species diversity on community phenotype and community invasibility. The microbial inocula were from a constructed community of eleven isolates, a low diversity community of contaminant species, and a complexity gradient of communities derived from industrial wastewater treatment plant activated sludge. An integrated approach using a combination of PCR-based phylogenetic analysis and quantification, community level physiological profiling (CLPP) and microarray analysis was employed to evaluate the diversity, phenotypic potential and invasibility of inoculated communities. The ability of the inocula to persist upon inoculation into a plant-based graywater waste processing system was evaluated over the course of six studies running for 13 days each. The resistance of each population to invasion by a competitor (*Pseudomonas fluorescens* 5RL) was determined for 10 days post-invasion using quantitative PCR analysis and cultural cell counts. The phenotypic potential of the inocula was evaluated by measuring surfactant degradation and through Biolog and BD oxygen biosensor CLPP analysis. Differences in community structure between treatments and the effects of invasion within treatments were evaluated using a 16S-23S rDNA ISR array. Differences in community genotype (array analysis), phenotype (surfactant degradation and CLPP) and invasibility were observed for communities of varying structural diversity. The highest diversity treatment was the least invaded and was able to degrade surfactant to a greater extent than other complexity gradient treatments. Lower diversity complexity gradient treatments were similar in terms of functional potential (as measured by CLPP), but were impaired in degradation of surfactant. The constructed

community was phenotypically diverse and even degraded surfactant to a greater extent than any of the other communities. However, this community was invaded to a greater extent than any of the other communities, the invader persisted in the population, and one member of the community was completely displaced upon invasion. These results suggest that species richness improves system performance by increasing community persistence, resistance to invasion and phenotypic performance.

INTRODUCTION

The relationship between structural diversity, functional diversity and ecosystem stability is of great interest in ecology. Insight into the interaction between these factors may make it possible to better predict ecosystem function, thus improving process control for ecological and biotechnological applications (3). Microbial communities are highly versatile and dynamic and therefore, provide a good model system for evaluation of diversity-stability hypotheses (25). McGrady-Steed et al. (52) used constructed communities to create a diversity gradient in several different combinations. Using these artificial communities of aquatic eukaryotic species, they found that biodiversity enhanced system predictability (52). In separate studies, researchers used fumigation to evaluate the effect of loss of species on functional diversity in soil (18, 37). These researchers found that loss of microbial diversity did not directly correlate to loss in functional capacity, rather specific functional measures were found to be more important and loss of function in specific functional groups (nitrifiers, methane oxiders) was more indicative of loss of diversity than overall loss of function (18, 37). Dilution to extinction has also been used to create diversity gradients. In this approach, dilutions of a diverse

community are used to initiate new systems and re-growth of the community produces populations of progressively lower diversity, but similar cell density. This approach has been used in rhizosphere and bioreactor studies and has been shown to produce communities of different structural and functional potential (23, 29, 33).

PCR-based techniques using primers specific for a particular genotype provide a high level of resolution for microbial identification and characterization (2). More recently, real-time quantitative PCR has been used to detect and quantify PCR products as they are generated during each PCR cycle (35). TaqMan oligonucleotide probes, commonly used in real-time analysis, are complementary to a target nucleic acid sequence and have a fluorophore attached at the 5' end and a quencher at the 3' end. The 5'-nuclease activity of the Taq polymerase displaces the probe during PCR extension, separating the fluorophore (reporter) from the quencher. The result is an increase in fluorescence proportional to the amount of amplicon produced. Real-time PCR is a high-throughput method with a wide detection range and high specificity given proper probe design and optimization of amplification conditions (35). Real-time PCR is commonly used for detection of medically and environmentally significant organisms or groups of organisms (2, 6, 9, 39, 60).

Microarray technology is a valuable tool for high-throughput analysis of genetic differences among species and in response to experimental treatments (5, 21, 42). However, the technology has only recently been applied to environmental systems (13, 16, 49, 68, 69). Recently, an array containing plasmids with16S–23S rDNA intergenic spacer (ISR) probe sequence inserts was developed for analysis of mixed microbial communities (15). These 16S-23S rDNA ISR sequences were obtained from an industrial

wastewater treatment plant (WWTP) sludge community. The higher sequence variability of the ISR compared to 16S rRNA gene sequences provides greater specificity for hybridization. The array had a high level of specificity for evaluation of environmental samples with a strong signal from labeled targets that closely matched arrayed probe sequences (greater than 99% similarity in 16S rDNA sequence) and very little background from labeled target sequences of less than 95% similarity in the 16S rDNA sequence of the arrayed probe (15).

Biolog community level physiological profiling (CLPP) was developed by Garland and Mills (32) as a means to characterize differences among microbial populations on the basis of substrate utilization. In this method, commercially available microtiter plates (Biolog GN; Biolog, Inc. Hayward, CA) containing 95 different carbon substrates are directly inoculated with environmental samples. The phenotypic potential of microbial mixtures is quantified by measuring the reduction of tetrazolium violet to a colored formazan (28). Patterns of carbon source utilization by mixed communities are analyzed using multivariate statistical methods. CLPP analysis of carbon source utilization patterns is a rapid, high-throughput method with significant resolving power (25). Different communities are clearly distinguished following principal component analysis (PCA) suggesting that differences in patterns of carbon source utilization reflect real differences in community composition (27). Biolog CLPP has been used to distinguish communities from plant, soil and water ecosystems (24, 28, 38, 44, 66). More recently, a CLPP method has been developed which may provide more functionally relevant information (34). The BD Oxygen Biosensor System (BD Biosciences, Bedford, MA) is used to detect shifts in substrate utilization by mixed microbial communities in

either a 96 or 384-well microplate format. The improved system provides substrate at lower concentrations and results in detectable signal over shorter incubation periods, thereby minimizing biases associated with cell growth and selective enrichment (34).

In this research, a plant-based graywater waste processing system was used as model system to evaluate the effect of species richness on community phenotype and community invasibility. Graywater degradation via plant-microbe interactions in the root zone (rhizosphere) has been evaluated for use on long-term space missions proposed by the National Aeronautics and Space Administration (NASA) (7, 30, 36, 48). The high cost of re-supplying food and water, coupled with the need for self-sufficiency for astronauts on long-term space missions necessitates the development of safe, effective methods for food production and waste processing system. Recycling wastewater through biological life support systems (BLSS) designed for plant production represents an ideal method for re-supplying water at substantial cost savings (36). Graywater, the waste from showers, sinks, laundry, and dishwashing will make up the single largest waste stream on long-term space missions (up to 25 L person^{-1} day^{-1}) (7). In a closed system, it is estimated that plant production for one person would produce 40 L of atmospheric condensate per day (31). Therefore, it should be possible to incorporate wastewater processing into hydroponic plant production to recycle water for non-hygenic uses. However, a significant concern of this approach is the establishment of robust and reliable microbial communities in the rhizosphere to accomplish wastewater treatment as well as to ward off invasive species and microbial pathogens. Therefore, the ability of inoculants to persist, resist invasion by outside species and degrade organic matter that

may otherwise accumulate in the system will be vital to ensure reproducible, reliable and resilient system function.

The goal of this research was to evaluate how species diversity affects phenotypic potential and resistance to invasion by a competitor in a model plant-based graywater waste processing system. Communities of varying structural diversity were created by artificially combining rhizosphere isolates, by using dilution/extinction of industrial WWTP activated sludge, and by growing contaminant species that colonize sterile wheat roots. The ability of the inocula to persist upon inoculation into the rhizosphere was evaluated over the course of six studies running for 13 days each. The resistance of each population to invasion by a competitor (*Pseudomonas fluorescens* 5RL) was determined using quantitative real-time PCR analysis and cultural cell counts. The phenotypic potential of the inocula was evaluated by measuring surfactant degradation and through CLPP. Differences in community structure were evaluated using the 16S-23S rDNA ISR array. The integrated approach chosen in this study incorporates the analysis of genotypic diversity, phenotypic diversity and community dynamics to achieve a more complete view of microbial community processes.

MATERIALS AND METHODS

Environmental conditions and plant cultural techniques

Wheat (*Triticum aestivum* L. cv. Apogee) was grown in a 1.8 x 2.4 m walk-in growth chamber (EGC, Inc. Chargin Falls, OH) located at Kennedy Space Center (KSC), Florida. 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 23 ºC. Relative humidity (RH) was maintained at 70% for the duration of the study. Atmospheric CO_2 was maintained at 1200 ppm with an infrared gas analyzer (LiCor Model 6252, LiCor Corp, Lincoln, NE). Wheat seeds were sterilized using a combination of mercuric chloride and hydroxylamine hydrochloride as previously described (1). Surface sterilized seeds were placed onto sterile filter paper (Whatman qualitative, 10 cm) moistened with sterile distilled water in 100 mm glass petri dishes. Following sterilization, wheat seeds were refrigerated at 4 ºC for 4 days. The seeds were moved into the plant growth chamber for 8 days in conditions described above. After 8 days, seedlings were randomly selected and placed into slits in autoclaved foam plugs and transferred into sterile wide-mouthed vessels containing approximately 500 mL of filter sterilized, modified ½ strength Hoaglands nutrient solution (30) with 5mM MES (Sigma, St. Louis MO) (54) 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. Plant growth vessels were placed on a rotating shaker table (75 rpm) to provide aeration.

Inocula Preparation

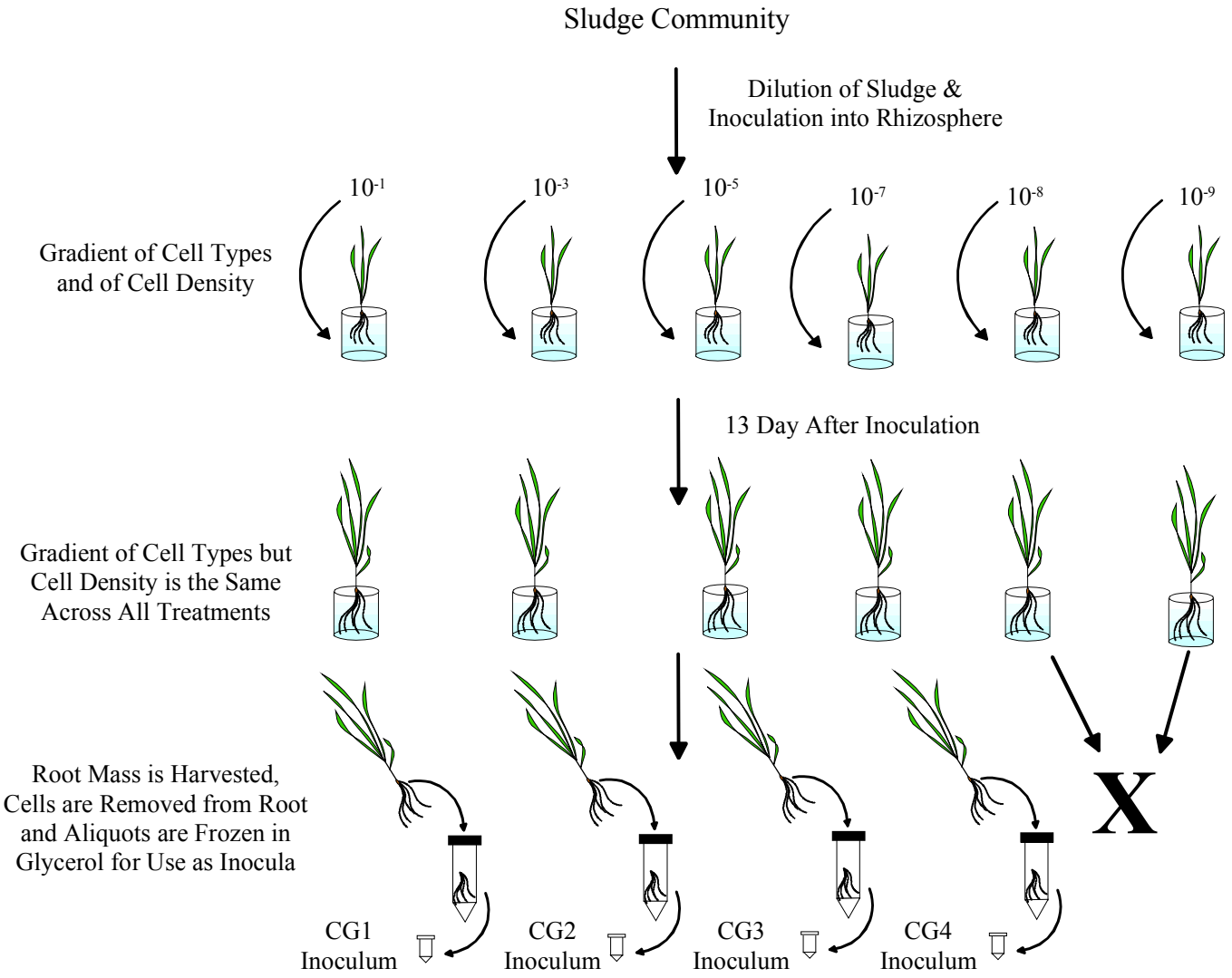
Six different microbial communities were used as rhizosphere inocula; a constructed community, a community of contaminant species and an industrial WWTP sludge community complexity gradient (CG1, CG2, CG3, CG4). The constructed community was prepared as previously described (14). The constructed community consists of eleven isolates phylogenetically similar to identified clones from a graywater rhizosphere 16S rDNAclonal library. The isolates include members of the alpha (one isolate), beta (three isolates) and gamma (three isolates) subdivisions of proteobacteria,

three isolates from the *Cytophaga-Flavobacterium* (CF) cluster from the *Cytophaga-Flavobacterium-Bacteriodes* (CFB) division and one *Paenibacillius* isolate from the Firmicutes division. Ten of the isolates from the constructed community were grown separately in R2A broth (0.5 g/L yeast extract, 0.5 g/L proteose peptone #3, 0.5 g/L casamino acids, 0.5 g/L dextrose, 0.5 g/L starch, 0.3 g/L sodium pyruvate, 0.3 g/L K_2HPO_4, 0.05 g/L $MgSO_4$) for 18 hr. *Cytophaga hutchinsonii* was grown for four days on Dubos media (14) with sterile filter paper (Whatman #1 Qualitative, Kent, UK) as a source of cellulose. Each isolate was centrifuged at 16,000 X *g* for 15 min to pellet cultures, supernatant was removed and cells were resuspended in ½ strength Hoaglands nutrient solution to obtain cultures of similar OD at 600 nm (corresponding to total cell densities of 2×10^8 to 5×10^{10} cells). Each culture was mixed in equal portions (10 mL each) to form the constructed community mixture. This mixture was aliquoted (1 mL) into freezer tubes and frozen at –80 ºC with a final concentration of 12% (v/v) sterile glycerol. Samples for total, culturable and molecular analysis were taken from the pure cultures and the final constructed community.

The complexity gradient (CG) was prepared from organisms in an industrial WWTP activated sludge sample collected in July 2001. Influent wastewater from this plant contains primarily low-molecular weight organic acids and short-chain alcohols resulting from the manufacture of fibers, plastics and chemicals (8, 20, 46). The composition of the sludge community was characterized by 16S rDNA sequence analysis as previously described (14). To obtain a gradient of microbial complexity (i.e., a gradient in species richness) without a gradient in cell density, dilutions of the industrial WWTP sludge were inoculated into the rhizosphere of hydroponically grown wheat as

shown in Fig. 1. Simultaneously, triplicate axenic plants were prepared and left uninoculated. The resulting rhizosphere population from the uninoculated samples (consisting of contaminant species) was designated the propitious community (i.e., community of potential or chance). To enrich for surfactant degraders, 15 ppm polyoxyethylene 10 lauryl ether (Pol; Sigma-Aldrich, St. Louis, MO) was added to each treatment in the complexity gradient and to the uninoculated plant growth vessels. On day 13, microbial suspensions were prepared from each treatment in the complexity gradient and from the propitious community by placing the plant root into filter sterilized nutrient solution (25 mL) containing glass beads (3 mm dia., Fisher Scientific, St. Louis MO) and hand shaking for 3 min. Rhizosphere suspensions were frozen at -80 °C in one mL aliquots with a final concentration of 12% (v/v) sterile glycerol. A 16S rDNA clonal library was prepared, plasmid extracted and analyzed by restriction fragment length polymorphism (RFLP) screening of clones and sequencing, as previously described (14). Sequence analysis of clones from a 16S rDNA clonal library prepared from the propitious community inoculum suggests that there were only 3 dominant types, *Enterobacter* sp. (4 clones), *Pseudomonas* sp. (2 clones), and *Burkholderia* sp. (4 clones). This is supported by species diversity analysis on R2A media that showed a predominance of only 2 to 3 morphotypes throughout each experiment. Samples were taken from the complexity gradient plants for microbiological analysis and for evaluation of overall phenotypic potential. Phenotypic potential was determined from the number of carbon sources used by each community upon inoculation of dilutions of each community (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) into Biolog Ecoplates (Biolog, Hayward, CA). The Ecoplates contain 31 carbon sources repeated three times on each plate. Mean number of positive

Figure 1. Preparation of complexity gradient treatments from industrial WWTP sludge. Sterile wheat plants were prepared as described in methods and inoculated in triplicate with 1 mL each of dilutions (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-8}, 10^{-9}) of industrial WWTP sludge prepared in filter sterilized plant nutrient solution. Rhizosphere was harvested from all treatments and analyzed for shoot and root weight, total and culturable cell numbers and CLPP as described in methods. Based on analysis of plant and microbiological data, four populations (originating from the 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} sludge dilutions) were chosen and were designated CG1, CG2, CG3, and CG4 and were used as inocula in all other studies.


Sludge Community
Dilution of Sludge &
Inoculation into Rhizosphere
Gradient of Cell Types
and of Cell Density
10-1
10-3
10-5
10-7
10-8
10-9
13 Day After Inoculation
Gradient of Cell Types but
Cell Density is the Same
Across All Treatments
Root Mass is Harvested,
Cells are Removed from Root
and Aliquots are Frozen in
Glycerol for Use as Inocula
X
CG1
Inoculum
CG2
Inoculum
CG3
Inoculum
CG4
Inoculum

responses was determined after 4 days of incubation at room temperature. Evaluation of data from the complexity gradient study (Table 1) indicated that cell density was similar for all treatments. Phenotypic potential was reduced in the high dilution treatments (10^{-8} and 10^{-9}) to a level similar to the propitious community, indicating that these treatments had a community structure similar to the contaminant community. Based on this analysis, communities from the 10^{-1}, 10-3, 10^{-5} and 10^{-7} treatments (designated CG1, CG2, CG3, and CG4) and the propitious community were chosen for further analysis.

Plant growth studies

A total of six plant growth studies were performed. On day 0 of each experiment, CG1, CG2, CG3, CG4 and propitious inocula (1.0 mL) were added from the preserved samples, directly into the root zone of four separate plant growth vessels. Based on initial microbiological analysis (Table 1), the constructed community mixed inocula was diluted 1:100 and then added (1 mL) to each of four plant growth vessels to obtain a final cell density similar to that of the other treatments. Each experiment lasted 13 days. On days 3, 5, 7 and 13 one plant from each treatment was removed for plant and microbiological analyses. The plant growth experiments were repeated three times with no invader added. The invasibility of the respective

inocula was tested in three other plant growth experiments. In these experiments, *Pseudomonas fluorescens* 5RL, containing the *luxCDABE* gene cassette with a naphthalene-inducible promoter, was added on day 3 of the experiment as an invader of the established inocula.

Table 1. Results of total and cultrable cell counts of prepared inocula

Treatment	Sludge Dilution	Total Cells (10^6 cells mL^{-1})	Cultable Cells (10^6 cells mL^{-1})	Biolog Ecoplates[a]	Root Dry Wt (mg)	Shoot Dry Wt (mg)
CG1	10^{-1} Dilution	22.3 ± 3.17	21.4 ± 0.21	30	57.6 ± 2.62	136.2 ± 12.6
CG2	10^{-3} Dilution	46.0 ± 53.42	24.7 ± 5.20	29	73.7 ± 30.5	160.1 ± 48.7
CG3	10^{-5} Dilution	46.0 ± 1.52	41.9 ± 33.0	21	108.1 ± 31.1	156.8 ± 13.8
CG4	10^{-7} Dilution	13.6 ± 11.2	8.70 ± 8.37	18	43.1 ± 3.9	115.5 ± 17.3
N/A	10^{-8} Dilution	11.7 ± 12.6	9.47 ± 9.08	16	85.9 ± 51.6	135.1 ± 40.9
N/A	10^{-9} Dilution	12.2 ± 8.68	19.1 ± 22.7	17	65.5 ± 31.5	128.0 ± 41.4
Propitious	N/A	2.73 ± 1.93	1.73 ± 1.62	16	45.0 ± 10.2	103.2 ± 26.6
Constructed	N/A	1,630	7,300	N/A	N/A	N/A

N/A = not applicable

[a]Mean number of carbon sources utilized (of triplicate samples)

Surfactant analysis

Graywater was simulated by the addition of the nonionic surfactant Pol to plant nutrient solution. Simulated graywater (SGW) was added to the plant nutrient solution on day 3 of each experiment at a final concentration of between 7 and 11 ppm. Plant nutrient solution amended with SGW was sampled on day 3 (10 minutes after SGW addition) and on days 5, 7 and 13. Samples (1 mL) were taken from plant nutrient solution and filtered through a 0.2 µm nylon Acrodisc HPLC syringe filter (Gelman Sciences, AnnArbor MI) into an HPLC autosampler vial and stored at -25 ºC until analysis. Surfactant concentration was characterized using reversed-phase gradient HPLC in conjunction with electrospray ionization quadrupole ion trap mass spectrometry (ESI-MS) as described by Levine et al. (47). Surfactant data were normalized to day 3 concentrations to account for variability in the concentration of surfactant added to each treatment. For statistical analysis, surfactant data were transformed by squaring normalized values to meet requirements for normal distribution and homogeneity of variance.

Plant measurements, total and culturable cell counts

Suspensions of rhizosphere organisms from each treatment were prepared by placing the plant root into filter sterilized nutrient solution (25 mL) containing glass beads and hand shaking for 3 min. The resulting cell suspensions were analyzed for total cell density using acridine orange staining and epifluorescent microscopy (41). Cultural cell density was analyzed by serial dilution and spread plating onto R2A media (Difco, Detroit, MI). Colony forming units (CFU) were enumerated following incubation at 25 ºC for 3 days. Wheat root dry weight and shoot dry weight was determined after plant material was dried at 70 ºC for 72 h.

Community level physiological profiling (CLPP)

Biolog GN Plates. For each sample, a 10^{-1} dilution of the rhizosphere suspension was prepared in 0.85% NaCl and inoculated into Biolog GN microplates (Biolog, Hayward, CA). The Biolog GN plates contain 95 wells each with a separate sole C source, and a control well without a C source. A redox-sensitive tetrazolium dye was reduced as a result of microbial respiration, and accumulates as insoluble formazan. Color formation in each of the 96 wells was read every 2 h measuring the OD_{590} using a Biotek EL 320 microplate reader. Data were normalized using a blank-corrected average well color development of 0.30 absorbance units and analyzed using principal component analysis (PCA) as previously described (26, 32).

BD Oxygen Biosensor System. The BD Oxygen Biosensor System (BD Biosciences, Bedford, MA) was used to detect shifts in substrate utilization by the rhizosphere communities by incubation of mixed community samples in the presence of 12 different carbon sources. The BD plates consisted of a 96 well plate containing an O_2-sensitive fluorophore, 4, 7-diphenyl-1,10-phenathroline ruthenium (II) chloride immobilized within a hydrophobic, oxygen-permeable silicon matrix (67). The dye was quenched in the presence of oxygen, but fluorescence increased in response to oxygen depletion. Carbon sources tested included 4 sugars (glucose, rhamnose, arabinose, and xylose), 4 amino acids (lysine, alanine, aspartic acid and glycine), 2 organic acids (sodium acetate and proprionic acid), polyethylene glycol and a control with water. Substrate solutions were prepared, plates inoculated and the response to oxygen depletion (i.e., respiration of the substrate) was measured with an automated fluorescence plate

reader as described by Garland et al. (34). Time to peak responses were normalized for background and used for subsequent analyses.

Extraction of DNA for molecular analysis

Rhizosphere suspensions (10 to 15 mL) for molecular analysis were filtered onto Supor filters (Gelman Sciences, AnnArbor, MI) and stored at –80 ºC until analysis. Mixed-community DNA was extracted using the Ultra Clean™ Soil DNA Isolation kit (MoBio Laboratories, Solana Beach, CA). Extractions were carried out according to manufacturers instructions, except bead tubes were placed in a Fast Prep FP120 (QBiogene, Carlsbad, CA) for 30 s at a speed of 5.5 m/s instead of vortexing. The concentration of extracted DNA was quantified using the PicoGreen ds DNA quantification kit (Molecular Probes, Eugene, OR). Community profiles were obtained for all extracted rhizosphere samples by PCR amplification of the 16S-23S rDNA from mixed community samples and electrophoresis of PCR products in 2% agarose gels using the primers and PCR conditions previously described (15).

Detection of invader using real-time PCR assay and culturable cell counts

Culturable *Pseudomonas fluorescens* 5RL cells were detected on selective media by plating dilutions of rhizosphere suspensions on LB agar (1 L contains 10 g tryptone, 5 g yeast extract, 10 g NaCl, 17 g agar, pH 7.5) with salicylate (50 μg ml^{-1}) and tetracycline (15 μg ml^{-1}). Additionally, a real-time PCR assay was developed for detection of an 83 bp sequence within the *luxE* portion of the *luxCDABE* gene cassette present in *Pseudomonas fluorescens* 5RL. The real-time PCR assays were run on the DNA Engine Opticon continuous fluorescence detection system (MJ Research, Inc., Waltham, MA). The primers and the dual-labeled Black Hole Quencher™ probe were prepared by

Biosearch Technologies, Inc. (Novato, CA). The *luxE* gene sequence was amplified from samples using primers luxEf (5'TATTGCACGTGATCGTCTTA3') and luxEr (5'TGCTCAAACCAATCTCCA3'). The TaqMan probe (LuxE-BHQ; 5' 6-FAM(ACGCTTGCTTGGTTCTGTCAACTTC) BHQ-1 3') was derived from a sequence between the primers. The real-time PCR assays were performed in Qiagen HotStart Taq Master Mix (Qiagen, Valencia, CA) in a total volume of 25 µl with 5 mM $MgCl_2$, 500 nM of primers, 100 nM probe, 0.5 to 10 ng of sample DNA or dilutions of plasmid pCR 2.1 vector (Invitrogen, Carlsbad, CA) carrying the 83bp *luxE* PCR product sequence as standard (from 10^2 to 10^8 copies). The PCR program was 2 min at 50 ºC, 10 min at 95 ºC, 49 cycles at 95 ºC for 15 s and 61 ºC for 30 s. Baseline values averaged over cycles 2 thru 10 were subtracted from all values. Threshold was set at 0.005 relative fluorescence units. Assays were performed using duplicate samples at two different concentrations, and all PCR runs included standards and control reactions without template.

Community analysis using 16S-23S rDNA ISR array

A plasmid-based 16S-23S rDNA ISR array was prepared as previously described (15). Briefly, the ISR array was composed of probes containing plasmid with 16S-23S rDNA ISR inserts spotted in 50% DMSO at a final concentration between 400 ng/µl and 550 ng/µl. The ISR array contains 160 plasmids from an industrial WWTP 16S-23S rDNA ISR library produced from the same activated sludge used to generate the CG treatments. Each member of the constructed community as well as isolates from rhizosphere samples, and selected clones from a 16S-23S rDNA ISR library from the propitious community were also included on the array. Each glass slide contained triplicate ISR arrays. TA vector with no insert, dimethyl sulfoxide (DMSO; Sigma, St.

Louis, MO) and water blanks served as negative controls. Labeled 16S-23S rDNA ISR sequences from mixed community rhizosphere samples (days 5 and 13) from each treatment were hybridized to the ISR array. 16S-23S rDNA ISR sequences from mixed community DNA samples (2-5 ng) were amplified and labeled with Cy3 labeled dCTP (Amersham Pharmacia, Peapack, NJ) using techniques described by Cook et al. (15). The kanamycin gene from the pCR®2.1-topo vector was used as reference DNA by PCR incorporation of Cy5-dCTP (Amersham Pharmacia, Peapack, NJ) into a portion of the gene (15). The Cy3 labeled target and the Cy5 labeled reference standard were mixed in equal portions and hybridized to the array simultaneously. The arrays were hybridized, washed and analyzed as described previously (15). Signal intensity and local median background for each slide were determined by using Imagene 4.0 (BioDiscovery, Inc., Los Angeles, CA) analysis software. Signal intensities were corrected for background and negative control fluorescence to obtain the final hybridization intensity. Corrected signal intensities for Cy3 and Cy5-labeled samples were $\log_{10}$ transformed. The normalized signal intensity of each sample spot was calculated by dividing the log transformed intensity of the sample spot by the corresponding intensity of the reference DNA spot (Cy3-Sample/Cy5-Reference). Points were set to missing in the analysis if they possessed a Cy-3 to Cy-5 ratio: (1) greater than 1 (signifying that the sample intensity was greater than the reference), or (2) lower than 3 times the standard deviation of the slide mean (this removed extreme outliers that were generally negative and could artificially drive the statistical analysis of the data). Values with negative normalized intensities were set to zero signifying the absence of hybridization.

Statistical analysis

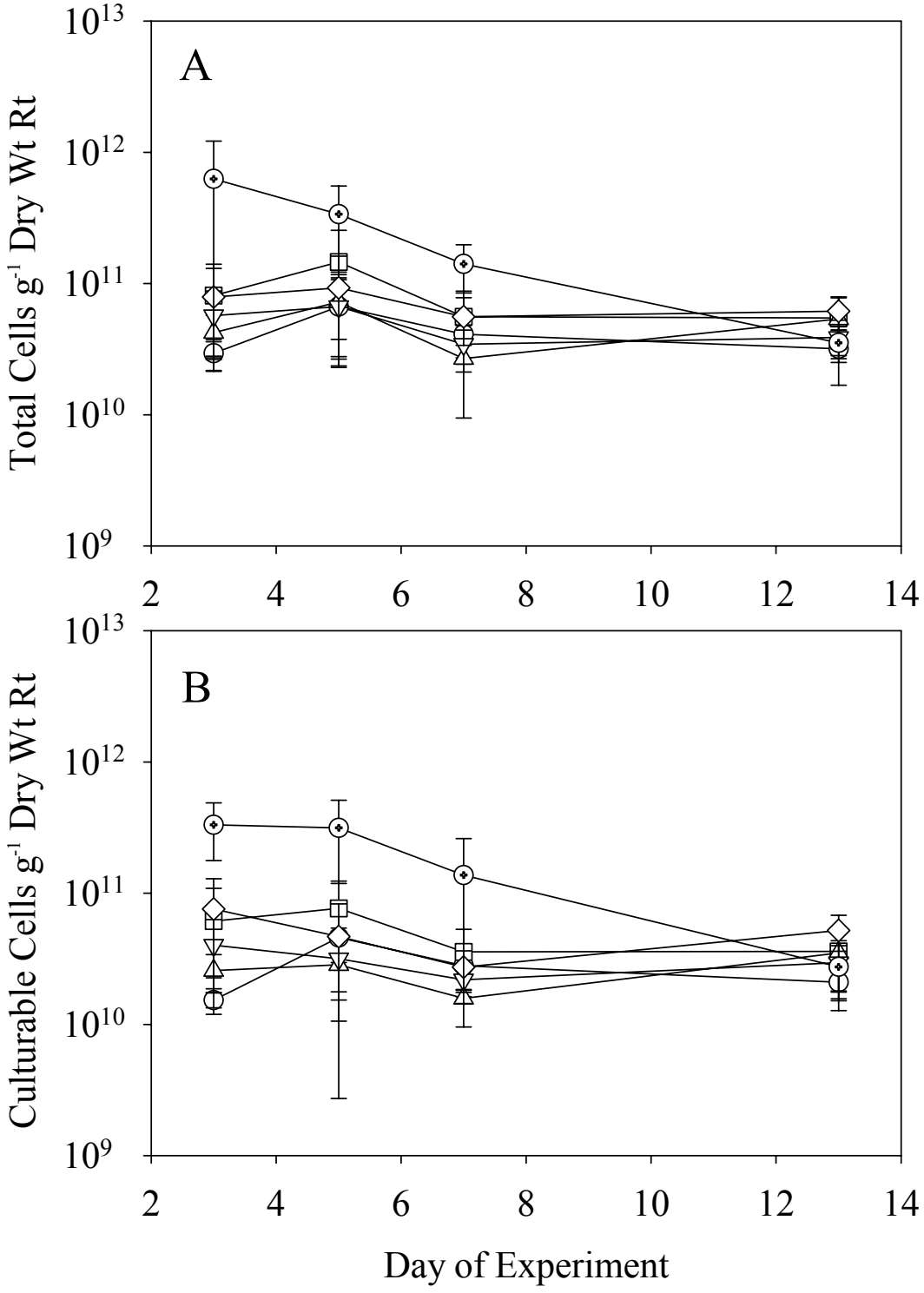
Total and culturable cell counts, plant measurements, invader, and array data were $\log_{10}$ transformed to improve the normal distribution and homogeneity of variance for the data. Multivariate analysis of variance (MANOVA) of log transformed data from microbiological and plant measurements from all experiments was used to determine whether there were significant differences among bacterial communities. Communities were considered significantly different if $P < 0.05$ based on Wilks' lambda F statistic. Significant differences among treatments were then further assessed for each sample day and for the effect of invasion; Tukey's post hoc tests were used to determine significant differences ($P < 0.05$) between individual treatments. Differences in CLPP among communities were evaluated using PCA and principal component scores were further analyzed by comparing principal component scores among treatments using ANOVA. Array data were analyzed by Univariate ANOVA with Tukey's post hoc test to evaluate the effects of day, treatment and invasion. All statistical analyses were carried out using SPSS version 11.0 (SPSS, Chicago, IL).

RESULTS

Inocula cell density

There were no significant differences in total or culturable cell density among experiments with or without invader added ($P = 0.05$), therefore data for each treatment from each of the six experiments (three with invader and three without invader) were averaged (Fig. 2a and Fig. 2b). There were no significant differences in culturable or total cell density among inocula from the constructed, CG1, CG2, CG3 and CG4 communities

Figure 2. Total (A) and culturable (B) cell counts for each treatment. Samples were taken on days 3, 5, 7 and 13. All values are reported as the average of six replicate experiments. Bars represent standard deviation. Constructed community ( ), CG1 ( ), CG2 ( ), CG3 ( ), CG4 ( ), and Propitious community ( ).

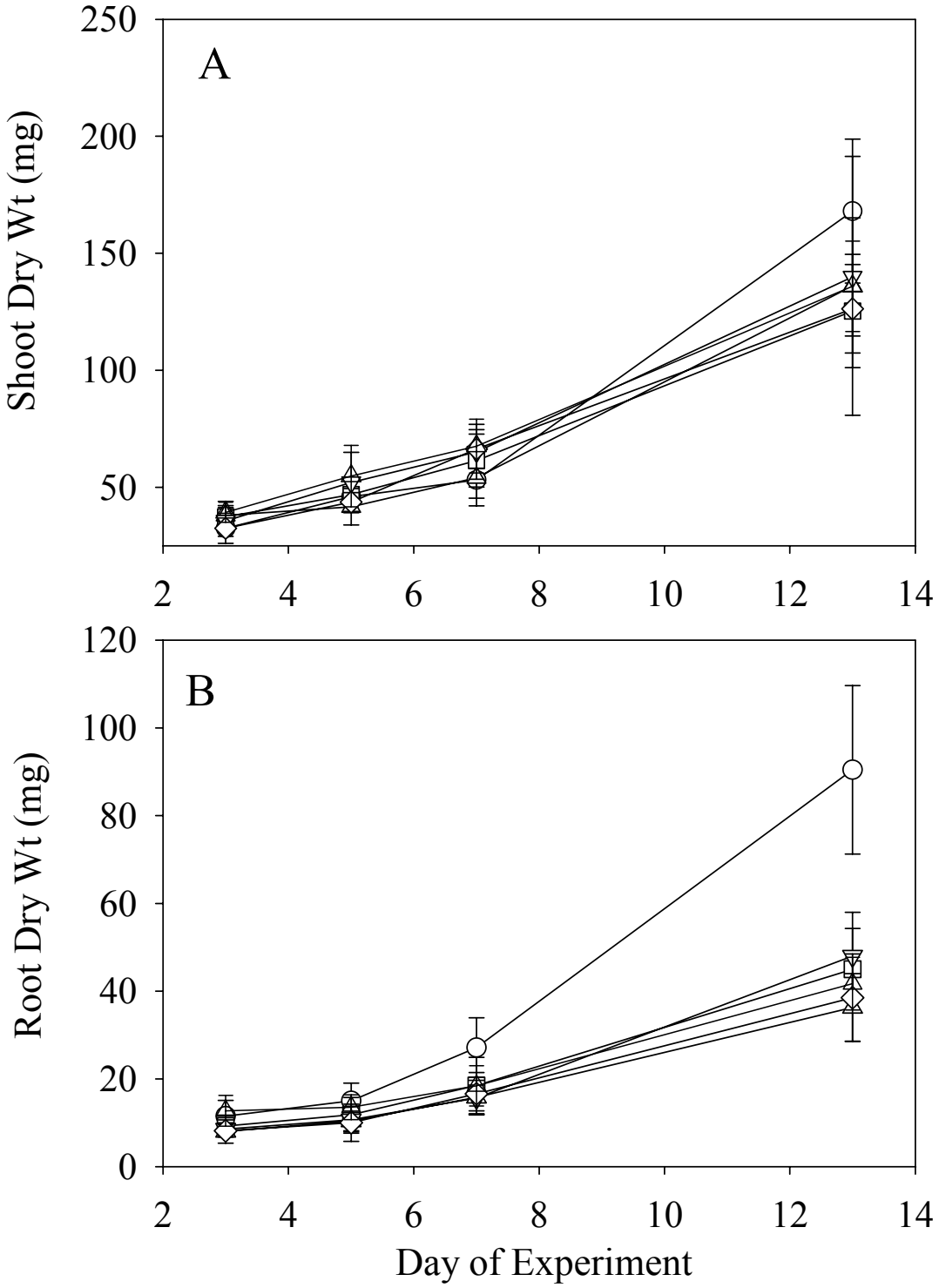

A
B
Total Cells g-1 Dry Wt Rt
Culturable Cells g-1 Dry Wt Rt
10^13
10^12
10^11
10^10
10^9
2
4
6
8
10
12
14
Day of Experiment

as measured on days 3, 5, 7 and 13 of each experiment ($P = 0.05$)(Fig. 2a and Fig. 2b). However, total and culturable cell density for the propitious community was significantly higher than that of the other treatments on days 3, 5 and 7 of each experiment ($P < 0.005$). On the final day of sampling (day 13) there was no difference in culturable or total cell density among any of the treatments. These results suggest that, with the exception of the propitious community, the overall density of inoculated organisms was similar at the onset of each experiment (1.5×10^{10} to 7.6×10^{10} culturable cells and 3.0×10^{10} to 8.1×10^{10} total cells g^{-1} dry weight root) and cell density per gram dry weight of root remained stable for the duration of the experiments (2.1×10^{10} to 5.2×10^{10} culturable cells and 3.2×10^{10} to 6.2×10^{10} total cells g^{-1} dry weight root after 13 days). Total and culturable cell densities for the propitious community were between 15% and 40% higher than the next highest treatment until the final day of sampling. Interestingly, at most sample points, the lowest diversity complexity gradient community (CG4) was, although not significantly higher, the second highest treatment in terms of culturable and total cell density.

Plant growth responses

There were no significant differences in shoot or root dry weight among experiments with or without invader added ($P = 0.05$), therefore data for each treatment from each of the six experiments (three with invader and three without invader) were averaged (Fig. 3a and Fig. 3b). Root dry weight was the same for all treatments on days 3 and 5 (Fig. 3a). By day 7, the root dry weight of plants inoculated with the constructed community was higher than that of plants inoculated with CG2, CG3, CG4 and the propitious communities ($P < 0.05$). Root dry weight of plants inoculated with the

Figure 3. Shoot dry weight (A) and root dry weight (B) for plants from each treatment. Samples were taken on days 3, 5, 7 and 13. All values are reported as the average of six replicate experiments. Bars represent standard deviation. Constructed community ( ), CG1 ( ), CG2 ( ), CG3 ( ), CG4 ( ), and Propitious community ( ).


A
Shoot Dry Wt (mg)
250
200
150
100
50
2
4
6
8
10
12
14
B
Root Dry Wt (mg)
120
100
80
60
40
20
0
2
4
6
8
10
12
14
Day of Experiment

constructed community was not significantly different than that of plants inoculated with the highest diversity complexity gradient community (CG1; $P < 0.05$). On day 13, root dry weight was significantly higher for plants inoculated with the constructed community than any of the other treatments (Fig. 3a). There were no significant differences ($P < 0.05$) in shoot dry weight for any of the treatments at any of the sample points (Fig. 3b).

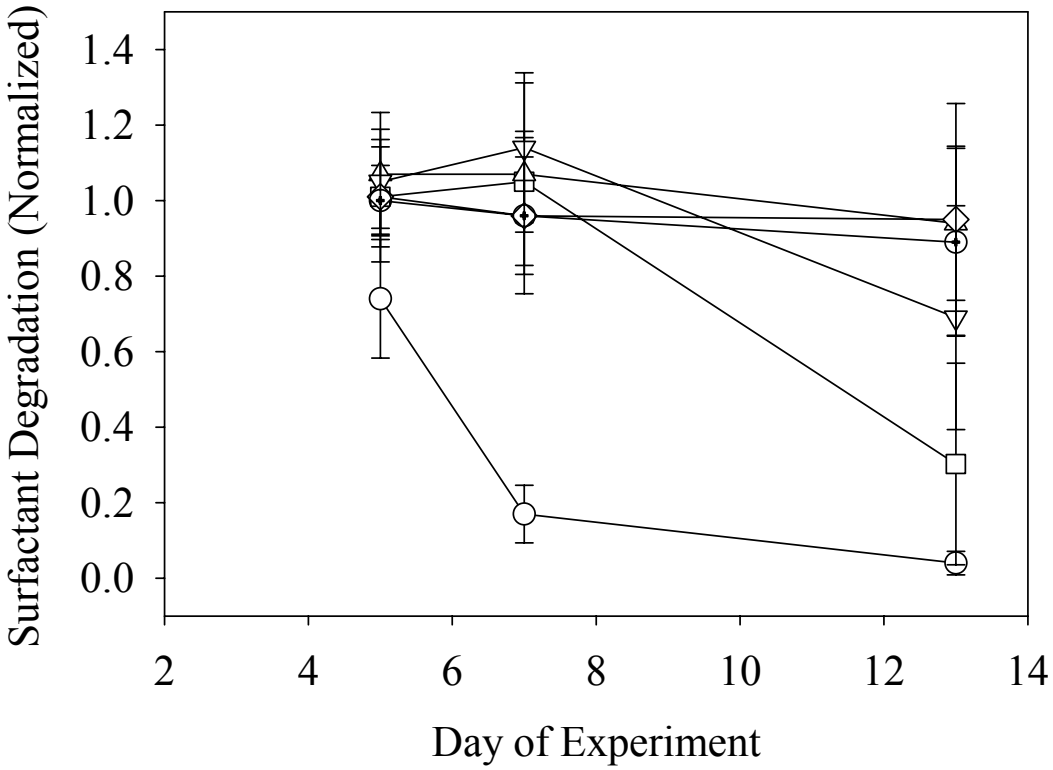
Surfactant degradation

There were no significant differences in surfactant degradation among experiments with or without invader added ($P = 0.05$), therefore data for each treatment from each of the six experiments (three with invader and three without invader) were averaged (Fig. 4). Surfactant was degraded more rapidly and more completely in the rhizosphere of plants inoculated with the constructed community than in the rhizosphere of plants inoculated with other communities (Fig. 4). The concentration of surfactant in plants inoculated with the constructed community was significantly lower than the concentration of surfactant in the rhizosphere of plants inoculated with complexity gradient treatments CG2 and CG4 and the propitious community throughout the experiment ($P < 0.05$). On day 7, surfactant concentration in the roots of plants inoculated with the constructed community was significantly lower than all other treatments ($P < 0.001$). By day 13, surfactant concentration in plants inoculated with the constructed community was not significantly lower than the concentration in the rhizosphere of plants inoculated with CG1 or CG3 communities ($P < 0.05$).

Detection of invader using real-time PCR assay and culturable cell counts

Pseudomonas fluorescens 5RL (5RL) is a bioluminescent bioreporter organism that contains an inducible *luxCDABE* gene cassette resulting in light production upon

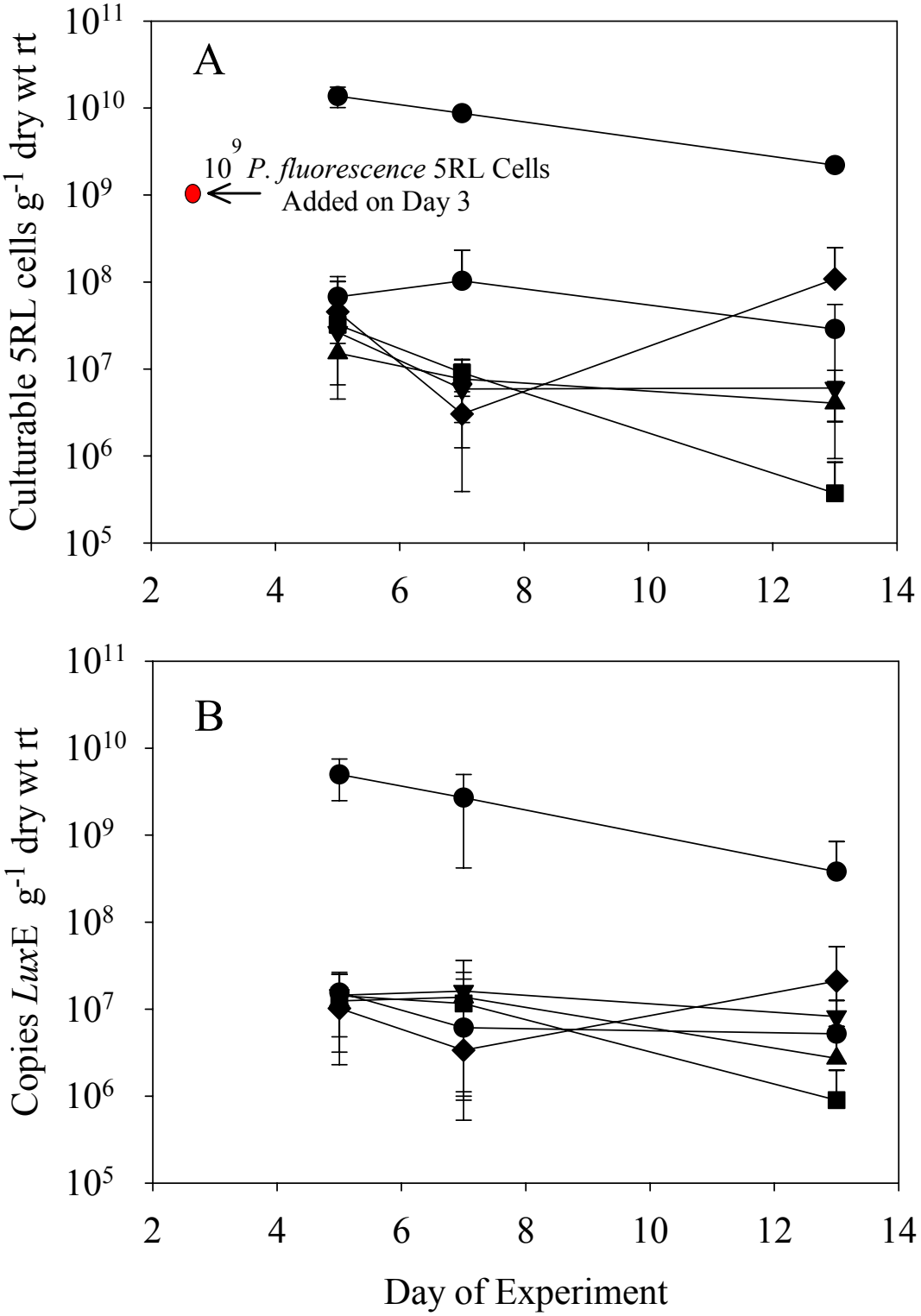
Figure 4. Surfactant degradation for each treatment. Surfactant degradation was normalized to the surfactant concentration as measured on day 3 of each experiment. Samples were taken on days 3, 5, 7 and 13. All values are reported as the average of six replicate experiments. Bars represent standard deviation. Constructed community ( ), CG1 ( ), CG2 ( ), CG3 ( ), CG4 ( ), and Propitious community ( ).


Surfactant Degradation (Normalized)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
10
12
14
Day of Experiment

exposure to naphthalene or the inducing metabolite, salicylate (45). The *lux* cassette in 5RL was used as a marker to detect the presence of the invader in rhizosphere samples. Two different methods were used to detect the density of the invader; culturable cell counts and quantitative PCR analysis via real-time detection of product accumulation. 5RL was cultured on selective media containing salicylate and tetracycline. Although some background growth was observed, invader colonies were readily detected via light production. The detection limit on plates was 10^2 cells mL^{-1} or 10^3 cells g^{-1} dry weight root. No bioluminescent cells were observed in any treatment before invader was added. Upon inoculation with 5RL, the concentration of the invader was significantly higher in the rhizosphere of plants inoculated with the constructed community than in all other treatments over the course of the 13 days ($P < 0.001$; Fig. 5a). In contrast, the concentration of the invader in the rhizosphere of plants inoculated with CG1 (the most diverse treatment) was lower than all other treatments throughout the experiment, but was significantly lower than that of constructed, CG3 and CG4 treatments ($P < 0.05$) and less than CG2 at the $P = 0.06$ level on the final day of sampling.

When the concentration of invader was evaluated by real-time PCR, considerable background was observed in day 3 samples (taken before invader was added) from the invaded experiments. Background for all samples averaged 8×10^7 copies g^{-1} dry weight root, but also varied considerably with a standard deviation of 1×10^8 copies g^{-1} dry weight root. Background is presumed to be due to contamination of pipettors that may have occurred during extraction of samples inoculated with 5RL. This is assumed because no background was detected by real-time PCR in samples from uninvaded experiments (less than 3 cells per PCR reaction). Once pipettors were thoroughly cleaned

Figure 5. Invader cell concentration determined by culturable cell counts or real-time quantitative PCR. Invader culturable cell numbers (A) and Invader (*luxE* copies) concentration as determined by real-time PCR (B) for each treatment. Samples were taken on days 3, 5, 7 and 13. Culturable cells numbers are reported as the average of triplicate experiments. Real-time PCR values are the average of duplicate samples at two different concentrations. Bars represent standard deviation. Constructed community (#), CG1 (!), CG2 (%), CG3 (&), CG4 ("), and Propitious community (⊙).

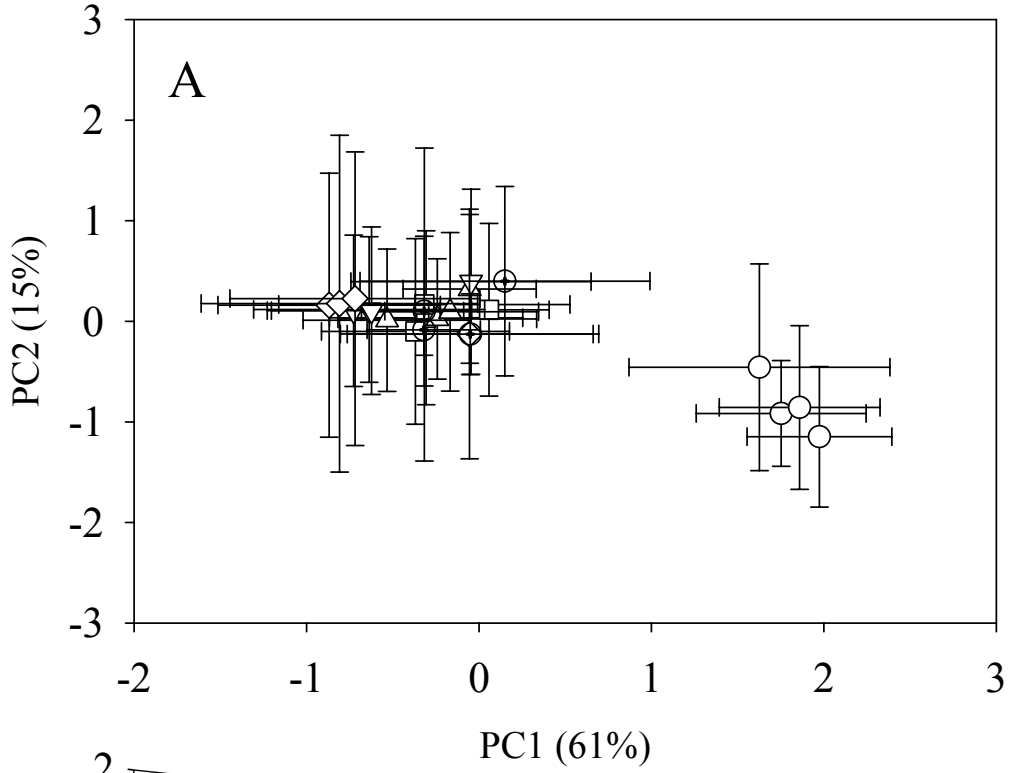

A
10^9 *P. fluorescence* 5RL Cells
Added on Day 3
Culturable 5RL cells g^{-1} dry wt rt
10^{11}
10^{10}
10^9
10^8
10^7
10^6
10^5
2
4
6
8
10
12
14
B
Copies *Lux*E g^{-1} dry wt rt
Day of Experiment

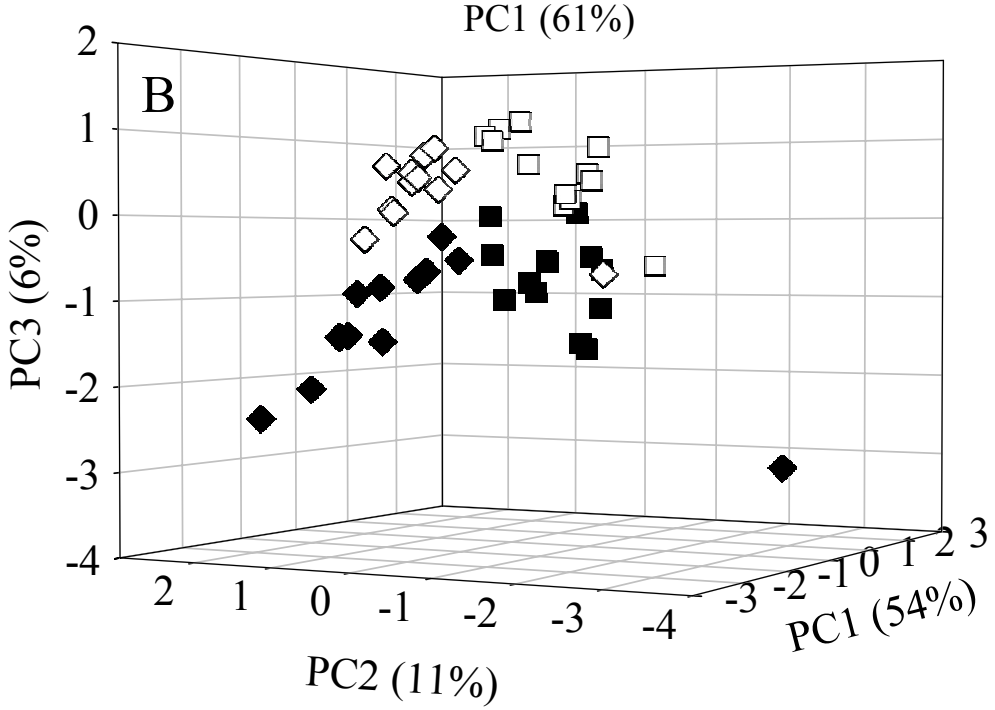
and only filter tips used for extractions, background was eliminated in control samples. Excluding the background encountered in day 3 samples, the trend for the density of 5RL in all treatments was the same as determined by cultural techniques (Fig. 5b). The concentration of 5RL in the rhizosphere of plants inoculated with the constructed community was significantly higher than all other treatments on Days 5 and 7 ($P < 0.005$). On day 13, the concentration of 5RL in the rhizosphere of plants inoculated with the constructed community, as determined by real-time PCR, was only significantly different than that of plants inoculated with CG1 ($P < 0.05$).

Community level physiological profiling (CLPP): Biolog

PCA of the CLPP data separated the constructed community from all other the other treatments (Fig. 6a). The first principal component (PC1), reflecting the primary effect in the data (61% of the variance), separated the constructed community from the other communities. ANOVA on the scores from the first three PCs showed that the separation on PC1 was highly significant ($P < 10^{-13}$). PC2 was not significantly different among any of the treatments ($P < 0.05$). PC3 (which explains 4% of the variation) separated treatments over a gradient, with CG1 and the constructed community forming one group, the constructed community and CG3 in a second group, CG2 and the propitious community in a third group and CG4 in a fourth group. PCA of Biolog data resulting from the complexity gradient treatments showed that PC1 was not significantly different for any of the communities. However, PC2 produces three homogenous subsets, one groups CG1 and CG2, a second distinguishes CG4 from the other treatments, and the third separates CG3 from the other treatments ($P < 0.001$). PC3 reflects differences among the three invaded and the three uninvaded experiments, however, this difference is

Figure 6. Principal component analysis of Biolog data. Data from all treatments combined (A); Constructed community ( )), CG1 ('), CG2 (+), CG3 (,), CG4 (( ), and Propitious community (<). Samples were taken on days 3, 5, 7 and 13. Points represent the average of six replicate experiments. Bars represent standard deviation. Principal component analysis of Biolog data from analysis of carbon source utilization among the complexity gradient communities; figures shows the highest and lowest complexity gradient treatments (B); CG1, not invaded ('), CG1, with invader added (!), CG4, not invaded (( ), CG4 with invader added ("). Samples were taken on days 3, 5, 7 and 13. Points represent the average of triplicate experiments. Bars represent standard deviation.


A
PC2 (15%)
PC1 (61%)
3
2
1
0
-1
-2
-3
-2
-1
0
1
2
3


B
PC3 (6%)
PC2 (11%)
PC1 (54%)
2
1
0
-1
-2
-3
-4

significant on day three suggesting that the effect is the result of differences among the two groups of experiments rather than the result of invasion. This separation is illustrated for the most complex treatment (CG1) and the least complex treatment (CG4) (Fig. 6b).

Community level physiological profiling: BD Oxygen biosensor (BD)

Data from CLPP profiles obtained using the BD oxygen biosensor system indicated that the predominant effect was the difference between the constructed community and the other treatments (Fig. 7). ANOVA on the first three PCs suggested that the affect detected on PC1 is highly significant ($P < 10^{-13}$). In this case, the first PC also separates CG1 from the other treatments ($P < 0.005$). Correlation of the PCs to specific loading factors (i.e., carbon sources) indicated that the complexity gradient communities (particularly CG1) more strongly utilized carbohydrates (specifically, arabinose, xylose and rhamnose). In contrast, the constructed community pattern was negatively correlated with carbohydrate utilization and more positively correlated with utilization of amino acids (specifically, glutamic acid) and proprionic acid (an organic acid). Data from the complexity gradient treatments indicated that PC1 separated CG1 from all other treatments ($P < 0.05$) and CG4 from all other treatments ($P < 0.01$; Fig. 8). In this case, PC3 separated treatments based on invasion. Differences between PCA plots visualized with invasion (Fig. 8a) and without invasion (Fig. 8b) indicate that, although not significant on PC1 and PC2 ($P < 0.05$), invasion resulted in a shift in the pattern of carbon source utilization. Interestingly, the effect of invasion on PC3 was correlated with glycine utilization. The most diverse treatment and the least invasible (CG1) had no change in glycine utilization (negatively correlated with PC3), while the other complexity gradient treatments (CG2, CG3 and CG4) had strongly positive increases in glycine

Figure 7. Principal component analysis of BD Oxygen Biosensor data for all treatments; Constructed community ( )), CG1 ('), CG2 (+), CG3 (,), CG4 (( ), and Propitious community (<). Samples were taken on days 3, 5, 7 and 13. Points represent the average of six experiments. Bars represent standard deviation.

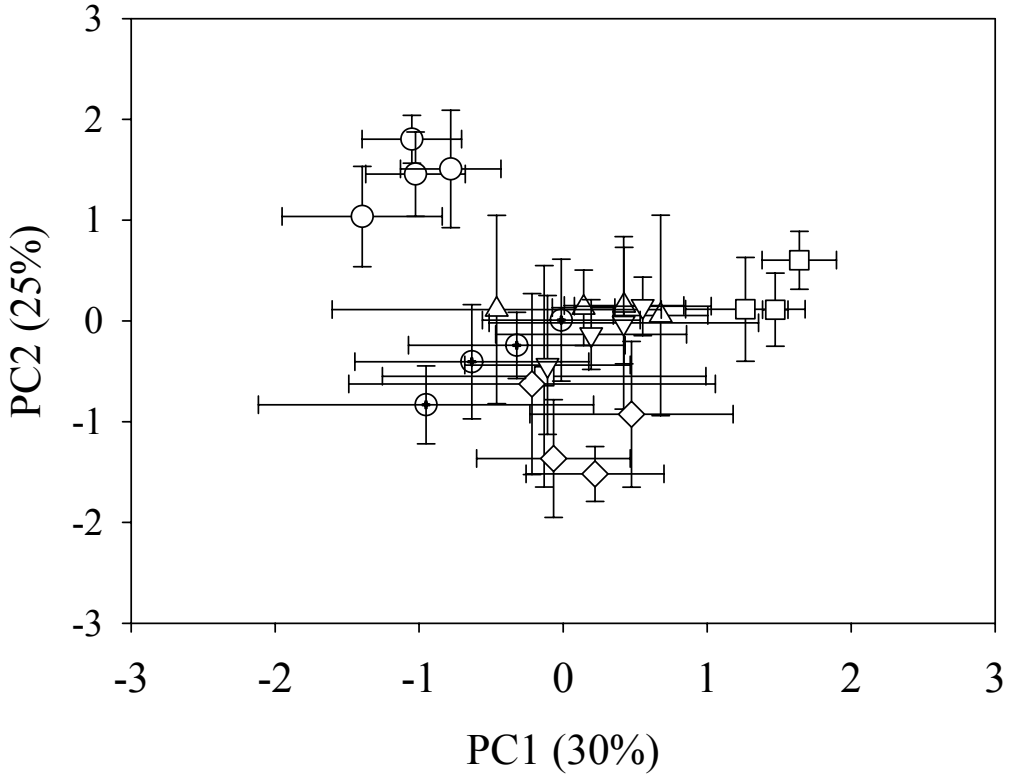
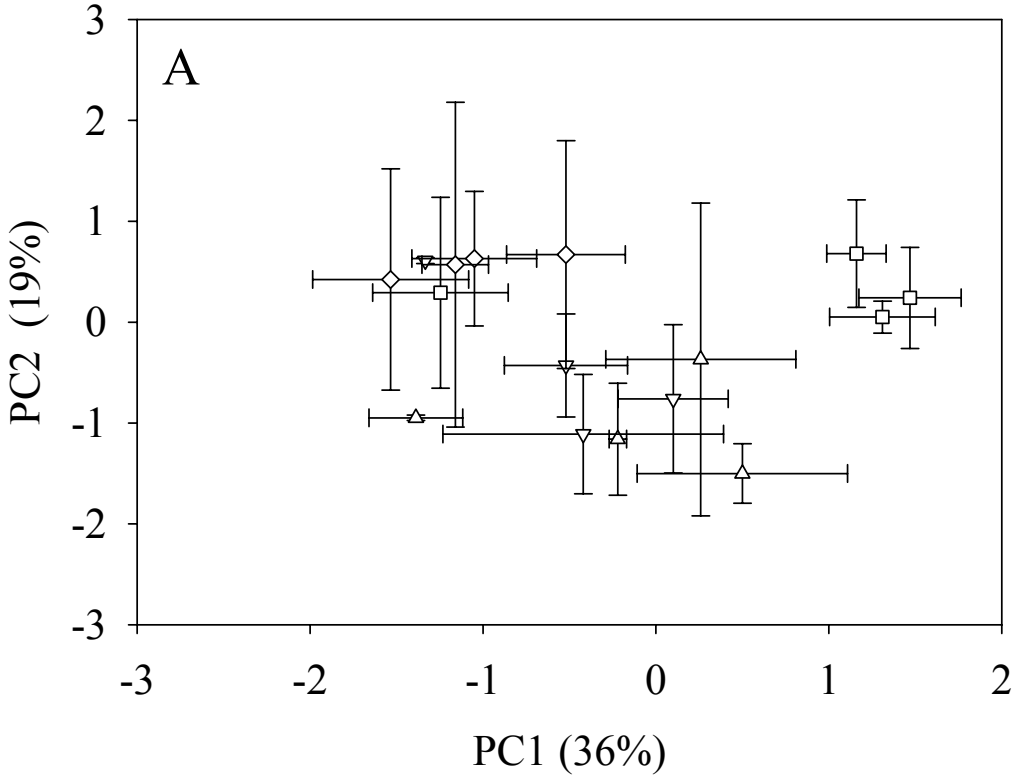
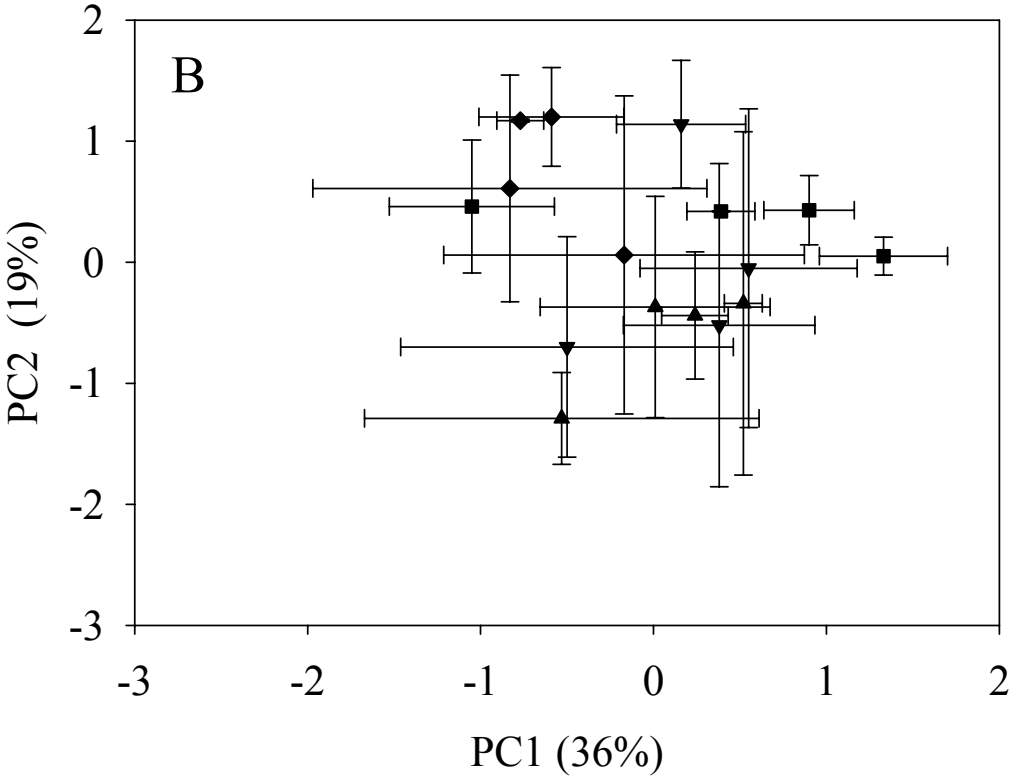

3
2
1
0
-1
-2
-3
PC2 (25%)
-3
-2
-1
0
1
2
3
PC1 (30%)

Figure 8. Principal component analysis of BD Oxygen Biosensor data for the complexity gradient treatments. Samples plotted without invader (A); CG1 ('), CG2 (+), CG3 (,), and CG4 (( ); or with invader (B); CG1 (!), CG2 (%), CG3 (&), CG4 ("). Samples were taken on days 3, 5, 7 and 13. Points represent the average of triplicate experiments. Bars represent standard deviation.


A
3
2
1
0
-1
-2
-3
PC2 (19%)
-3
-2
-1
0
1
2
PC1 (36%)


B
2
1
0
-1
-2
-3
PC2 (19%)
-3
-2
-1
0
1
2
PC1 (36%)

utilization with invasion. This effect was not apparent when the propitious and constructed community samples were included in the analysis.

Community analysis using 16S-23S rDNA ISR array

Overall analysis. Hybridization of labeled rhizosphere samples to ISR arrays was very reproducible among triplicate arrays on each slide (Fig. 9) and hybridization produced strong, distinctive hybridization patterns that corresponded to the overall diversity of organisms present in the respective treatments (Fig. 9 and Fig. 10). Normalization of spot intensity from the sample to the intensity of the reference sequence (kanamycin) present on every spotted plasmid on the array minimized slide to slide variability due to differences in the amount of arrayed probe material and some differences in hybridization. Additionally, this normalization procedure reduced within slide variability (for replicate spots) due to differential hybridization occurring over the surface of the slide. Overall coefficient of variation (CV) between replicate spots on a single slide was fairly high (40%) with a high standard deviation (54%). However, spots with strong hybridization signals (hybridization ratio of 0.75 or greater), had very low CVs (5% ± 4%). Similarly, the CV for replicate slides was 40% ± 41% for all spots, but only 9% ± 6% for the strongest spots. These results indicate that variability was highest among spots with low overall fluorescence, but was very consistent among strongly positive responses.

Treatment differences. Univariate analysis of ISR array hybridization differences between treatments suggests that, CG1 (the most diverse sludge gradient community) was significantly different from all other samples ($P < 0.001$;Fig. 10). The two lowest diversity complexity gradient communities (CG3 and CG4) formed another

Figure 9. Reproducibility of hybridization for triplicate arrays on one slide. Hybridization of Cy5 labeled kanamycin gene to the array (A); the gene is present in the TA vector portion of every spot on the array. Figure shows three of four subarrays of the array and triplicate arrays on one slide. Hybridization of Cy3 labeled rhizosphere probe to the same array (B) showing three of four subarrays of the array and triplicate arrays.

(A)



(B)

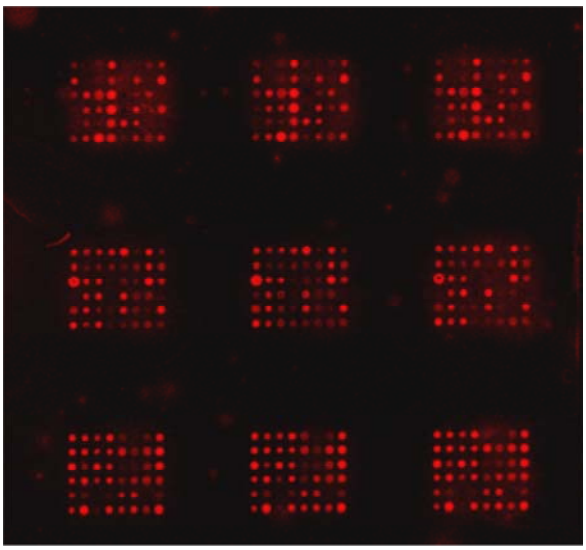
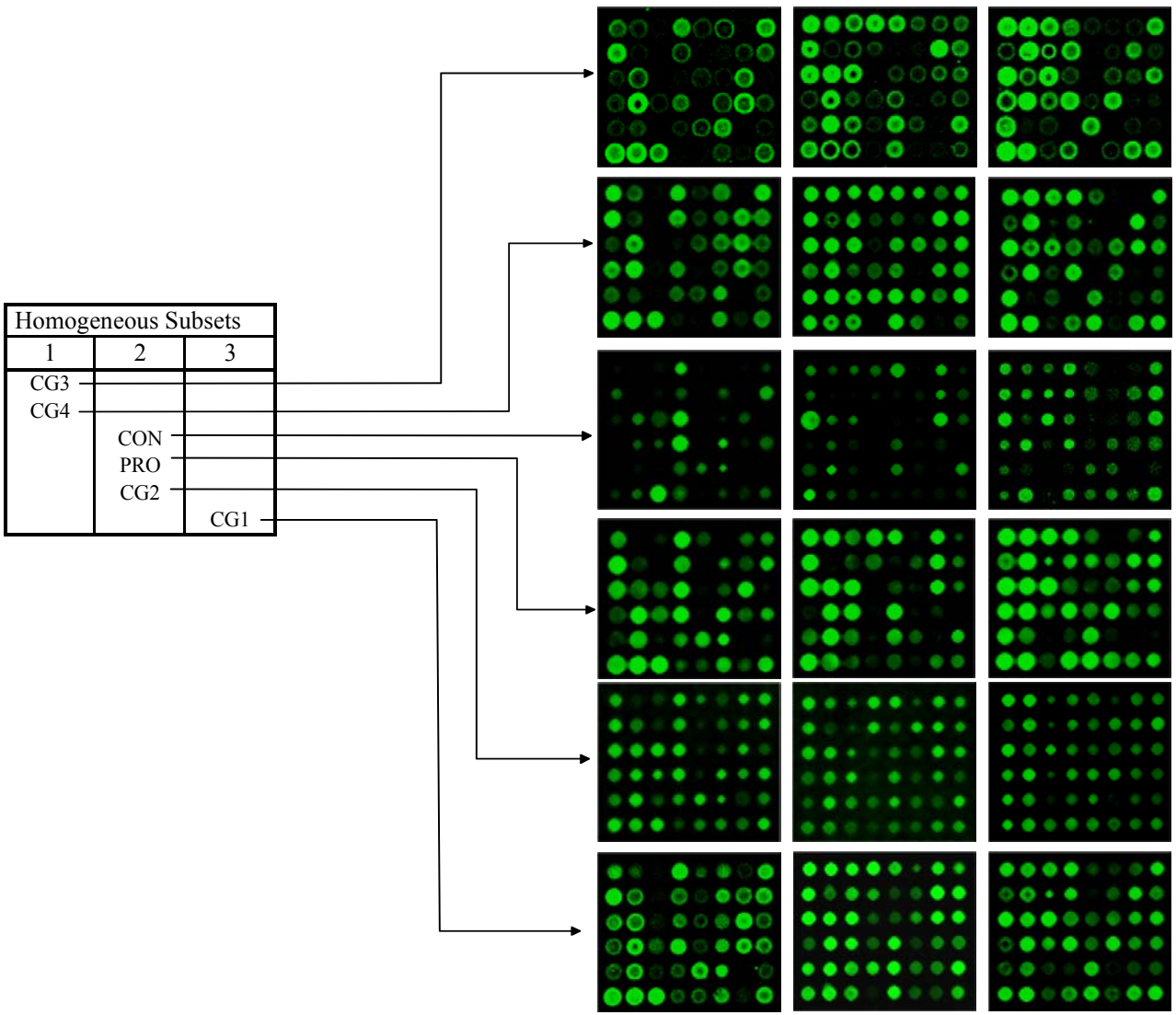


Figure 10. Separation of treatments based on hybridization to ISR array. Table shows homogeneous treatments (based on Univariate ANOVA). Arrows lead to arrays from matching treatment. Arrays shown represent 3 subarrays of one array and one of triplicate arrays on one slide from each treatment.


Homogeneous Subsets
1
2
3
CG3
CG4
CON
PRO
CG2
CG1

group and the propitious community, the constructed community and CG2 formed a third group. Both treatment and invasion were found to be significant, however, there were interactions between treatments, days, and invasion. Therefore, data were separated by invasion and day and evaluated by post hoc Tukeys tests for treatment effects (Fig. 11). The separation of CG1 from the other CG treatments (except on day 5 of uninvaded experiments) was highly significant ($P < 0.001$). The CG2 and CG3 treatments grouped with each other or fell out intermediate to each other, but separate from the CG1 treatment. The propitious community tended to be separate from the other treatments early (on day 5; $P < 0.01$), but by day 13 it grouped with the CG treatments or the constructed community. The constructed community always grouped with the lowest diversity complexity gradient community (CG4).

Within treatment effects of surfactant addition and invasion. The effect of surfactant addition was evaluated by ANOVA of individual treatments based on day. Only data from un-invaded samples was evaluated to avoid interaction effects of day and invasion. Samples were evaluated by differences between day 5 and day13 since surfactant degradation was only significantly different for the CG treatments on day 13, suggesting that the major shift in community structure must have occurred after day 5. Based on this analysis, day 5 was significantly different than day 13 for the propitious community, CG1, and CG2 ($P < 10^{-6}$). CG3, CG4 and the constructed community were not different at the $P = 0.01$ level. The effect of invasion was evaluated for each treatment separately using ANOVA. Results suggest that invasion had a significant effect on the community profile on days 5 and 13 of the constructed community and CG2, CG3, and CG4 ($P < 10^{-5}$). The effect of invasion was measured in the community profile for the

Figure 11. Homogenous treatment groups based on Univariate ANOVA. Different days (5 and 13) with and without invasion and with Tukey's post-hoc analysis of treatment differences.

Not Invaded

Day 5

Homogeneous Subsets			
1	2	3	4
CG3			
	CG1		
	CG4		
	CON		
		CG2	
			PRO

Day13

Homogeneous Subsets			
1	2	3	4
CG3			
PRO	PRO		
CG4	CG4	CG4	
	CG2	CG2	
		CON	
			CG1

Invaded

Day 5

Homogeneous Subsets			
1	2	3	4
PRO			
	CG2		
	CG3		
	CG4	CG4	
		CON	CON
			CG1

Day13

Homogeneous Subsets			
1	2	3	4
CG4			
CON			
	CG3		
	CG2	CG2	
		PRO	PRO
			CG1

propitious community only on day 5 ($P < 0.0001$). The effect of invasion was only significant on the community profile for CG1 on day 13 ($P < 10^{-26}$). Further analysis on a probe by probe (or organism by organism) basis for the constructed community suggests that two spots directly related to the constructed community were highly correlated to the effect of invasion. The spots corresponded to the predominant isolate from plated rhizosphere samples from the constructed community treatment and the corresponding spot of the pure culture of the isolate, *Pseudomonas putida* ($P < 0.007$ and $P < 0.01$, respectively).

Persistence of species. Qualitative analysis of presence/absence of specific organisms was used to evaluate the persistence of members of the constructed community 5 and 13 days post inoculation (Fig. 12). Two members of the constructed community, *Cytophaga hutchinsonii* and *Flexibacter* sp., both members of the *Cytophaga-Flavobacterium* (CF) cluster of the *Cytophaga-Flavobacterium-Bacteriodes* division, were not detected in any rhizosphere samples by the ISR array. Eight other members of the constructed community were detected consistently in both day 5 and day 13 samples (Fig. 12). *Pseudomonas putida*, a gamma proteobacterium, was present in day 5 and day 13 in samples that were not invaded and in day 5 samples that were invaded, but was not in day 13 samples that were invaded. This is in agreement with the statistical analysis of the data for this spot on the array. The mean number of positive responses was reduced in all treatments upon invasion, with the exception of a slight increase in the number of positive responses for the propitious community on day 13 following invasion (Fig. 13). This also agrees with the statistical analysis of the data that shows that the greatest effect of invasion was measured on day 13. These results suggest that most members of the

Figure 12. Distribution of eleven constructed community members in rhizosphere samples. Samples were on days 5 and 13 after inoculation and with and without invasion; Frequency distribution was for presence or absence of a positive response on the ISR array for triplicate samples measured from strong positive; Cytophaga-flavobacterium cluster = CF. Members of the constructed community are designated C1 to C11; *Rhizobium leguminosarium* (C1), *Ralstonia eutropha* (C2), *Burkholderia cepacia* (C3), *Aquaspirillum metamorphum* (C4), *Frateuria aerantia* (C5), *Paenibacillus amylolyticus* (C6), *Xanthomonas axonopodis* (C7), *Chryseobacterium joosteii* (C8), *Flexibacter* sp. (C9), *Pseudomonas putida* (C10), *Cytophaga hutchinsonii* (C11).

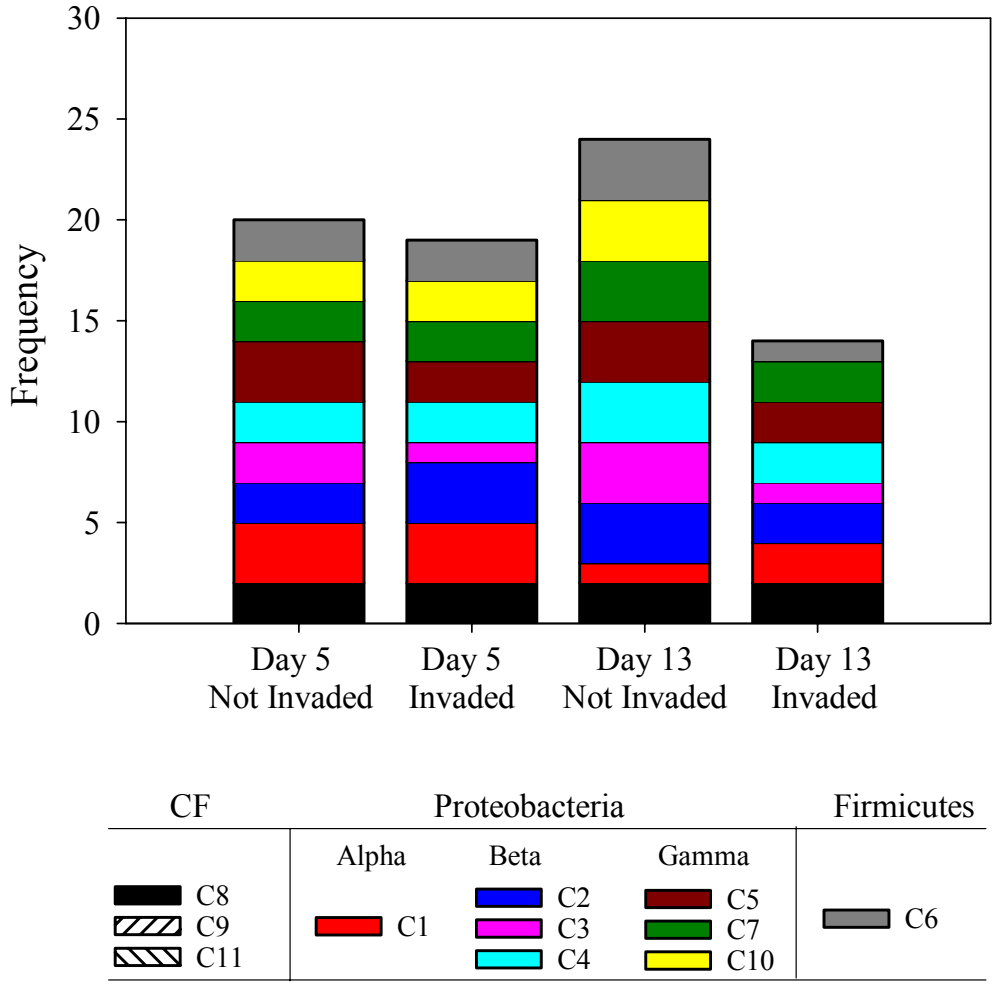
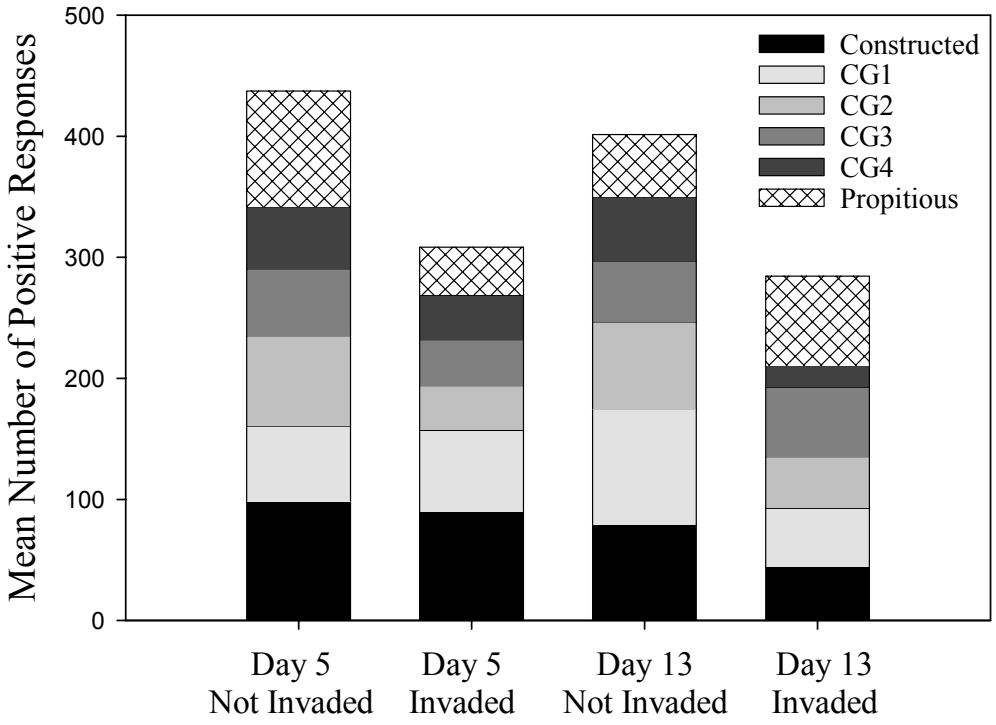

30
25
20
15
10
5
0
Frequency
Day 5
Not Invaded
Day 5
Invaded
Day 13
Not Invaded
Day 13
Invaded
CF
Proteobacteria
Firmicutes
Alpha
Beta
Gamma
C8
C9
C11
C1
C2
C3
C4
C5
C7
C10
C6

Figure 13. Mean number of positive responses for each treatment as determined by ISR array analysis. Responses were measured as the presence or absence of a positive response on triplicate ISR arrays on days 5 and 13 after inoculation and with and without invasion.


Mean Number of Positive Responses
500
400
300
200
100
0
Constructed
CG1
CG2
CG3
CG4
Propitious
Day 5
Not Invaded
Day 5
Invaded
Day 13
Not Invaded
Day 13
Invaded

constructed community remained in the rhizosphere for the duration of the experiments, but invasion did have a measurable affect on the community structure of the constructed community as well as each of the other treatments.

DISCUSSION

Diversity gradient

Six separate microbial communities, of different structural diversities, were inoculated into a model plant-based graywater waste processing system to evaluate the effect of changes in species richness on the ability of the community to establish in the rhizosphere, resist invasion and process surfactant. A dilution/extinction approach was used to re-grow microbial inocula from dilutions (10^{-1} to 10^{-7}) of the original industrial WWTP sludge. The four chosen communities (CG1 to CG4) had similar cell density, but differed in community structure as measured by CLPP analysis (Table 1). The propitious community was composed of contaminant species that colonized the root zone of sterilized seeds. A limited number of phylotypes were found in this treatment (*Enterobacter* sp., *Burkholderia* sp., and *Pseudomonas* sp.). Other researchers have found that these species are early rhizosphere colonizers with *Enterobacter* sp. often dominant in the endorhiza, suggesting that some portion of the contaminant species may have been present inside the seed cover and unaffected by seed sterilization (51). The constructed community was composed of isolates that were phylogenetically similar to sequences of clones from a 16S-23S rDNA clonal library produced from a community of rhizosphere organisms growing in the presence of SGW (14). The eleven isolates included in the constructed community were all related to organisms that are competitive root colonizers,

many were closely related to organisms that have plant growth promoting properties, and six of the isolates grew well on surfactant (14).

Total and culturable biomass

Evaluation of total and culturable cell density for each of the inocula suggested that the starting cell density was the same for all treatments (Fig. 2). However, within three days of inoculation, significantly higher cell density was detected in the propitious treatment than in the other treatments (Fig. 2). This difference remained significant until the final day of sampling. This increase in microbial biomass resulting from low overall diversity is not uncommon and is postulated to reflect the release of the population from interspecific competition (18, 23, 37). Furthermore, the fact that this community grew, in a relatively short time frame to a high cell biomass suggests that organisms re-grown in this treatment were representative of early colonizers. Ecological theory predicts that early colonizers will be dominated by fast growing, generalist species with a broad-niche width and high investment of energy in reproduction (50). This is supported by phylogenetic data that suggests that the community was dominated by species that are early rhizosphere colonizers (51). Additionally, BD CLPP peak height data suggests that although the cell density was considerably higher in this treatment, the extent of response (peak height) was lower in the propitious treatment than in the other treatments (data not shown). Since BD oxygen biosensor plates have a fluorescence response proportionate to the reduction in DO, reduced peak height, but similar pattern and rate of carbon source utilization, suggests that more of the carbon used by this community was assimilated than respired. The total and culturable cell densities were similar among the other treatments

(constructed, CG1, CG2, CG3 and CG4) and changed little (per gram dry weight of root) over the time course of the experiments (Fig. 2).

Surfactant degradation

Surfactant was added to simulate graywater because surfactant represents the major source of carbon input into the system from graywater addition. Garland et al. (30) found that carbon input by surfactant addition into a plant-based hydroponic graywater waste processing system over the course of a 70 day wheat study was approximately equal to that extruded by the plants assuming 5% net primary productivity (NPP) was lost from the plant as exudate. In this study, the amount of carbon input by surfactant addition (3.5 mg to 5.5 mg) was double the amount of plant exuded carbon assuming 5% NPP (1.5 mg to 3.5 mg). Surfactant addition had a negative impact on the root dry weight of plants inoculated with organisms that did not completely degrade the surfactant (Fig. 3). This may be due to the fact that surfactants are surface-active agents that increase the permeability of the plant cell wall causing root leakage. Therefore, in treatments with low degradation of surfactant (CG2, CG4 and propitious treatments especially) an increase in total biomass may have resulted from increased carbon exudation. However, no increase in biomass was detected suggesting that either the root leakage was not pronounced, that the surfactant impacted microbial biomass as well as plant biomass, or that the additional carbon was respired rather than assimilated into biomass.

Surfactant was not degraded completely in the rhizosphere of plants inoculated with the CG treatments (Fig. 4), suggesting that the sludge community was not enriched for surfactant degraders despite addition of 15 ppm surfactant to the rhizosphere of plants used to re-grow each CG community in the dilution/extinction experiment. Only the

community re-grown from the lowest dilution (CG1) degraded surfactant to a significant degree and degradation was detected only on the final day of sampling. Therefore, enrichment either failed to select for surfactant degraders or inoculation of the communities into new rhizosphere systems caused a shift in community structure that required the population of surfactant degraders to be selected again following inoculation into a new plant-based graywater waste processing system. Stoffels et al. (59) grew an enrichment culture from an industrial wastewater community on Solvesso100 (a pollutant resulting from crude oil reforming and other industrial processes and containing a mixture of aromatic compounds with limited water solubility) for 48 days. When the enrichment culture was used to initiate a new bioreactor for processing the same pollutant, the dominant population from the enrichment was different than the dominant bacteria found in the final reactor. In fact, the enrichment culture was dominated by gamma proteobacteria while the final community was dominated by beta and alpha subclass proteobacteria (59). In this study, enrichment of the complexity gradient communities on surfactant prior to inoculation into new rhizosphere systems may have had a similar effect on the community structure. Array analysis also indicated a significant shift in community structure from day 5 to day 13 in the CG1 treatment. Preliminary analysis of structural data from the array analysis of the CG1 treatment suggests that this shift was correlated to an increase in overall diversity from a community dominated by CF division species to a more evenly distributed community consisting of CF division organisms, and alpha, beta and gamma proteobacteria (data not shown).

Surfactant was degraded more completely in the rhizosphere of plants inoculated with the constructed community than in the rhizosphere of the other plants (Fig. 4). Six different isolates included in the constructed community were capable of growing on the surfactant (14). Based on array analysis, five of the six organisms known to degrade the surfactant were detected over the course of the 13-day experiments. Furthermore, no significant shift in the constructed community profile was detected by array analysis. These results agree with other researchers who suggest that assembly of artificial communities of organisms predisposed to degradation of targeted compounds for bioremediation (19, 40, 58) and biocontrol (11, 56, 64) in rhizosphere systems is often successful when competition from indigenous organisms is low.

Resistance to invasion

Pseudomonas fluorescens 5RL was chosen as an invader because: (1) this strain survives in the rhizosphere (17), (2) *Pseudomonas fluorescens* strains have plant growth promoting properties (64), (3) the presence of the *lux* cassette allows monitoring of the organism in the rhizosphere through measurement of light production on plates and through detection of the *luxE* using real-time PCR (63) and (4) the *lux* cassette is inducible suggesting that its presence will not produce an additional metabolic drain on the cell while it was in the rhizosphere (45). Resistance to invasion was used as a model for ecosystem stability because resistance to invasion is a fundamental measure of community intransience (57). However, resistance to invasion (i.e., competition among species) is of special importance in environmental systems where inoculants have limited success in competition with indigenous flora (65). The commercial application of inoculants to control pathogens or for bioremediation has been limited by the lack of

consistency of results in field application. Inoculants often survive and grow in sterilized soils, but decline in competition with the indigenous rhizosphere flora (4, 53). The high invasibility of the constructed community is illustrative of this issue. The constructed community was phenotypically diverse on the same order as the CG treatments, and was able to degrade surfactant to a greater extent than any of the other treatments. However, the community was invaded to a greater extent than any of the other communities, the invader persisted in the community, and even displaced one member of the community (*Pseudomonas putida*).

Although the constructed community was invaded to the greatest extent, other significant differences in invasibility were detected (Fig. 5). There was a gradient in terms of invasibility with the constructed community being most susceptible, followed by the lowest diversity CG treatment (CG4) and the propitious community. The invader quickly died off in the highest diversity CG treatment (CG1), supporting the theory that resistance to invasion is a metapopulation response in which communities with narrow resource utilization and strong interactions among members form a barrier to invasion (10, 57, 61). The organism in the constructed community most closely related to the invader (*P. putida*) was displaced upon invasion, as measured by the array analysis suggesting that interspecific competition also played a role in invasion success in the constructed community (Fig. 12).

CLPP analysis

Biolog CLPP and BD CLPP were used to evaluate community structure among treatments based on patterns of carbon source utilization. Both CLPP methods separated the constructed community from all of the other communities (Fig. 6 and Fig. 7). This

affect was stronger than the effect of invasion, time or surfactant addition. Interestingly, the communities showed no difference in the number or rate of carbon sources utilized. Plots of the positive responses in Biolog plates after 48 h incubations showed that all treatments utilized approximately the same number of carbon sources (data not shown). Additionally, the time to peak measure for BD response, used by Garland et al. (34), was not effective for discriminating of these communities, because all of the communities had similar rates of response, but differed in the extent and type of carbon sources used. Therefore, the difference between the constructed community and the other communities was not correlated to a diminished phenotypic response, but instead reflected differences in the type of carbon sources utilized. This difference in carbon utilization strategy between the constructed community and the other communities may reflect a physiological difference resulting from the constructed communities greater ability to degrade surfactant or it may reflect a fundamental difference between the culturable organisms in the constructed community and the mixture of organisms in the other treatments.

The BD CLPP method also separated the highest diversity complexity gradient treatment (CG1) on the first PC (Fig. 7). Furthermore, analysis of data from only the complexity gradient treatments, permitted separation of differences among treatments along a gradient with CG1 in one group, CG2 and CG3 in a second and CG4 separated in a third group. Biolog CLPP did not discriminate between treatments on the first PC, but separated CG1 and CG2 into one homogeneous subset, CG3 in a second and CG4 into a fourth on PC2 (Fig. 6B). The effect of invasion was reflected in the loading factors for the third PC using BD CLPP analysis and, although not significant on PC1 and PC2,

differences among invaded and uninvaded samples were evident (Fig. 8). In this case, BD CLPP provided better resolution for separation of differences among treatments than did the Biolog CLPP method. Lower substrate concentrations, shorter incubation periods and the use of ecologically relevant substrates that are freshly made and inoculated one the same day as the sample may make the BD CLPP method more reflective of *in situ* community difference.

ISR array analysis

Community structure was evaluated using a 16S-23S rDNA ISR array consisting of 16S-23S rDNA ISR sequences of cloned organisms from the industrial WWTP sludge, consortium members, propitious community clones and from selected rhizosphere isolates. The specificity of the ISR array was previously demonstrated for analysis of industrial WWTP clonal libraries (15). In this study, the ISR array was used in a community fingerprinting capacity to characterize similarities and differences among treatments. Subsequent analysis provided specific phylogenetic information about shifts occurring in response to surfactant addition and invasion by 5RL (Fig. 12 and Fig. 13). Therefore, a genetic measure of differences in community structure and information about specific organisms responsible for those differences were obtained.

The major difference among treatments was the separation of CG1 from all other treatments (Fig. 10). CG1 was distinct from the CG2 and CG3 treatments regardless of days after inoculation and the effect of invasion (Fig. 11). Franklin et al. (23) used numerical simulations and batch bioreactor cultures to show that in unevenly distributed communities with high dominance, the first dilution along a gradient results in the removal of a large number of types. The remaining population would be more evenly

distributed and differences among subsequent dilutions would be small until the dilution factor exceeds the original number of types of organisms in the community (23). In this study, separation of the highest diversity treatment (CG1) from the other CG treatments by ISR array analysis and BD CLPP analysis suggests that the initial sludge community was unevenly distributed with some highly dominant species. The CG1 community was able to degrade diverse carbon sources including surfactant and was able to resist invasion from an outside species. The effect of subsequent dilutions (CG2 and CG3) resulted in no consistent differences, however, the lowest diversity CG treatment (CG4) was different based on BD CLPP analysis, was invaded to a greater extent than the other CG treatments, and lacked the ability to degrade surfactant. These results suggest that this community was significantly different from the other communities both genotypically and phenotypically.

The community structure of the constructed community shifted over time and in response to invasion. This response was detected, among members of the constructed community, and nonspecifically via changes in hybridization patterns over the remainder of spotted 16S-23S rDNA ISR probes on the array. Preliminary analysis suggested that these differences were in hybridization intensity among alpha and beta proteobacteria that may be closely related to members of the constructed community (data not shown). However, another significant difference was in spots correlating to *Enterobacter* sp. from the propitious treatments. Hybridization to this spot was eliminated in day 13 samples from invaded treatments. Although it is not known if these organisms were part of the constructed community, it seems likely that if they were contaminant species present

within the seed coat that they may have been incorporated into the community initially and were eliminated upon invasion.

Evaluation of the presence or absence of members of the constructed community indicated that nine of the eleven members of the constructed community were established and persisted over the course of the 13-day experiments (Fig. 12). Two CF group isolates were not detected in any of the samples from the constructed community or in any of the other treatments (i.e., less than 2 of 3 experiments showed a positive response). It is difficult to determine if these organisms were unable to become established in the rhizosphere or if ISR array hybridization conditions were not conducive to detection of these organisms. CF group isolates are not commonly used as rhizosphere inocula although these organisms are commonly identified in rhizosphere systems, so it is difficult to determine if inoculation is an effective means of introducing these organisms into a plant growth system (12, 43). The industrial WWTP library had a large number of CF group clones; therefore it is possible that non-specific hybridization resulted in reduced response on the spots correlating to the constructed community CF isolates. This is unlikely for two reasons (1) hybridization to the ISR array was very specific, especially for unrelated CF group organisms (15) and (2) hybridization to the spot on the array corresponding to the third CF member of the constructed community, *Chryseobacterium joosteii,* was strong and consistently positive.

CONCLUSION

Differences in species diversity corresponded to differences in phenotypic potential (surfactant degradation and CLPP) and differences in the level of resistance to

invasion. Communities in the complexity gradient all had similar patterns of carbon source utilization as determined by BIOLOG and BD profiles. However, only the highest diversity complexity gradient community (CG1) responded to the addition of surfactant. These results support the theory that when species diversity is high in a community, changes in microbial diversity will not necessarily produce concomitant changes in community function (18, 22, 37). The loss of structural diversity is instead reflected in the loss of more specific functional parameters (i.e., surfactant degradation in this case) (37). Similarly, array analysis suggested that invasion had a significant effect on community structure in each of the treatments, but lack of measurable phenotypic response via CLPP suggested that shifts in population structure at the individual level might have more of an effect than at the population level (3). This also suggests that perturbations such as the invasion event may destabilize individual species, but diversity stabilizes the population as a whole (22, 62). Invasion of the constructed community and replacement of one community member suggests that this community lacked the structural diversity necessary for the community to respond to perturbations.

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PART V

SUMMARY

Microorganisms are the fundamental transformers of energy and microbial degradation is as fundamental as primary production for the long-term functioning of an ecosystem (28). Microorganisms break down organic matter and play an important role in biogeochemical cycling. Microorganisms mediate many important agricultural processes including nutrient recycling, degradation of toxic compounds, and control of plant pathogens (4, 5, 15, 19). Microbial consortia are used in industrial processes and to degrade environmental contaminants (13, 14, 24). The use of beneficial microorganisms to suppress diseases caused by plant pathogens would reduce the need for and accumulation of environmentally damaging pesticides (2). NASA is considering using microbial inocula for initiation of systems used for food production and waste processing on long-term and deep space missions. However, the commercial application of microbial inoculants has been limited by a lack of consistency of results in field application. To achieve practical ecological and biotechnological benefits from microbial processes, it will be necessary to assure reproducible, reliable and resilient functioning of inoculated systems.

Elucidating the relationship between structural diversity, functional diversity and ecosystem stability has been a great challenge in ecological studies. Ecological theories suggest simultaneously that diversity increases (17, 27), decreases (21), or is unrelated (1, 10) to ecosystem function. Microbial systems serve as good models for evaluation of diversity-stability hypotheses. Microbial communities are highly versatile and dynamic, microorganisms can be easily propagated, and have short generation times and large population sizes (22). Controlled environmental systems are a convenient tool for investigating microbial ecosystems (25). Model systems can be used to address specific

ecological questions in a controlled environment with fewer confounding factors. Therefore, this research focused on the use of a model plant-based graywater waste processing system to evaluate the effect of microbial diversity on community phenotype and community invasibility. The principal hypothesis of this research was that a constructed community of rhizosphere organisms, constructed using parameters described in this research, will be as effective as an undefined industrial activated sludge community in terms of its ability to: establish in the rhizosphere, persist over time, resist invasion, and degrade organic compounds.

The constructed or defined community of organisms was compared to undefined microbial inocula. The eleven isolates included in the constructed community were similar to organisms that are competitive root colonizers and to organisms that have plant growth promoting properties. However, most of the isolates in the constructed community were not complete matches to clones from the graywater rhizosphere clonal library. It is not uncommon in environmental systems to find disparity between the cultured and the uncultured portion of the population (7, 12). Results suggest that it may not be as important to find isolates that are identical to organisms in the rhizosphere as to obtain representative organisms which fill functional niches in the system and compete effectively with invader species (3). For example, Blumenroth et al. (3), in a comparison of the survival of indigenous and nonindigenous inoculants in sediment, found that indigenous isolates did not perform better than nonindigenous strains. Other research suggests that strong rhizosphere colonizers (especially *Bacillus*, *Pseudomonas*, and *Xanthomonas*) establish well in roots over diverse environments and irrespective of the strain origin (18). Therefore, isolates were included in the constructed community based

on their relationship to organisms that are effective rhizosphere colonizers, which compete effectively with potential human or plant pathogens, and based on their ability to degrade surfactant.

The mixed inocula approach was used based on results that suggest that inoculant mixtures (rather than single species) added to the rhizosphere for purposes of biocontrol are the most successful in terms of long-term survival of inoculants and overall ability to effect changes in rhizosphere structure and plant growth (6, 23). The use of complex mixtures of organisms is also supported by general ecological theory that suggests that more diverse populations are also more stable (16, 20). Therefore, the most prudent means of achieving the ultimate goals of reproducible, reliable and resilient functioning of inoculated systems may be to approximate the structure of indigenious populations.

In this research, differences in species diversity corresponded to differences in phenotype and differences in the level of resistance to invasion. Communities in the complexity gradient were phenotypically diverse. However, only the highest diversity complexity gradient community was able to degrade surfactant. These results support the theory that when community richness is high, changes in microbial diversity will not necessarily produce concomitant changes in community function (8, 10, 11). The loss of structural diversity is instead reflected in the loss of more specific functional parameters (i.e., surfactant degradation in this case) (11). Similarly, array analysis suggested that invasion had a significant effect on community structure in each of the treatments, but lack of measurable phenotypic response via CLPP suggested that shifts in population structure at the individual level may have more of an effect than at the population level

(1). This also suggests that perturbations such as the invasion event may destabilize individual species, but diversity stabilizes the population as a whole (10, 26).

The constructed community effectively degraded surfactant and invasion did not affect the phenotypic potential of the community or the ability of the community to degrade surfactant. However, at the community level, susceptibility of the constructed community to invasion and loss of a community member suggests that this community lacked the structural diversity to respond to extreme events in a resilient fashion. Pimm (21) suggests that the difficulty with which a community is invaded is a measure of the community's stability (persistence). When more species are present and interactions are stronger among species, the community is harder to invade (21). Based on this assessment, the constructed community was not persistent and community interactions may have been weak in comparison to the other inocula, including the propitious community, which were all invaded to a lesser extent than the constructed community. The susceptibility of the constructed community to invasion has two potentially negative implications for application to closed systems (1) loss of ecosystem function through removal or replacement of key process species and (2) susceptibility to invasion by potential human or plant associated pathogenic organisms.

Key process species are theorized to produce an overall functional and structural framework for a complex self-organizing community of organisms (10). In the presence of intense competition, key species buffer the effects of environmental perturbation, an essential trait in environmental systems. However, distinct environmental conditions correspond to unique community assemblages that may be correlated to a few key processes and resultant, distinctive key process species. Community history or assembly

mechanics may also influence the trajectory of the community and resultant community properties (9). Consequently, the ultimate community may have properties that are unpredictable regardless of the simplicity or complexity of the community (9, 10). The most prudent means of achieving the ultimate goals of reproducible, reliable, and resilient functioning from inoculated systems may be to use structurally and functionally diverse inocula (i.e., undefined communities) that will be resilient in the face of rare and extreme events that might otherwise compromise ecosystem function.

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VITA

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