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Evaluating Diazotrophy, Diversity, and Endophytic Colonization Ability of Bacteria Isolated from Surface-Sterilized Rice

By

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ABSTRACT

EVALUATING DIAZOTROPHY, DIVERSITY, AND ENDOPHYTIC COLONIZATION ABILITY OF BACTERIA ISOLATED FROM SURFACE-STERILIZED RICE

By

Jon R. Stoltzfus

Information about the nitrogen fixing potential, diversity and sites of colonization of endophytic bacteria from rice are needed to expand our understanding of a new area of microbial ecology and plant microbe interactions and lay the foundations for future studies aimed at using biologically fixed nitrogen to replace nitrogen fertilizers. A collection of 142 bacteria isolated from mechanically-abraded, surface-sterilized rice roots was studied. Polymerase Chain Reaction (PCR) mediated gene amplification using degenerate primers derived from highly conserved regions of the nitrogenase *nifD* gene revealed 20 isolates harboring *nifD* gene sequences. Southern hybridization analysis confirmed the presence of *nif* genes in 19 of these isolates. The diazotrophic nature of these 19 isolates was confirmed using Acetylene Reduction Assays (ARA). Examination of genetic diversity using amplified ribosomal DNA restriction analysis (ARDRA) fingerprints and rep-PCR genomic fingerprints with

Gelcompar software revealed 56 unique ARDRA fingerprints and 71 unique rep-PCR genomic fingerprints. Clusters of similar combined fingerprints, consisting of 37, 15, 12, and 9 non-diazotrophic bacteria, as well as two clusters each containing 4 diazotrophic bacteria, were found. Analysis of partial Small Subunit (SSU) ribosomal RNA (rRNA) gene sequences revealed the presence of isolates with similarity to strains from the alpha-, beta-, and gamma subdivisions of the Proteobacteria, and to members of the Bacillaceae and Microbacteriaceae. Many of the ARDRA fingerprints and/or SSU rRNA gene sequences of these bacteria were highly similar to those of other bacteria previously isolated from the rhizosphere of rice. Two isolates from the collection and *Sinorhizobium meliloti*, a control, were tagged with the biomarker *gus* or *gfp*, and the colonization of rice tissue was examined. *In situ* visualization of colonization of three week old inoculated rice seedlings revealed no endophytic colonization of rice tissue by these bacteria. However, clumps of bacteria, as well as individual cells, could be visualized on the surface of the roots. On very rare occasions an isolated epidermal cell filled with bacteria was observed.

DEDICATION

To all the bacteria and trees that gave their lives to produce this thesis.

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CHAPT

INTRO

CHAPTER 1

INTRODUCTION

Use of biologically-fixed nitrogen to replace chemical nitrogen fertilizers is an important goal in agriculture since current cropping systems require large inputs of fertilizer nitrogen and therefore are not sustainable. The recent isolation of endophytic nitrogen-fixing bacteria from Brazilian sugar cane varieties capable of high sustainable yields without input of nitrogen fertilizer has suggested that endophytic diazotrophic bacteria may be an efficient means for supplying graminaceous crops with biologically-fixed nitrogen (Ladha et al., 1997; James and Olivares, 1998). However, it is not clear to what extent endophytic bacteria colonize crop plants such as rice. This work presents data derived from studies aimed at increasing our understanding of the diversity of bacteria that colonize rice, with special emphasis on diazotrophs. The information presented not only expands our basic understanding of microbial ecology and plant microbe interactions, but is also fundamental for further studies designed to use endophytic bacteria to supply rice and other crops with biologically-fixed nitrogen.

IMPORTANCE OF NITROGEN IN RICE PRODUCTION

Increased rice (*Oryza sativa* L.) production is clearly essential to meet rising global food demands (Hossain and Fischer, 1995; Peoples et al., 1995). To meet this demand in a sustainable manner the amount of industrially produced fertilizer nitrogen used needs to be reduced (Bohlool et al., 1992; Ladha et al., 1997). One method of achieving this is the use of diazotrophic bacteria which would make biologically-fixed nitrogen available to rice for plant growth.

DIAZOTROPHIC BACTERIA ASSOCIATED WITH RICE

A considerable amount of information is available about the nature of diazotrophic bacteria found closely associated with rice. In the early 1970s, acetylene-reduction assays (ARA) were used to demonstrate the occurrence and activity of nitrogen-fixing organisms in flooded rice fields (Yoshida and Ancajas, 1971; 1973a; 1973b; Dommergues et al., 1973). Many of these early studies lacked proper controls, or were performed using imprecise techniques, causing considerable disagreement about the actual amount of nitrogen fixation taking place (Hirota et al., 1978). Nevertheless, the advantages of cereal crops that are able to fix their own nitrogen have been obvious to many scientists (Postgate, 1974). In efforts to achieve this goal, extensive attempts to isolate the bacteria

responsible for this nitrogen fixation have revealed the presence of a broad diversity of diazotrophs inhabiting the rhizosphere, most of which remain poorly studied or uncharacterized.

The first diazotrophic bacteria isolated from the rhizosphere in the mid 1970s was called *"Spirillum"* and was found to be common in the rhizosphere of grasses, including rice (Day and Döbereiner, 1976; Döbereiner et al., 1976; Lakshmi Kumari et al., 1976). The ability of this bacterium to supply nitrogen to plants has been of considerable interest. Renamed *Azospirillum,* the association between this bacterium and plants has been studied intensively (Fendrik et al., 1995; Vande Broek and Vanderleyden, 1995).

Continued isolation revealed additional diazotrophic bacteria associated with rice plants. Watanabe et al. (1979) examined different media for isolation of nitrogen-fixing bacteria from rice and found numerous distinct nitrogen-fixing Gram-negative rod-shaped bacteria, suggesting that *Azospirillum* might not be the most common diazotroph associated with rice. They studied the percentage of diazotrophic bacteria isolated from different plant tissues and suggested the lower stem as a possible site for

nitrogen fixation (Watanabe and Barraquio, 1979). In their study, 81% of bacterial isolates from roots that had been shaken with glass beads to remove exogenous bacteria were diazotrophic. Isolation experiments using a semi-solid medium made with exudates from germinating rice seedling as the carbon source revealed members of the family Enterobacteriaceae as the most common nitrogen-fixing rhizosphere bacteria, followed by *Azospirillum,* and *Pseudomonas* spp. (Bally et al., 1983). Nitrogen-fixing *Pseudomonas diazotrophicus*, *Enterobacter cloacae*, and *Klebsiella planticola* strains were isolated from the rhizosphere of Philippino rice (Ladha et al., 1983; Watanabe et al., 1987). In an extensive study, Oyaizu-Masuchi and Komagata (1988) reported the isolation of >1000 nitrogen-fixing bacteria from rice roots. Seventy-four of the diazotrophs with the highest acetylene-reducing activity were classified using a variety of biochemical and morphological tests. Bacteria isolated included *Xanthobacter autotrophicus*, three species of *Azospirillum*, three species from Enterobacteriaceae, *Alcaligenes* sp., *Protomonas*-like bacteria, and *Azotobacter*-like bacteria. However, 52 of the 74 isolates could not be assigned to any known species and 30 were not similar to any of the reference genera. These 52 bacteria were

morphologically and phenotypically diverse, falling into 7 distinct groups (Oyaizu-Masuchi and Komagata, 1988).

Bacteria isolated using current culture techniques represent only a small portion of the number of bacteria observed by microscopy (Amann et al., 1995). Therefore, in addition to the culturable bacteria, it is likely that uncultured nitrogen-fixing bacteria also contribute to the diversity of diazotrophs in the rice rhizosphere. To determine the diversity of non-culturable diazotrophs, phylogenetic studies of nitrogen-fixing bacteria associated with rice roots using PCR primers designed to amplify fragments from *nif* genes have been carried out (Ueda et al., 1995a; 1995b). The sequences of 16 *nifD* and 23 *nifH* PCR products thus generated distinct from sequences of nitrogen-fixing genes from culturable bacteria, and were found to form seperate branches in a phylogenetic tree (Ueda et al., 1995a; 1995b). This provides further evidence for the notion that many of the nitrogen-fixing bacteria from the rice rhizosphere have not yet been characterized.

The studies summarized above revealed a diversity of diazotrophic bacteria associated with rice roots but have provided little insight into the

diversity of bacteria that colonize internal rice tissues. Watanabe and Barraquio (1979) attempted to remove rhizoplane bacteria by vigorously shaking the rice tissue with glass beads prior to isolation. While enriching for bacteria from the "inner rhizoplane", this isolation undoubtedly included bacteria which adhere tightly to the surface of the root. Other isolation protocols made no effort to distinguish between bacteria from the interior of the root and bacteria from the rhizoplane and rhizosphere, nor to determine colonization sites. The lack of information about the diversity of bacteria specifically isolated from internal tissues leaves a gap in our understanding of an important niche in microbial ecology. Information on bacteria that occupy this niche is needed, since bacteria from this niche may present an opportunity to increase the supply of biologically-fixed nitrogen to rice.

SUPPLYING NITROGEN TO RICE

For bacteria to effectively supply crop plants with nitrogen they must have an adequate carbon supply to sustain the energy-intensive process of nitrogen fixation. In addition, the intracellular oxygen tension must be high enough for efficient nitrogen fixation without inactivating the oxygen-sensitive nitrogenase enzyme, and the nitrogen fixed by the bacteria must be transferred from the bacteria to the plant (Kennedy and

Tchan, 1992; Kennedy et al., 1997). There must also be enough bacteria to produce the nitrogen needed, which will be affected by the three conditions mentioned above, as well as the total nitrogen demand of the crop plant.

Problems with nutrient transfer between rhizosphere bacteria and plants are illustrated by the interactions between *Azospirillum* and various cereal crops. Colonization of wheat by *Azospirillum brasilense* is limited to the surface of the root, with occasional intercellular colonization of the cortex (Schank et al., 1979; Levanony et al., 1989; Bashan and Levanony, 1989; Kennedy et al., 1998). Plants gain little nitrogen from bacterial nitrogen fixation when inoculated with *Azospirillum* (Nayak et al., 1986; Christiansen-Weniger and van Veen, 1991; Kennedy et al., 1998). The addition of a carbon source to the rhizosphere increases the amount of nitrogen fixation (Christiansen-Weniger and van Veen, 1991) . This indicates that rhizosphere bacteria are limited for carbon and therefore not efficient sources for biological nitrogen fixation (BNF). Nitrogen supplied to the plant can be increased by the use of ammonium-excreting mutants of *Azospirillum* (Christiansen-Weniger and van Veen, 1991; Christiansen-Weniger 1992), however, these bacteria compete poorly in field soil. This indicates that in the rhizosphere the bacteria use most of

the nitrogen they fix for their own growth, rather than supplying nitrogen to the plant.

The intimate association between plant and endophyte may provide more suitable conditions for nutrient transfer between bacteria and their host than the association between rhizosphere bacteria and plants. *Acetobacter diazotrophicus*, an endophytic diazotroph isolated from sugar cane, excretes fixed nitrogen (Cojho et al., 1993), does not survive well in soils (James and Olivares, 1998), colonize the interior of the sugar cane and appear to supply the host with nitrogen (James and Olivares, 1998). This provides an example of a plant host providing protection from rhizosphere competition, and simultaneously allowing an ammonium-excreting bacterium to flourish and provide nitrogen to the host. Based on this example, it is conceivable that genetically modified ammonium-excreting endophytic bacteria could effectively supply the host plant with nitrogen.

The ambient oxygen tension required for efficient nitrogen fixation varies between diazotrophs with optimal oxygen tensions ranging from 0.3% to 6.5% (Vande Broek et al., 1996). For effective nitrogen fixation bacteria

require enough oxygen for efficient respiration, but low concentrations of free oxygen to protect the oxygen-sensitive nitrogenase enzymes. Sites in the interior of plant tissue, especially flooded rice, may provide the environment required for efficient nitrogen fixation. The apparently high rates of nitrogen fixation by endophytic diazotrophs in sugar cane (Urquiaga et al., 1992) and the de-repression of *nif* genes by *Azoarcus* (Egener et al., 1998) and *Alcalegenes faecalis* (Vermeiren et al., 1998) following internal colonization of rice roots suggest that conditions favorable for nitrogen fixation, including low oxygen tension, must exist inside the plant tissue.

The number of bacteria needed to provide enough biologically-fixed nitrogen to reduce or eliminate fertilizer inputs will depend heavily on the efficiency of nitrogen fixation and nutrient transfer. In sugar cane, estimated population sizes for *A. diazotrophicus* range from 10^3 to 10^7 bacteria per gram fresh weight of roots (Boddey, 1995). Studies of BNF in Brazilian sugarcane cropping systems suggest contributions of 39 kg N ha^{-1} $year^{-1}$ to over 170 kg N ha^{-1} $year^{-1}$ (Boddey, 1995). It is unclear if this nitrogen is fixed by *A. diazotrophicus* alone or in conjunction with other endophytic diazotrophs. However, it has been suggested that BNF

by endophytes contributes enough nitrogen for maximum yields, while requiring minimal inputs of chemical nitrogen fertilizer (Boddey et al., 1995).

The intimate interactions between endophytic bacteria and their host may provide the potential for significant contributions of biologically-fixed nitrogen to rice growth and yield. Nature provides us with several examples of bacteria living internally in eukaryotic host tissues and supplying the hosts with nitrogen or other nutrients. The *Rhizobium*/legume symbiosis has been the most widely studied example of the former (Long, 1989; van Rhijn and Vanderleyden, 1995). A similar symbiosis between *Frankia* and actinorhizal plants has also been studied, but to a lesser degree (Benson and Silvester, 1993). The symbiosis between *Anabaena-Azolla* and *Nostoc-Gunnera* are examples of cyanobacteria supplying eukaryotic hosts with biologically-fixed nitrogen (Peters and Meeks, 1989; Bergman et al., 1992). As mentioned above, endophytic bacteria present in selected sugar cane varieties appear to contribute enough fixed nitrogen to the plant host so that no chemical nitrogen fertilizer is needed for maximum yields (Lima et al., 1987; Boddey et al., 1991; Urquiaga et al., 1992; James et al., 1994).

It has been proposed that diazotrophic bacterial endosymbionts of shipworms (teredinid mollusks) allow these marine invertebrates to utilize wood as a principal food source (Distel et al., 1991), suggesting that the bacteria supply some nitrogen to the eukaryotic host. Thioautotrophic and methanotrophic bacterial endosymbionts of other marine invertebrates supply their hosts with the energy needed for life (Cavanaugh 1994; Distel et al., 1995). Chloroplasts and mitochondria are also ancient endosymbiotic bacteria that have evolved into organelles (Gary, 1989). In each of these cases, the bacterial symbiont provides the eukaryotic host with biochemical pathways not readily available in the host cell.

As discussed above, bacteria can supply a eukaryotic host with nutrients. However, diazotrophic endophytes of rice currently do not supply enough biologically fixed nitrogen to sustain high yields. Molecular biology allows scientists to manipulate bacteria in very specific ways, making it theoretically possible to engineer a bacterium with enhanced endophytic interactions with its crop host. One example is the use of genetically engineered endophytic bacteria to provide corn with insect resistance. *Clavibacter xyli* subsp. *cynodontis* is capable of endophytic establishment

in maize xylem. Inoculation of corn with this bacterium engineered to express the gene encoding CryIA(c) insecticidal protein reduced European corn borer damage by 60% (Lampel et al., 1994). In the future it should also be possible to enhance diazotrophic endophytes to supply their host with more biologically fixed nitrogen.

At present our knowledge of the complex interaction between endophytes and rice is limited and much of the information needed to use diazotrophic endophytic bacteria to replace chemical nitrogen fertilizer is still lacking (Quispel, 1992). The diversity of rice endophytes and the niches they colonize have not been determined. Therefore, more studies that focus on determining the types of endophytic bacteria that associate with rice, the nature of the interactions between host and endophyte, and the sites of endophytic colonization are clearly needed.

DEFINITION OF ENDOPHYTIC BACTERIA

Disagreements exist about the definition of "endophytic" bacteria. Quispel (1992) restricts his definition of endophytic organisms to non-pathogenic microorganisms that spend a considerable part of their life cycle within the plant host, either in inter- or intracellular locations. Other definitions of endophytes also exclude pathogens (Misaghi and

Donndelinger, 1990; Frommel et al., 1991; Fisher et al., 1992). However, Kloepper et al. (1992) does not exclude pathogens, nor will I in this thesis, because of the difficulty distinguishing between pathogenic and non-pathogenic interactions, especially when considering "quiescent" pathogens and variations in plant reactions to an "endophyte" depending on plant genotype (James and Olivares, 1998).

Baldani et al. (1997) define obligate endophytes as those that survive only inside the plant host, while facultative endophytes can also survive in the soil. Distinguishing between these two classes in a robust manner is difficult. However, this distinction may be useful for identifying bacteria most suited for future use in crop inoculations, because "obligate" endophytes many form more intimate associations with the plant. Kloepper et al. (1992) does not included bacteria colonizing only the epidermis. I have also chosen to exclude this type of colonization as it is probable that many opportunistic rhizosphere/rhizoplane bacteria occupy this niche. Therefore, in this thesis endophytic bacteria will be defined as bacteria capable of survival and multiplication either inter- or intracellularly, in plant tissues internal to the epidermis.

ENDOPHYTIC BACTERIA ASSOCIATED WITH PLANTS

Bacterial endophytes have been isolated from healthy plants including onion and potato (Frommel et al., 1991), corn (Fisher et al., 1992; McInroy and Kloepper, 1995; Palus et al., 1996), cotton (Misaghi and Donndelinger, 1990; McInroy and Kloepper, 1995), Kallar grass (Reinhold-Hurek and Hurek, 1998a; 1998b), sugar cane (James and Olivares, 1998), C-4 grasses (Kirchhof et al., 1997) and rice (Barraquio et al., 1997; see below). A non-fluorescent *Pseudomonas* sp. has been shown to colonizes the vascular tissues of potato (Frommel et al., 1991), *A. diazotrophicus* and *Herbaspirillum* spp. colonize the vascular tissue of sugar cane (James and Olivares, 1998) and *Azoarcus* can colonize the cortex and vascular tissue of rice and Kallar grass (Hurek et al., 1994). Very little is known about the sites of colonization of other natural bacterial endophytes.

ENDOPHYTIC BACTERIA ASSOCIATED WITH RICE

The presence of endophytic bacteria is suggested by the isolation of bacteria from surface-sterilized rice tissues. Using surface sterilization protocols and most probable number estimates, Barraquio et al. (1997) calculated the population of putative endophytic bacteria in rice to be between 10^5 to 10^8 g dry wt^{-1}. The endophytic bacteria population size

varied with the rice genotype examined. Less than 10% of the putative endophytic bacteria were diazotrophic. The presence of endophytic bacteria is supported by electron micrographs of field-grown rice that revealed the presence of bacteria with diverse morphologies in the interior of rice roots (Yanni et al., 1997).

In addition to the evidence suggesting endophytic colonization of field grown plants, studies carried out in the laboratory suggest that certain bacteria can infect rice tissue endophytically. Barraquio et al. (1997) have used the *gus* marker gene to visualize subepidermal colonization of rice roots by *Herbaspirillum seropedicae*, a diazotroph found associated with many graminaceous crops (Baldani et al., 1986). You and Zhou (1988) have described the colonization of cortical rice root cells by *Alcaligenes faecalis* A15 using light and electron microscopy. However, *A. faecalis* A15 cells marked with the *gus* marker gene were only found in epidermal cells of rice roots (Vermeiren et al., 1998). *A. faecalis* colonizing the epidermal cells of rice express the *gus* gene under the control of the *nifH* promoter, suggesting that the bacteria are fixing nitrogen during colonization of roots (Vermeiren et al., 1998). However, no evidence was presented that the rice plants benefited from this

nitrogen fixation activity. A diazotrophic bacterium belonging to the genus *Azoarcus* has been isolated from the roots of Kallar grass and shown to be able to invade the cortex and vascular system of rice roots and occupy dead plant cells (Hurek et al., 1994). *Azoarcus* in the intercellular spaces of the cortex and in dead epidermal cells expresses the *gfp* gene under the control of the *nifH* promoter, suggesting the bacteria are fixing nitrogen (Egener et al., 1998; Reinhold-Hurek and Hurek, 1998b). Inoculation with *Azoarcus* has also been observed to increase rice plant growth, however, it has been demonstrated that the observed growth increase was not due to nitrogen fixation by the bacteria (Hurek et al., 1994).

There is also evidence that some rhizobia can colonize rice tissue. Cocking et al. (1995) have reported the formation of short thick lateral roots, intercellular infection pockets, and nitrogen fixation activity following inoculation of rice or wheat with *Azorhizobium caulinodans* ORS571 or *Rhizobium* ORS310. These bacteria are capable of forming root and stem nodules on *Sesbania rostrata* and *Aeschynomene indica*, respectively. The ability of *A. caulinodans* and other rhizobia to cause short, thick lateral roots, colonize intercellular spaces between the

epidermis and cortex at the point of lateral root emergence, and occupy apparently dead cortical cells has been confirmed (Reddy et al., 1997; Webster et al., 1997). However, the claim that these bacteria supply fixed nitrogen to the rice plant has not been substantiated. Another rhizobial strain, *Rhizobium leguminosarum* bv. trifolii, has been isolated from field grown rice rotated with Egyptian berseem clover, the legume host of *R. leguminosarum* bv. trifolii. These bacteria were shown to be able to colonize the upper portion of the rice root (Yanni et al., 1997). Colonization of rice by these bacteria stimulates plant growth. As in the cases described above, this plant growth stimulation is most likely not due to nitrogen fixation (Yanni et al., 1997).

These studies show that diazotrophic bacteria do interact intimately with rice roots, and in some cases have endophytic colonization patterns. However, the mechanisms underlying the ability of these bacteria to colonize rice roots in an endophytic manner are poorly understood, and in none of these cases has it been shown that the rice plant benefits from nitrogen fixed by the bacteria, nor has intracellular colonization of living plant cells been demonstrated. Moreover, comprehensive studies on the diversity of endophytic bacteria, a careful analysis of the ability of

bacteria to colonize rice roots endophytically, and a detailed characterization of the invasion mechanisms is still lacking.

SUMMARY

It is important to realize that nitrogen fixation in cereal crops is a long term project, and while potentially great benefits exist for the future, immediate results should not be expected (de Bruijn et al., 1995; Ladha et al., 1997). Progress has been made in understanding the interactions of specific nitrogen-fixing bacteria with cereals and their contribution to crop yield. However, more information is needed about the bacteria capable of invading rice roots, including the diversity of such bacterial endophytes, modes of bacterial invasion, sites of bacterial colonization, competitiveness of endophytes in colonizing the roots, endophytic population sizes and the ability of the bacteria to fix nitrogen and transfer it to the plant. Previous studies have concentrated on identifying diazotrophic bacteria from the rhizosphere of rice roots or looked at the ability of one type of bacterium to colonize rice and supply it with nitrogen. A comprehensive study of the diversity of rice endophytes and the sites of endophytic colonization has not been undertaken.

This study begins to address the genetic diversity of endophytic bacteria associated with rice and the sites they colonize. In Chapter 2 putative rice endophytes are isolated from mechanically-abraded, surface-sterilized rice tissue and preliminary characterization of their genetic diversity and diazotrophy is carried out. Chapter 3 describes the development of *nifD*-specific PCR primers and the use of these primers to identify diazotrophic bacteria in a collection of putative rice endophytes. In Chapter 4, the genetic diversity of 142 putative endophytic bacteria is evaluated using amplified ribosomal DNA analysis (ARDRA) fingerprinting, rep-PCR genomic fingerprinting, and small subunit (SSU) ribosomal RNA (rRNA) gene DNA sequencing. In Chapter 5, the utility of the green fluorescent protein (GFP) as a biomarker/bioreporter is reviewed. Chapter 6 demonstrates the use of two biomarkers, GUS and GFP, to study the sites and levels of rice colonization using two putative rice endophytes and one *Rhizobium* control strain. These data provide insight into the diversity of bacteria isolated from surface-sterilized rice tissue and the utility of biomarkers for observing colonization of rice tissue by bacteria, and will be useful in the planning and success of future experiments aimed at using endophytic bacteria to supply rice with biologically-fixed nitrogen.

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CHAPTER 2

ISOLATION OF ENDOPHYTIC BACTERIA FROM RICE AND ASSESSMENT OF THEIR POTENTIAL FOR SUPPLYING RICE WITH BIOLOGICALLY FIXED NITROGEN

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ABSTRACT

The extension of nitrogen-fixing symbioses to important crop plants such as the cereals has been a long-standing goal in the field of biological nitrogen fixation. One of the approaches that has been used to try to achieve this goal involves the isolation and characterization of stable endophytic bacteria from a variety of wild and cultivated rice species that either have a natural ability to fix nitrogen or can be engineered to do so. Here we present the results of our first screening effort for rice endophytes and their characterization using acetylene reduction assays (ARA), genomic fingerprinting with primers corresponding to naturally

occurring repetitive DNA elements (rep-PCR), partial 16S rDNA sequence analysis and PCR-mediated detection of nitrogen fixation (*nif*) genes with universal *nif* primers developed in our laboratory. We also describe our efforts to inoculate rice plants with the isolates obtained from the screening, in order to examine their invasiveness and persistence (stable endophytic maintenance). Lastly, we review our attempts to tag selected isolates with reporter genes/proteins, such as beta-glucuronidase (*gus*) or green fluorescent protein (*gfp*), in order to be able to track putative endophytes during colonization of rice tissues.

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple in the diet of over 40% of the world's population making it the most important food crop currently produced (Hossain and Fischer, 1995). Much of this rice is grown in countries where rapidly growing populations, coupled to limited amounts of land and scarce resources, make high yields per hectare with reduced inputs essential to avoid food shortages. Crop productivity is based on numerous variables including weather, soil type, moisture, and nutrients. One of the most important factors in the generation of high yields from modern rice crops is nitrogen fertilizer. Without the addition of (fertilizer) nitrogen the yield of the present varieties is drastically limited. While biological nitrogen fixation in wetland rice fields contributes significantly to the long term fertility of these systems, it is not enough to produce maximum yields. Studies show that biological nitrogen fixation in flooded rice paddies can yield up to 50 kg N $ha^{-1}/crop^{-1}$ (Roger and Ladha, 1992). This biologically-fixed nitrogen has been sufficient to maintain traditional flooded rice systems for thousands of years. However, the productivity of these sustainable systems is low, with yields less than four tons per hectare. Modern agriculture has increased rice yields to five to eight tons per hectare, but requires the

input of 60 to 100 kg/ha^{-1} of fertilizer to supply additional nitrogen. Global population estimates predict the need for a 70% increase in rice production over the next thirty years. The use of current agronomic practices to generate this increase would require even larger inputs of fertilizer nitrogen.

The use of high levels of nitrogen fertilizers in crop production has several drawbacks. Most nitrogen fertilizer is produced via the Haber-Bosch process. This process requires large amounts of natural gas, coal, or petroleum, all non-renewable energy source. In addition it produces CO_2, a gas implicated in the greenhouse effect. The chemical production of nitrogen fertilizer is also expensive, and in developing countries the additional costs often exceed the means of farmers, limiting the yield potential of their crops. Once chemical fertilizers are applied, additional problems can arise. Roughly one third of the nitrogen applied is used by the crop. The non-assimilated nitrogen from farming systems has been implicated in nitrate contamination of ground water supplies, a potential health hazard. In addition, excess nitrogen can also lead to production of nitrous oxide (N_2O), a potent "greenhouse" gas. Therefore, crop systems requiring large additions of fertilizer nitrogen are non-sustainable

systems, since they require the use of non-renewable natural resources and can contribute to health hazards and environmental pollution.

Decreasing the amount of industrially produced fertilizer nitrogen needed in agricultural systems is an important goal of agricultural scientists in general (Bohlool et al., 1992). In the case of sustainable rice production, in particular, one important aim is to replace industrially fixed nitrogen with biologically fixed nitrogen. According to a National Research Council Report (1994), an estimated 100-175 million metric tons of biologically fixed nitrogen is produced annually. Most of this nitrogen is fixed by the legume/*Rhizobium* symbiosis. This is significantly more than the 10 million metric tons produced by lightning and 80 million tons produced in 1989 by industrial processes. Therefore, it is obvious that (symbiotic) biological nitrogen fixation has great potential for supplying nitrogen to crops.

However, the delivery of biologically fixed nitrogen to plants such as rice has been problematic. For example, supplying biologically fixed nitrogen through associative nitrogen-fixing bacteria or green manuring has some of the same drawbacks that have been observed with industrially

produced fertilizers. All three methods rely on plant uptake of nitrogen from the soil nitrogen pool. In order to meet the nitrogen requirements of the plant, a large excess nitrogen pool is needed. Green manure systems using *Sesbania rostrata, Aeschynomene afraspera,* or *Azolla* produce yields comparable to fertilized control plots (Ladha et al., 1992; Diekmann et al., 1996; Kumarasinghe et al., 1986). However, the percentage of the nitrogen from green manure incorporated by the rice plant is similar to that from fertilizer nitrogen (Clément et al., 1995; Kumarasinghe et al., 1986) and, therefore, nitrogen losses in green manure systems can be similar to those in fertilized systems (Harris et al., 1994). In legume crops this problem is avoided because the endo-symbiotic relationship with *Rhizobium* supplies biologically fixed nitrogen directly to the plant, without requiring an excess pool of soil nitrogen, and the ammonia produced is rapidly and efficiently assimilated by the host plant.

Three approaches to achieve a more direct transfer of biologically fixed nitrogen to rice plants have been proposed: 1. Development of novel symbiotic interactions resulting in the formation of nitrogen-fixing nodules or nodule-like structures on rice roots; 2. Identification of stably

maintained diazotrophic endophytic bacteria in rice tissues; and 3. Direct incorporation/expression of the required complement of nitrogen fixation genes in(to) rice (Kush and Bennet, 1992; Ladha and Peoples, 1995; de Bruijn et al., 1995; Ladha et al., 1997). While each of these methods has distinct merits, much of the basic knowledge needed to successfully develop any of these systems is still lacking. The development of nodules on legumes is a highly developed program involving specialized genes in both the plant and bacteria (Fisher and Long, 1992). While great progress has been made toward characterizing the genes involved in this process and the functions of the proteins they express, there are still large gaps in our knowledge, and extensive hurdles to be overcome (de Bruijn et al., 1995). Exogenously applied hormones or cell wall degrading enzymes cause hypertrophies to form on the roots of several non-leguminous crops (Kennedy and Tchan, 1992). It has been reported that these structures can be invaded by diazotrophs, and that in some cases an increase in nitrogen uptake by the plant can be observed. However, these structures lack the complexity and specificity of nodules. More knowledge about the plant genes involved in nodulation and symbiotic nitrogen fixation and the function of their gene products is needed before the development of true nodulation of cereals can be seriously considered

(de Bruijn et al., 1995; Reddy et al., 1997; Kennedy et al. 1997; Webster et al., 1997). The development of plants containing nitrogen fixing genes in their own genome may be even more complex (de Bruijn et al., 1995; Dixon et al., 1997). While most of the genes necessary for nitrogen fixation in bacteria are well characterized, the transfer of the genes to the plant genome, along with the appropriate expression of all these genes is beyond our current technical ability. In addition, the creation of the proper environment, in terms of oxygen concentration/supply, energy provision to the bacteria, and efficient ammonia assimilation within plant cells may also present serious problems (de Bruijn et al., 1995; Dixon et al., 1997).

Therefore, the employment of endophytic nitrogen-fixing bacteria may involve the fewest technical challenges. Moreover, this approach has already been shown to be successful in the case of sugar cane (Boddey et al., 1995; Kirchhof et al., 1997) and Kallar grass (Reinhold-Hurek and Hurek, 1997). In addition, a beneficial natural endophytic association of rhizobia and rice grown in rotation with clover has recently been documented (Yanni et al., 1997). By using naturally occurring endophytes that colonize a niche in which conditions appropriate for

nitrogen fixation exist, we would have 'allowed evolution to do some of the work for us'. Even if a stably endophytic microbe was to be identified that did not have the capacity to fix nitrogen, the process of introducing and expressing the *nif* gene complement in such an endophyte would be significantly easier than engineering the rice plant itself to fix nitrogen. However, it is clear that first some basic knowledge about the presence, predominance and stability of endophytic bacteria in different rice tissues must be obtained. Below we report some recent results of our ongoing study on rice-endophyte associations, that has the following objectives: a) isolating putative endophytic bacteria from diverse rice varieties grown in different soil types and assessing their diversity, b) developing molecular probes for the detection of putative N_2-fixing endophytes, and c) studying the internal colonization of rice tissue by putative endophytic bacteria.

MATERIA

sciation

MATERIAL AND METHODS

Isolation of Endophytic Bacteria.

The isolation of bacteria from 8 varieties of rice grown in the greenhouse in pots containing one of five soil types was carried out at IRRI (Malarvithi, 1995; Figure 2-1). The rice tissue was collected at the heading stage as follows. Plants were carefully removed from pots, washed to remove all soil and separated into stems and roots. The outer layer of the stems was removed, the stems were washed with tap water and deionized water, cut into sections 2-3 cm long, and dried on absorbent towels. Roots were cleaned thoroughly with tap water, rinsed with deionized water, and drained on absorbent towels. All tissues were surface-sterilized in the following manner. Ten grams of tissue was shaken for thirty minutes in a 500 ml Erlenmeyer flask containing 250 ml sterile deionized water and 25 grams of glass beads. The tissue was transferred aseptically to a sterile beaker, washed two times with sterile distilled water, and sterilized using 0.2% $HgCl_2$ (30 seconds for roots, 60 seconds for stems). The tissue was washed 6 times with sterile distilled water, cut into small pieces and homogenized in a Warring blender containing 90 ml sterile distilled water. Serial dilutions were prepared and spread on

plates containing bacterial growth media or tubes containing semisolid media described by Malarvithi (1995) and Barraquio et al. (1997).

The isolation of bacteria using an *in planta* selection step to reduce the number of non-endophytes (Figure 2-2) was also carried out at IRRI. Initial isolation was carried out on field grown rice plants (variety IR72) as described above with the following modifications. The duration of surface sterilization of tissue was varied from 0 to 120 seconds. One hundred microliters of macerate from the 10^{-1} or 10^{-2} dilution were inoculated onto three day old aseptically grown rice seedlings generated as follows: Rice seeds (variety IR24, IR42, or Lemont) were gently dehulled, placed in 70% ethanol for five minutes, washed with sterile distilled water, sterilized by addition of 0.2% $HgCl_2$ for four minutes or a fresh solution of 30% Clorox (17.5 ml sterile distilled water, 7.5 ml Clorox Bleach, 30 µl tween 20) for 45 minutes, and washed six times with sterile distilled water. Seeds were placed on plates containing TY medium (Beringer, 1974) and incubated at 30°C for two days to allow germination and check for contamination. Seeds showing no contamination were placed in 25 mm X 200 mm tubes containing either 25 ml of semisolid modified Fahraeus medium (Fahraeus, 1957) or sterile sand watered with Fahraeus

medium. Seedlings grown in sand were watered with Fahraeus medium on a regular basis during the remainder of the growth period.

Bacteria were isolated from the inoculated seedlings as follows. Seedlings were grown from 23 to 25 days before re-isolation of the bacteria and carefully removed from the growth tubes to keep the roots intact. The roots and shoots were separated and the shoots were cut into small sections. The tissues were placed in 125 ml Erlenmeyer flasks containing 10 ml of sterile distilled water and 5 grams of glass beads, shaken for 30 minutes, and washed with 10 ml sterile distilled water. The tissue was sterilized by adding 10 ml of 0.2% $HgCl_2$ for 30 seconds or 30% Clorox solution for 15 minutes and washed six times with sterile distilled water. Tissues were macerated in one milliliter sterile distilled water and 20 µl of macerate was spread onto plates containing Tryptic Soy Agar (Difco, Detroit, MI, USA), 0.1% Tryptic Soy Agar, TY medium or Davis and Mingolini minimal medium (Atlas, 1997).

Acetylene Reduction Assays

Acetylene reduction assays were performed at IRRI using malate semi-solid nitrogen-free media and standard protocols (Barraquio et al., 1997).

PCR Amplification of nifD-Specific DNA Fragments

Using sequence data from known diazotrophs (obtained from medline), the following universal *nifD* primers were derived: FdB261 (5'-TGGGGICCIRTIAARGAYATG-3') and FdB260 (5'-TCRTTIGCIATRTGRTGNCC-3'). These primers were synthesized at the MSU macromolecular facility and tested using a reference collection of known nitrogen-fixing and non-nitrogen-fixing bacteria (Chapter 3). A DNA fragment 390 bp in length was amplified from all diazotrophic bacteria tested, using the whole cell ERIC-PCR conditions previously described (Louws et al., 1996; Rademaker and de Bruijn, 1997). The DNA sequence of the amplified DNA fragment from *Mesorhizobium loti* NZP2235 was determined by automated fluorescent sequencing of the purified DNA at the MSU-DOE-PRL Plant Biochemistry Facility using the ABI Catalyst 800 for Taq cycle sequencing and the ABI 373A Sequencer and shown to be highly homologous to *nifD* sequences of a variety of diazotrophs. The identity of the PCR amplified fragments from the reference collection of diazotrophic microbes was confirmed by standard Southern hybridization (Maniatis et al., 1982), using the amplified *M. loti nifD* fragment as a probe.

rep-PCR Genomic Fingerprinting

Whole cell rep-PCR of bacteria and computer analysis of the resulting fingerprints was carried out as described by Louws et al. (1996) and Rademaker and de Bruijn (1997).

16S rRNA Gene Sequencing and Phylogenetic Studies

Two highly conserved eubacterial 16S rDNA primers (8F and 1492R) were used to amplify segments of the 16S rRNA genes of the members of the test collection (see Results and Discussion), using rep-PCR conditions. The PCR amplification products were purified using ultra-Free columns (Millipore, Bedford, MA, USA). Partial 16S rDNA sequences were obtained using primers 8F and 519RB using the ABI373A DNA sequencer (see above). The sequences obtained were submitted to the SSU RDP Database at the University of Illinois using Netscape Navigator and analyzed using the Similarity Rank method described by Maidak et al. (1994).

Introduction of Transposons into Putative Endophytes and Detection of GUS Activity

Transposons containing the beta-glucuronidase (*uidA; gus*) marker gene were introduced into the genome of the rice isolates by conjugation with *Escherichia coli* strains harboring narrow host range plasmids carrying the

respective transposons. Detection of Gus activity was carried out as described by Wilson et al. (1995).

RESULTS AND DISCUSSION

Isolation of Rice Endophytes

In order to isolate putative endophytes of rice, two distinct approaches were used. The first approach involved the use of root and stem tissues of diverse varieties grown in different soil types, as described in the Material and Methods (Figure 2-1). One hundred thirty-three of these isolates were randomly selected for further study. In an attempt to enrich for invasive/endophytic bacteria a second approach incorporating an *in planta* selection step was utilized (see Material and Methods and Figure 2-2). This procedure led to the isolation of ~300 microbial isolates, of which 175 were brought to MSU for further analysis. Two of the isolates from this collection [R061.S1.3. and R032.S2.3] were randomly chosen and included in the study presented below (Test Collection).

Diversity of Putative Endophytes in Rice Tissues

The diversity of a collection of one hundred thirty-three putative endophytic microbes isolated using the approach outlined in Figure 2-1 was assessed using rep-PCR genomic fingerprinting (Versalovic et al., 1994; Louws et al., 1996; Rademaker and de Bruijn, 1997). Both REP

and ERIC primers were employed and the resulting fingerprints were combined and analyzed using the Gelcompar software package, as described by Rademaker and de Bruijn (1997). The dendrogram derived from this analysis is shown in Figure 2-3. The results presented reveal a large degree of diversity in these putative endophytes, although some distinct clusters of closely related strains could be observed (Figure 2-3). One large cluster of thirty-two similar fingerprints stands out in the dendrogram. The origin of these bacterial isolates is interesting from a standpoint of cosmopolitanism verses endemism. The thirty-two isolates in this cluster were isolated from eleven of the rice variety/soil types tested. Bacteria were isolated from plants grown in all five soil types, and from six of the eight rice varieties used. The largest number of similar fingerprints in the cluster that originated from the same variety/soil combination was eight. The diversity of their origins suggests that the bacteria found in this cluster are cosmopolitan, being found in a wide distribution of geographically removed soil types and being able to colonize a number of different rice genotypes. Three strains from the large cluster [-R6a(126), R33(120) and R45(42)] were selected for further analysis (Test Collection; see below).

Nitrogen Fixation by and Presence of *nif* Genes in the Endophytic Isolates

The nitrogen fixing ability of the one hundred thirty-three putative endophytic bacteria from the first collection (Figure 2-1) was examined using the acetylene reduction assay (ARA). In addition, highly conserved DNA primers for PCR mediated detection of *nifD* genes were developed and used to screen the collection for the presence of *nif* genes. In the case of diazotrophic bacteria, PCR amplification of genomic DNA using these primers generally leads to the generation of a single 390 bp fragment, which has been shown to be *nifD*-specific by examining a reference collection of thirty-three well characterized bacteria selected from our laboratory strain collection. This collection included twenty-four species of nitrogen-fixing bacteria and nine non-nitrogen-fixing bacteria. The identity of the amplified fragment was further confirmed by determining the DNA sequence of the amplified fragment from *M. loti* NZP 2235 and by Southern blot analysis (Chapter 3). Seventeen of the one hundred thirty-three strains fingerprinted (Figure 2-3) displayed a characteristic amplification fragment when tested using the *nifD* primers. Of the seventeen strains shown to contain *nifD* using PCR, thirteen were found to reduce acetylene (Nif plus). Four additional isolates, which were scored as *nifD* minus in the PCR reactions, were nevertheless found to be

ARA plus, suggesting that they contain highly divergent *nifD* genes, or alternative *nif* genes. Figure 2-4 shows fingerprints and *nifD* PCR results for ten bacteria which were both *nifD* plus and Nif plus, four bacteria that were *nifD* plus and Nif minus, and four bacteria that were *nifD* minus and Nif plus. The remaining one hundred twelve strains were scored as *nifD* minus and Nif minus. Three of the isolates that were found to be both *nifD* and Nif plus [T105, R90(8), R100(64)] and one isolate that was *nifD* plus but Nif minus [R22(88)] were chosen to be included in the Test Collection (see below).

Composition of a "Test Collection" and Preliminary Characterization of its Members

A "Test Collection" of bacteria was composed for further studies (see Table 2-1). The nine isolates noted above [-R6a(126), R33(120), R45(42), T105, R22(88), R90(8), R100(64), R032.S2.3 and R061.S1.3] were included, as well as five previously characterized "control" bacteria. *Escherichia coli* DH5α was chosen as a negative control because it is unlikely that this bacterium would colonize rice roots endophytically. *Azorhizobium caulinodans* ORS571 was chosen because it has been reported to invade the roots of wheat and rice (Cocking et al., 1995; see also Webster et al., 1997; Reddy et al., 1997). *Sinorhizobium meliloti*

1021 was included to compare a *Rhizobium* with *Azorhizobium caulinodans* ORS571 in its ability to colonize rice roots. *Azoarcus indigens* LMG9092 and *Herbaspirillum seropedicae* Z67 were included since these two bacteria have previously been reported to invade rice tissues (Reinhold-Hurek and Hurek, 1987).

In order to further characterize the endophytic isolates from rice, a partial DNA sequence of their 16s rDNA was determined. The DNA sequences obtained were submitted to the SSU RDP database at University of Illinois using the Similarity Rank function (Maidak et al., 1994). The results of this analysis are shown in Table 2-1. The most similar 16s rDNA sequence in the database for each of the "control" bacteria was found to be "itself", except for the case of *Herbaspirillum seropedicae* Z67, where 16S rDNA sequences were not yet available for comparison.

Re-colonization of Rice Tissue by Putative Endophytes

To study the endophytic nature of the bacteria in the Test Collection, experiments were performed to verify that the same bacteria inoculated to sterile rice seedlings could be re-isolated from these seedlings (to fulfill Koch's postulate), and to examine their endophytic competence (infection and persistence characteristics). Gnotobiotically grown rice

seedlings were inoculated with each of the bacteria from the test collection and bacteria were re-isolated as described in Material and Methods (Figure 2-5). The identity of the colonies was verified by comparing the ERIC-PCR fingerprints of these bacteria with the ERIC-PCR fingerprint of the original inocula. In some cases, no bacteria could be re-isolated from the inoculated seedlings. In other cases a large number of bacteria (>25,000), could be re-isolated from a single seedling.

As expected, no bacteria were re-isolated from seedlings inoculated with *Escherichia coli.* Surprisingly, no bacteria could be re-isolated from seedlings inoculated with either *Herbaspirillum seropedicae* or *Azoarcus indigens*. In the case of the rice strains and other control strains large differences in numbers of re-isolatable bacteria were observed. However, large differences in the number of bacteria re-isolated from different seedlings inoculated with the same bacteria were also observed. In spite of these variations, certain endophytic isolates [R22(88), R33(120), R45(42), and R061.S1.3] appeared to be considerably more aggressive colonizers than others. The most aggressive colonizer [R22(88)] was found to cause stunting of seedling growth and therefore may be perceived by the plant as potential pathogen. Presently, the experimental

parameters of these tests are being further standardized and the experiments are being repeated using sufficient replicas to ensure statistically significant results.

The analysis of bacteria re-isolated from seedlings inoculated with *Azorhizobium caulinodans* ORS571, R22(88), R100(64), -R6a(126), R33(120), R45(42), R032.S2.3, and R061.S1.3 using rep-PCR genomic fingerprinting (see Figure 2-5 for the experimental approach) revealed that Koch's postulate was fulfilled, in that bacteria with the same fingerprint as the original inoculum strain could be re-isolated (Data not shown).

Use of Marker Genes to Track Endophytic Bacteria in Rice Tissues

To track bacteria during colonization of rice roots and subsequent inter- or intracellular persistence, specific marker gene constructs were selected (Figure 2-6). A number of different marker genes are available for the tracking of genetically modified organisms (Jansson, 1995). Initially, we focused on the use of the beta-glucuronidase(*uidA; gus*) gene, since Gus activity can be easily detected using a colorometric Gus enzyme assays or *in situ* staining methods (Wilson et al., 1995). In order to obtain stably

tagged strains, we used the Tn*5gus* transposon pCAM111 or pCAM121 kindly provided by Kate Wilson (Wilson et al., 1995). One or the other of these transposons was introduced into 8 out of the 14 test collection strains, and Gus activity of the tagged bacteria could be verified. The remaining six strains exhibit multiple intrinsic antibiotic resistances, and modified transposons/suicide vectors are being developed to tag these strains.

Initial results with a limited number of tagged strains indicate that global colonization patterns of *gus* tagged microbes in rice roots can indeed be monitored by *in situ* Gus staining (see Chapter 6). However, the work required for sectioning tissues in order to localize individual bacteria using Gus activity may be prohibitive to the screening of large sample numbers. Therefore, in collaboration with the laboratory of J. Jansson at Stockholm University, we have developed Tn*5* derived transposons carrying the gene encoding the green fluorescent protein (*gfp*), originally derived from the jellyfish *Aequorea* (Chalfie et al., 1994). Using the constitutively expressed Tn*5gfp* marker transposon, we have shown that GFP is stably and highly expressed in single cells of *Pseudomonas fluorescence*, and that single *gfp* tagged cells can be detected on the root hairs of plants

using laser confocal microscopy (Tombolini et al., 1997; Unge et al., 1998). The use of fluorescent laser scanning confocal microscopy allows optical sectioning reducing the work required to screen large sample numbers. We are presently attempting to tag the rice endophytes and control strains with this marker gene and use the resulting isolates to study rice plant colonization.

Do the Proper Physiological Conditions Exist in Rice Tissues to De-repress *nif* Genes Harbored by Endophytic Bacteria?

Even when stably maintained diazotrophic endophytes (endosymbionts) of rice are identified, it still remains to be shown that the proper physiological environment exists at the site(s) of colonization to allow expression of the *nif* genes and functioning of nitrogenase (de Bruijn et al., 1995; Dixon et al., 1997). As a first step in this analysis we have made use of a plasmid carrying a translational fusion of the 5' end of the *Azospirillum brasilense nifH* gene (including its promoter region) to *gus* (Vande Broek et al., 1992; 1996). Preliminary results show that selected endophytic strains from the test collection harboring the *nifH-gus* fusion do express the reporter gene in the free living state under low oxygen tensions, as expected (Vande Broek et al., 1996). These strains will be used to infect rice seedlings, and *in situ* staining of infected tissues for

Gus activity will be performed in order to determine if and where the proper physiological conditions exist for *nif* gene de-repression, and therefore, by inference, for nitrogen fixation *per se*, although the latter correlation has not always been found to hold true (Vande Broek et al., 1996).

CONCLUSIONS

In this paper we have described preliminary results from our first screens for and characterization of naturally occurring diazotrophic endophytes of rice. The approach we have used here differs from strategies to assess the potential for nitrogen fixation in rice we have reported previously (de Bruijn et al., 1995; Yanni et al., 1997). The latter strategies are based on the assessment of the potential of known rhizobia to induce (nitrogen-fixing) nodule-like structures or root hypertrophies on rice roots, or the ability of specific naturally-occurring endophytic rhizobia to stimulate rice growth under different nitrogen regimes and display acetylene reduction activity, respectively. As a basis for the screen described here, we reasoned (hypothesized) that rather than, or in addition to, attempting to induce or modify known rhizobia to engage in symbiotic or endophytic interactions with rice plants resulting in biological nitrogen fixation for plant growth enhancement, we would try to isolate and characterize naturally occurring, stable endophytic microbes from a variety of different modern and more primitive varieties of rice, and subsequently determine their nitrogen fixation potential or genetically modify them to fix nitrogen. A major difference between this study and that of Yanni et al. (1997) was the strategy used to isolate diazotrophs from the rice endophytic

community. Whereas Yanni et al. used an appropriate legume trap host to specifically enumerate and isolate endophytic rhizobia from rice roots, this study used various plating media to isolate a more diverse collection of microbes belonging to the bacterial community that colonizes rice roots. In fact, these studies are complementary and resulted in the isolation of a large collection of rhizobial and non-rhizobial native microbes that are capable of developing endophytic relationships with rice.

The results presented here, and those described by Barraquio et al. (1997) indicate that a large diversity of apparently diazotrophic (~10%) and non-diazotrophic endophytic bacteria can be isolated from rice tissues, some of which are capable of re-colonizing their host when re-inoculated onto sterile rice seedlings. Past isolations of nitrogen-fixing bacteria from rice roots have already revealed a broad diversity of diazotrophs inhabiting the rice rhizosphere (Lakshmi Kumari et al., 1976; Watanabe and Barraquio, 1979; Bally et al., 1983; Ladha, 1986; Oyaizu-Masuchi et al., 1988; Yanni et al., 1997). In addition, recent studies based on the molecular phylogeny of the DNA sequences generated by PCR amplification of nitrogen fixing genes found in the rice rhizosphere

also suggest an even broader range of different rhizosphere diazotrophs (Ueda et al., 1995a; 1995b). However, the ability of these bacteria to colonize rice tissue endophytically and de-repress genes needed for nitrogen fixation remains virtually unstudied.

Having developed the tools to mark and track some of the promising stable endophytes and monitor the physiological environment in their colonization niches with regard to expressing *nif* genes, we are now able to further test our hypothesis that natural or genetically modified rice endophytes may be useful to achieve the long term goal of developing rice-microbe interactions capable of supplying biologically fixed nitrogen for higher yields, in the absence of additional chemical fertilizer. In addition, information gained during these studies may yield interesting new insights into basic mechanisms of plant-microbe interactions and microbial colonization of plant tissues in general, and be helpful in designing strategies for the discovery and adaptation of nitrogen-fixing bacteria that could provide nitrogen to other major cereal crops such as maize and wheat.

ACKNOWLEDGMENTS

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Bacteria	Most Similar 16S rRNA	Sab	2nd Most Similar 16S rRNA	Sab
Escherichia coli DH5α	*Escherichia coli*	0.945	*Escherichia coli* subsp. K-12 str. MG1655	0.945
Sinohizobium meliloti 1021	*Sinorhizobium xinjiangensis*	0.949	*Sinohizobium meliloti*	0.949
Azorhizobium caulinodans ORS571	*Azorhizobium caulinodans*	0.972	*Aquabacter spiritensis* str. SPL-1	0.791
Azoarcus indigens LMG 9092	*Azoarcus indigens* str. VB32	0.929	*Pseudomonas* sp. str. K172 clone K1	0.780
Herbaspirillum seropedicae Z67	*Zoogloea ramigera*	0.762	*Telluria chitinolytica*	0.688
T105-IRRI collection	*Azorhizobium caulinodans*	0.852	*Aquabacter spiritensis* str. SPL-1	0.809
R22(88)-IRRI collection	*Pseudomonas cepacia* str. G4	0.952	*Burkholderia gladioli* subsp. pathovar gladioli	0.899
R90(8)-IRRI collection	*Agrobacterium vitis*	0.896	*Agrobacterium vitis*	0.869
R100(64)-IRRI collection	*Agrobacterium vitis*	0.878	*Agrobacterium vitis*	0.878
-R6a(126)-IRRI collection	*Azospirillum* sp*	0.913	*Pseudomonas putida* str. mt-2	0.826
R33(120)-IRRI collection	*Azospirillum* sp*	0.919	*Pseudomonas putida* str. mt-2	0.801
R45(42)-IRRI collection	*Azospirillum* sp*	0.913	*Pseudomonas putida* str. mt-2	0.826
R032.S2.3-JRS selection	*Bacillus subtilis* str. 168	0.839	*Bacillus subtilis* str. 168	0.835
R061.S1.3-JRS selection	*Bacillus megaterium*	0.880	*Bacillus megaterium*	0.822

Table 2-1 Partial SSU rDNA Similarities

Bacteria listed in the first column were used to test various method for evaluation of colonization. Partial SSU rDNA sequences from these bacteria were submitted to the SSU RDP database (version 5.0) at University of Illinois using Similarity Rank (version 2.5). Bacteria with first and second most similar SSU rDNA sequences in the are shown. The similarity rank function of the SSU RDP finds the database sequences that are most similar to a sequence submitted by the user. The program counts the number of unique 7 base oligomers in the submitted sequence that are common to each sequence in the data base. The Sab value is the result expressed as the number of shared 7 base oligomers divided by either the number of unique oligomers in the submitted sequence or the database sequence, whichever is lower. This is not a phylogenetic analysis, but allows assessment of the similarities between the sequence. *This bacteria is given the name *Azospirillum* in the RDP database. However, it is not closely related to other *Azospirillum* based on phylogenetic trees made using SSU rDNA sequences.

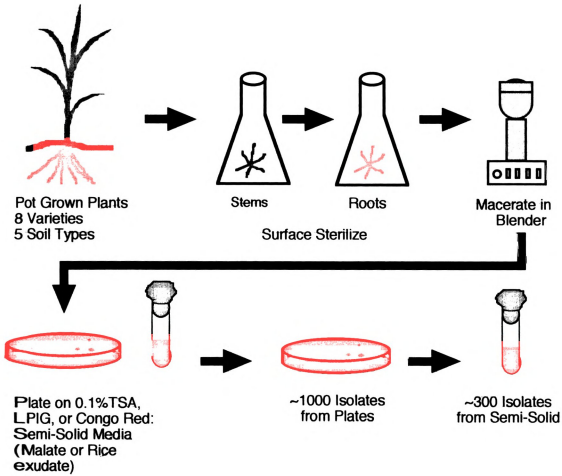


Figure 2-1 Isolation of Putative Rice Endophytes

Scheme to isolate putative endophytic bacteria from rice tissues. Surface sterilization eliminates/reduces the presence of non-endophytic bacteria and maceration releases endophytic bacteria from the tissues. Over 1000 bacteria were isolated at IRRI using this procedure .

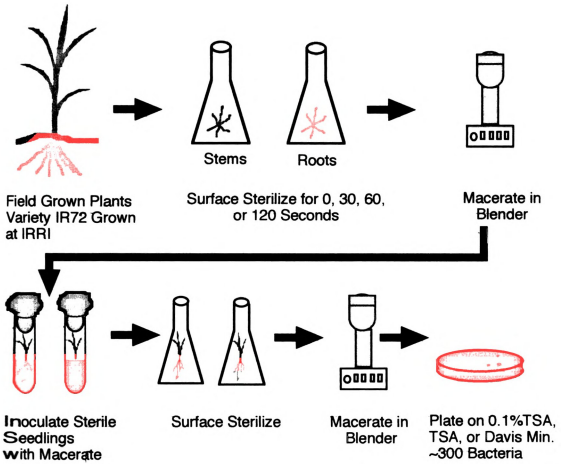


Figure 2-2 Isolation of Endophytic Bacteria Using a Re-infection Step

Scheme adds an in planta selection step to eliminate non-endophytic bacteria. Approximately 300 bacteria were isolated at IRRI using this procedure.

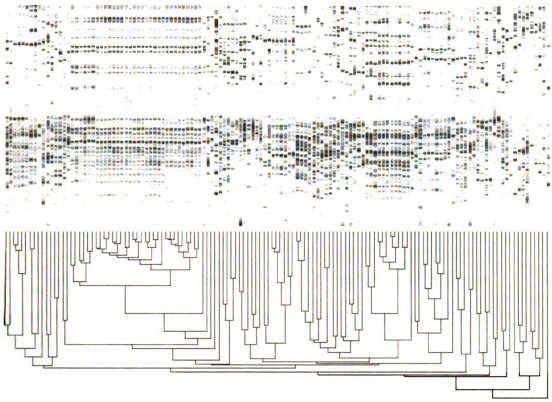


Figure 2-3 Gel Profiles and Dendrogram of rep-PCR Generated Genomic Fingerprints of Putative Endophytic Bacteria Isolated from Rice Roots

The dendrogram of 133 putative endophytes from rice generated from the combined BOX- and ERIC fingerprints using the Gelcompar 4.0 program is shown The fingerprint patterns revealed a high degree of diversity, as well as several clusters of related bacteria. Analysis was performed using Pearson correlation applied to the densitometric curves formed by the fingerprints followed by clustering analysis using the unweighted pair-grouping method, using arithmetic averages (UPGMA).

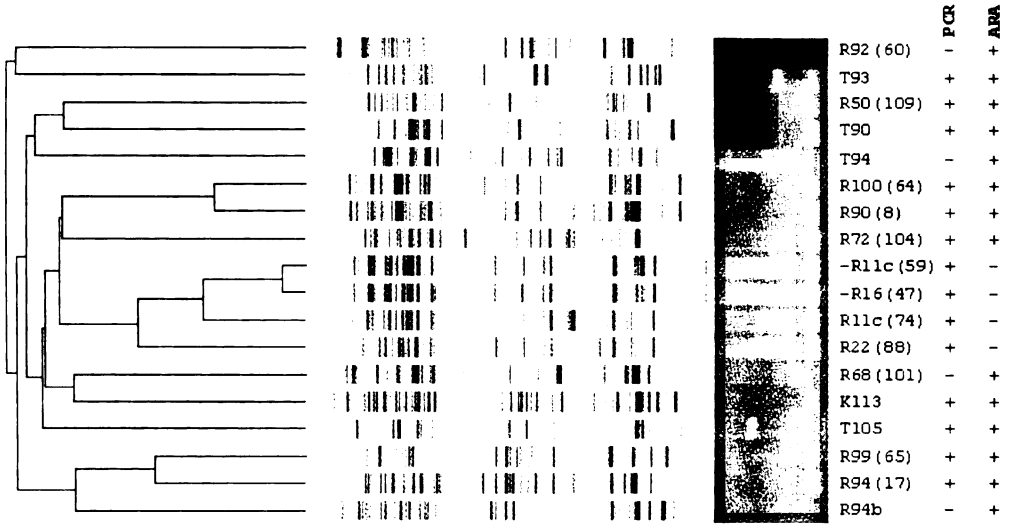


Figure 2-4 rep-PCR Genomic Fingerprints and *nifD*-PCR Analysis of Putative Diazotrophic Endophytes

The dendrogram of the rep-PCR genomic fingerprints of 18 putative nitrogen-fixing strains is shown. The dendrogram was generated using the combined BOX and ERIC and REP fingerprint patterns and the Gelcompar 4.0 software program. This dendrogram shows both the diversity of organisms, as well as clustering of related bacteria. A representative gel on which *nifD* -PCR fragments were electrophoretically separated is shown below the fingerprints. (+) indicates bacteria from which an amplified *nifD* fragment could be obtained. (-) indicates bacteria from which no amplified *nifD* PCR fragment could be obtained. Results from the acetylene reduction assays are also presented. (+) indicates bacteria capable of reducing acetylene in semi-solid malate media. (-) indicates bacteria which did not reduce acetylene in semi-solid malate media.

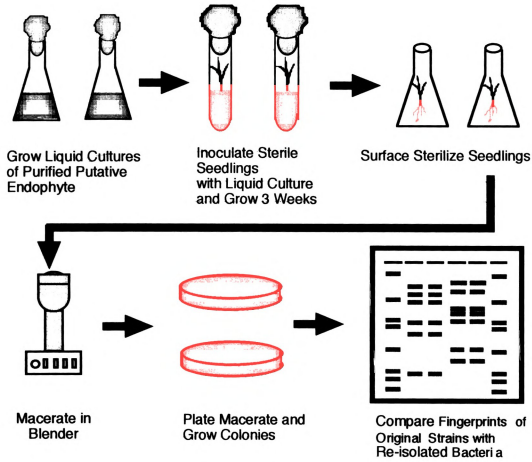


Figure 2-5 Re-infection Potential of Putative Endophytes

The scheme for re-infecting rice plants with and re-isolation of putative endophytes is shown. This experiment was carried out to fulfill Koch's postulate that true endophytes should be re-isolatable after infection of gnotobiotically grown rice seedlings. After re-isolation the identity of the bacteria was confirmed using rep-PCR genomic fingerprinting.

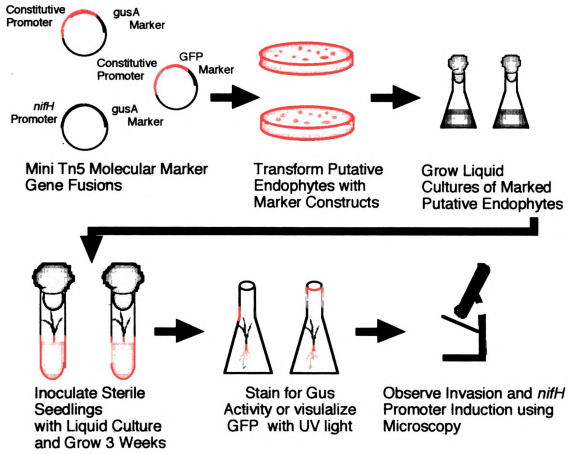


Figure 2-6 *In planta* Visualization of Endophyte Colonization in Rice

The scheme for the utilization of the molecular markers beta-glucuronidase (GUS) and green fluorescent protein (GFP) to characterize endophyte invasion of rice tissues is shown. A constitutive promoter fused to the *gus* or GFP reporter gene will be used to visualize colonization. A *nifH* promoter fused to the *gus* reporter gene will be used to monitor *nif* gene expression during colonization.

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CHAPTER 3

DEVELOPMENT AND USE OF POLYMERASE CHAIN REACTION (PCR) PRIMERS FOR THE RAPID DETECTION OF NITROGEN-FIXING BACTERIA

ABSTRACT

A set of PCR primers corresponding to highly conserved regions of the nitrogen fixation *nifD* gene, encoding the α-subunit of the nitrogenase MoFe-protein, was developed using amino acid sequence data derived from a variety of different diazotrophs. This primer set was shown to amplify a characteristic 390 bp DNA fragment from diverse genera of diazotrophs. Southern hybridization and DNA sequence analysis confirmed that the 390 bp fragments corresponded to the *nifD* gene. This primer set was used to screen a collection of 142 bacteria isolated from surface-sterilized rice tissue. Twenty potential nitrogen-fixing bacteria were identified. Genomic Southern hybridization confirmed the presence of *nif* genes in 19 of these bacteria. The ability of these 19 bacteria to fix nitrogen was verified using acetylene reduction assays. This primer set was also used to detect the presence of diazotrophic bacteria in laboratory and field grown rice plants.

INTRO

INTRODUCTION

Biological nitrogen fixation constitutes an essential part of the global nitrogen cycle and contributes more fixed nitrogen to agricultural production than any other source (Bockman, 1997). However, in the case of wetland rice available levels of natural biologically fixed nitrogen are not sufficient for maximum yield and additional nitrogen must be supplied in the form of industrially produced fertilizer or green manure (Ladha and Kundu, 1997). It is an important goal of agricultural scientists to decrease the amount of industrially-produced fertilizer nitrogen needed by increasing the amount of biologically-fixed nitrogen available to rice crops (Introduction to Chapter 2; Bohlool et al., 1992; Ladha and Kundu, 1997). Recent studies have shown the presence of diazotrophic bacteria colonizing the internal tissues of non-legume plants such as sugar cane and Kallar grass (James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998a; 1998b). In the case of sugar cane these endophytic bacteria have been proposed to be able to supply the plants with enough nitrogen for sustained high yields (Boddey, 1995).

Currently, little information is available about diazotrophic bacteria that colonize the cortex and/or vascular tissue of field grown rice plants. Studies have been carried out to determine the numbers and types of diazotrophic bacteria present in the rhizosphere of rice (Lakshmi Kumari et al., 1976; Watanabe and Barraquio, 1979; Thomas-Bauzon et al., 1982; Bally et al., 1983; Ladha et al., 1983; Watanabe et al., 1987; Oyaizu-Masuchi and Komagata, 1988). These studies relied on the use of selective media and acetylene-reduction assays (ARA) to detect rice-associated bacteria with nitrogen-fixing capability (Watanabe and Barraquio, 1979; Bally et al., 1983; Oyaizu-Masuchi and Komagata, 1988). Unfortunatley, the use of nitrogen-free media is prone to error since some bacteria which do grow are nevertheless unable to fix nitrogen and instead grow by scavenging trace amounts of mineral nitrogen from the media. ARAs are time consuming and may not detect diazotrophic bacteria if the proper physiological growth conditions, including proper carbon source, oxygen concentration, and nitrogen levels are not used for the assay.

PCR technology (Saiki et al., 1988) has revolutionized screening of large collections of uncharacterized bacteria for DNA sequences of interest (Steffan and Atlas, 1991). For example, universal PCR primers have been developed that allow detection of *Listeria monocytogenes* by amplification of a DNA fragment from the haemolysin gene (Furrer et al., 1991) and *Agrobacterium* by amplification of a DNA fragments from *vir* and *ipt* genes (Hass et al., 1995; Sawada et al., 1995). Ueda and coworkers assessed the diversity of diazotrophic bacteria associated with rice by amplifying and sequencing *nifH* and *nifD* DNA fragments from total DNA isolated from the rhizosphere of rice. (Ueda et al., 1995a; 1995b). However, at the onset of the work described here, PCR primers suitable for screening large collections of bacteria to identify nitrogen-fixing strains had not yet been developed. Therefore we set out to develop a quick, inexpensive, reliable method to screen large bacterial collections for nitrogen-fixing strains.

The ability of all known diazotrophic organisms to convert atmospheric N_2 into biologically usable forms depends on the activity of the enzyme nitrogenase. The nitrogenase complex contains two

components: an iron-containing homodimer and a molybdenum- and iron-rich $\alpha 2\beta 2$ tetramer. The genes encoding these proteins are *nifH*, *nifD*, and *nifK*, respectively (Kim and Rees, 1994) Therefore, the presence of DNA sequence corresponding to these genes in a given bacteria would suggest that the microbe may have the potential to fix nitrogen and constitutes a useful and rapid pre-screen for diazotrophs. Some diazotrophic bacteria contian additional nitrogen fixing genes which encode alternate forms of nitrogenase which utilize different metal cofactors. Sequence comparisons indicates that the *nifD* PCR primers would not amplifiy DNA fragments from these genes. However, all diazotrophs which are known to contain alternate forms of nitrogenase also harbor *nifD* (Chisnell et al., 1988; Schuddekopf et al., 1993; Thiel et al., 1993; Zinoni et al., 1993). Therefore, the *nifD* primers would still be useful for detecting these bacteria. However, additional undiscovered methods of nitrogen-fixation which do not rely on nitrogenase may be present in nature. These primers would not detect diazotrophhic organisms which utilize non-nitrogenase systems.

Here we describe the generation of a degenerate PCR primer set corresponding to the *nifD* gene that can be used to amplify a 390 bp DNA fragment from *nifD*, allowing specific detection of this nitrogen-fixing gene from diverse genera of diazotrophic bacteria. This primer set was used to identify novel nitrogen-fixing strains from a collection of bacteria isolated from surface-sterilized rice. This primer set was also used to detect *nif*D gene sequences in total DNA isolated from rice tissue.

MATERIAL AND METHODS

Bacterial Strains

The bacterial strains used to examine the efficacy of the *nifD*-PCR primers are listed in Table 3-1. The 142 stains isolated from surface-sterilized rice tissue and screened with the *nifD*-PCR primers are described in Chapter 4 of this work and listed in Appendix 1. The 35 strains characterized using ARA and Southern hybridization are listed in Table 3-2. Plasmid pRS2 carries the *Azorhizobium caulinodans nifHDK* genes (Elmerich et al., 1982).

Media and Growth Conditions.

E. coli DH5α harboring pRS2 was cultured at 37°C on LB media containing 100mg/l ampicillin. All other bacteria used in this study were routinely cultured at 28°C on TY media (Beringer, 1974).

PCR Template Preparation and Amplification

Screening of bacteria using *nifD*-PCR was carried out using ERIC-PCR conditions as described by Louws et al. (1996) and Rademaker and de Bruijn (1997), with the following modification. Template for PCR was generated by filling a 1 μl disposable loop with a bacterial colony

grown on a plate. The contents of the loop were resuspended in 100 μl sterile distilled H_20 (sdH_20) in a 1.5 ml microfuge tube, boiled for 10 min, incubated at 37°C for 1 hour with 5 μg of RNAase A and stored at –20°C. One microliter of this mixture was used as template for PCR reactions. Whole cells or purified DNA were used to test the efficacy of the *nifD* primers with different template types.

Prior to DNA isolation, rice tissue was subjected to vigorous shaking with glass beads to remove rhizoplane bacteria (Watanabe and Barraquio, 1979). Rice DNA was isolated from rice tissue following the protocol of Ueda et al. (1995a; 1995b) with the following modifications: Following treatment with RNase A, samples were phenol/chloroform extracted, isopropanol precipitated, and resuspended in TE. The DNA was quantified using ethidium bromide staining, as described by Sambrook et al. (1989). 200 ng of the rice DNA was used as template for PCR reactions.

nifD-PCR Primer Design

Degenerate oligonucleotides FdB261 5'-TGGGGICCIRTIAARGAYATG-3', and FdB260 5'-TCRTTIGCIATRTGRTGNCC-3 were synthesized by

the Macromolecular Structure, Synthesis and Sequencing Facility at Michigan State University (N = any base; I = inosine; Y = T or C; R = A or G). The sequence was derived from an alignment of amino acid sequences from the alpha subunit of the nitrogenase MoFe-protein (NifD; Figure 3-1) using the pile-up function from the GCG software package (Genetics Computer Group, University Research Park, Madison, WI, USA) at Michigan State University. The primer set (FdB261/FdB260) was designed to amplify a fragment of approximately 390 bp in length from the 3' end of the *nifD* gene. A schematic of the primer design is presented in Figure 3-2.

DNA Analyses of *nifD*-PCR Products

Visualization of the PCR products was achieved by adding two microliters of loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 15% ficol type 400 in 10xTAE) to the total reaction mixture and separating one fourth to one half of this mixture on 2.0% agarose gels in TAE buffer. The gels were stained with ethidium bromide, and DNA fragments were visualized on a transilluminator. The size marker used was the 100 bp ladder of Boehringer Mannheim (Indianapolis, IN, USA).

DNA sequencing of the 390 bp fragment amplified from *M. loti* NZP2235 DNA was carried out using automated fluorescent sequencing as performed by the MSU DNA Sequencing Facility using an ABI Catalyst 800 for Taq cycle sequencing and an ABI 373A Sequencer for the analysis. Prior to sequencing the PCR reaction was run on a 2.0% agarose gel and the 390 bp fragment was excised and extracted from the agarose using phenol/TE. The DNA was further purified using a Millipore 30,000 ultrafree NC column (Millipore, Bedford, MA, USA).

Southern hybridization analysis of the amplified DNA fragments from the strains listed in Tables 3-1 and 3-2 was performed using the DIG DNA labeling and detection (Boehringer Mannheim, Indianapolis, IN, USA). The purified DNA fragment amplified from *M. loti* NZP 2235 was used as hybridization probe.

Southern Hybridization Analysis of Total Genomic DNA

Bacterial genomic DNA was isolated using the MasterPure Genomic DNA Purification Kit from Epicenter Technologies (Madison, WI, USA) according to the protocol provided by the manufacturer. DNA

digestion, separation, and transfer to Hybond-N nylon membranes (Amersham International, Little Chalfont, Buckinghamshire, England) were carried out according to standard protocols (Sambrook et al., 1989). A 4 Kb DNA fragment containing *A. caulinodans nifHDK* was excised from plasmid pRS2 (Elmerich et al., 1982) by digestion with *Bgl*I and *Sal*I run on an agarose gel and purified using the Qiaex II Gel Extraction Kit from Qiagen (Madison, WI, USA). Probe preparation was carried out according to standard protocols, using ^{32}P labeled dATP. Prehybridization and hybridization reactions were carried out at 65°C using buffer containing 5x Denhardts solution, 4x SSC, 1% SDS, and 100 μg/ml salmon sperm DNA (Sambrook et al., 1989). The membranes were washed three times at low stringency (65°C in buffer containing 4x SSC and 0.1% SDS).

Acetylene Reduction Assays

The bacteria listed in Table 3-2 were examined for nitrogenase activity using acetylene reduction assays. Bacteria were grown to late exponential phase in liquid TY media at 28°C with shaking. The cells in one milliliter of culture were pelleted in a microfuge and the supernatant was removed. The cells were washed two times with nitrogen-free LSO (Elmerich et al., 1982) and resuspended in

nitrogen-free LSO to a final OD_{600} of 0.8. Vacutainer tubes containing 3 ml of nitrogen free LSO plus 0.2% agar were inoculated with 30 µl of this suspension. Following incubation at 28°C for 24 hours, the tubes were stoppered, 1 ml of headspace was removed and replaced by 1 ml of acetylene. The tubes were returned to 28°C and 50 µl of headspace was extracted following 24 and 48 hours of incubation. Samples were analyzed using a Varian Model 3700 Gas Chromatograph equipped with a Propack N 80/100 2m x 1/8" Stainless steel column (Varian, Palo Alto, CA, USA) and a flame ionization detector. The injector temperature was set to 190°C, the column temperature was set to 69°C, and the detector temperature was set to 250°C. Output from the gas chromatograph was measured with a Perkin-Elmer R 100A recorder (Perkin-Elmer, Norwalk, CT, USA). The ethylene peak height was compared to a standard ethylene curve to determine the moles of ethylene produced per tube.

Growth, Sterilization and Inoculation of Rice Seedlings.

Dehulled rice seeds were sterilized as follows. Dehulled seeds rice (Variety IR42 from International Rice Research Institute stocks, the Philippines) were immersed in 70% EtOH for 4 minutes with gentle

agitation followed by washing in sdH_2O, immersion in 0.1% $HgCl_2$ for 4 minutes with gentle agitation, and six washes with sdH_2O. After sterilization, seeds were placed on plates containing Nutrient Agar (Difco) and incubated for 2 days at 30° Celsius to allow germination and check for contamination. Non-contaminated seedlings were transferred to sterile test tubes (2.5 cm x 20 cm) containing ~40 ml sand. Seedlings were watered with 10-15 ml liquid Fahraeus media (Fahraeus, 1957) after planting, and, as necessary, during growth. Seedlings were grown at 28° Celsius with 16 hours light and 8 hours dark. For inoculation, bacterial cultures were grown in liquid TY media to late log phase. Seedlings inoculated with 100 µl of this liquid culture two to three days after transfer to the tubes.

RESULTS

The objective of this study was to establish a rapid, inexpensive protocol for identifying putative nitrogen-fixing bacteria. The Polymerase Chain Reaction (PCR) was used to rapidly screen phylogenetically diverse bacteria for the presence of the nitrogen fixation *nifD* gene. Based on the known amino acid sequences of the nitrogenase molybdenum-iron protein alpha chain (Figure 3-1), oligonucleotides corresponding to conserved regions of the *nifD* gene were designed and synthesized (Figure 3-2). The two opposing oligonucleotides thus generated, dB261 and dB260, correspond to sequences located at the 3' end of the *nifD* gene. Amplification of a 390 bp product would be expected when oligonucleotides dB261 and dB260 are used as PCR primers in reactions containing *nifD* sequences.

PCR Amplification of *nifD* Genes

The ability of primers dB261 and dB260 to detect *nifD* sequences from different template types including whole cells (Versalovic et al., 1994) was tested using purified DNA, single colonies grown on agar plates and resuspended in PCR buffer, and cells grown in liquid

culture, washed, and resuspended in sdH_2O. Results were similar each type of template tested. All three types of templates prepared from the diazotrophic control bacteria *Sinorhizobium meliloti* L5-30, *Sinorhizobium meliloti* 1021, *Mesorhizobium loti* NZP 2037, *Mesorhizobium loti* NZP 2235, and *Azorhizobium caulinodans* ORS 571 yielded PCR products of the expected size (390 bp; data not shown). All three types of templates prepared from the non-nitrogen-fixing control bacteria *Escherichia coli* DH5α, *Agrobacterium tumefaciens* C58, *Xanthomonas campestris* pv vesicatoria, and *Pseudomonas syringae* pv. tomato failed to yield a PCR product of 390 bp. In some cases minor amplification products of other sizes were observed as faint bands in the agarose gel (Data not shown). However, these minor fragments were easily distinguished from the major 390 bp fragment amplified from the nitrogen-fixing strains.

The ability of the dB261/dB260 primer set to detect *nifD* sequences from a wide range of nitrogen-fixing bacteria was further examined using the bacterial strains listed in Table 3-1. Of the 23 nitrogen-fixing bacteria tested, 21 yielded PCR products in the expected size range (390 bp; Figure 3-3A). One of the two

nitrogen-fixing bacteria which failed to yield a clear PCR product (*Enterobacter cloacae*) produced a low abundance PCR product in the expected size range. The other nitrogen-fixing bacterium which failed to yield a PCR product of the expected size was *Enterobacter* sp Eu F26.12 (Figure 3-3A). No PCR product of 390 bp was observed with any of the 9 non-nitrogen-fixing bacteria tested. *Pseudomonas fluorescens* was originally included as a non-nitrogen-fixing control strain. However, a low abundance fragment of the appropriate size was observed. A strain background check revealed that the lineage of this strain is unclear, and it is not clear if it is diazotrophic.

Characterization of PCR Fragments Using DNA Sequencing and Southern Hybridization Analysis

The identity of one of the PCR products was confirmed by DNA sequencing. The 390 bp PCR product from *Mesorhizobium loti* NZP2235 DNA was purified and sequenced as described in the Material and Methods. The dB261/dB260 primer set was used to determine the central portion of the DNA sequence. The translation of the derived DNA sequence revealed a polypeptide with high identity to the nitrogenase molybdenum-iron protein α chain (*nifD*)

sequences used to design the primers (Figure 3-1). Blast analysis of both nucleotide- and amino acid sequences confirmed the identity of the *nifD* fragment (Data not shown).

The identity of the 390 bp PCR product from the other strains was confirmed by Southern hybridization. The amplified DNA from the strains listed in Table 3-1 was electrophoresed on an agarose gel and transferred to a membrane, as described in Material and Methods. The purified PCR product from *Mezorhizobium loti* NZP2235 was labeled and used as a probe. The probe hybridized to the 390 bp PCR product amplified from all nitrogen-fixing bacteria tested (Figure 2-3 B) indicating that these fragments contain significant homology to *nifD* sequences. In addition, the probe hybridized to some PCR products outside the 390 bp range. This was only observed in the case of a few of the nitrogen-fixing bacteria. Very weak hybridization signals were observed in the lanes containing *Pseudomonas fluorescens* and *Enterobacter cloacae* DNA products. Longer exposure of the hybridization filters confirmed this result (Data not shown). The probe did not hybridize to *nifD*-PCR reactions

from non-nitrogen-fixing bacteria, nor to PCR products generated using REP-PCR primers (Data not shown).

Screening a Collection of Rice Associated Bacteria for the Presence of *nifD* DNA Sequences

A collection of 142 unidentified bacteria isolated from surface-sterilized rice tissue was screened for strain harboring *nifD* gene sequences using primers dB261/dB260. A PCR product of approximately 390 bp was reproducibly generated from twenty of these bacteria (Table 3-2). An example of this analysis is shown in Figure 3-4. The identity of the PCR products was confirmed using Southern blot hybridization, as described in Material and Methods (Data not shown). The *nifD* probe was found to hybridize to each fragment observed in the 390 bp range, confirming that these PCR products contain *nifD* sequences.

Confirming the Presence of *nif* Genes by Southern Hybridization of Total Genomic DNA

Genomic DNA for Southern analysis was isolated from 35 of the rice isolates. The 35 isolates were selected based on the observation that in initial screening done at IRRI they either produced ethylene in acetylene reduction assays (ARA) or contained *nifD* gene sequences

in PCR experiments (Chapter 2). Southern hybridization experiments were carried out using a probe containing the *nifHDK* region from *Azorhizobium caulinodans* ORS571, as described in Material and Methods. Hybridization signals were detected in 19 of the 35 isolates, as shown in Table 3-2. An example of the hybridization analysis is shown in Figure 3-5.

Acetylene Reduction Capacity as an Indicator of Nitrogen Fixing Activity

The ability of the above described 35 rice isolates to fix nitrogen was assessed using ARA. The results of this analysis are shown in Table 3-2. Isolates that failed to produce ethylene were scored as negative (-). The 19 isolates that produced, on average, more than 5 nmoles of ethylene and produced ethylene in all replicates tested were scored as positive (+). These 19 isolates were found to correspond to the same 19 shown to harbor *nif* genes by Southern hybridization. Isolates which produced some ethylene(> 6 nmole) in one or more experiment, but failed to produce ethylene in at least one replicate, were scored as positive/negative (+/-).

Direct Detection of Rice-Associated Nitrogen-Fixing Bacteria

The utility of primers dB261/dB260 to directly detect nitrogen-fixing bacteria associated with rice plants under laboratory and field conditions was examined. Sterile rice seedlings (IR42) were inoculated with previously identified nitrogen-fixing rice isolates R22(88), R100(64), and R90(8). After four weeks of growth, DNA template was prepared from infected and uninfected rice tissues as described in Material and Methods, and used for PCR with the dB261/dB260 primer set. A PCR product of 390 bp was observed in DNA from inoculated-, but not from uninoculated plants (Figure 3-6), suggesting the presence of bacteria harboring *nifD* gene sequences. In addition, field grown rice plants (IR72 and IR24) were examined for the presence of bacteria harboring *nifD* gene sequences. Tissue from roots and aerial portions of field grown plants were harvested and DNA was isolated as described in Material and Methods. A 390 bp PCR product was observed in DNA samples from both roots and aerial portions of both varieties (Figure 3-6). A 390 bp PCR product was also amplified from DNA isolated from field grown plants following vigorous shaking with glass beads, or shaking with glass beads and

surface sterilization (Data not shown). This indicates that the rice tissues indeed contain bacteria harboring *nifD* gene sequences.

DISCUSSION

nifD-PCR Primers for Rapid and Specific Identification of Diazotrophic Bacteria.

We have shown here that the *nifD*-PCR primer set, developed based on highly conserved regions of NifD, is useful for the rapid identification of diazotrophs in large collections of environmental microbial isolates. Use of the *nifD* primer set in PCR resulted in the generation of a ~390 bp fragment from the DNA of 21/23 diazotrophic test bacteria examined. DNA sequence and Southern hybridization analysis confirmed that the ~390 bp PCR products observed contain *nifD* gene sequences. From one of the diazotrophic bacteria tested, *E. cloacae,* only a minor PCR product of the appropriate size could be generated, showing a weak hybridization signal with the *nifD* probe. Lowering the annealing temperature of the PCR reaction from 52°C to 40°C increased the intensity of the 390 bp PCR products, including the fragment from *E. cloacae*, but also increased the number and intensity of background fragments, making it more difficult to distinguish diazotrophic and non-diazotrophic bacteria (Data not shown). The other nitrogen-fixing

bacteria, *Enterobacter* sp Eu F26.12, showed no PCR product at either annealing temperature or with Southern hybridization analysis.

PCR with the *nifD* primer set failed to generate distinct ~390 bp fragments from DNA of non-diazotrophic test bacteria, although faint fragments corresponding to minor PCR products of different sizes were observed in some cases. In the case of *P. fluorescens*, a minor PCR product of the expected size was observed, which shared homology with *nifD* sequences. This was surprising, since *P. fluorescens* has not been reported to carry *nif* genes. It is possible that the isolate used is a contaminate, or that in rare cases our PCR based identification protocol yields a false positive result.

When a large collection of bacteria isolated from surface-sterilized rice tissue was screened for diazotrophs using the *nifD* primer set, ~390 bp DNA fragments were generated from 20 of these isolates. The fragments hybridized to *nifD* sequences in all cases. Southern hybridization analysis of total DNA confirmed the presence of *nif* genes in the genome of 19 of these bacterial isolates. The same 19

bacterial isolates were able to reduce acetylene, indicating the production of an active nitrogenase enzyme complex.

The one isolate, K113, from which a ~390 bp fragment was generated, but in which no *nif* genes were detected or acetylene reduction observed, is similar to two other isolates, R100(64) and R90(8), both of which are positive in *nifD*-PCR, Southern hybridization, and ARA experiments. It is possible that K113 is a diazotrophic strain carrying *nif* genes that are different enough from the *nifHDK* genes of *A. caulinodans* used as a probe, that under the conditions employed, no hybridization signals could be observed, and that the culture conditions used for ARA experiments were not appropriate for this particular isolate. Alternatively, it is possible that K113 is not a diazotrophic strain and that the *nifD*-PCR product is an artifact. However, the fact that ≥95% of the isolates from which a ~390 bp fragment was generated were shown to be diazotrophs by Southern analysis and ARA clearly shows the utility of the *nifD* primer based PCR screening protocol.

The *nifD* Primer Set Rapidly Identifies Diazotrophic Bacteria in Large Collections of Environmental Isolates

The *nifD*-PCR primers were able to identify 19 diazotrophic bacteria from a collection of 142 bacteria isolated from surface-sterilized rice plants. Each of the 19 isolates that was identified by the *nifD*-PCR primers showed acetylene reduction activity and hybridization to *nif* genes in genomic Southern analyses. Ten bacterial isolates which showed acetylene reduction activity in preliminary experiments carried out at IRRI did not reduce acetylene in our tests, and four of the isolates which reduced acetylene during the screens at IRRI showed weak acetylene reduction in some of the replicate experiments at MSU. The characteristic ~ 390 bp fragment was not generated from DNA isolated from these bacteria, nor were *nifHDK* hybridization signals observed.

As pointed out above for the *E. cloacae* case, it is possible that these bacteria carry *nif* genes which have diverged to the point where no hybridization with the *nifHDK* gene from *A. caulinodans* would be observed under the conditions used and that, therefore, the *nifD*-PCR primers also do not anneal. It is also possible that these bacteria are able to fix nitrogen using a novel nitrogenase enzyme. Some isolates

formed dense pellicles when grown in a nitrogen free semi-solid medium, even in replicate tubes lacking acetylene reduction activity. This suggests that these bacteria are either good scavengers of traces of nitrogen or are nitrogen-fixing.

NifD-PCR Detects Novel Diazotrophs

The *nifD*-PCR primers were able to detect diazotrophs from highly diverse genera, include eleven genera of diazotrophic test bacteria and nine genera of bacteria isolated from surface-sterilized rice tissue.

nifD-PCR Directly Detects Diazotrophs in Environmental Samples

The *nifD*-PCR primers were also able to detect nitrogen-fixing bacteria directly in environmental samples, such as infected plant tissues. A PCR product of the expected size was detected in the total DNA of plants inoculated with diazotrophic bacteria, but not in DNA from aseptic, uninoculated plants. This suggests that these primers may be useful tools for tracking nitrogen-fixing bacteria during re-inoculation tests under laboratory conditions.

CONCLUSIONS

On the basis of the results presented above, we conclude that the *nifD* primers and PCR provide a quick and reliable method for identifying nitrogen-fixing bacteria from a wide range of genera. While other groups have designed PCR primers capable of amplifying *nif* genes from environmental samples, the utility of these primers for identifying nitrogen-fixing bacteria was not reported (Ueda et al., 1995a; 1995b). The *nifD*-PCR primers we developed permit the specific amplification of *nifD* from a variety of template types including purified DNA, colonies from plates, liquid culture and environmental samples, making them suitable for a variety of purposes. Because the *nifD*-PCR primers are able to amplify a specific fragment from a wide variety of diazotrophic bacteria, they are useful for rapid screening of large collections of isolates cultured from a specific ecological niche. In addition the *nifD*-PCR primers allow the detection of diazotrophs in environmental samples, without culturing bacteria.

An important aspect of increasing the amount biologically fixed nitrogen available to agricultural crops is the isolation and

identification of novel types of nitrogen-fixing bacteria. The ability of the *nifD*-PCR primers to rapidly and reliably identify nitrogen-fixing bacteria in a collection of uncharacterized isolates has been shown in this study. This will facilitate isolation of diazotrophic bacteria from a variety of environments, including the rhizoplane and internal tissues of crop plants. Isolation of diazotrophs from the internal tissues of crop plants will lead to a better understanding of the ecology of this niche and provide knowledge important for the long term goal of replacing industrial nitrogen fertilizer with biological nitrogen fixation.

TABLE 3-1

Strain	Nitrogen Fixing	*nifD* PCR Product
Agrobacterium rhizogenes 15834[1]	-	-
Agrobacterium tumefaciens C58[1]	-	-
Alcaligenes eutrophus[1]	-	-
Clavibacter michiganensis subsp *sepedonicus* SS#43[2]	-	-
Corynebacterium flaccumfaciens[1]	-	-
Escherichia coli DH5α[1]	-	-
Pseudomonas fluorescens[1]	Unknown	+/-
Pseudomonas syringae pv *tomato* ATCC 10862[2]	-	-
Xanthomonas campestris pv *campestris*[2]	-	-
Xanthomonas campestris pv *vesicatoria* ATCC 35937[2]	-	-
Anabaena sp strain PCC 7120[3]	+	+
Azoarcus indigens LMG9092[1]	+	+
Azorhizobium caulinodans ORS571[1]	+	+
Azospirillum brasilense Sp7[1]	+	+
Azotobacter DSM 2285[4]	+	+
Bradyrhizobium japonicum[1]	+	+
Enterobacter spEu F26.12[4]	+	-
Enterobacter cloacae[4]	+	+/-
Frankia CESI 5[5]	+	+
Herbaspirillum seropedicae Z67[4]	+	+
Klebsiella planticola KpM5A1[4]	+	+
Rhizobium leguminosarum bv *trifolii*[1]	+	+
Rhizobium leguminosarum bv *viciae* JIM 1402[1]	+	+
Rhizobium leguminosarum bv. *viciae* AVH1[1]	+	+
Mesorhizobium loti NZP 2037[1]	+	+
Mesorhizobium loti NZP 2235[1]	+	+
Sinorhizobium meliloti 1021[1]	+	+

TABLE 3-1 Continued

Sinorhizobium meliloti L5-30[1]	+	+
Rhizobium sp USDA31[1]	+	+
Rhizobium sp JRG2[1]	+	+
Rhizobium sp TAL380[1]	+	+
Rhizobium sp WBM13[1]	+	+
Rhizobium trifolii[1]	+	+

Table 3-1 Bacterial Strains and PCR Fragments Generated with the *nifD* Primer Set dB261/dB260.

List of bacteria used to test *nifD*-PCR primers. For Nitrogen Fixing a (+) indicates strain s reported to be diazotrophic and a (-) indicates strains not reported to be diazotrophic. For *nifD* PCR Product a(+) indicates the presence of a ~390 bp PCR product, a (-) indicates no ~390 bp PCR product, and a (+/-) indicates a faint ~390 bp PCR product in reactions containing that bacteria as a template. Source of bacteria: [1]Lab Collection, [2] Kind gift from Dr. Frank Louws, [3]Kind gift from Dr. Peter Wolk, [4]IRRI Collection, [5] Kind gift from Dr. Marcia Murry.

Table 3-2

Isolate	PCR	Southern	ARA
K46	-	-	-
R29(91)	-	-	-
R68(101)	-	-	-
R82(128)	-	-	-
R94b	-	-	-
R96(115)	-	-	-
S2	-	-	-
T14	-	-	-
T94	-	-	-
T96(a)	-	-	-
R75(50)	-	-	+/-
R92(60)	-	-	+/-
R95(7)	-	-	+/-
R97(106)	-	-	+/-
-R14(5)	-	-	+/-
K113	+	-	-
-R11c(59)	+	+	+
-R16(47)	+	+	+
K103	+	+	+
K107	+	+	+
R100(64)	+	+	+
R11c(74)	+	+	+
R22(88)	+	+	+
R48b	+	+	+
R62b(117)	+	+	+
R72(104)	+	+	+
R81(90)	+	+	+
R90(8)	+	+	+
R94(17)	+	+	+
R99(65)	+	+	+
T105	+	+	+
T60	+	+	+
T62	+	+	+
T90	+	+	+
T93	+	+	+

Table 3-2 Results of *nifD*-PCR, Southern Hybridization and ARA experiments for 35 Rice Isolates

Table showing results of *nifD*-PCR, Southern, and ARA analysis for 35 rice isolates. For *nifD*-PCR (-) indicates no amplification of a 390 bp DNA fragment, (+) indicates the presence of a 390 bp DNA fragment. For Southern analysis (-) indicates lack of hybridization with *nifHDK* from *A. caulinodans*, (+) indicates hybridization with *nifHDK* from *A. caulinodans*. For ARA (-) indicates no ethylene production, (+/-) indicates low levels of ethylene production in some but not all tubes tested, and (+) indicates higher levels of ethylene production in all tubes tested.

```
KLEBSIELLA PNEUMONIAE                          FGPIKDMAHI
ANABAENA SP. (STRAIN PCC 7120)                 WGPIKDMIHI
BRADYRHIZOBIUM JAPONICUM                       WGPIKDMVHI
RHIZOBIUM SP. COWPEA (STRAIN IRC78)            WGPIKDMVHI
AZOSPIRILLUM BRASILENSE                        WGPIKDMIHI
FRANKIA SP. (STRAIN ARL3)                      WGPVKDMVTI
MEZORHIZOBIUM LOTI NZP2235

SHGPAGCGQY SRAERRNYYT GVSGVDSFGT LNFTSDFQER DIVFGGDKKL
SHGPVGCGYW SWSGRRNYYV GVTGINSFGT MHFTSDFQER DIVFGGDKKL
SHGPVGCGQY SWGSRRNYYV GTTGIDSFVT LQFTSDFQEK DIVFGGDKKL
SHGPVGCGQY SWGSRRNYYV GTTGIDSFVT LQFTFDFREK DIVFGGDKKL
SHGPVGCGYY SWSGRRNYYV GDTGVDKLGT MHFTSDFQEK DIVFGGDKKL
SHGPVGCGQY SWATRRNYAH GHLGVDNFTA MQITTDFQEK DIVFGGDPKL
                 NYYV GTTGIDTFVT MQFTSDFQEK DIVFGGDKKL

SKLIEEMELL FPLTKGITIQ SECPVGLIGD DISAVANASS KAL.DKPVIP
TKLIEELDVL FPLNRGVSIQ SECPIGSIGD DIEAVAKKTS KQI.GKPVVP
DKILDEIQEL FPLNNGITIQ SECPVGLIGD DIEAVSRAKS KEYGGKTIVP
VKILDEIQEL FPLNNGITIQ SECPIGLIGD DIEAVSRAKS KEYGGKTIVP
HKVIEEINEL FPLVNGISIQ SECPIGLNGD DIEGVSKAKS EEL.GKPVVP
EQGLDEIVEL FPLAKGISVQ SECPIGLIGD DIEAVARVSS R..LDIPVMR
EKIIDEIEDL FPLSGGISVQ S

VRCEGFRGVS QSLGHHIAND
LRCEGFRGVS QSLGHHIAND
VRCEGFRGVS QSLGHHIAND
VRCEGFRGVS QSLGHHIAND
VRCEGFRGVS QSLGHHIAND
VRCEGFRGVS QSLGHHIAND
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Figure 3-1 Amino Acid Alignment of Mo-Fe Protein Sequences

Amino acid alignment of the translation products of *nifD* genes. The six sequences shown, along with eight others, were obtained from Medline and used to design degenerate oligonucleotide primers for *nifD*- PCR. The underlined amino acid sequences were used to design the primers. The translation of the DNA sequence of the *nifD*–PCR product from *Mezorhizobium loti* NZP 2235 is shown in bold.

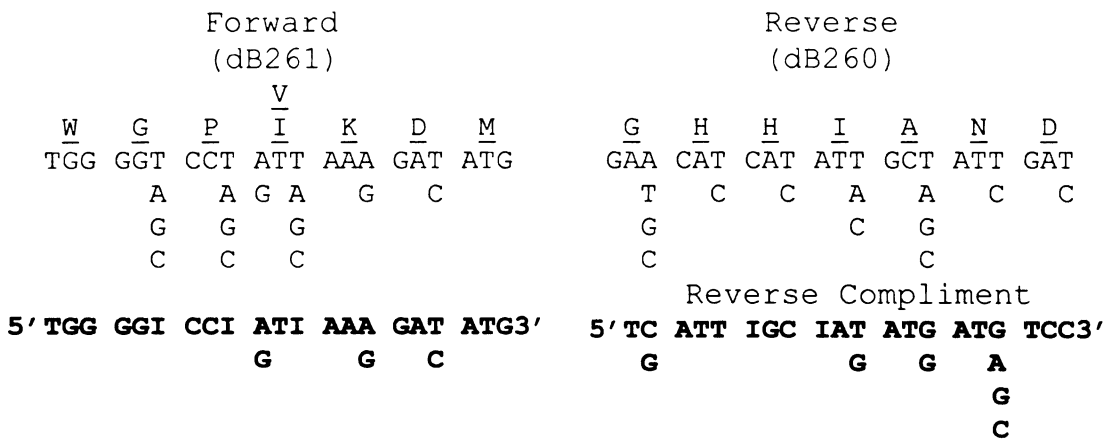


Figure 3-2 *nifD*-PCR Primers

Diagram of primer design for the *nifD* primers. The amino acid sequence used to design the primers is underlined. All possible nucleic acid codons are shown below each amino acid. The final primers are shown in bold with I representing inosine and nucleotides listed below showing degeneracy.

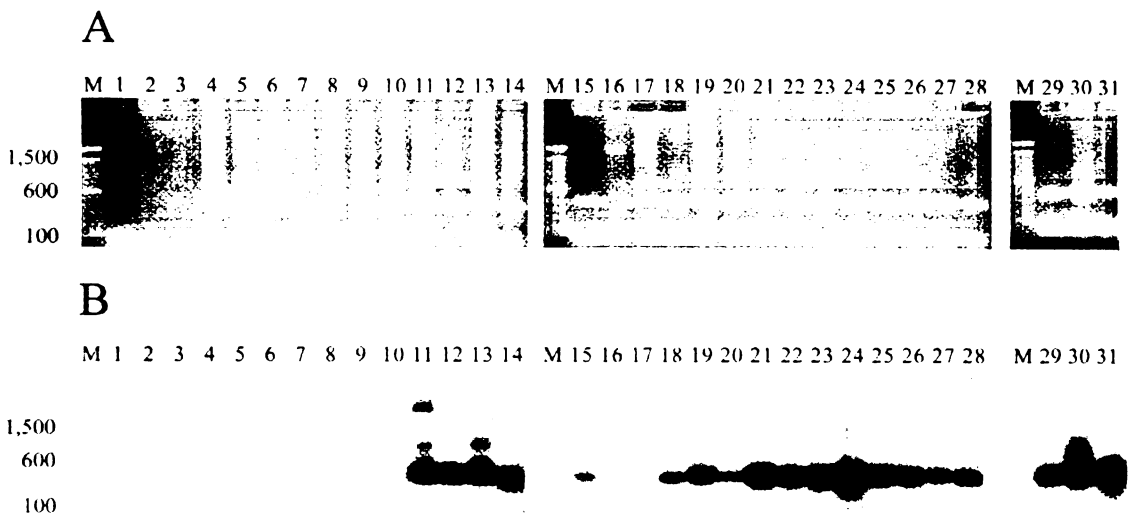


Figure 3-3 *nifD*-PCR Products Visualized by Agarose Gel Electrophoresis and Identified by Southern Hybridization

(A) Polymerase chain reaction (PCR) product generated with oligonucleotide primers corresponding to *nifD* genes (*nifD*-Primers) from liquid culture of known bacteria. A photograph of an ethidium bromide stained 2.0% agarose gel is shown. "M" indicates the size marker lanes (100 bp ladder from Boehringer Mannheim) The numbers above the lanes correspond to the following strains: 1, water; 2, *Agrobacterium rhizogenes* 15834; 3, *Agrobacterium tumefaciens* C58; 4, *Alcaligenes eutrophus*; 5, *Corynebacterium flaccumfaciens*; 6, *Clavibacter michiganensis* sub. sp. sepedonicus SS#43; 7, *Escherichia coli* DH5α; 8, *Pseudomonas fluorescens*; 9, *Pseudomonas syringae* pv. tomato ATCC 10862; 10, *Xanthomonas campestris* pv. vesicatoria ATCC 35937; 11, *Azoarcus indigens* LMG 9092; 12, *Azorhizobium caulinodans* ORS571; 13, *Azospirillum brasilense* sp7; 14, *Azotobacter* DSM 2285; 15, *Bradyrhizobium japonicum*; 16, *Enterobacter* spEu F26.12; 17, *Enterobacter cloacae*; 18, *Frankia* CESI 5; 19, *Herbaspirillum seropedicae* Z67; 20, *Klebsiella planticola* KpM5A1; 21, *Rhizobium leguminosarum* bv trifolii; 22, *Rhizobium leguminosarum* bv viciae JIM 1402; 23, *Rhizobium leguminosarum* bv viciae AVH1; 24, *Mesorhizobium loti* NZP2235; 25, *Sinorhizobium meliloti* 1021; 26, *Sinorhizobium meliloti* L5-30; 27, *Rhizobium* sp USDA31; 28, *Rhizobium* sp JRG2; 29, *Rhizobium* sp TAL380; 30, *Rhizobium* sp WBM13; 31, *Rhizobium trifolii*. (B) Southern blot of gel A with *nifD* probe verifying the identity of the PCR product.

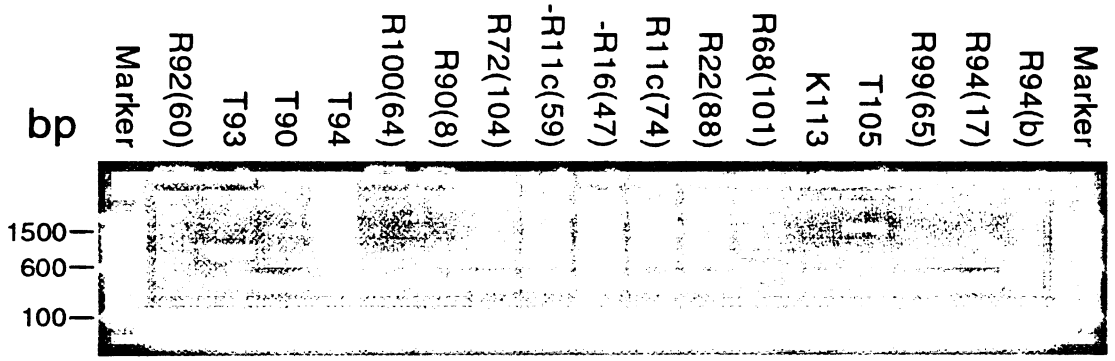


Figure 3-4 Agarose Gel Electrophoresis of *nifD*-PCR Products from Rice Isolates

Products of *nifD*-PCR performed on rice isolates. A ~390 bp fragment indicates the presence of the *nifD* gene in the bacterial genome. Products of sizes other than ~390 bp did not hybridize to *nifD* gene sequences in Southern analysis unless a prominent 390 bp band was also present (see Figure 3-3).

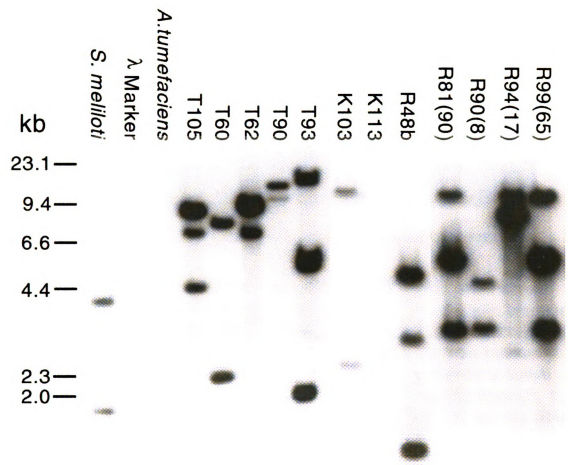


Figure 3-5 Southern Blot of Bacterial DNA Probed with *nifHDK*.

Low stringency Southern blot of bacterial DNA probed with the *nifHDK* region from *Azorhizobium caulinodans* ORS 571. Hybridization indicates the bacteria contain some of the genes necessary for nitrogen fixation. DNA from *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* C58 was included as positive and negative controls.

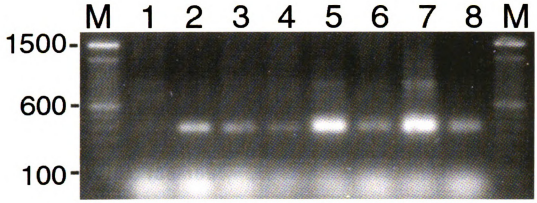


Figure 3-6 Agarose Gel Electrophoresis of *nifD*-PCR Products from Rice Tissues

Polymerase chain reaction (PCR) products generated with *nifD*-Primers and DNA isolated from rice tissue. A photograph of an ethidium bromide stained 2.0% agarose gel is shown on which the *nifD*-PCR products from DNA isolated from rice was separated. "M" indicates the size marker lanes (100 bp ladder from Boehringer Mannheim). The numbers above the lanes correspond to DNA isolated from the following rice tissues: 1, Variety IR42 grown gnotobiotically from sterilized seeds; 2, Variety IR42 grown gnotobiotically from sterilized seeds and inoculated with strain R22(88); 3, Variety IR42 grown gnotobiotically from sterilized seeds and inoculated with strain R00(64); 4, Variety IR42 grown gnotobiotically from sterilized seeds and inoculated with strain R90(8); 5, Roots of field grown variety IR72; 6, Stems and leaves of field grown variety IR72; 7, Roots of field grown variety IR24; 8, Stems of field grown variety IR24. All DNA was prepared as described in Material and Methods.

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CHAPTER 4

DIVERSITY OF A COLLECTION OF BACTERIA ISOLATED FROM SURFACE-STERILIZED RICE TISSUE

ABSTRACT

A collection of 142 bacteria isolated from surface-sterilized rice tissue was analyzed for its genetic diversity using amplified Ribosomal DNA restriction analysis (ARDRA) fingerprinting, rep-PCR genomic fingerprinting and small subunit (SSU) ribosomal RNA (rRNA) gene sequencing. Analysis of fingerprints using Gelcompar software revealed 71 unique rep-PCR genomic fingerprints and 56 unique ARDRA fingerprints. Clusters of similar combined fingerprints, consisting of 37, 15, 12, and 9 non-diazotrophic bacteria, as well as two clusters each containing 4 diazotrophic bacteria, were found. Analysis of SSU rRNA gene sequences from selected isolates in the collection revealed similarities to bacteria from the alpha-, beta-, and gamma subdivision of the Proteobacteria, and members of the Bacillaceae and Microbacteriaceae. Several of the bacteria analyzed displayed ARDRA fingerprints and/or contained SSU rRNA gene sequences similar to bacteria previously isolated from the rhizosphere of rice.

INTRODUCTION

The presence and role of endophytic diazotrophs in graminaceous plants has been the subject of several recent studies (James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998a; 1998b). The presence and genetic diversity of diazotrophs in the rhizosphere and rhizoplane of rice has also been investigated in some detail (Lakshmi Kumari et al., 1976; Watanabe and Barraquio, 1979; Thomas-Bauzon et al., 1982; Bally et al., 1983; Ladha et al., 1983; Watanabe et al., 1987; Oyaizu-Masuchi and Komagata et al., 1988; Ueda et al., 1995a; 1995b). However, the presence and genetic diversity of diazotrophic endophytes in field grown rice has remained relatively unevaluated (For a definition of "endophytes" see Chapter 1). In this chapter we extend our previous preliminary analysis of diazotrophic bacteria associated with rice by presenting a detailed molecular phylogenetic characterization of a collection of bacteria we isolated from surface-sterilized rice tissue (Chapter 2).

rep-PCR genomic fingerprinting was initially used to assess the genetic diversity of bacteria isolated from mechanically-abraded, surface-sterilized rice plants (Chapter 2). rep-PCR genomic fingerprints have been found to be extremely useful for distinguishing bacteria at the

subspecies- and strain- specific level, and phylogenetic relationships derived from such fingerprints are highly correlated to DNA-DNA homology levels (Vinuesa et al, 1998; Rademaker and de Bruijn, 1997; Rademaker et al., 1999). However, ARDRA fingerprints are clearly more suitable for determining the relationships between bacteria at higher taxonomic levels (Moyer et al., 1996; Rademaker and de Bruijn, 1997). Therefore, the genetic diversity of isolates in a previously described, large collection of putative rice endophytes (Stoltzfus et al., 1997; Chapter 2) was examined over a wider phylogenetic range by linearly combining ARDRA and rep-PCR fingerprints as described by Vinuesa et al. (1998). Similarities between selected isolates in this collection and previously characterized bacteria were examined by analyzing partial DNA sequences of the SSU rRNA genes. The combined analysis permitted a direct comparison with bacteria previously isolated from the rhizosphere of rice and endophytic bacteria isolated from other graminaceous plants.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions

E. coli HB101 cells harboring pUCNC were cultured at 37°C on LB media containing 100μg/l ampicillin. The other bacterial strains used in this study and their mode of isolation are described in Chapter 2. A complete list of the characteristics of the bacteria studied, including soil type, rice variety and rice tissue from which they were isolated, and the type of medium used for their isolation, is presented in Appendix I. These bacteria were cultured for DNA template preparation on TY media at 28°C (Beringer, 1974).

PCR Reactions

Generation of rep-PCR genomic fingerprints and computer analysis of the resulting fragment patterns were carried out as described by Louws et al. (1996), Rademaker and de Bruijn (1997) and Rademaker et al. (1998) with the following modifications: Template for PCR was prepared by filling a 1 μl disposable loop with cells from a bacterial colony grown on a plate. The contents of the loop were resuspended in 100 μl sterile distilled water in a 1.5 ml microfuge tube, boiled for 10 min, incubated at 37°C for 1 hour with 5 μg of RNAase A and stored at –20°C. One microliter of the resulting extract was used as template for PCR reactions.

ARDRA and SSU Sequencing

Two highly conserved eubacterial SSU rRNA gene primers (8F and 1492R; Weisburg et al., 1991) were used to amplify the SSU rRNA genes from the bacteria tested using ERIC-PCR amplification conditions (Rademaker and de Bruijn, 1997).

For ARDRA, 7.5 μl sterile distilled water, 1.5 μl digestion buffer (supplied by Boehringer Mannheim, Indianapolis, IN, USA), and 1 μl of enzyme were added to 5 μl of the PCR reaction and the mixture was incubated for 1.5 hours at 37°C for *Rsa*I and *Msp*I and 65°C for *Taq*I. Following digestion 2 μl of loading buffer was added and the digestion products were separated by overnight gel electrophoresis on a 2.0% agarose gel in 0.5x TAE Buffer and visualized on a UV transilluminator. Documentation and fragment pattern analysis were carried out as described by Louws et al. (1996), Rademaker and de Bruijn (1997), and Rademaker et al. (1998).

For DNA sequence analysis, the rDNA PCR amplification products were purified using ultra-Free columns (Millipore, Bedford, MA, USA) or Wizard PCR preps DNA purification systems (Promega, Madison, WI, USA). Partial SSU rRNA gene sequences were obtained using primers 8F and 519RB

(Weisburg et al., 1991). The DNA sequence was determined by automated fluorescent sequencing at the MSU DNA Sequencing Facility using the ABI Catalyst 800 for *Taq* cycle sequencing and the ABI 373A or ABI 377 Sequencer. The DNA sequences obtained were subjected to NCBI Blast Analysis (Altschul et al., 1997).

Sesbania rostrata Plant Growth and Inoculation

Growth and infection of *Sesbania rostrata* plants were carried out in sterile test tubes, as described by Pawlowski et al. (1987).

Southern Hybridization Analysis of Total Genomic DNA

Bacterial genomic DNA was isolated using the MasterPure Genomic DNA Purification Kit from Epicenter Technologies (Madison, WI, USA) according to the protocol provided by the manufacturer. DNA digestion, separation, and transfer to Hybond N nylon membranes (Amersham International, Little Chalfont, Buckinghamshire, England) were carried out according to standard protocols (Sambrook et al., 1989). A 390 bp DNA fragment amplified from the *A. caulinodans nifD* gene was purified using a Qiaex II Gel Extraction Kit from Qiagen (Madison, WI, USA) and used as a probe for *nifD*. A 395 bp DNA fragment containing *A. caulinodans nodC* gene sequence was excised from plasmid pUCNC (K. Goethals Ph.D. thesis,

Universiteit Gent) by digestion with *Hinc*III, run on an agarose gel and purified using a Qiaex II Gel Extraction Kit from Qiagen (Madison, WI, USA). Probe preparation was carried out according to standard protocols using ^{32}P labeled dATP. Prehybridization and hybridization reactions were carried out at 65°C using buffer containing 5x Denhardts solution, 4x SSC, 1% SDS, and 100 μg/ml salmon sperm DNA (Sambrook et al., 1989). The membranes were washed three times at low stringency (65°C in buffer containing 4x SSC and 0.1% SDS).

Computer Assisted Pattern Analyses

Gelcompar 4.1 software (Applied Maths, Kortrijk, Belgium) was used to analyze linear combined data from *Rsa*I, *Msp*I, and *Taq*I ARDRA digests; ERIC-, REP-, and BOX- PCR reactions; or all six fingerprints, as described by Rademaker et al. (1998). Cluster analysis was performed using a densitometric curve based UPGMA algorithm (Sneath and Sokal, 1973).

RESULTS

Phylogenetic Diversity of Bacteria Isolated from Surface-sterilized Rice Plants

Two collections of bacteria isolated from surface-sterilized rice tissues, as described in Chapter 2 (Figure 2-1 and 2-2), were initially included in this analysis. However, when tested using Gram staining, the bacteria from the second collection (Figure 2-2) were found to include a high percentage of Gram-positive bacteria. The SSU rRNA gene sequence from two of these bacteria was determined and found to be similar to sequences from *Bacillus*. Because many Gram-positive bacteria form spores which could be resistant to our surface-sterilization protocol, the decision was made not to study these isolates in detail. Rather, we focused on studying the genetic diversity of a collection of 142 bacteria from the first collection (Figure 2-1) using combined ARDRA and rep-PCR fingerprints in conjunction with Gelcompar computer assisted pattern analysis. The results from the analysis of the linearly combined *Rsa*I, *Msp*I and *Taq*I ARDRA, and ERIC-, REP- and BOX-PCR genomic fingerprints are shown in Figure 4-1. This analysis revealed both the presence of clusters of similar bacteria, as well as a variety of bacteria with unique fingerprints, as discussed below.

Since the triple ARDRA and rep-PCR fingerprint samples were generated on multiple independent gels, two internal controls were included in the combined pattern analysis. *Azorhizobium caulinodans* was included in each ARDRA and rep-PCR experiment and used as a control for variations in conditions between different PCR reactions, digestions, and gel electrophoretic runs. In addition, the DNA size marker lanes from each gel were included as controls to observe the variation between gels. The red line in Figure 4-1 represents the level of variation between *A. caulinodans* fingerprints derived from different reactions and run on different gels. The variations in a cluster of 23 DNA size marker lanes is indicated by the letter M in Figure 4-1. Interestingly, we found that the combined fingerprint patterns of four of the rice isolates closely matched the *A. caulinodans* "internal control" strains ("F"; Figure 4-1).

Gelcompar analysis, in conjunction with visual inspection of original fingerprints of the 142 rice isolates in this collection, revealed 56 unique combined *Rsa*I, *Msp*I and *Taq*I ARDRA fingerprints and 71 unique combined ERIC-, REP- and BOX-PCR genomic fingerprints. Unique fingerprints were defined as those exhibiting less similarity than the seven replicate control fingerprints of *A. caulinodans*. Fingerprint patterns exhibiting more

similarity than the replicate *A. caulinodans* fingerprints in the Gelcompar analysis but which showed clear differences in fragment patterns by visual inspection were also counted as unique.

Eight clusters were identified that contained four or more isolates with combined ARDRA and rep-PCR fingerprints more similar to each other than the combined fingerprints of replicates of *A. caulinodans* ("A-H"; Figure 4-1 and Table 4-1). These clusters are described in more detail below.

SSU rRNA Gene Sequence Similarities

A partial SSU rRNA gene DNA sequence was determined for those bacteria in the collection of rice isolates that had previously been shown to be able to reduce acetylene, or to contain *nifD* DNA sequences by PCR analysis (Chapters 2 and 3) as well as for selected bacteria from the largest fingerprint clusters ("A-H"; Figure 4-1). These DNA sequences were compared to sequences in the GenBank, EMBL, DDBJ, and PDB databases using NCBI Blast analysis (Altschul et al., 1997). The bacteria from this collection were found to have SSU rRNA genes sharing similarities with 15 different genera of bacteria (Table 4-2).

Characterization of Clusters of Bacteria with Similar Fingerprints

Cluster analysis of combined ARDRA and rep-PCR fingerprints using Gelcompar software revealed the presence of groups of similar bacteria which were isolated from different rice varieties grown in different soil types. The clusters of bacteria with fingerprints with a higher similarity level than the replicate fingerprints of *A. caulinodans* are shown in Table 4-1, which also summarizes the number of soil types, rice varieties, and soil type/rice variety combinations from which the strains were isolated. Groups A, B, E and G exemplify isolates with similar ARDRA fingerprints that have a single dominant rep-PCR genomic fingerprint pattern (Table 4-1; Figures 4-1, 4-2, 4-3, 4-6). For bacteria in these groups the genotype of the strain may be important for interactions with rice. Groups C, D, F and H exemplify isolates with similar ARDRA fingerprints but distinct rep-PCR genomic fingerprints (Table 4-1; Figure 4-1, 4-4 , 4-5, 4-7). For these strains the genotype of the strain may not be as important in interactions with rice.

Characterization of Clusters Containing Non-Diazotrophic Bacteria

The largest group ("A"; Table 4-1; Figures 4-1, 4-2) was found to contain 37 bacteria isolated from five different soil types and six different rice

varieties, representing 11 soil type/rice variety combinations. None of the bacteria in this cluster were found to be diazotrophic by either ARA or *nifD*-PCR analysis (Chapter 3). These bacteria display combined ARDRA fingerprints highly similar to the combined ARDRA fingerprint for *Pseudomonas fluorescence*. The only difference between the fingerprints is a 100 bp shift in one fragment from the *Rsa*I digest. The DNA sequence for the SSU rRNA genes from bacteria in this cluster are most similar to those from *Pseudomonas rhodesiae* and *P. marginalis* (Table 4-2). Within this group, three distinct combined rep-PCR genomic fingerprints were observed. Thirty two isolates displayed the same rep-PCR genomic fingerprint, four isolates a second rep-PCR genomic fingerprint, and one isolate a unique rep-PCR genomic fingerprint. However, the rep-PCR genomic fingerprint of *P. fluorescence* is clearly distinct from the fingerprints of all 37 rice isolates. This data indicates that the bacteria in this group are related to the fluorescent pseudomonads. However, more taxonomic data will be required to determine a more precise phylogenetic position of these isolates.

Group B (Table 4-1; Figure 4-1, 4-3) was found to contain 15 bacteria isolated from three different soil types and three different rice varieties,

representing four soil type/rice variety combinations. None of the bacteria in this cluster were found to be diazotrophic by either ARA or *nifD*-PCR analysis (Chapter 3). The ARDRA fingerprints for the 15 bacteria in this cluster are highly similar. However, the fingerprints of two of the bacteria display a minor difference from the fingerprints of the other 13. In these two fingerprints a 900 bp fragment is present in the *Rsa*I fingerprints and a of a 400 bp fragment is absent in the *Msp*I fingerprints. The ARDRA fingerprints of the bacteria in this cluster are very similar to the ARDRA fingerprints of *Klebsiella planticola* and *Enterobacter cloacae*. The ARDRA fingerprint of *K. planticola* differs from the ARDRA fingerprints of the 13 rice isolates only by the absence of a 400 bp fragment in the *Msp*I fingerprint and differs from the ARDRA fingerprints of the two rice isolates only by the absence of a 900 bp *Rsa*I fragment. The ARDRA fingerprint of *E. cloacae* differs from the ARDRA fingerprints of the 13 rice isolates only by the absence of a 400 bp *Msp*I fragment and the presence of a 600 bp fragment in the *Taq*I digest. It differs from the ARDRA fingerprints of the two rice isolates only by the absence of a 900 bp *Rsa*I fragment and the presence of a 600 bp *Taq*I fragment. The partial SSU rRNA gene DNA sequence from R95(7), a bacteria in the group of 13 rice isolates, is most similar to *Pantoea* spp.

and *Enterobacter dissolvens,* both belonging to the Enterobacteriaceae. Of the isolates in group B that display identical ARDRA fingerprints, ten display one rep-PCR genomic fingerprint pattern and three a second rep-PCR genomic fingerprint pattern. The two isolates with a distinct ARDRA fingerprint also have a distinct rep-PCR genomic fingerprint. The rep-PCR genomic fingerprints for *K. planticola* and *E. cloacae* are distinct from each other and all of the isolates in this group. This suggests that the bacteria in this group are related to Enterobacteriaceae. However, more multiphasic taxonomic data will be required to determine a more precise phylogenetic position in this family.

Group C (Table 4-1; Figures 4-1, 4-4) was found to contain 12 isolates from four soil types and five rice varieties, representing nine soil type/rice variety combinations (Figure 4-4). Ten of the isolates in this group have identical ARDRA fingerprints, as explained below. None of the bacteria in this cluster were found to be diazotrophic by ARA or *nifD*-PCR analysis (Chapter 3). When analyzed using Gelcompar, only eight of the isolates in this group displayed combined fingerprints with higher similarity than the replicate fingerprints of *A. caulinodans.* However, visual inspection of the original gels revealed that the ARDRA fingerprints of strains K9 and

K46 were identical to the ARDRA fingerprints of the eight isolates in this cluster. Apparently, uneven staining of the gels caused the loss of a fragment during computer analysis which resulted in less similar fingerprints. Thus strains K9 and K46 are considered as part of the cluster with the eight other isolates. The ARDRA fingerprint of K96 differs from that of these 10 bacteria only by the absence of a 700 bp fragment in the *Taq*I digest. The ARDRA fingerprint for K6 differs from the ARDRA fingerprints of these 10 isolates by the absence of a 300 bp fragment and the presence of a 100 bp fragment in the *Msp*I fingerprint. Generally, the isolates in this cluster have very diverse rep-PCR genomic fingerprint patterns. Only two of the ten isolates with identical ARDRA fingerprints were found to have identical rep-PCR genomic fingerprint patterns (Figure 4-4). The partial sequence of the SSU ribosomal RNA gene from two isolates in this group, T14 and K46, are most similar to the SSU rRNA gene DNA sequence of *Aureobacterium kitamiense*, a polysaccharide producing *Microbacterium* isolated from the wastewater of a beet sugar factory (Table 4-2).

Group D (Table 4-1; Figures 4-1, 3-5) was found to contain nine bacteria isolated from five soil types and three rice varieties, representing seven

soil type/rice variety combinations. None of the bacteria in this cluster were found to be diazotrophic by ARA or *nifD*-PCR analysis (Chapter 3). Minor variations in the ARDRA fingerprints were observed in this cluster. Five isolates displayed a major 900 bp fragment, while the other four isolates contained minor fragments of both 900 bp and 700 bp in their *Taq*I fingerprints. The isolates in this group display three distinct rep-PCR genomic fingerprints. Visual inspection of the fingerprints revealed two strains, R97(106) and R97b(34), with ARDRA fingerprints highly similar to ARDRA fingerprints of the other isolates in this cluster. However, these strains were not included in this cluster by Gelcompar analysis. The partial SSU rRNA gene DNA sequence for two isolates in this cluster, strains R75(50) and R97(106), were most similar to SSU rRNA gene DNA sequence of *Alcaligenes* and *Bordetella*, both members of the family Alcaligenaceae.

Characterization of Clusters Containing Diazotrophic Bacteria

Gelcompar analysis of the combined ARDRA and rep-PCR fingerprints revealed only two groups (containing more than two isolates) of diazotrophic bacteria with identical ARDRA fingerprints. These two groups are described below.

The first group of diazotrophic isolates (Group E) was found to contain four strains isolated from two soil types and two rice varieties, representing two soil type/rice variety combinations (Table 4-1; Figures 4-1, 4-6). All bacteria in this group were found to reduce acetylene and harbor *nif* genes using PCR and Southern blot analysis (Chapter 3). The combined ARDRA and rep-PCR fingerprints of these bacteria were identical. The partial DNA sequences of the SSU rRNA gene of isolates in this group were most similar to the SSU rRNA gene DNA sequence of *Burkholderia* (Table 4-2). The ARDRA fingerprints for these isolates share many fragments with the ARDRA fingerprint for M130, a putative endophytic *Burkholderia* sp. isolated from rice in Brazil (Boddey et al., 1995; Hartman et al., 1995). This suggests that the bacteria in this cluster belong to the genus *Burkholderia*.

The second group of diazotrophic bacteria (Group F) was found to contain four bacteria isolated from three soil types and three rice varieties, representing four soil type/rice variety combinations (Table 4-1; Figure 4-1, 4-7). All bacteria in this group were found to reduce acetylene and harbor *nif* genes using PCR and Southern blot analysis (Chapter 3). These bacteria were shown to have ARDRA fingerprints

identical to those of *A. caulinodans* (Figure 4-7). The complete DNA sequence of the R94(17) SSU rRNA gene was determined and found to be more than 99% identical to the DNA sequence of the *A. caulinodans* SSU rRNA gene (Table 4-2). *A. caulinodans* forms root and stem nodules on the tropical legume *Sesbania rostrata*. However, isolates R81(90), R94(17) and R99(65) were unable to nodulate *S. rostrata* when inoculated to plants (Data not shown).

The relationship between isolates in Group F and *A. caulinodans* was further investigated using restriction fragment length polymorphism (RFLP) analysis. No polymorphisms were found between R94(17) and *A. caulinodans* genomic DNA digested with *Bam*HI, *Hind*III, or *Eco*RI and hybridized with the *A. caulinodans nifD* gene. Likewise, genomic DNA from strains R90(8) and R99(65) digested with *Bam*HI and *Hind*III and hybridized with the *A. caulinodans nifD* gene showed RFLP patterns similar to those of *A. caulinodans*. However, polymorphisms between these two stains and *A. caulinodans* were detected when genomic DNA was digested with *Eco*RI and hybridized with the *A. caulinodans nifD* gene (Figure 4-8). No hybridization signal was detected with the *A. caulinodans nodC* gene in Southern hybridization analysis of genomic DNA from R81(90), R94(17)

and R99(65) (Figure 4-8). The fourth bacterium in the cluster, R62b(117) was not examined for nodulation properties or for hybridization with *nodC*. However, hybridization to genomic DNA from R62b(117) was detected using *nifHDK* from *A. caulinodans* (Chapter 3). This suggests that the bacterial isolates in this group are very closely related to, but distinct from, *A. caulinodans*.

DISCUSSION

Genetic Diversity of Putative Endophytic Bacteria

Bacteria isolated from mechanically-abraded, surface-sterilized rice roots displayed diverse ARDRA and rep-PCR fingerprints. 71 unique rep-PCR genomic- and 56 unique ARDRA fingerprints were observed among the 142 isolates studied (Figure 4-1). Both gram-positive and gram-negative bacteria were represented in the collection of rice isolates analyzed (Table 4-2). While it has not been rigorously shown that these bacteria are true endophytes (See definition in Chapter 1; Chapter 6), these bacteria clearly appear to have an intimate interaction with rice plants. The genetic diversity of the isolates analyzed can be explained by several equally plausible explanations. It is possible that these bacteria are present in numerous unique ecological or physiological 'niches' associated with rice plants and the genetic diversity of bacteria simply reflects niche diversity. It is also possible that these bacteria are present in a few unique niches, and the genetic diversity of the bacteria indicates that a variety of bacteria can colonize those few niches.

Specificity of Rice Plant/Bacterial Interactions

The importance of plant and bacterial genotypes in plant colonization gives us insight into the specificity of these interactions. While the data

from our study does not contain the numbers needed for proof, it does indicates that in many cases the interactions we observed between rice plants and putative endophytes are non-specific. The rice plant genotype does not appear essential for colonization by specific bacteria in our collection. Every cluster containing four or more bacteria harbored isolates from several rice varieties and soil types (Table 4-1), indicating that each type of bacteria isolated is capable of interacting with a broad variety of rice genotypes. Likewise, in some cases the bacterial genotype does not appear essential for colonization of a specific rice genotype, since bacterial isolates with a similar SSU rDNA gene structure, but diverse genomic fingerprints, were found to colonize the same rice variety. Groups C and D both contain bacteria which were isolated from the same rice variety grown in the same soil type with identical ARDRA-, but diverse rep-PCR fingerprint patterns.

However, in selected cases, the bacterial genotype may be important for interactions with rice plants. Groups A, B, E and G all contain bacteria with a single prominent rep-PCR genomic fingerprint. This could indicate that for some types of bacteria a specific genotype confers an advantage when interacting with the rice plants.

SSU rRNA Gene DNA Sequence Analysis

The robust characterization of novel bacterial isolates requires combined data from multiple taxonomic approaches (Polyphasic taxonomy; Vandamme et al., 1996). However, the SSU rRNA gene DNA sequence provides a significant amount of phylogenetic information (Woese, 1987) and permits the preliminary identification of bacteria isolated from the environment (Amann et al., 1995). Partial SSU rRNA gene DNA sequences indicate that selected isolates from this collection are related to endophytes previously isolated from rice, bacteria previously isolated from the rhizosphere of rice and legume symbionts.

Similarities to Endophytic Bacteria

The SSU rRNA genes from several diazotrophic isolates in the collection are very similar to those of putative endophytic bacteria previously isolated from rice. Isolates from group E are diazotrophic and have SSU rRNA genes similar to those of *Burkholderia vietnamiensis* and M130. *B. vietnamiensis* was isolated from rice tissue and is the only nitrogen-fixing species of *Burkholderia* (Gillis et al., 1995). M130 is a putative endophytic *Burkholderia* isolated from rice in Brazil (Boddey et al., 1995; Hartman et al., 1995). The diazotrophic strain R48b has SSU rRNA genes similar to those of *Azoarcus* sp6a3, a diazotrophic endophyte of Kallar

grass (Hurek et al., 1997b). Some strains of *Azoarcus* are able to colonize rice endophytically (Hurek et al., 1994). While the isolation of *Azoarcus* bacteria from rice has not been previously reported in the literature, *Azoarcus*-like strains have been recently isolated from rice plants grown in Nepal (Barbara Reinhold-Hurek, personal communication). In addition a *nifH* sequence derived from DNA isolated from rice roots in Japan (Ueda et al., 1995b) has been found to be similar to *Azoarcus nifH* sequences (Hurek et al., 1997a). Our isolation of *Burkholderia*- and *Azoarcus* like bacteria in conjunction with information from previous studies clearly suggests that these bacteria colonize rice endophytically and should be targets for further studies in this area of research.

Interestingly, no bacteria with SSU rRNA genes similar to *Herbaspirillum* spp. were found in this collection. *Herbaspirillum* has been reported as a common diazotrophic endophyte of graminaceous plants, including rice (Baldani et al., 1986; James and Olivares, 1998). The absence of *Herbaspirillum*-like isolates from this collection does not prove that this type of bacteria are not present in Philippino rice varieties, but it does suggest that they are not present in high numbers.

Similarities to Rice Rhizosphere Bacteria

Some diazotrophic bacteria in this collection have SSU rRNA genes similar to those of *Xanthomonas flavus*, *Pseudomonas* and *Azospirillum*, diazotrophic bacteria commonly isolated from the rhizosphere or rice (Ladha et al., 1982; Bally et al., 1983; Watanabe et al., 1987; Oyaizu-Masuchi and Komagata, 1988; Reding et al., 1991). It is unclear if these bacteria are novel endophytic strains or simply rhizosphere bacteria that "escaped" surface sterilization.

Non-diazotrophic isolates in this collection also have SSU rRNA genes similar to those of diazotrophic bacteria isolated from the rhizosphere of rice. Isolates from the largest cluster, Group A, are non-diazotrophic and have SSU rRNA genes similar to those of the fluorescent pseudomonads. Nitrogen-fixing pseudomonads have been reported as common rice rhizosphere bacteria (Watanabe et al., 1987). Isolates in Group B, have SSU rRNA genes similar to those of the family Enterobacteriaceae. However, unlike the members of Enterobacteriaceae previously isolated from rice (Ladha et al., 1983), none of these isolates were diazotrophic. Isolates from group D have SSU rRNA genes similar to those of Alcaligenaceae. On member of Alcaligenaceae, *Alcaligenes faecalis* A15,

is a nitrogen-fixing strain isolated from surface-sterilized rice capable of colonizing rice plant epidermal cells (You and Zhou, 1988; Vermeiren et al., 1998). The role of these non-diazotrophic bacteria as endophytes and their importance for future studies in this area of research is unclear.

Similarities to Legume Symbionts

The isolates in group F have SSU rRNA gene sequences very similar to those of *A. caulinodans*, the unusual stem- and root nodulating symbiont of the tropical legume *Sesbania rostrata* (Dreyfus and Domergues, 1981). However, they clearly differ from *A. caulinodans* since they do not harbor the *nodC* gene and are unable to form nitrogen-fixing nodules on *S. rostrata*. To our knowledge, this is the first report of the isolation of *Azorhizobium*-like bacteria from field grown rice. However, previous studies have shown that *A. caulinodans* is capable of colonizing rice under laboratory conditions (Christiansen-Weniger, 1996; Webster et al., 1997; Khokhar and Qureshi, 1998). Moreover, *A. caulinodans* is thought to be a promising candidate for supplying rice with biologically fixed nitrogen since, unlike other rhizobia, it is able to fix nitrogen independent of its legume host (Gebhard et al., 1984) and can tolerate relatively high levels of O_2 during nitrogen fixation (Ratet et al., 1989). If the isolates in group F share such characteristics with *A. caulinodans* they are excellent

candidates for further studies on the use of bacteria to supply biologically fixed nitrogen to rice.

In addition to the isolates sharing similarities with *Azorhizobium*, ten isolates in this collection have SSU rRNA genes similar to those of *Rhizobium, Bradyrhizobium* , and *Agrobacterium* species. Five of these isolates are diazotrophic, including two with SSU rRNA genes similar to those from *Agrobacterium*. *Agrobacterium* is closely related to *Rhizobium*, and when *nif* and *nod* genes from *Rhizobium* are introduced into *Agrobacterium* it can interact with legumes and fix nitrogen (Hirsch et al., 1985; Martinez et al., 1987; Novikova and Safronova, 1992). As discussed below, the interactions between these bacteria and rice plants deserve further investigation.

Several authors have suggested extending nodulation to rice as a method of supplying rice with biologically fixed nitrogen (Ladha et al., 1997; Reddy et al., 1997; Kennedy et al., 1997). When examining this possibility, the ability of *Rhizobium* and related species to interact with rice is of keen interest. For example, *Rhizobium leguminosarum* has been isolated from surface-sterilized rice grown in Egypt and shown to colonize

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rice grown under gnotobiotic conditions (Yanni et al., 1997). However, the production of nitrogen-fixing nodules on legumes is a complex process requiring the development of specialized structures by the plant host (van Rhijn and Vanderleden, 1995; Long, 1989), a process that clearly does not take place in the interaction between rhizobia and rice (de Bruijn et al., 1995; Reddy et al., 1997 Webster et al., 1997;). Therefore, further investigations of the interactions between putative rhizobial endophytes from rice, which may be well adapted for rice colonization, are clearly needed and will provide information useful for future attempts to extend nodulation to rice and other cereal crops.

Other SSU rRNA Gene Similarities

Two Isolates from Group C have SSU ribosomal DNA gene sequences similar to those of *Aureobacterium kitamiense*. Other members of the genus *Aureobacterium* have been redefined as the genus *Microbacterium* (Takeuchi and Hatano, 1998), However, *A. kitamiense* has never been formally renamed. *A. kitamiense* is a polysaccharide producing bacterium isolated from the wastewater of a beet sugar factory (Table 4-2). To our knowledge, there are no other reports of *Microbacterium* isolated from the rhizosphere or tissues of rice.

The partial SSU rRNA gene DNA sequences from the other isolates which were analyzed show similarity to *Bacillus*, *Comamonas* , *Brenvundimonas* and *Caulobacter*. Since *Bacillus* spp. form spores, it is quite likely that this isolate was protected from surface sterilization due to spore formation rather than endophytic colonization. These strains are not of lesser interest as candidates for nitrogen-fixing endophytes because they do not fix nitrogen nor were they isolated from different rice varieties and soil types.

Obligate and Facultative Endophytes

It has been proposed that endophytic bacteria should be divided into obligate and facultative endophytes (Baldani et al., 1997). Facultative endophytes can survive in the soil and on the plant surface as well as in the interior of the plant. *Azospirillum* strains capable of colonizing the interior of the plant could be considered facultative endophytes because they also are commonly found in the rhizosphere (Baldani et al., 1997). However bacteria such as *Herbaspirillum* spp. and *Burkholderia* spp. seem to be found only inside plant tissues and could be considered to be obligate endophytes. Assuming that bacteria isolated from mechanically-abraded, surface-sterilized rice plants are capable of colonizing the

interior of the plant to some extent, the bacteria in this collection can be divided into obligate and facultative endophytes.

Bacteria in Groups A and B, the largest groups in the collection, as well as isolates with partial SSU rRNA sequences similar to those of *Azospirillum* spp. and *Xanthobacter* spp. should be tentatively classified as facultative endophytes, since Pseudomonads, Enterobacteriaceae, *Azospirillum* spp. and *Xanthobacter* spp. are commonly isolated from the rhizosphere of rice (Bally et al., 1983; Ladha et al., 1983; Watanabe et al., 1987; Oyaizu-Masuchi and Komagata, 1988; Reding et al., 1991).

Bacteria in Groups C, D, E, and those with SSU rRNA genes similar to those of *Azoarcus* spp. should be tentatively classified as obligate endophytes, since *Microbacterium* spp., *Alcaligenes* spp., *Burkholderia* spp. and *Azoarcus* spp. have not been isolated as common rhizosphere bacteria from rice. However, many of the common nitrogen-fixing isolates from the rhizosphere of rice have not been classified (Bally et al., 1983; Oyaizu-Masuchi and Komagata, 1988). Additional evidence that some of these isolates may be obligate endophytes comes from the observations that *Azoarcus* spp. have never been isolated from soil

(Reinhold-Hurek and Hurek, 1998a) and that *Burkholderia* spp. appear to require a living plant host for survival (Baldani et al., 1997). Investigation of bacteria from these groups may be most fruitful for increasing our understanding of rice-endophyte interactions.

Bacteria from Group F and those with partial SSU rRNA gene sequences similar to those of rhizobia and *Agrobacterium* spp. could be classified either as facultative- or obligate endophytes. These bacteria have not been reported as common isolates from the rhizosphere of rice. However, it is known that rhizobia and *Agrobacterium* spp. can survive in the soil. Better classification and careful study of colonization patterns are needed to determine what type of endophytic characteristics these bacteria display.

Pathogenic and Endophytic Bacteria

The difference between pathogen and endophyte is often quite subtle (James and Olivares, 1998). It is possible for two very similar bacteria to have drastically different interactions with plants. Different subspecies of *Clavibacter xyli*, distinct species of *Herbaspirillum*, and *Agrobacterium* spp./*Rhizobium* spp. are examples of bacteria having endophytic/symbiotic interactions with plants in some cases and

pathogenic interactions in others. In some cases the type of interaction may vary depending on the plant genotype or environmental conditions. Because of this, great care must be taken when proposing the use of endophytic bacteria as inoculum for supplying crops with biologically fixed nitrogen. Bacteria such as *Azoarcus* spp. or *Azorhizobium* spp., which have no close relatives known to cause plant disease, may be more suitable for these approaches than *Burkholderia* spp. or *Herbaspirillum* spp. which include phytopathogenic isolates.

CONCLUSIONS

This study shows that genetically diverse bacteria can be isolated from mechanically-abraded, surface-sterilized rice tissue. It reveals that many of these bacteria are similar to bacteria commonly isolated from the rhizosphere of rice, but others are not. It also demonstrates that a variety of nitrogen-fixing bacteria can be isolated from this niche. Some of these nitrogen-fixing bacteria, specifically those similar to *Azoarcus* spp., and *Azorhizobium* spp., are not commonly found in the rhizosphere of rice, can colonize rice under laboratory conditions, are not closely related to known plant pathogens, and have been isolated from surface-sterilized rice tissue at multiple sites. Further study of these bacteria will increase our understanding of plant-microbe interactions and may provide

new insights into the possibility of using nitrogen-fixing endophytes to supply rice with biologically fixed nitrogen.

Group	Strains	Soil Types	Rice Var.	Comb.	ARDRA	rep-PCR
A	37	5	6	11	1	3(32,4,1)
B	15	3	3	4	2(13,2)	3(10,3,2)
C	12	4	5	9	3(10,1x2)	11(2,1x10)
D	9	5	3	7	2(5,4)	3(3,3,3)
E	4	2	2	2	1	1
F	4	3	3	4	1	3(2,1x2)
G	4	2	1	2	1	1
H	4	3	4	4	1	4(1x4)

Table 4-1 Clusters of Isolates with Similar Combined Fingerprints

This table contains information on the eight clusters containing four or more isolates with combined fingerprints more similar than the replicate fingerprints of *A. caulinodans*. The columns contain the following information: Groups, the letter of the group shown in Figure 4-1; Strains, the number of strains in each group; Soil Types, the number of soil types in which plants containing these strains where grown; Rice Var., the number of rice varieties from which these strains were isolated; Comb., the number of soil type/rice variety combinations from which these strains were isolated; ARDRA, the number of unique ARDRA fingerprints present the cluster; rep-PCR, the number of unique rep-PCR genomic fingerprints present the cluster. The numbers in parenthesis indicate the number of strains within each cluster displaying unique fingerprints.

Table 4-2

Strain	Genus	Species	Red.	Bases	Identical/Total	% Identity
Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae						
R95(7)	*Pantoea*	sp.	2	424	394/398	98
	Enterobacter	*dissolvens*	2	424	394/398	98
Bacteria; Proteobacteria; alpha subdivision; Rhizobiaceae group; Rhizobiaceae						
K113	*Agrobacterium*	*vitis*	2	447	443/446	99
	Agrobacterium	*vitis*	2	447	442/446	99
R100(64)*	*Agrobacterium*	*vitis*	3	448	434/448	96
	Agrobacterium	*vitis*	3	448	434/448	96
R90(8)*	*Agrobacterium*	*vitis*	2	395	374/389	96
	Agrobacterium	*vitis*	2	395	374/389	96
R68(101)	Unidentified α proteobacterium		2	392	355/375	94
	Agrobacterium	*stellulatum*	2	392	355/375	94
Bacteria; Proteobacteria; alpha subdivision; Ancylobacter group						
R94(17)*	*Azorhizobium*	*caulinodans*	3	1447	1433/1435	99
	Azorhizobium	*caulinodans*	3	1447	1443/1448	99
R99(65)*	*Azorhizobium*	*caulinodans*	1	355	353/353	100
	Azorhizobium	*caulinodans*	1	355	353/353	100
R81(90)*	*Azorhizobium*	*caulinodans*	2	450	438/440	99
	Azorhizobium	*caulinodans*	2	450	428/429	99

Table 4-2 Continued

Strain	Genus	Species	Red.	Bases	Identical/Total	% Identity
Bacteria; Proteobacteria; alpha subdivision; Ancylobacter group						
T62*	α proteobacterium	TV6-2b	2	437	415/418	99
	Xanthobacter	*flavus*	2	437	405/407	99
T105*	α proteobacterium		2	427	409/410	99
	Xanthobacter	*flavus*	2	427	402/403	99
Bacteria; Proteobacteria; alpha subdivision; Bradyrhizobium group						
T90*	*Bradyrhizobium*	*sp*	2	442	428/428	100
	Bradyrhizobium	*japonicum*	2	442	427/428	99
T93*	*Bradyrhizobium*	*lupini*	2	447	445/445	100
	Bradyrhizobium	*japonicum*	2	447	445/445	100
T94	*Bradyrhizobium*	*japonicum*	1	371	370/371	99
	Bradyrhizobium	*lupini*	1	371	370/371	99
Bacteria; Proteobacteria; alpha subdivision; Rhodospirillaceae						
K103*	*Azospirillum*	*parooensis*	2	439	427/441	96
	Azospirillum	*lipoferum*	2	439	394/427	92
R72(104)*	*Azospirillum*	*lipoferum*	2	440	414/424	97
	Azospirillum	*lipoferum*	2	440	410/424	96

Table 4-2 Continued

Strain	Genus	Species	Red.	Bases	Identical/Total	% Identity
Bacteria; Proteobacteria; alpha subdivision; Rhizobiaceae group; Rhizobiaceae						
K107*	*Rhizobium*	*leguminosarum*	1	497	470/498	94
	Rhizobium	*tropici*	1	497	456/485	94
R92(60)	*Rhizobium*	*etli*	2	447	424/446	95
	Rhizobium	sp	2	447	393/406	96
R94b	*Rhizobium*	sp	2	447	392/407	96
	Rhizobium	*gallicum*	2	447	422/447	94
Bacteria; Proteobacteria; alpha subdivision;						
S2	*Brevundimonas*	sp.	1	338	330/339	97
	Caulobacter	*intermedius*	1	338	330/339	97
Bacteria; Proteobacteria; beta subdivision; Burkholderia group						
R22(88)*	*Burkholderia*	sp	3	482	475/476	99
	Burkholderia	*cepacia*	3	482	474/476	99
-R11c(59)*	*Burkholderia*	*vietnamiensis*	1	258	258/258	100
	Burkholderia	*vietnamiensis*	1	258	258/258	100
R11c(74)*	*Burkholderia*	*cepacia*	1	456	440/448	98
	Burkholderia	*vietnamiensis*	1	456	440/448	98

Table 4-2 Continued

Strain	Genus	Species	Red.	Bases	Identical/Total	% Identity
Bacteria; Proteobacteria; beta subdivision; Alcaligenaceae						
R75(50)	*Alcaligenes*	sp.	1	454	444/450	98
	Alcaligenes	*xylosoxidans*	1	454	446/454	98
R97(106)	*Bordetella*	sp.	1	373	330/333	99
	Alcaligenes	sp.	1	373	330/333	99
Bacteria; Proteobacteria; beta subdivision; Rhodocyclus group						
R48b*	uncultured bacterium SJA-52		2	482	480/483	99
	Azoarcus	sp. 6a3	2	482	450/450	100
Bacteria; Proteobacteria; beta subdivision; Comamonadaceae						
R96(115)	Unidentified β proteobacterium		1	479	472/479	98
	Comamonas	sp.	1	479	462/466	99
Bacteria; Firmicutes; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae						
T14	*Aureobacterium*	*kitamiense*	2	483	475/479	99
	Microbacterium	*laevaniformans*	2	483	464/473	98
K46	*Aureobacterium*	*kitamiense*	1	434	423/434	97
	Aureobacterium	*kitamiense*	1	434	423/434	97

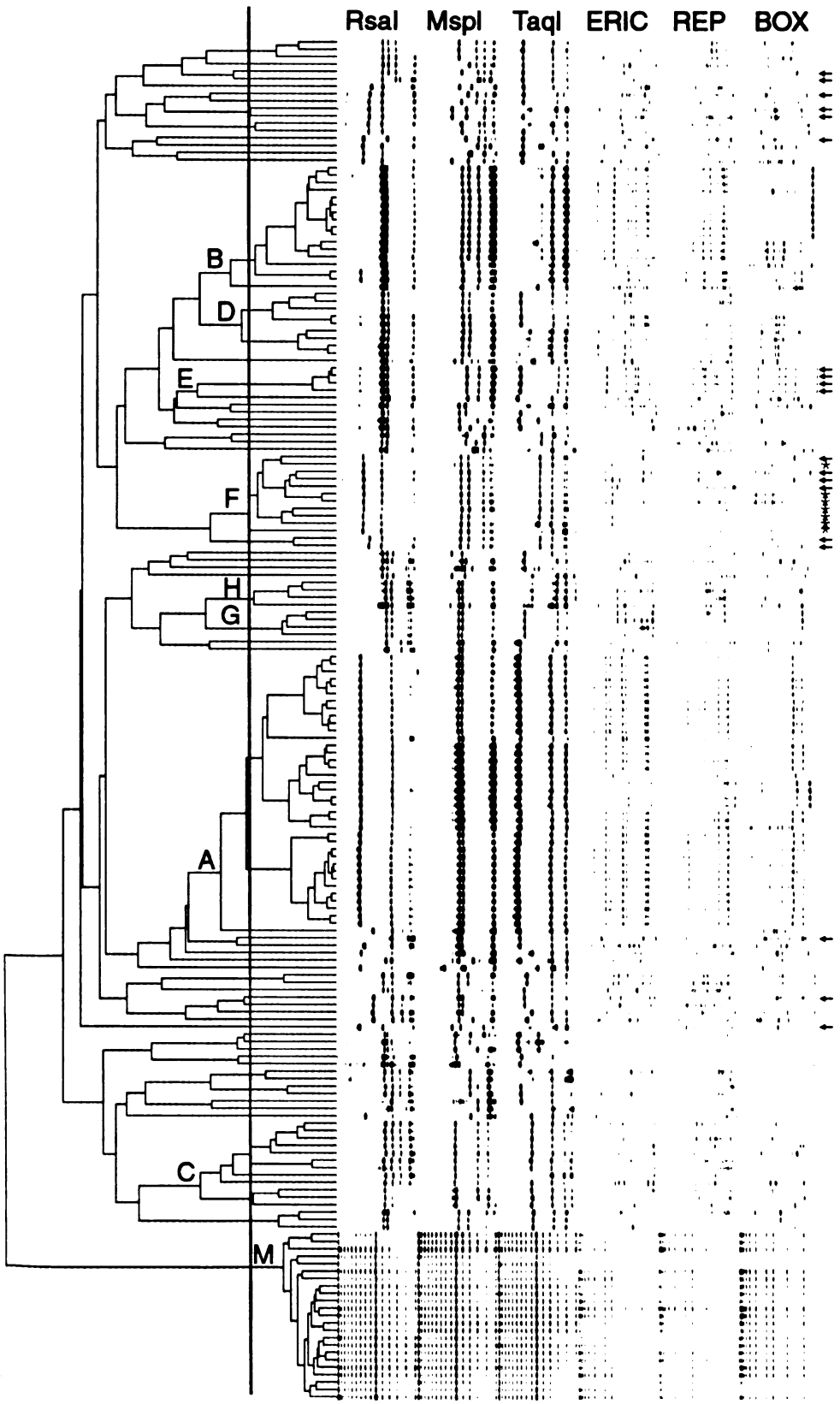
nucleotide/the number of nucleotides compared; and %, the percentage of identical nucleotides. The taxonomy of each group of similar isolates is shown above that group.

Table 4-2 Continued

Strain	Genus	Species	Red.	Bases	Identical/Total	% Identity
Bacteria; Proteobacteria; gamma subdivision; Pseudomonas group; Pseudomonas						
R33(120)	*Pseudomonas*	*marginalis*	2	474	473/474	99
	Pseudomonas	*rhodesiae*	2	474	472/474	99
R45(42)	*Pseudomonas*	*marginalis*	2	475	474/475	99
	Pseudomonas	*rhodesiae*	2	475	473/475	99
-R6a(126)	*Pseudomonas*	*marginalis*	2	475	474/475	99
	Pseudomonas	*rhodesiae*	2	475	473/475	99
-R14(5)	*Pseudomonas*	*rhodesiae*	1	292	284/292	97
	Pseudomonas	*marginalis*	1	292	284/292	97
T60*	*Pseudomonas*	sp. B13	3	487	478/489	97
	Pseudomonas	*stutzeri*	3	487	457/464	98
Bacteria; Firmicutes; Bacillus/Clostridium group; Bacillaceae						
R29(91)	*Bacillus*	*pumilus*	1	496	470/475	98
	Bacillus	*pumilus*	1	496	478/495	96

Table 4-2 SSU rRNA Gene Sequence Similarities

Table showing bacteria from the database with SSU rRNA gene sequences most similar to the SSU rRNA gene sequences from rice isolates. The table lists; strain, the rice isolate number; genus and species, the genus and species of bacteria from the database; Red., the number of times the SSU rRNA gene was sequenced for each isolate; Bases, the number of nucleotides in the sequence for each isolate; I/T, the number of identical nucleotide/the number of nucleotides compared; and %, the percentage of identical nucleotides. The taxonomy of each group of similar isolates is shown above that group.


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Figure 4-1 Dendrogram Showing Genetic Diversity of Rice Isolates

This dendrogram was created using Gelcompar to show clusters of similar isolates based on linearly combined *Rsa*I, *Msp*I and *Taq*I ARDRA fingerprints and ERIC-, REP-, and BOX-PCR genomic fingerprints of 142 rice isolates, 12 soil/rhizosphere bacteria, and seven replicates of *A. caulinodans* (marked with a red asterisk). Also included as a reference are 23 marker lanes (M). The red line shows the level of similarity between the seven *A. caulinodans* fingerprints. Letters A-H indicate the groups listed in Table 4-1. The position of the letter indicates the branches shown in Figures 4-2 to 4-7, which include the fingerprints from previously isolated soil/rhizosphere bacteria.

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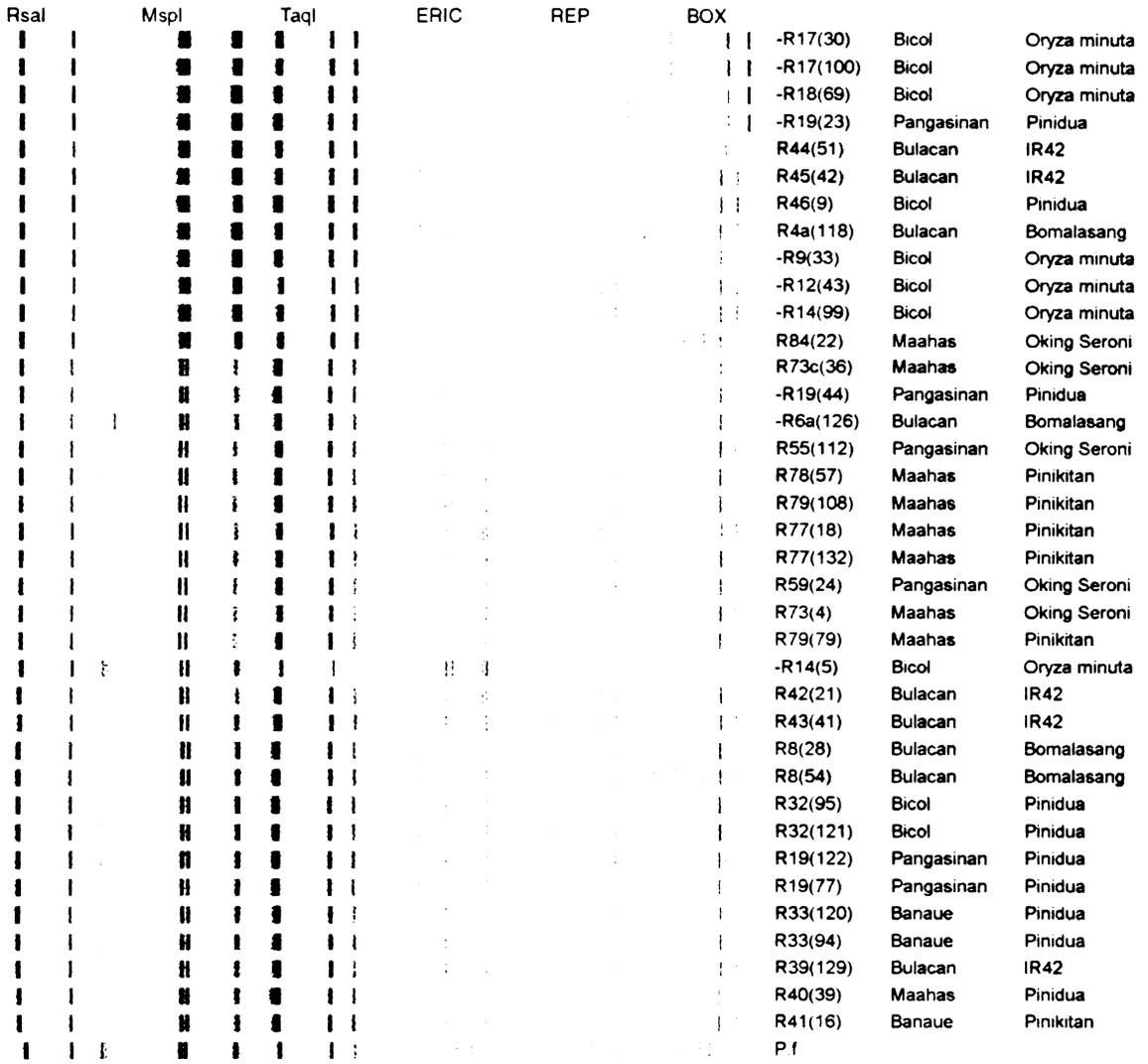


Figure 4-2 Combined Fingerprints for Group A isolates

Shown here are the combined ARDRA and rep-PCR fingerprints of the 37 isolates in Group A, plus the combined fingerprint of *Pseudomonas fluorescens* (P.f.). The strain designation, soil type and rice variety from which the strain was isolated are indicated in the three vertical columns.



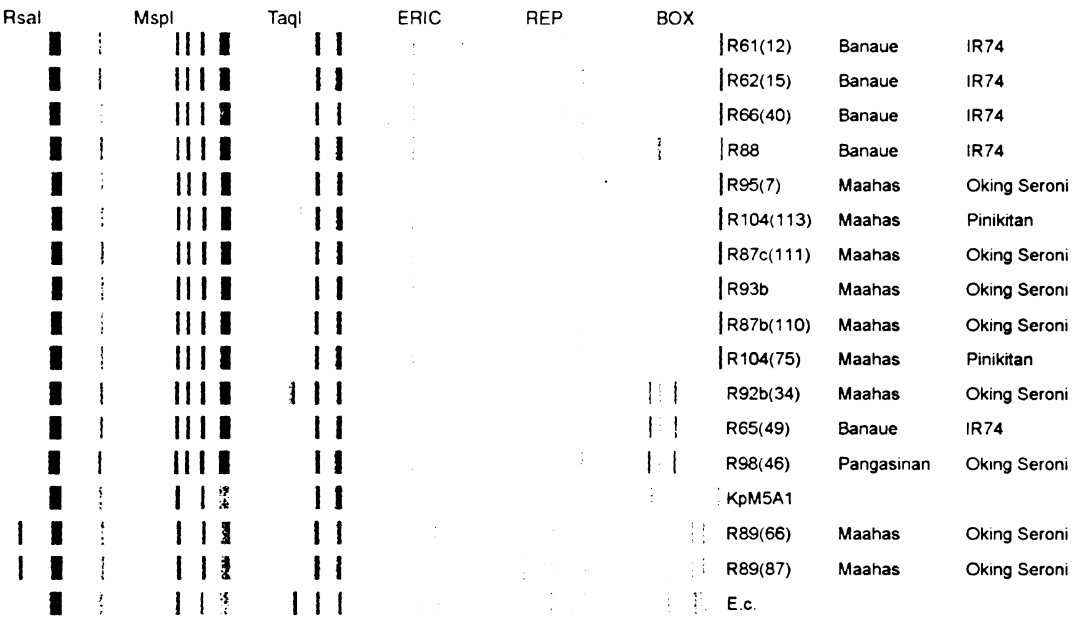


Figure 4-3 Combined Fingerprints for Group B isolates

Shown here are the combined ARDRA and rep-PCR fingerprints of isolates from group B, plus the combined fingerprints of *Klebsiella planticola* (KpM5A1) and *Enterobacter cloacae* (E.c.). The strain designation, soil type and rice variety from which the strain was isolated are indicated in the three vertical columns.

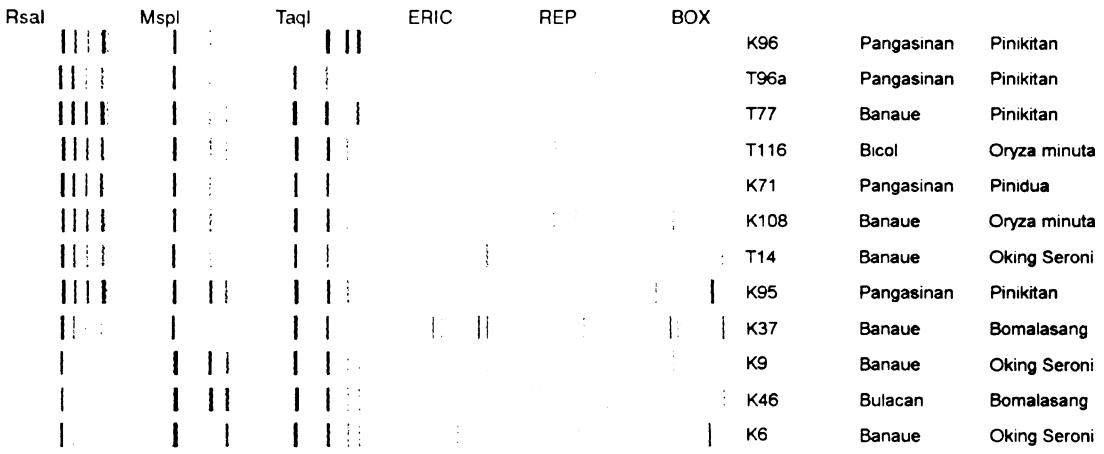


Figure 4-4 Combined Fingerprints for Group C Isolates

Shown here are the combined ARDRA and rep-PCR fingerprints of the isolates in group C. The strain designation, soil type and rice variety from which the strain was isolated are indicated in the three vertical columns.

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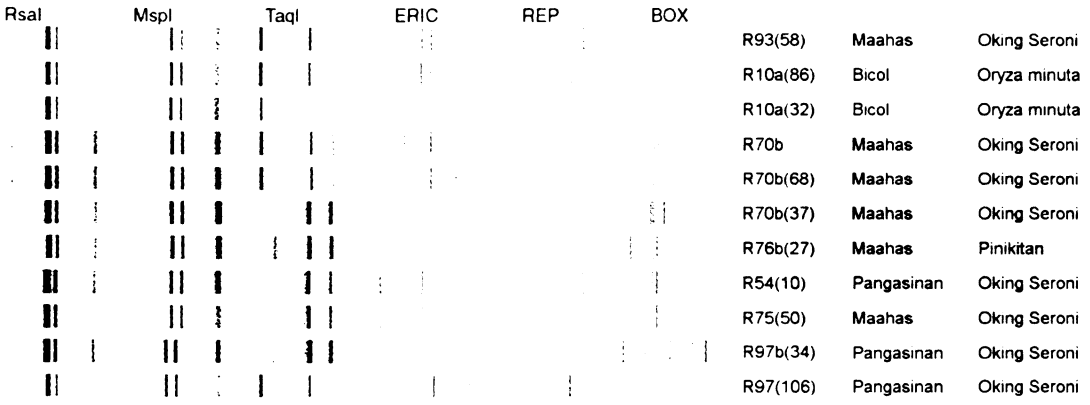


Figure 4-5 Combined Fingerprints for Group D Isolates

Shown here are the combined ARDRA and rep-PCR fingerprints of the 10 isolates in group D plus two isolates with similar ARDRA fingerprints (strains R97b(34) and R97(106). The strain designation, soil type and rice variety from which the strain was isolated are indicated in the three vertical columns.

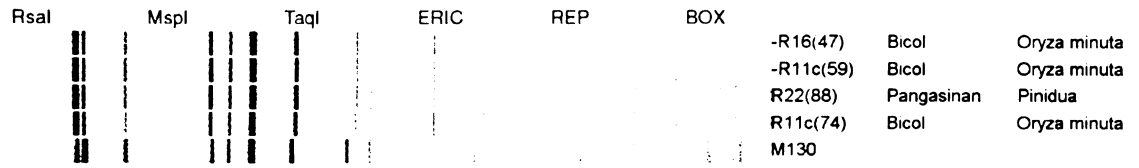


Figure 4-6 Combined Fingerprints for Isolates in Group E

Shown here are the combined ARDRA and rep-PCR fingerprints of the isolates in group E. The strain designation, soil type and rice variety from which the strain was isolated are indicated in the three vertical columns.

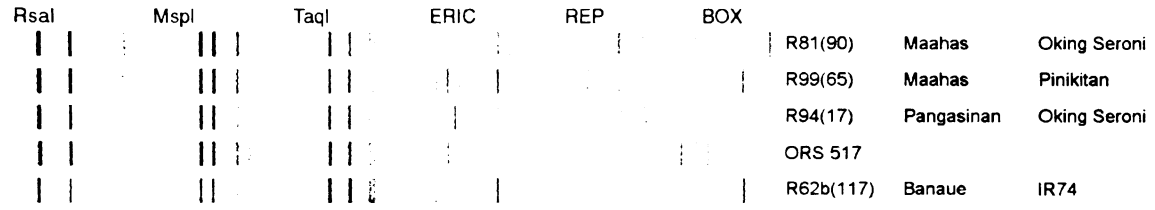


Figure 4-7 Isolate Fingerprints Similar to *Azorhizobium*

Shown here are the combine ARDRA and rep-PCR fingerprints of four isolates with ARDRA fingerprints identical to *Azorhizobium caulinodans* (ORS 571). The rep-PCR genomic fingerprints for these isolates contain many distinct fragments, indicating diverse genomic arrangements. The strain designation, soil type and rice variety from which the strain was isolated are indicated in the three vertical columns.

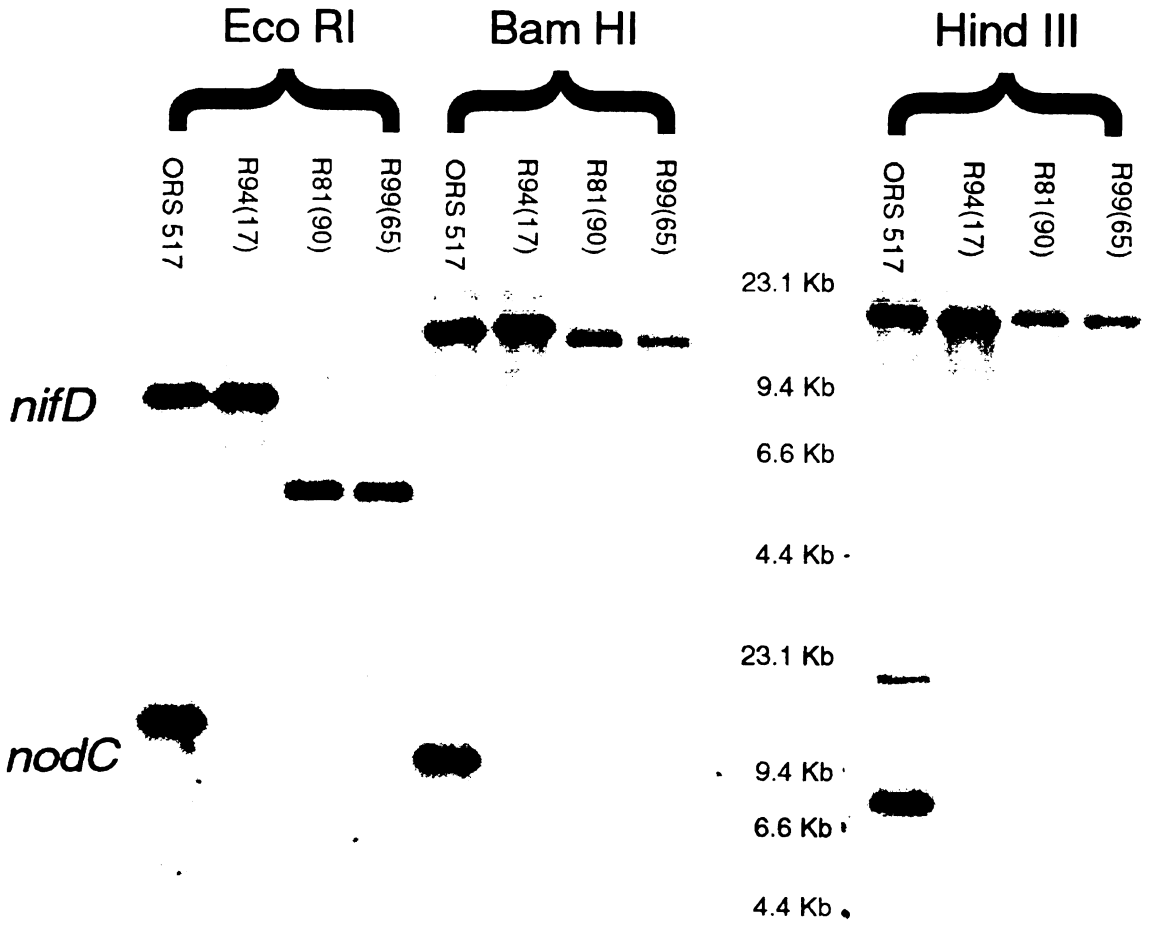


Figure 4-8 Southern Blots of Genomic DNA from Isolates Similar to *Azorhizobium caulinodans*

Shown here are Southern blots of genomic DNA from three isolates similar to *Azorhizobium caulinodans* (ORS 571). The genomic DNA was digested with the restriction enzyme *Eco*RI, *Bam*HI, or *Hind*III and hybridized with a *nifD* or *nodC* gene probe.

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CHAPTER 5

USING GFP AS A BIOMARKER FOR MICROBIAL ECOLOGY

INTRODUCTION

Since the cloning of the Green Fluorescent Protein (GFP) gene from the jellyfish *Aequorea victoria* (Prasher et al., 1992), and its expression in a variety of other organisms (Chalfie et al., 1994), GFP has rapidly become an important biomarker/bioreporter in a wide variety of eukaryotic and prokaryotic organisms. The utility of GFP as a bioreporter/biomarker lies mainly in the simplicity of the biochemical reaction generating fluorescence. GFP expression and fluorescence does not depend on the addition of co-factors or additional substrates, and only requires oxygen briefly for the auto-oxidation of GFP. When GFP is excited with light of the proper wavelength it autofluoresces. Visualization of this fluorescence can be used to monitor gene expression, to localize proteins, to isolate novel genes, or to track tagged cells (Cubitt et al., 1995; Gerdes and Kaether, 1996; Misteli and Spector, 1997). Because of these advantages, GFP is replacing some of the traditional biomarkers/bioreporters used in molecular microbial ecology (Akkermans

et al., 1995, 1996, 1998; Tombolini and Jansson, 1998; Unge et al., 1998; Jansson and de Bruijn, 1999).

GENETIC IMPROVEMENT OF GFP

Wild-Type GFP (wtGFP)

Wild-type GFP is a 27 kDa monomer consisting of 278 amino acid residues (Prasher et al., 1992). In *Aequorea victoria* GFP serves to convert blue chemiluminescence, generated by the protein aequorin, into green light (Prasher, 1995). This conversion is accomplished by means of an internal protein p-hydroxybenzylideneimidazolinone chromophore formed by the cyclization of three amino acid residues of GFP, Ser-Tyr-Gly, followed by the oxidation of the Tyr residue. These essential three residues are located at positions 65-67 of the GFP protein (Cody et al., 1993). The formation and activation of the GFP chromophore does not require additional enzymes or cofactors, and therefore heterologous expression of GFP in numerous organisms has been shown to result in fluorescence (Cubitt et al., 1995). The wtGFP absorbs light with an excitation maximum of 395 nm, with a second excitation peak at 470 nm. It fluoresces with an emission maximum of 510 nm (Morise et al., 1974; Ward et al., 1980).

Disadvantages of wtGFP

The excitation/emission characteristics of GFP have important implications for its use as a biomarker/bioreporter. The 470 nm excitation peak allows observation of wtGFP fluorescence with a standard fluorescein isothiocyanate (FITC) excitation-emission filter set, or with the 488 nm line of an argon laser, equipped with appropriate filters. This allows the detection of GFP using fluorescence-activated cell sorting (FACS), flow cytometry, epifluorescent microscopy and laser-scanning confocal microscopy (LSCM).

However, the fluorescent emission of the wtGFP when excited with higher wavelength light is less intense than its emission when excited with lower wavelength light. This renders detection of the low levels of fluorescence emitted from wtGFP expressed in some bacteria problematic. On the other hand, excitation of wtGFP with higher wavelength light causes less photobleaching than excitation with lower wavelength light, making higher wavelength light a better choice for exciting GFP (Chalfie et al., 1994). One possible reason for the low fluorescence emission levels observed when wtGFP is expressed in selected bacteria is that much of the wtGFP produced is found in inclusion bodies in an insoluble non-fluorescent form

(Heim et al., 1994). GFP derivations with better excitation/emission spectra and higher fluorescence levels, due in part to better solubility, have increased the utility of GFP as a biomarker/bioreporter.

IMPROVING GFP FLUORESCENCE

Background

GFP from the sea pansy *Renilla reniformis* contains the same chromophore as GFP from *Aequorea victoria*. However, the GFP from *R. reniformis* has a single excitation peak at 498 nm. This difference is presumably due to the difference in amino acid residues surrounding the chromophore in the mature protein (Ward, 1980). Neither the isolated chromophore, nor the denatured protein at neutral pH, are fluorescent (Cody et al., 1993). These data suggested that changing the protein environment around the chromophore of GFP could change the excitation/emission characteristics of GFP.

Mutants of GFP

A number of methods to generate GFP mutants with altered excitation-emission spectra and increased emission intensities have been used to increase the utility of GFP as a biomarker/bioreporter (Table 5-1). Heim et al. (1994) used hydroxylamine treatment and error-prone PCR to

generate mutant cDNAs encoding new versions of GFP. Colonies of *E. coli* harboring mutant cDNAs were screened for changes in emission color and brightness when excited with 375 vs 495 nm light. Five mutants were identified in this screen. Three of these mutants displayed significant alterations in excitation maxima, with little or no effect on emission spectra. These three mutants exhibited an increased fluorescence level (1.8 x higher). None of these three mutants contained changes in the chromophore-forming residues. The other two mutants both harbored substitutions at position 66, which forms part of the chromophore, and exhibited blue fluorescence. The excitation maxima of one of these mutants was increased.

Site-directed mutagenesis of the serine residue at position 65 yielded four mutants of GFP with single excitation peaks in the 470-490 nm range and an increased level of light emission intensity (≤6X; Heim et al., 1995). The S65T mutant GFP was found to fold four times faster than wtGFP, making it useful for applications requiring rapid detection of fluorescence following protein production. The faster folding may also partially explain the increased emission levels from the mutant protein.

Additional mutagenesis of primary GFP mutants yielded versions of GFP with multiple mutations and enhanced emission properties. Two variants had lower wavelength emission than wtGFP, making it possible to obtain three distinct color emissions. Fluorescence resonance energy transfer (FRET) between two version of GFP was also demonstrated (Heim and Tsien, 1996).

Delagrave et al. (1995) used optimized combinatorial mutagenesis of amino acid residues 64-69 to create mutations in the GFP chromophore. The mutant proteins were screened for GFP with an increased excitation maximum using Digital Imaging Spectroscopy (DIS). These mutant proteins were named Red-Shifted GFP (RSGFP). No comparison of their fluorescence intensity with wtGFP was carried out. Ehrig et al. (1995) identified two GFP mutants using a strain of *E. coli* deficient in DNA polymerase proofreading. One GFP mutant was not fluorescent when excited with 395 nm light; the other mutant was not fluorescent when excited with 470 nm light. Neither of the corresponding mutations mapped to the chromophore.

Cormack et al. (1996) used a codon-based mutagenesis scheme to mutate amino acid residues in positions 55-74 and a FACS machine to isolate red shifted GFP mutants with increased emission (≤30x wtGFP). They found that 90-100% of the mutant protein was soluble, which probably contributes to the increased emission level. The authors also suggested that the mutation may result in faster chromophore formation and increased 488 nm excitation. Using DNA shuffling, Crameri et al. (1996) isolated a non-redshift mutant of GFP that remains soluble and has emission levels 18 times that of wtGFP. These mutants have greatly increased the utility of GFP as a biomarker/bioreporter in bacterial systems.

Variants that change the stability of GFP in bacteria have also been developed. Andersen et al. (1998) added short peptide sequences to GFP that cause bacterial proteases to degrade the protein at different rates, generating GFP variants with short half lives. These mutant GFPs allow temporal gene expression studies.

Mutants of GFP designed to optimize expression in non-bacterial systems are also available. Many of these mutants have codon usage optimized

for a particular organism. GFP mutants have been developed for yeast (Cromack et al., 1997), plants (Sheen et al., 1995; Chiu et al., 1996; Haseloff et al., 1996; Rouwendal et al., 1997; Davis and Vierstra et al., 1998) and mammals (Zolotukhin et al., 1996; Anderson et al., 1996; Zhang et al., 1996; Yang et al., 1996; Siemering et al., 1996; Kimata et al., 1997; Stauber et al., 1998). Two of these mutants have been optimized for use at 37°C (Siemering et al., 1996; Kimata et al., 1997), which may be useful in bacterial species that grow at higher temperatures.

Therefore, it is now possible for researchers to select from a number of GFP variants with different excitation and emission maxima and fluorescence levels (Table 5-1). Patterson et al. (1997) compared wtGFP with four mutant versions of GFP and concluded each version has advantages and disadvantages, depending on the application.

GFP AS A BIOMARKER/BIOREPORTER IN BACTERIA: GENERAL CONSIDERATIONS

Advantages of GFP

A variety of systems are now available for use as biomarkers/bioreporters (Jansson and de Bruijn, 1999). GFP is easier to use than most of these

monitoring systems and has characteristics that allow it to perform novel bioreporter functions. The advantages described below have lead to the rapid adoption of GFP as a biomarker/bioreporter.

GFP production requires expression of a small single gene product independent of additional cofactors (Chalfie et al., 1992). This facilitates DNA manipulations and construction of vectors utilizing GFP as a biomarker/bioreporter. It also allows subcellular localization of a specific protein by fusing the protein with GFP. Because GFP requires no additional substrate for fluorescence (Chalfie et al., 1992), detection is basically non-invasive, requiring no pre-detection processing and allowing rapid and accurate *in vivo*/*in situ* analysis. It also allows detection of tagged bacteria regardless of the energy status of the cells (Tombolini et al., 1997). A wide variety of detection methods suitable for analysis of bacteria expressing GFP are available (see below). This allows optimization of experimental design to answer relevant biological questions without the compromises sometimes associated with biomarkers that require special detection protocols. The intense green fluorescence produced by GFP in most bacteria allows robust, accurate detection of the tagged bacteria in environmental samples (Unge et al.,

1998). In some cases the use of image enhancement software is needed to distinguish the fluorescence of tagged bacteria from soil and root autofluorescence (Gage et al., 1996; Tombolini et al., 1997).

Problems with GFP

Despite the many advantages of GFP, no biomarker/bioreporter is perfect. The major problems encountered when using GFP in bacteria are variable fluorescence, absence of detectable fluorescence, and/or toxicity of the protein itself. These problems vary from bacteria to bacteria and are often overcome by the use of mutant versions of GFP or different promoters and Ribosomal Binding Sites. However, several attempts may be needed to optimization fluorescence in new species or strains of bacteria.

Clonal populations of bacteria expressing GFP sometimes exhibit variable levels of fluorescence. For example, Burlage et al. (1996) observed broad variability in fluorescence of clonal populations of *Pseudomonas putida* tagged with wtGFP. Moreover, Matthysse et al. (1996) observed variable fluorescence in clonal populations of *E. coli* expressing GFPmut2 grown on agar plates.

Tombolini et al. (1997) and Olofsson et al. (1998) observed that populations of *E. coli* cells containing identical plasmids encoding wtGFP gave rise to a non-fluorescent subpopulation. The reasons for the observed variations in GFP fluorescence have not been elucidated.

At times, there is no detectable fluorescence in bacteria containing GFP, even when the *gfp* gene is controlled by a promoter known to be active in that bacterium. For example, Kremer et al. (1995) observed blue color in all *E. coli* cells harboring a plasmid with the *lacZ* gene controlled by a heat shock promoter (*hsp60*). However, when the *lacZ* gene in this plasmid was replaced with the *gfp* gene, no fluorescence was observed. Egener et al. (1998) observed fluorescence in *Azoarcus* sp BH72 harboring the gene encoding GFPmut2 under the control of the *nifH* promoter. However, they could not detect fluorescence in bacteria harboring the genes encoding wtGFP or PII expressed from the same promoter. We have observed similar results using the vector pRL765gfp (Tombolini et al., 1998) to tag uncharacterized soil bacteria. This vector harbors the *gfp* gene and the kanamycin resistance gene *npt*, under control of the same promoter, P*psbA*. Bacteria isolated from rice and transformed with this vector were resistant to kanamycin, but no fluorescence was

observed (Chapter 6). While the exact reasons for lack of detectable fluorescence have not been elucidated, the use of a mutant GFP with higher fluorescence levels may overcome this problem (Egener et al., 1998).

Toxicity of GFP can also be a problem when trying to obtain detectable fluorescence levels in bacteria. *E. coli* cells containing wtGFP have been reported to grow 2-3 times slower than cells expressing the cycle III mutant version of GFP (Crameri et al., 1996). Miller and Lindow(1997) have reported that *E. coli* cells harboring high copy-number plasmids strongly expressing GFPmut1 were prone to cell lysis. Evidence that GFP can be toxic in mammalian cells has also been presented (Hanazono et al., 1997).

Detection of GFP

The rapid adoption of GFP as a bioreporter/biomarker has been considerably enhanced by the development of a variety of techniques for detecting GFP. These methods include visualization of bacterial colonies on plates using a transilluminator, visualization of colonies or individual cells using an epifluorescent microscope or laser-scanning confocal microscope, visualization of colonization patterns by tagged bacteria on

plant tissue using fluorescence stereomicroscopy, counting fluorescent cells using flow cytometry or FACS, screening bacterial collections using fluorescent microtiter plate readers, and quanitation of fluorescence using spectrofluorimetry. Several reviews detailing these techniques are available (Unge et al., 1997; Khodjakov et al., 1997; Tombolini and Jansson, 1999). A filter set has been developed that optimizes detection of GFP (Zylka and Schnapp, 1996). More specialized techniques for observing GFP-mediated fluorescence have also been described. Scanning near-field optical/atomic force microscopy has been used for spatial localization of tagged bacteria in liquid (Tamiya et al., 1997) and video-endoscopy can be used to detect GFP expression *in situ* (Flotte et al., 1998). Specialized software can create three-dimensional images from optical sections made from GFP labeled samples using a laser scanning confocal microscope. Because GFP detection is non-invasive, real time and time-lapse videos can be used to track cells (or proteins) labeled with GFP (Gerdes and Kaether, 1996). Studies using different GFP mutants have been carried out using double and even triple labeling allowing simultaneous detection of multiple targets (Heim and Tsien, 1996; Yang et al., 1996; Lybarger et al., 1998; Yang et al., 1998).

Delivery and Maintenance of the *gfp* gene

The *gfp* gene can be delivered to bacteria using the same methods developed for use with other biomarkers/bioreporters, e.g. electroporation, conjugation and transduction. The *gfp* marker/reporter gene can be maintained in the cells on a plasmid or integrated into the bacterial chromosome, via transposons or homologous recombination. A comprehensive list of GFP vectors used as biomarkers/bioreporters and their delivery vehicles is presented in Table 5-2. A list of the bacterial species studied and the types of studies performed using these vectors is presented in Table 5-3.

GFP AS A BIOMARKER IN BACTERIA

Use of constitutive promoters or inducible promoters to express GFP in bacterial cells allows tracking of these cells in the environment and visualization of their location (see Unge et al., 1998). Constitutive expression can be obtained by cloning GFP into a vector containing a promoter known to be constitutively expressed in the bacterium of interest (Tombolini et al., 1997). Alternatively, constitutive expression can be obtained by random insertion of a promoterless GFP gene into the bacterial genome and screening for strains that remain fluorescent under a variety of physiological conditions (Burlage et al., 1996). Inducible

expression of GFP can be obtained using inducible promoters such as P*lac* or P*Φ10* (Christensen et al., 1996; Normander et al., 1998).

Tracking Bacteria in the Environment

Bacteria marked with GFP can be monitored for movement and survival in the environment. For example, Burlage et al. (1996) used a promoterless GFP transposon to obtain fluorescent *P. putida* strains and monitored their movement through columns of defined medium. They also performed similar experiments with *E. coli* cells harboring a plasmid containing the *gfp* gene under control of the inducible P*lac* promoter. In addition, Leff and Leff (1996) used *E. coli* cells expressing GFP constitutively to monitor survival of the tagged bacteria in stream water. Heuermann and Haas (1998) and Josenhans et al. (1998) used GFP as a fluorescent tag in *Helicobacter*. Tombolini et al. (1997) used a Tn*5* derivative transposon containing the *gfp* gene expressed from a constitutive promoter to tag *P. fluorescens* cells, and showed that the tagged cells could be detected by flow cytometry, "black light blue" lamp illumination, epifluorescent microcopy and laser-scanning confocal microscopy. In addition, dual tagging experiments using GFP and Lux allow simultaneous tracking of tagged bacteria cells and monitoring of their energy status (Unge et al., 1999).

GFP can also be used to monitor transfer of plasmids between bacteria. Christensen et al. (1996) used a plasmid containing GFP controlled by an inducible promoter, P*Φ10*, to monitor plasmid transfer on semi-solid surfaces. The donor strain of *P. putida* did not contain the T7 RNA polymerase gene therefore the T7 P*Φ10* promoter was inactive and the bacteria were not fluorescent. The recipient strain carried the T7 RNA polymerase gene integrated in its chromosome. The T7 RNA polymerase activates the P*Φ10* promoter and the bacteria harboring P*Φ10-gfp* are fluorescent. Thus, fluorescence resulted only when the plasmid was transferred to the recipient strain. Normander et al. (1998) used a similar system with GFP expression regulated by P*lac* to observe plasmid transfer in the phylloplane of bush bean and on polycarbonate filters.

Localizing Bacteria *in situ*

Bacteria marked with GFP can be visualized *in situ*, allowing identification of the niche they occupy. For example, Kremer et al. (1995) and Luo et al. (1996) followed infection of mice cells using *Mycobacterium bovis* marked with GFP. The infection of human macrophages and epithelial cells by *Mycobacterium avium* marked with GFP has also been observed (Parker and Bermudez et al. 1997). Valdivia et al. (1996) used GFP to visualize infection of live mammalian cells by *Salmonella typhimurium*,

Yersinia pseudotuberculosis and *Mycobacterium marinum*. Gage et al. (1996) used GFP to monitor early events in rhizobial infection and nodule formation on alfalfa using a *Sinorhizobium meliloti* isolate carrying a plasmid constitutively expressing GFP. Bloemberg et. al. (1997) used a plasmid-borne GFP to distinguish tagged *Pseudomonas aeruginosa* from untagged *Burkholderia cepacia* bacteria attached to an abiotic surface. They also used GFP to monitor *P. fluorescens* bacteria associated with the roots of tomato seedlings. The biocontrol strain *P. chlororaphis* MA 342, which controls fungal disease on cereal crops, was chromosomally tagged with two copies of the *gfp* gene to allow visualization of the pattern of colonization of the cells on barley seeds by fluorescence stereomicroscopy. GFP-marked *E. coli* and *Serratia marcescens* cells have also been used to study flocculation in activated sludge (Olofsson et al. 1998). We have also used bacteria marked with a transposon containing GFP to monitor the interactions between rice roots and endophytic bacteria (Chapter 6).

Monitoring Gene Expression in the Environment

Biomarking can be combined with bioreporting to obtain spatial and temporal information on gene expression *in situ*. For example the urea-inducible promoter from the *ureD* gene fused to the *gfp* gene has been

used to infer the location of *Proteus mirabilis* urease gene expression during infection of the mouse urinary tract (Zhao et al., 1998). A *nifH* promoter-*gfp* fusion has been used to monitor expression of nitrogen-fixing genes in *Azoarcus* sp. BH72 in soil and on rice roots (Egener et al., 1998). Moreover, Möller et al. (1998) used *P. putida* cells containing the *gfp* gene expressed from promoters controlling the expression of proteins involved in the biodegradation of toluene to visualize gene expression in flow chambers and observe the effects of various community members on gene induction.

GFP AS A BIOREPORTER IN BACTERIA

Localizing Protein Fusions

The fusion of GFP to a protein of interest and monitoring of the expression of the fusion *in vivo* permits cellular localization of the fusion protein (Cubitt et al., 1995; Gerdes and Kaether, 1996; Misteli and Spector, 1997; Jansson and de Bruijn, 1999). Although GFP loses its fluorescence when truncated (Cubitt et al., 1995), the full-length protein can be used successfully in both C-terminal and N-terminal protein fusions (Wang and Hazelrigg, 1994). A flexible linker may be required to maintain the function of both fused proteins (Glaser et al. 1997). In bacteria, GFP

has been used extensively as a bioreporter to study cell division in *E. coli* and *Bacillus subtilis.*

Ma et al. (1996) have created fusions of the cell division proteins FtsZ and FtsA to GFP and reported that both C-terminal and N-terminal fusions of FtsZ with GFP showed midcell localization and polymerization. However, the fusion proteins did not function properly in mediating cell division. C-terminal fusions of the FtsA protein with GFP also showed proper midcell localization. FtsZ and FtsA interactions have also been examined with GFP fusions in *Agrobacterium tumefaciens* and *S. meliloti* (Ma et al. 1997). Hale and de Boer (1997) used a C-terminal fusion of the ZipA protein with GFP to localize ZipA in a ring structure at the division site, but this fusion protein also failed to function properly in cell division. Raskin and de Boer (1997) found that C-terminal fusions of MinE with GFP were biologically active and formed a central ring in an FtsZ-independent manner. Yu et al. (1998) used truncations of FtsK fused to GFP to determine that as little as 15% of the FtsK protein's N-terminus is needed for proper targeting.

GFP has also been used to study proteins involved in septation during vegetative division and sporulation in *Bacillus*. Barak et al. (1996) reported that a SpoIIE-GFP fusion was localized to septum formation sites in *Bacillus megaterium*. However, one study using wtGFP in a SpoIIE-GFP fusion found reduced sporulation in *B. subtilis* (Aringoni et al., 1995). Edwards and Errington (1997) used a DivIV A-GFP fusion to visualize sites of old, nascent, and possibly future vegetative cell division in *B. subtilis*. Ju et al. (1997) used GFP fused to pro-σ^E to localize this fusion protein and determine in which compartment of *B. subtilis* pro-σ^E processing takes place. Wu et al. (1998) used the GFPmut1 variant to construct a SpoIIE-GFP fusion. This fusion appeared to be fully functional during sporulation and enabled the localization of SpoIIE during septation.

The localization of proteins involved in chromosome behavior has also been studied using GFP. For example, a SpoOJ-GFP fusion was created that resulted in a functional protein which permitted the visualization of chromosomal partitioning in live *B. subtilis* cells (Lin et al., 1997; Glaser et al., 1997). Webb et al. (1997) used a GFP-LacI fusion to localize specific regions of the *B. subtilis* chromosome in vegetative and sporulating cells by inserting a *lacO* cassette at specific sites in the chromosome. The

same system was used to compare chromosome and plasmid behavior during replication of *E. coli* (Gordon et al. 1997; Kim and Wang, 1998). Finally, Kohler and Ma (1997) used a HBsu-GFP fusion to visualize the location of this essential histone-like protein in *B. subtilis*.

Monitoring Gene Expression

The expression of GFP from a promoter of interest and visualization of fluorescence also permits the monitoring of gene expression in vivo (Cubitt et al., 1995; Misteli and Spector 1997; Jansson and de Bruijn, 1999). For example, the localization of gene expression during sporulation in *B. subtilis* was investigated using promoters controlled by various sigma factors fused to GFP (Webb et al., 1995; Arigoni et al., 1995). Lewis and Errington (1996) used GFP to localize expression directed by the pre-spore-specific promoter of the *dacF* gene and the mother-cell-specific promoter of the *sopIV* A gene in *B. subtilis*. GFP has been used to study the expression of various promoters from *Mycobacterium* during infection of macrophages (Dhandayuthapani et al., 1995; Via et al., 1996). Collins et al. (1998) used GFP as a reporter of metabolic activity in *Mycobacterium tuberculosis*. Finally, Chrast-Balz and van Huijsduijnen (1996) used GFP controlled by the *tetA* gene promoter to evaluate tetracycline antagonists.

Isolating Environmentally Regulated Genes

Promoterless GFP constructs can also be used to screen for promoters that are induced under a specific set of physiological conditions. For example, Kremer et al. (1995) inserted fragments of the *M. bovis* genome in front of the *gfp* gene and found that 3 to 5% of the chimeric genes showed differing levels of fluorescence on agar plates. In an effort to isolate genes important for *S. typhimurium* survival during infection of acidic macrophages, Valdivia and Falkow (1996) used a similar strategy to find promoters induced under acidic conditions. Suarez et al. (1997) have developed both plasmid and mini-Tn*5*-based promoterless GFP vectors. *P. putida*, *Pseudomonas* spB13 and *Alcaligenes eutrophus* were mutated with mini-*Tn5::gfp*. Up to 5% of the bacteria with chromosomal integration of the mini-Tn*5* were fluorescent, displaying varied levels of expression. Moreover, Handfield et al. (1998) developed a promoterless GFP vector that allows rapid selection for active promoters by fusing aspartate β-semialdehyde dehydrogenase with GFP and using it as a selectable marker in Asd^- strains. Josenhans et al. (1998) constructed a mini-Tn*3* based promoterless *gfp* transposon for use in *Helicobacter pylori* and *H. mustelae*. Other promoterless GFP vectors have also been

described, but their utility has not yet been demonstrated (Podbielski et al., 1996; Kalogeraki and Winans, 1997).

Another example of the utility of GFP as a bioreporter is the construction of pGreenscript (Inouye et al., 1997). In this vector GFP replaces the β-galactosidase (*lacZ* gene) as the marker for insertion of DNA in the multiple cloning site of the popular cloning vector pBluescript.

CONCLUSIONS

The large number of studies employing GFP as a biomarker/bioreporter clearly demonstrate the value and versatility of GFP. While problems with GFP as a biomarker/bioreporter in bacteria have been encountered, many have been overcome by using mutant versions of GFP, adding appropriate translation initiation sites, or adding flexible linkers to protein fusions. The many successful studies using GFP and the continued development of new techniques incorporating GFP will undoubtedly lead to the use of GFP in more species of bacteria to answer diverse questions in molecular microbial ecology.

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Table 5-1

GFP name	Residues mutatated	Excitation (nm)[a]	Emission (nm)[b]	Relative intensity[c]	Ref.
wtGFP	None	395/470	509	1	Chalfie et al. 1994
H9	S202F	398	511	1.17	Heim et al. 1994
P9	I167V	471/396	502	1.66	Heim et al. 1994
P11	I167T	471/396	502	1.88	Heim et al. 1994
P4	Y66H	382	448	0.57	Heim et al. 1994
W	Y66W	458	480	ND	Heim et al. 1994
S65-T	S65T	490	510	6	Heim et al. 1995
Cycle 3	F100S, M145T, V164A	385	510	18	Cameri et al. 1996
RSGFP4	F64M, S65G, Q69L	490	505	ND	Delagrave et al. 1995
GFPmut1(EGFP)	F64L, S65T	488	507	30-50	Cormack et al. 1996
GFPmut2	S65A, V68L, S72A	481	507	80-100	Cormack et al. 1996
GFPmut3	S65G, S72A	501	511	70-80	Cormack et al. 1996
GFPT203I	T203I	400	512	ND	Ehrig et al. 1995
GFPE222G	E222G	481	506	ND	Ehrig et al. 1995
GFPmut3*	S2R, S65G,S72A	501	511	70-80	Anderson et al. 1998
gfp(AAV)	GFPmut3* with instability tail added	501	511	70-80	Anderson et al. 1998
gfp(LVA)	GFPmut3* with instability tail added	501	511	70-80	Anderson et al. 1998
gfp(LAA)	GFPmut3* with instability tail added	501	511	70-80	Anderson et al. 1998
gfp(ASV)	GFPmut3* with instability tail added	501	511	70-80	Anderson et al. 1998
S65A	S65A	ND	505	2	Inouye et al. 1997
S65G	S65G	ND	513	1	Inouye et al. 1997

Table 5-1 continued

GFP name	Residues mutatated	Excitation (nm)[a]	Emission (nm)[b]	Relative intensity[c]	Ref.
P4-3	Y66H, Y145F	381	445	1	Heim and Tsien 1996
W7	Y66W, N146I, M153T, V163A, N212K	433	475	1	Heim and Tsien 1996
V2	Y66W, I123V, Y145H, H148R, M153T, V163A, N212K	432	480	1	Heim and Tsien 1996
P4-1	S65T, M153A, K238E	504	514	ND	Heim and Tsien 1996

ND - no data was given in the paper sited

[a]Different methods were used to determine peak excitation. See the reference for details.

[b]Different methods were used to determine peak emission. See the reference for details.

[c]Different methods were used to determine relative intensity. See the reference for details.

Table 5-1 GFP Mutants

List of GFP variants giving the mutant name, amino acid changes, excitation wavelength, emission wavelength, and relative intensity to wtGFP.

Table 5-2

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pJBA 114	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (LAA)	T0-T1	Km	Andersen et al. 1998
pJBA115	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (LAA)	T0-T1	Km	Andersen et al. 1998
pJBA116	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (LVA)	T0-T1	Km	Andersen et al. 1998
pJBA117	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (LVA)	T0-T1	Km	Andersen et al. 1998
pJBA118	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (AAV)	T0-T1	Km	Andersen et al. 1998
pJBA119	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (AAV)	T0-T1	Km	Andersen et al. 1998
pJBA120	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (ASV)	T0-T1	Km	Andersen et al. 1998
pJBA121	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3* (ASV)	T0-T1	Km	Andersen et al. 1998
pJBA28	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3*	T0-T1	Km	Andersen et al. 1998
pJBA29	mini-Tn*5*	Pa1/04/03 (P*lac*)	RBSII	GFPmut3*	T0-T1	Km	Andersen et al. 1998
pCW27	plasmid(pBluescript derivative)	*spoIIE*	spoIIE	wtGFP	ND	Ap	Arigoni et al. 1995
pCW28	plasmid(pBluescript derivative designed for chromosomal integration)	*spoIIE*	spoIIE	wtGFP	ND	Ap, Km	Arigoni et al. 1995
pIIE-GFP-T	plasmid(designed for chromosomal integration)	*spoIIE*	translational fusion w / spoIIE	wtGFP	ND	Ap, Cm	Barak et al. 1996

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pGB3	plasmid(pUC18 derivative)	P*lac*	ND	GFPmut2	ND	Ap, TC	Bloemberg et al. 1997
pGB5	plasmid(pKEN derivative)	P*tac*	ND	GFPmut2	ND	Ap, TC	Bloemberg et al. 1997
pSMC2	plasmid(pKEN derivative)	P*tac*	ND	GFPmut2	ND	Ap	Bloemberg et al. 1997
Tn*5*GFP1	Tn*5*	P*lac*	ND	wtGFP	ND	Tc, Cm, Gm	Burlage et al. 1996
pTU#58	plasmid	T7 P*Φ10* promoter	ND	wtGFP	ND	Ap	Chalfie et al. 1994
ptetA-GFP	plasmid(pBK-CMV derivative)	Tn *10 tetA* promoter	translational fusion w / tetA	Cycle III	Transcriptional stop from pASK85-D1.3	Km	Chrast-Balz et al. 1996
pSM1387	mini-Tn*5*	promoterless	ND	wtGFP	ND	Km	Christensen et al 1996
pSM1373	mini-Tn*5*	T7 P*Φ10* promoter	ND	wtGFP	ND	Km	Christensen et al. 1996
TOL-gfp (RP1::GFP)	broad host range plasmid (TOL derivative)	T7 P*Φ10* promoter	ND	wtGFP	ND	Km	Christensen et al. 1996
pGFP	plasmid	P*lac*	ND	wtGFP	ND	Ap	Clontech
p*hsp60-gfp*	plasmid(pMV261 derivative)	*hsp60*	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
p*Mlaphc-gfp*	plasmid(pMV206 derivative)	*aphC*	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
p*Mlaphc-gfp/oxyR*	plasmid(pMV206 derivative)	*aphC*	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
p*Mtahpc-gfp*	plasmid(pMV206 derivative)	*aphC*	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
p*Mtahpc-gfp/oxyR*	plasmid(pMV206 derivative)	*aphC*	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
p*mtrA-gfp*	plasmid(pMYGFP1 derivative)	*mtrA*	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
pMYGFP1	plasmid(pMV206 derivative)	promoterless	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
pMYGFP2	plasmid(pMV206 derivative)	promoterless	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
p*tbprc3-gfp*	plasmid(pMYGFP1 derivative)	*tbprc3*	ND	wtGFP	ND	Km	Dhandayuthapani et al. 1995
pSG1044	plasmid(designed for chromosomal integration)	*divIV*	translational fusion w / DivIV	S65T	ND	Ap, Cm	Edwards and Errington 1997
pBHFN35	plasmid (pUC18 derivative designed for chromosomal integration)	*nifH*	translational fusion w / NifH	GFPmut1	ND	Ap	Egener et al. 1998
pTB93F	broad host range plasmid (from bordetella)	P*trp* (from salmonella)	ND	S65T	ND	Sp, Cm	Gage et al. 1996
pTB93G	broad host range plasmid (from bordetella)	P*trp* (from salmonella)	ND	wtGFP	ND	Sp, Cm	Gage et al. 1996
pSG1517[a]	plasmid(pBluescript derivative designed for chromosomal integration)	*spoOJ*	translational fusion w / SpoOJ	S65T	ND	Ap, Cm	Glaser et al. 1997
pSG20	plasmid(pBAD-18 derivative)	P*ara*BAD	optimal E.coli RBS	ND	ND	Ap	Gordon et al. 1997
pCH50	Phage	P*lac*	translational fusion w / ZipA	S65T	ND	ND	Hale and de Boer 1997

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pIV ET-GFP	plasmid(suicide vector pGP704 derivative)	promoterless	ND	GFPmut3	ND	Cb	Handfield et al. 1988
pGreenscript	plasmid(pBluescript derivative)	P*lac*	ND	S65-A	ND	Ap	Inouye et al. 1997
pF1	plasmid(pFEL-1 derivative designed for chromosomal integration)	*dacF*	translational fusion w / SigE55	wtGFP	ND	Cm	Ju et al. 1997
pVIK165[a]	plasmid (R6K suicide vector)	none	ND	S65T	ND	Km	Kalogeraki and Winans 1997
pSopB-GFP	plasmid(pASLR2 derivative)	P*tac*	translational fusion w / SopB	ND	ND	Ap	Kim and Wang 1998
pPK9C2	plasmid(pQE 9 derivative)	T5 promoter	translational fusion w / Hbsu	wtGFP	ND	Ap	Kohler and Marahiel 1997
pPK9C8	plasmid(pDH88 derivative designed for chromosomal integration)	P*spac*	translational fusion w / Hbsu	wtGFP	ND	Ap, Cm	Kohler and Marahiel 1997
pPKC2	plasmid	ND	translational fusion w / Hbsu	wtGFP	ND	Ap	Kohler and Marahiel 1997
pGFM-11	plasmid (with mycobacterial replication ori)	*hsp60*	*hsp60* RBS	wtGFP	ND	Km, Sm	Kremer et al. 1995
pGFM-12	plasmid (with mycobacterial replication ori)	none	*hsp60* RBS	wtGFP	ND	Km, Sm	Kremer et al. 1995

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pSG1125	plasmid(pBluescript derivative designed for chromosomal integration)	None (for C-terminal translational fusions)	ND	wtGFP	ND	Ap, Cm	Lewis and Errington 1996
pSG1135	plasmid(pBluescript derivative designed for chromosomal integration)	None (for C-terminal translational fusions)	ND	Y66H	ND	Ap, Cm	Lewis and Errington 1996
pSG1137	plasmid(pBluescript derivative designed for chromosomal integration)	None (for C-terminal translational fusions)	ND	S65T	ND	Ap, Cm	Lewis and Errington 1996
pSG1141[a]	plasmid(pBluescript derivative designed for chromosomal integration)	*spoIVA*	translational fusion w / SpoIVA	S65T	ND	Ap, Cm	Lewis and Errington 1996
pSG1147	plasmid(pBluescript derivative designed for chromosomal integration)	*dacF*	translational fusion w / DacF	S65T	ND	Ap, Cm	Lewis and Errington 1996
pDL50B	plasmid(pGEMcat derivative)	*spoOJ*	translational fusion w / SpoOJ	ND	ND	Cm	Lin et al. 1997
pAG[a]	plasmid(pBC derivative)	P*lac*	translational fusion w / FtsA	GFPmut2	ND	Ap	Ma et al. 1996
pCSK100	plasmid(pBC derivative)	P*lac*	ND	GFPmut2	ND	Ap	Ma et al. 1996
pGZ	plasmid(pBC derivative)	P*lac*	ND	GFPmut2 fused to ftsZ	ND	Ap	Ma et al. 1996

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pZG[a]	plasmid(pBC derivative)	P*lac*	translational fusion w / FtsZ	GFPmut2	ND	Ap	Ma et al. 1996
p519gfp	broad host mob+ plasmid	P*lac*	T7 gene10	GFPmut2	ND	Nm	Matthysse et al. 1996
p519gfp	broad host mob+ plasmid	P*npt2*	T7 gene10	GFPmut2	ND	Nm	Matthysse et al. 1996
pTn3gfp	Tn*3*	none	T7 gene10	GFPmut2	ND	Ap	Matthysse et al. 1996
pUTmini-Tn5gfp	mini-Tn*5*	none	T7 gene10	GFPmut2	ND	Tc, Ap	Matthysse et al. 1996
pGFPLSD	plasmid (pUC1813 derivative)	P*lac*	lacZ Shine-Delgarno	wtGFP	ND	Ap	Miller and Lindow 1997
pGreen	plasmid (pUC1813 derivative)	P*lac*	lacZ Shine-Delgarno	GFPmut1	ND	Ap	Miller and Lindow 1997
pGreenTIR	plasmid (pUC1813 derivative)	P*lac*	Shine-Delgarno & spacer (TIR)	GFPmut1	ND	Ap	Miller and Lindow 1997
pVIGreen	low copy broad host range plasmid (pVSP61 derivative)	P1 (iron promoter)	lacZ Shine-Delgarno	GFPmut1	ND	Ap	Miller and Lindow 1997
pVILSD	low copy broad host range plasmid (pVSP61 derivative)	P1 (iron promoter)	lacZ Shine-Delgarno	wtGFP	ND	Ap	Miller and Lindow 1997
pVITIR	low copy broad host range plasmid (pVSP61 derivative)	P1 (iron promoter)	Shine-Delgarno & spacer (TIR)	GFPmut1	ND	Ap	Miller and Lindow 1997

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pVSP61TIR	low copy broad host range plasmid (pVSP61 derivative)	P*lac*	Shine-Delgarno & spacer (TIR)	GFPmut1	ND	Ap	Miller and Lindow 1997
pJBA26	mini-Tn*5*	Pm	ND	GFPmut3b*	T1-T0	Sm, Sp	Moller et al 1998
pJBA30	mini-Tn*5*	Pu	T5 RBSII	GFPmut3b*	T1-T0	Km	Moller et al. 1998
TOL::gfpmut3b	broad host range plasmid (TOL derivative)	Pa1/04/03 (P*lac*)	T5 RBSII	GFPmut3b	T1-T0	Km	Normander et al. 1998
pFWgfp	plasmid (streptococci suicide vector)	none	ND	wtGFP	tt1-tt4	Sp	Podbielski et al. 1996
pDR112[a]	plasmid	P*lac*	translational fusion w / MinCDE	GFPmut2	ND	Km	Raskin and de Boer 1997
pDR123[a]	plasmid	P*aadA*	translational fusion w / MinCDE	GFPmut2	ND	Km	Raskin and de Boer 1997
pAG408	mini-Tn*5*	none	E.coli *atpE* gene	Cycle3	ND	aphA-3 and Gm	Suarez et al. 1997
pASV2	plasmid (pBluescript derivative)	λphage Pr and Pl in tandem	E.coli *atpE* gene	wtGFP	rrnB T1 (before promoter)	Ap	Suarez et al. 1997
pASV3	plasmid (pBluescript derivative)	none	E.coli *atpE* gene	wtGFP	rrnB T1 (before promoter)	Ap	Suarez et al. 1997
pASV4	plasmid (pBluescript derivative)	none	E.coli *atpE* gene	wtGFP + CAT	rrnB T1 (before promoter)	Ap	Suarez et al. 1997

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pRL765gfp	Tn*5*	P*psbA*	T7 gene 10	P11	ND	Km	Tombolini et al. 1998
pUTgfp	mini-Tn*5*	P*psbA*	T7 gene 10	P11	ND	Km	Tombolini et al. 1998
pUTgfplux	mini-Tn*5*	P*psbA*	T7 gene 10	P11	ND	Km	Unge et al. 1999
pFVP25[b]	plasmid(pED350 derivative)	none	ND	GFPmut3	ND	Ap	Valdivia and Falkow 1996
pFVP25.1	plasmid(pFPV25 derivative)	*rpsM*	ND	GFPmut3	ND	Ap	Valdivia and Falkow 1996
pFPV1	plasmid (pBR322 derivative)	P*lac*	ND	wtGFP	ND	Ap	Valdivia et al. 1996
pFPV2	plasmid (mycobacterial expression vector)	*hsp60*	ND	wtGFP	ND	Km	Valdivia et al. 1996
pAT3	plasmid(pBluescript derivative designed for chromosomal integration)	*gerE*	translational fusion w / GerE	wtGFP	ND	Km	Webb et al. 1995
pCW13	plasmid(pBluescript derivative designed for chromosomal integration)	*cotE*	translational fusion w / CotE	wtGFP	ND	Cm	Webb et al. 1995
pCW15	plasmid(pBluescript derivative designed for chromosomal integration)	*csfB*	translational fusion w / CsfB	wtGFP	ND	Km	Webb et al. 1995
pCW33	plasmid(pBluescript derivative designed for chromosomal integration)	*sspE-2G*	translational fusion w / SspE	wtGFP	ND	Ap, Km	Webb et al. 1995
pCW37	plasmid(pBluescript derivative designed for chromosomal integration)	*cotE*	translational fusion w / CotE	wtGFP	ND	Cm	Webb et al. 1995

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pCW8	plasmid(pBluescript derivative)	promoterless	ND	wtGFP	ND	Ap	Webb et al. 1995
pAT11	plasmid(pDG795 derivitave for integration into the ThrC locus)	*spoVG*	translational fusion w / SpoVG	fused to lacI	ND	MLS	Webb et al. 1997
pSG1151	plasmid(pBluescript derivative designed for chromosomal integration)	None (for C-terminal translational fusions)	ND	GFPmut1	ND	Ap, Cm	Wu et al. 1998
pSG1902	plasmid(pBluescript derivative designed for chromosomal integration)	*spoIIE*	translational fusion w / SpoIIE	GFPmut1	ND	Ap, Cm	Wu et al. 1998
pK3G	plasmid(pBluescript derivative)	P*tac*	translational fusion w / FtsK	GFPmut2	ND	Ap	Yu et al. 1998
pURE-RD-GFP[a]	plasmid(pBluescript derivative)	*ureRD*	translational fusion w / UreD	GFPmut2	ND	Ap	Zhao et al. 1998
pGBS	plasmid(pBluescript derivative)	promoterless	ND	GFPmut2	ND	Cb	Ma et al. 1997
pGBC	plasmid(pBC derivative)	promoterless	ND	GFPmut2	ND	Cam	Ma et al. 1997
pAEG1	plasmid(pBC derivative)	*ftsA*	translational fusion w / FtsA	GFPmut2	ND	Cam	Ma et al. 1997
pZEG1[c]	plasmid(pBC derivative)	*ftsZ*	translational fusion w / FtsZ	GFPmut2	ND	Cam	Ma et al. 1997

Table 5-2 continued

Vector Name	Type	Promoter	Translation Initiation	GFP Variant	Terminators	Selectable Marker	Reference
pAEG2[c]	plasmid(pBluescript derivative)	*ftsA*	translational fusion w / FtsA	GFPmut2	ND	Cb	Ma et al. 1997
pZEG2[c]	plasmid(pBluescript derivative)	*ftsZ*	translational fusion w / FtsZ	GFPmut2	ND	Cb	Ma et al. 1997
pAEG3[c]	plasmid(broad host range IncP derivative)	*ftsA*	translational fusion w / FtsA	GFPmut2	ND	Cb, Tr	Ma et al. 1997
pZEG3[c]	plasmid(broad host range IncP derivative)	*ftsZ*	translational fusion w / FtsZ	GFPmut2	ND	Cb, Tr	Ma et al. 1997
pWES4	plasmid(pMV261 derivative)	*hsp60*	ND	wtGFP	ND	Km	Parker and Bermudez 1997
pMV306 (*hsp60/gfp*)	plasmid(pMV306 derivative designed for chromosomal integration)	*hsp60*	ND	wtGFP	ND	Km	Parker and Bermudez 1997
pYL GFP	plasmid(pMOD-12 derivative)	*hsp70*	ND	wtGFP	ND	Km	Luo et al.1996
pYL mutGFP	plasmid(pMOD-12 derivative)	*hsp70*	ND	S65T	ND	Km	Luo et al.1996

[a]Derivatives of this plasmid containing truncations of the protein fused to GFP are also available

[b]Derivatives of this plasmid with acid inducible promoters are available

[c]This plasmid contains *ftsA* from *E.coli*. Other version containing *ftsA* from *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* are also available.

ND - no data was given in the paper sited

Table 5-2 GFP Vectors

Vectors designed to utilize GFP as a biomarker/bioreporter in bacteria. The table lists the name of the vector, the type of vector, the promoter used to express GFP, any translation initiation site mentioned in the paper, the GFP variant used, any terminators mentioned in the paper and the selectable marker on the vector.

Table 5-3

Organism	Type of study	Type of Vector	Vector Name	GFP mutant	Reference
***Agrobacterium tumefaciens* A136**	Localize proteins involved in septum formation and study their interactions	plasmid	pAEG3, pZEG3, and related plasmids	GFPmut2	Ma et al. 1997
Alcaligenes eutrophus	Create promoter fusions	mini-Tn*5*	pAG408	Cycle 3	Suarez et al. 1997
***Azoarcus sp* BH72**	Observe *Azoarcus* expressing nif genes in soil and on rice roots	chromosomal integration	pBHFN35	GFPmut2	Egener et al. 1998
Bacillus megaterium	Localize proteins involved in sporulation septum formation	chromosomal integration	pIIE-GFP-T	wtGFP	Barak et al. 1996
Bacillus subtilis	Localize proteins involved in sporulation septum formation	chromosomal integration	pCW28	wtGFP	Arigoni et al. 1995
Bacillus subtilis	Localize proteins involved in septum formation	chromosomal integration	pSG1044	S65-T	Edwards and Errington 1997
Bacillus subtilis	Localize proteins involved in chromosomal partitioning	chromosomal integration	pSG1517	S65-T	Glaser et al. 1997
Bacillus subtilis	Localize proteins to determine where processing takes place	chromosomal integration	pF1	wtGFP	Ju et al. 1997
Bacillus subtilis	Localize Hbsu	chromosomal integration	pPK9C8	wtGFP	Kohler and Ma 1997
Bacillus subtilis	Localize gene expression and proteins involved in sporulation	chromosomal integration	pSG1141	S65-T	Lewis and Errington 1996
Bacillus subtilis	Localize gene expression and proteins involved in sporulation	chromosomal integration	pSG1147	S65-T	Lewis and Errington 1996
Bacillus subtilis	Localize proteins involved in chromosomal partitioning	chromosomal integration	pDL50B		Lin et al. 1997
Bacillus subtilis	Study the expression of genes regulated by sporulation	chromosomal integration	pAT3, pAT11, pCW13, pCW15, pCW33, pCW37	wtGFP	Webb et al. 1995

Table 5-3 continued

Organism	Type of study	Type of Vector	Vector Name	GFP mutant	Reference
Bacillus subtilis	Study chromosome partitioning	chromosomal integration	pSG1151		Webb et al. 1997
Bacillus subtilis	Localize proteins involved in sporulation septum formation	chromosomal integration	pSG1902	GFPmut1	Wu et al. 1998
Escherichia coli	Localize proteins involved in septum formation	phage	pCH50	S65-T	Hale and de Boer 1997
Escherichia coli	Localize proteins involved in septum formation	plasmid	pAG	GFPmut2	Ma et al. 1996
Escherichia coli	Localize proteins involved in septum formation	plasmid	pCSK100	GFPmut2	Ma et al. 1996
Escherichia coli	Localize proteins involved in septum formation	plasmid	pGZ	GFPmut2	Ma et al. 1996
Escherichia coli	Localize proteins involved in septum formation	plasmid	pZG	GFPmut2	Ma et al. 1996
Escherichia coli	Localize proteins involved in septum formation	plasmid	pDR112	GFPmut2	Raskin and de Boer 1997
Escherichia coli	Localize proteins involved in septum formation	plasmid	pDR123	GFPmut2	Raskin and de Boer 1997
Escherichia coli	Localize proteins involved in septum formation	plasmid	pK3G	GFPmut2	Yu et al 1998
Escherichia coli **01**	Monitor floc stability in activated sludge	plasmid	pGFP	wtGFP	Olofsson et al. 1998
Escherichia coli **DH5α**	Detection and counting of bacteria using GFP and monitoring their energy status using *lux*AB	mini-Tn*5*	pUTgfplux	P11	Unge et al. 1999
Escherichia coli **DH5α**	Correlate *ureD* expression with urea concentrations	plasmid	pURE-RD-GFP	GFPmut2	Zhao et al. 1998

Table 5-3 continued

Organism	Type of study	Type of Vector	Vector Name	GFP mutant	Reference
***Escherichia coli* JM105**	Localize proteins involved in septum formation and study their interactions	plasmid	pAEG1, pZEG1, and related plasmids	GFPmut2	Ma et al. 1997
***Escherichia coli* JM109**	Monitor persistence of bacteria in stream water using microscopy	plasmid	pGFP	wtGFP	Leff and Leff 1996
***Escherichia coli* K12**	Study chromosome and plasmid partitioning	plasmid	pSg20		Gordon et al. 1997
***Escherichia coli* K12 HB101**	Study plasmid partitioning	plasmid	pSopB-GFP	S65-T	Kim and Wang 1997
***Escherichia coli* NM522**	Track movement of bacteria through a column of sand	plasmid	pGFP	wtGFP	Burlage et al. 1996
***Escherichia coli* XL1-Blue**	Test tetrcycline analogues on the tc-repressor gene switch	plasmid	ptetA-GFP	Cycle 3	Chrast-Balz & van Huijsduijnen 1996
Mycobacterium avium	Observe infection of macrophages and epithelial cells	plasmid	pWES4	wtGFP	Parker and Bermudez 1997
Mycobacterium avium	Observe infection of macrophages and epithelial cells	chromosomal integration	pMV306(*hsp 60/gfp)*	wtGFP	Parker and Bermudez 1997
***Mycobacterium bovis* BCG**	Study expression patterns of different promoters in bacteria grown in macrophages	plasmid	p*hsp60-gfp*, p*Mlaphc-gfp*, p*Mtaphc-gfp*, p*mtra-gfp*, p*tbprc3-gfp*	wtGFP	Dhandayuthapani et al. 1995
***Mycobacterium bovis* BCG**	Used to test drug susceptibility, response to environmental signals and localize bacteria *in vivo*	plasmid	pGFM-11	wtGFP	Kremer et al. 1995
***Mycobacterium bovis* BCG**	Observe infection of cell line from mice	plasmid	pYL mutGFP	S65-T	Luo et al. 1996

Table 5-3 continued

Organism	Type of study	Type of Vector	Vector Name	GFP mutant	Reference
Mycobacterium marinum	Visualize bacteria infecting host cells and count infected cells using flow cytometry	plasmid	pFPV2	wtGFP	Valdivia and Falkow 1996
Mycobacterium smegmatis	Study expression patterns of different promoters in bacteria grown in macrophages	plasmid	p*hsp60-gfp*, p*Mlaphc-gfp*, p*Mtaphc-gfp*, p*mtra-gfp*, p*tbprc3-gfp*	wtGFP	Dhandayuthapani et al. 1995
Mycobacterium smegmatis	Test for fluorescence	plasmid	pGFM-11	wtGFP	Kremer et al. 1995
Mycobacterium smegmatis	Use as a promoter probe for bacterial promoters	plasmid	pGFM-12	wtGFP	Kremer et al. 1995
Mycobacterium tuberculosis	Use GFP as a marker to screen for antimicrobial agents	plasmid	pFPV2	wtGFP	Collins et al. 1998
Mycobacterium tuberculosis	Study expression patterns of different promoters in bacteria grown in macrophages	plasmid	p*mtra-gfp*, p*hsp60-gfp*	wtGFP	Via et al. 1996
Proteus mirabilis **HI4320**	Observe ureD gene expression by bacteria in bladders and kidneys of infected mice	plasmid	pURE-RD-GFP	GFPmut2	Zhao et al. 1998
Pseudomonas aeruginosa	Observe fluorescent intensity and plasmid stability and distinguish tagged bacteria in mixed cultures	plasmid	pSMC2	GFPmut2	Bloemberg et al. 1997
Pseudomonas fluorescens **WCS365**	Observe fluorescent intensity and plasmid stability and localize bacteria on plant roots	plasmid	pGB5	GFPmut2	Bloemberg et al. 1997
Pseudomonas putida **KT2442**	Create promoter fusions	mini-Tn*5*	pAG408	Cycle 3	Suarez et al. 1997

Table 5-3 continued

Organism	Type of study	Type of Vector	Vector Name	GFP mutant	Reference
***Pseudomonas putida* mt-2**	Track movement of bacteria through a column of sand	Tn*5* derivative	Tn5GFP1	wtGFP	Burlage et al. 1996
***Pseudomonas putida* R1**	Observe gene induction and bacterial location in a bioreactor	mini-Tn*5*	pJBA30	GFPmut3b	Moller et al. 1998
***Pseudomonas putida* R1**	Observe gene induction and bacterial location in a bioreactor	mini-Tn*5* pJBA26	pJBA26	GFPmut3b	Moller et al. 1998
***Pseudomonas* sp B13**	Create promoter fusions	mini-Tn*5*	pAG408	Cycle 3	Suarez et al. 1997
***Pseudomonas aeruginosa* PA01**	Create promoter fusions	plasmid	pIVET-GFP	GFPmut3	Handfield et al. 1998
***Pseudomonas fluorescens* A506**	Monitor bacteria in culture, soil samples, and on roots with flow cytometry and microscopy	Tn*5*	pRL765*gfp*	P11	Tombolini et al. 1997
***Pseudomonas fluorescens* AS12**	Monitor conjugal plasmid transfer in the phylloplane of bush bean	plasmid	TOL::gfpmut3b	GFPmut3b	Normander et al. 1998
***Pseudomonas fluorescens* SBW25**	Detection and counting of bacteria using GFP and monitoring their energy status using *lux*AB	mini-Tn*5*	pUTgfplux	P11	Unge et al. 1999
***Pseudomonas putida* KT2442**	Monitor plasmid transfer by conjugation	plasmid	TOL-gfp (RP1::GFP)	wtGFP	Christensen et al. 1996
***Sinorhizobium meliloti* 1021**	Visualize nodulation of *Medicago sativa* by bacteria	plasmid	pTB93F	S65-T	Gage et al. 1996
***Sinorhizobium meliloti* MB501**	Localize proteins involved in septum formation and study their interactions	plasmid	pAEG3, pZEG3, and related plasmids	GFPmut2	Ma et al. 1997

Table 5-3 continued

Organism	Type of study	Type of Vector	Vector Name	GFP mutant	Reference
Salmonella typhimurium	Visualize bacteria infecting host cells and count infected cells using flow cytometry	plasmid	pFPV1	wtGFP	Valdivia and Falkow 1996
Salmonella typhimurium	Find acid induced promoters and test for expression by bacteria in macrophages	plasmid	pFVP25	GFPmut3	Valdivia and Falkow 1996
Serratia marcesens	Monitor floc stability in activated sludge	plasmid	TOL-gfp (RP1::GFP)	wtGFP	Olofsson et al. 1998
Yersinia pseudo-tuberculosis	Visualize bacteria infecting host cells and count infected cells using flow cytometry	plasmid	pFPV1	wtGFP	Valdivia and Falkow 1996

Table 5-3 GFP Marked Bacteria

List of bacteria in which GFP has been used as a biomarker/bioreporter. The table lists the genus and species of bacteria used in the study, the type of vector, the name of the vector and the GFP variant carried on the vector.

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CHAPTER 6

STUDIES ON RICE ROOT COLONIZATION BY SELECTED BACTERIA USING GFP AND GUS AS MOLECULAR BIOMARKERS

ABSTRACT

The endophytic colonizing ability of nine bacterial stains isolated from mechanically-abraded, surface-sterilized rice tissue was evaluated. These strains were inoculated to sterile rice seedlings and could be re-isolated following three weeks of plant growth. Transposons harboring genes encoding a GUS or GFP biomarker were constructed and used to tag two of the nine bacterial strains previously isolated from rice tissues [R33(120) and R100(64)]. The utility of these biomarkers for *in situ* visualization of bacterial colonization of plant tissues was evaluated. Three dimensional reconstruction of optical sections generated via laser scanning confocal microscopy was used to visualize spatial relationships between bacteria expressing GFP and plant tissues. Colonization sites for the two tagged strains and *Sinorhizobium meliloti*, a control strain, were examined in detail using both GUS and GFP as biomarkers. Little internal colonization of rice tissue by these bacteria was observed although both clumps of bacteria and individual cells were observed on the surface of

infected rice roots. On very rare occasions an isolated epidermal cell harboring large numbers of R100::*gfp* bacteria was observed.

INTRODUCTION

As pointed out in Chapter 1 and 2, the long term goal of the experiments outlined in this thesis is to isolate and characterize stable "endophytic" bacteria from rice plants. Our expectation was that the isolation of bacteria from mechanically-abraded, surface-sterilized rice plant tissue (Chapter 2) would enrich for endophytic bacteria. However, it was also realized that some non-endophytic bacteria would 'escape' surface sterilization and be isolated along with the endophytic bacteria. Therefore, the ability of these bacteria to colonize plant tissue endophytically must be verified. To accomplish this we focused on re-infection of gnotobiotically grown rice seedlings with selected bacterial isolates (Chapter 2) and *in planta* microscopic localization of bacteria harboring biomarkers.

The isolation of a bacterial strain following re-infection of gnotobiotically grown rice seedlings demonstrates the ability of that strain to form an intimate association with the plant host, resulting in the protection of these bacteria from the sterilizing agent. However, this approach does not address the possibility that the bacteria reside on the plant surface and escape sterilization by the production of polysaccharide capsules or

other means. Nor does this method determine the sites of colonization. Therefore, more direct approaches are required to determine whether a strain is capable of colonizing the rice tissue endophytically, and the sites and extent of colonization.

In principle, it should be possible to visualize the colonization of rice by putative endophytes using light microscopy, fluorescence microscopy, and electron microscopy (You and Zhou, 1988; Hurek et al., 1994; Yanni et al., 1997; Reddy et al., 1997). However, unless the location of colonization can be predicted, it is difficult to find the bacteria by random scanning of tissues. Moreover, unless the plants are grown under gnotobiotic conditions or highly specific antibodies are available, the identity of the bacteria observed will remain questionable.

Recently different types of biomarkers have become available that facilitate tracking bacteria in the environment (Wilson, 1995; Unge et al., 1998; Jansson and de Bruijn, 1999; Chapter 5). The use of biomarkers facilitates the localization and unambiguous identification of endophytes in infected plant tissues (Hurek et al., 1994; Barraquio et al., 1997; Reddy et al., 1997; Webster et al., 1997; Vermeiren et al., 1998). In the

studies cited above, the biomarker β-galactosidase (LacZ) or β-glucuronidase (GUS) was employed. We chose to use the Green Fluorescent Protein (GFP) and GUS biomarkers as they offer distinct advantages such as low background and easy detection (Chapter 5). Both of these markers have been used successfully to detect tagged bacteria in (legume) plant tissues, such as those resulting from infection with rhizobia (Wilson et al., 1995; Gage et al., 1996). Here we will describe the development of transposon borne *gfp* and *gus* biomarker constructs, the tagging of selected (putative) endophytes and control strains, and a microscopic analysis of rice seedlings infected with the tagged stains.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions

The strains and plasmids used in this study are listed in Table 6-1 an 6-2 respectively. *Escherichia coli* S17-1 λpir strains, harboring plasmid pUT Tn*5*Km, pRL765*gfp* and pTnPS65-T were grown at 37°C on LB medium (Silhavy et al., 1984) supplemented with kanamycin (20 μg/ml). *E.coli* strain HB101, harboring the conjugal transfer helper plasmid pRK2013, was grown at 37°C on LB medium supplemented with kanamycin (20 μg/ml). *E. coli* strain DH5α harboring pTB93F, or pUC 4K and *E. coli* strain S17-1 λpir harboring plasmid pCAM121 were grown at 37°C on LB medium supplemented with carbenicillin (100 μg/ml), kanamycin (20 μg/ml), or spectinomycin (100 μg/ml), respectively. All other bacterial isolates (Table 6-1) were routinely grown at 28°C on TY medium (Beringer, 1974). Minimal media used for selection of transconjugants following the introduction of the biomarker transposons into bacteria were GTS medium (Kiss et al., 1979) containing 0.2% $(NH_4)_2SO_4$ as a nitrogen source, LSO medium (Elmerich et al., 1982) containing 1g/l $(NH_4)_2SO_4$ as a nitrogen source, and Davis and Mingolini minimal medium (Atlas, 1997), each supplemented with kanamycin (20 μg/ml).

Generation of Sterile Rice Seedlings, Bacterial Infection and Re-isolation

Seeds of the rice variety Lemont (accession # PI 475833, National Small Grains Research Facility, Aberdeen, ID, USA) were gently dehulled, incubated in 70% ethanol for five minutes, washed with sterile distilled water, treated with a fresh solution of 30% Clorox (17.5 ml sterile distilled water, 7.5 ml Clorox Bleach, 30 μl Tween 20) for 45 minutes, and washed six times with sterile distilled water. Seeds were placed on plates containing TY medium and incubated at 28°C for two days to allow germination. Seeds showing no bacterial or fungal contamination were placed in 25 mm X 200 mm tubes containing sterile sand watered with Fahraeus medium (Fahraeus, 1957). After two days the seedlings were inoculated with 100 μl of bacterial cultures grown for 24 hours in liquid TY medium at 28°C. Seedlings were watered with Fahraeus medium to keep the sand moist.

Bacteria were re-isolated from the inoculated rice seedlings as follows: Seedlings were grown from 14 to 16 days and carefully removed from the growth tubes to keep the roots intact. Each seedling was placed in a 15 ml conical tube (Sarstedt, Inc., Newton, NC, USA) containing 7 ml of sterile distilled water, shaken for 15 minutes, washed with 7 ml sterile

distilled water, treated with 7 ml of 30% Clorox solution for 15 minutes and washed six times with sterile distilled water. Each seedling was macerated in 1.5 ml microfuge tubes with sterile sand. Following maceration 1 ml distilled water was added and 20 μl of macerate was spread onto plates containing TY medium.

Generation of Sterile Rice Seedlings and Bacterial Infection for Microscopic Studies

Rice seedlings used for *in-situ* visualization of colonization by bacteria expressing GFP or GUS were generated and grown as follows: Two seeds of the rice variety Lemont, sterilized and germinated as described above, were placed between two "pillows" in a 10 cm x 10 cm x 15 cm pot. Pillows were constructed by folding a 15 cm x 40 cm piece of very fine spectrum cloth in half and heat sealing two edges, filling the resulting bag with 800 ml of vermiculite and heat sealing the top, as described by Szczyglowski et al. (1998). Each pot was watered with approximately 300 ml of 0.5x Hoaglands solution (Sigma, St. Louis, MO, USA) and placed in a growth chamber under 16 hour light at 28°C and 8 hours dark at 22°C. After two days, each seedling was inoculated with 1 ml of stationary phase culture of *gfp* or *gus* tagged bacteria and analyzed two to three weeks after inoculation.

Construction of *gfp* and *gus* Biomarker Transposons

All DNA manipulations were carried out using standard molecular biology protocols (Maniatis et al., 1982). Plasmids used in this study are listed in Table 6-2. A mini-Tn*5* transposon containing *gusA* under the control of the P*aph* promoter and a kanamycin resistance cassette was constructed as follows: The streptomycin and spectinomycin resistance cassette was removed from mTn*5*Ss*gusA21* (Wilson et al., 1995) by digestion with the restriction enzyme *Sma*I and replaced by a 1.3 Kb kanamycin resistance cassette from pUC 4K. The resulting vector was named p121TH1.

A mini-Tn*5* transposon containing a *gfp* gene encoding the GFPS65-T variant (Chapter 5) under the control of the P*trp* promoter and a kanamycin resistance cassette was constructed as follows: A 1 kb *Sma*I fragment from pTB93F (Gage et al., 1996), containing the *gfp* gene under control of the P*trp* promoter, was cloned into the *Sma*I site of pBluescript, digested with *Kpn*I and *Bam*HI, and the resulting fragment inserted into pUC18Not (Herrero et al., 1990). The resulting plasmid was digested with *Not*I and the *Not*I fragment inserted into the *Not*I site of pUT/mini-Tn*5* Km (de Lorenzo et al., 1990). The resulting vector was named pTnPS65-T (Figure 6-1).

Introduction of Biomarker Transposons into Bacteria.

The *gfp* and *gus* containing Tn*5* transposon derivatives were introduced into the target bacteria via conjugal transfer. *E. coli* strain S17-1 λpir, harboring the appropriate transposon, was grown overnight at 37°C in LB medium supplemented with 20 μg/ml kanamycin. *E. coli* strain HB101, harboring pRK2013 (Figurski and Helinski, 1979), was grown overnight at 37°C in LB medium supplemented with 20 μg/ml kanamycin. The recipient strains were grown at 28°C to late log phase in TY medium. Bacteria were washed 2x with TY medium, mixed in a 1:5 donor:recipient ratio, or a 1:1:5 donor:helper:recipient ratio, concentrated 5-fold and spotted on TY plates. Donor, helper, and recipient cells were spotted alone as controls. After 24 hr incubation at 28°C the spotted cell mixtures were resuspended in sterile distilled water and several dilutions were spread on plates containing a selective medium (see above).

Histochemical Staining for Gus Activity and Microscopy

Plants inoculated with bacteria harboring the gus marker gene were examined for β-glucosidase activity using 50μg/ml X-gluc (Gold Biotechnology, Inc., St. Louis, MO, USA) in the assay buffer, as described by Jefferson (1987). Blue color was observable after two hours of incubation. Samples were routinely incubated overnight to allow more intense color development. Samples were examined by eye, with a dissecting microscope (Wild 420, Wild Leitz, Heerbrugg, Switzerland) or with a light microscope (Zeiss Auxiophot, Carl Zeiss, Oberkochen, West Germany, or Leitz Laborlux S, Wild Leitz, Heerbrugg, Switzerland). Images were recorded using a Kodak Digital Science DC120 digital camera (Eastman Kodak Company, Rochester, NY, USA). Digital images were stored using Kodak DC120 software in conjunction with Photoshop 5.0 (Adobe Systems Incorporated, San Jose, CA, USA).

Fluorescent Microscopy

Plants inoculated with bacteria harboring transposon TnPS65-T were examined using an epifluorescent microscope (Zeiss 10 or Zeiss Axiophot, Carl Zeiss, Oberkochen, West Germany) equipped with a standard FITC filter set. Optical sections were made using laser scanning microscopy (Zeiss 10, Carl Zeiss, Oberkochen, West Germany or Meridian InSIGHT,

Genomis Solutions, Lansing, MI USA). Optical sections were recorded using the software provided by the manufacture. Non-confocal fluorescent images were recorded as described above. Three dimensional reconstruction and analysis of optical sections were carried out using Voxel View software (Virtual Images, Fairfield, IA, USA) on a Silicon Graphics 4D-30 Personal IRIS Workstation.

RESULTS

Re-isolation of Bacteria from Infected Rice Seedlings

Re-isolation experiments were carried out to determine if bacteria previously isolated from surface-sterilized rice tissue (Chapter 2) were able to re-colonize rice seedlings. Fourteen bacterial isolates were selected for this analysis (see Chapter 2, Composition of a "Test Collection"). The results of the re-isolation experiments are shown in Figure 6-2. The number of bacteria isolated from different seedlings re-inoculated with the same isolate showed some variability. No bacteria could be re-isolated from seedlings inoculated with TY medium, *E. coli*, *Azoarcus indigens*, *Herbaspirillum seropedicae* or strain R90(8). In the case of all other bacterial strains, bacteria could be re-isolated from at least one test seedling.

Tagging Bacteria with Molecular Biomarkers

Multiple attempts were made to obtain green fluorescent derivatives of bacteria by introducing the transposons from pRL765gfp or pTnPS65-T into 14 rice isolates and 9 previously described rice rhizosphere/endophytic reference strains. In selected cases *E. coli* cells, harboring the conjugation helper plasmid pRK2013, were added to the mating mixture to boost the transfer efficiency. Mating mixtures of each

strain were spread on plates containing different selective media (Material and Methods). Kanamycin resistant transconjugants were obtained in approximately 40% of the cases. Detectable green fluorescence was observed for only six of the kanamycin resistant transconjugants. Three transconjugant bacteria, *Enterobacter cloacae*, *Klebsiella planticola*, and R66(40), showed intense green fluorescence following the introduction of the transposon from pRL765gfp. However, the fluorescence was rapidly lost when the transconjugants were removed from kanamycin selection. Only three bacterial strains, *S. meliloti*::*gfp*, R33::*gfp* and R100::*gfp* displayed stable, detectable green fluorescence levels following introduction of transposon TnPS65-T (Figure 6-4).

Transposon Tn121TH1 was introduced into *S. meliloti*, R133(120) and R100(64) to obtain *S. meliloti*::*gus*, R33::*gus*, and R100::*gus* marked derivatives, all of which were found to have β-glucuronidase (Gus) activity.

In Situ Microscopic Evaluation of the Colonizing Ability of Biomarker Tagged Strains

Rice seedlings grown using the pillow system and the inoculated with the tagged strains were examined for colonization. The root systems of rice seedlings inoculated with *S. meliloti*::*gfp*, R33::*gfp*, and R100::*gfp* where observed using fluorescence microscopy. The root system from twelve seedlings was examined for each strain. Likewise, the root systems of rice seedlings inoculated with *S. meliloti*::*gus*, R33::*gus*, and R100::*gus* were examined following incubation with X-gluc. The root systems from four rice seedlings were examined for each strain. In addition, the root systems from three inoculated rice seedlings grown in test tubes were examined for each strain tagged with the GUS biomarker. Following staining, colonization patterns were observed by eye and using light microscopy.

When bacteria harboring the GUS biomarker were used to infect rice seedlings, blue staining, indicating the presence of large numbers of bacteria, could generally be observed without the aid of a microscope (Figure 6-6). The staining of plants grown in test tubes was more pronounced in all cases. The degree of staining was found to vary between seedlings inoculated with the same tagged bacterial isolate.

However, in general, no distinct differences in the degree and distribution of Gus staining was observed in rice seedlings inoculated with different tagged bacterial isolates. Gus staining was heaviest in primary and secondary roots, proximal to the seed. Infrequently, staining of root tips was also observed (Figure 6-6 A). Seeds were darkly stained in most cases (Figure 6-6 B). These observations suggest that bacteria colonize the root system primarily on the upper portions of the primary and secondary roots.

GFP mediated bacterial fluorescence could not be detected on rice roots using a UV transilluminator or a CCD camera equipped with proper filters for GFP excitation and emission detection. Fluorescence could only be detected using a 25x or greater immersion lens and an epifluorescent microscope. Therefore, the search for areas with a high degree of colonization required scanning the entire root system using an epifluorescent microscope, which proved to be a time consuming process. More fluorescent bacteria were observed on the upper portion of the primary and secondary roots of plants inoculated with strains expressing GFP, supporting the observations made using the tagged bacteria expressing GUS.

Colonization of rice roots inoculated with bacteria expressing GFP or GUS was further examined microscopically (Figure 6-7, 6-8, 6-9). Clumps of bacteria and individual bacteria cells, tagged with either Gus or GFP, could be observed on the root surface. Roots colonized by bacteria expressing GFP were examined using laser scanning confocal microscopy, which allows optical sectioning (Figure 6-8). A careful determination of spatial relationships between bacteria and the plant tissues was made using software that creates three dimensional images from optical sections (Figure 6-8, 6-9). Observations of the relationship between the plant and bacteria marked with GFP were facilitated by staining the plant with a fluorescent dye such as propidium iodide (Gage et al., 1996; Figure 6-7 E and F, 6-8, 6-9 E-H), which causes the edges of plant cells to emit red fluorescence when exited with blue light.

No endophytic colonization by GFP tagged *S. meliloti*, R33(120) or R100(64) bacteria was observed. Bacteria were found to colonize the rice root surface in clumps, often at the base of root hairs (Figure 6-7 A and F, 6-9 C and D). Individual GFP tagged cells could also be observed to be associated with root hairs and along the junctions of epidermal cells (Figure 6-7 D and E). No qualitative differences in colonization efficiency

were observed between the there different bacterial strains. On rare occasions (four cells in the 21 root systems examined) an epidermal cell harboring a large number of R100::*gfp* bacteria was observed (Figure 6-7 B, 6-8, 6-9 E and F).

DISCUSSION

Re-isolation of Bacteria from Infected Rice Seedlings

Isolation of bacteria from mechanically-abraded, surface-sterilized plant tissue has been proposed to lead to the isolation of bacteria which engage in intimate interactions with plants and, in particular, endophytic bacteria (See Chapter 1 for a definition). In order to examine this proposal, we selected a number of putative rice endophytes isolated using this approach and observed their ability to re-colonize rice tissues. Most of these putative endophytes could be re-isolated following inoculation and growth on gnotobiotic seedlings (Figure 6-2). However, no bacteria (C.F.U.'s) could be isolated from seedlings inoculated with control bacteria, *E. coli,* suggesting that bacteria not normally found to be endophytes of rice do not survive the surface sterilization protocol used.

Interestingly, two putative rice endophytes, *Herbaspirillum seropedicae* and *Azoarcus indigens* could not be isolated after inoculation and surface sterilization. This suggests that these bacteria do not colonize 3 week old Lemont rice seedlings endophytically, or that the surface sterilization protocol used was too harsh. However, putative endophytic bacteria from our collection were able to survive this same surface sterilization

protocol, suggesting that these bacteria are better protected from the sterilizing agent than *H. seropedicae* or *A. indigens*. This protection may be the result of more aggressive endophytic colonization. However, a large variation was observed in the number of re-isolated bacteria from different seedlings inoculated with the same strain, making quantitative interpretation of the data impossible.

Generation of Sterile Rice Seedlings and Bacterial Infection for Microscopic Studies

Seedlings grown in test tubes were smaller and less vigorous then those grown in pots, or in the 'pillow system'. Colonization levels on rice seedlings grown in test tubes and inoculated with bacteria expressing the GUS biomarker were found to be much higher than colonization levels on seedlings grown in the pillow system. This suggests that inoculation experiments carried out in test tubes result in artificially elevated colonization levels due to the fact that the plant is unhealthy and more susceptible to bacterial infection. The pillow system (Szczyglowski et al. 1998) allows rice seedling to grow in a manner that more closely reflects that of plants grown in the field and, therefore, may be more suitable for colonization studies.

In Situ Microscopic studies on Colonizing Ability of the Tagged Stains

A variety of molecular markers are now available that allow the detection and monitoring of bacteria in the environment (Akkermans et al., 1995; 1997; 1998; de Bruijn and Jansson, 1999). The GUS and GFP molecular markers were selected to facilitate the visualization of bacteria colonizing rice seedlings in our study. Both markers have been used successfully to study plant microbe interactions, for example during nodulation of legumes by rhizobia (Wilson et al., 1995; Gage et al., 1996).

Our initial studies revealed that each marker has its advantages. For example, colonization of plant tissue by bacteria expressing Gus could easily be detected at the macroscopic level by an intense blue color (Figure 6-3). This allowed the examination of colonization patterns using entire infected root systems (Figure 6-5). However, it was difficult to determine three dimensional spatial location of Gus tagged bacteria in relation to the surface of the root at the microscopic level. In addition, diffusion of the cleaved GUS enzyme substrate prior to dimerization was found to cause the appearance of blue color in areas not directly colonized by bacteria.

Visualization of bacteria expressing GFP was found to overcome both these problems. Since bacteria expressing the gene encoding GFP could be detected using fluorescent microscopy (Figure 6-4), laser scanning confocal microscopy could be used to generate optical sections of infected tissue and to create images that depicted three dimensional spatial relationships. In addition GFP does not diffuse, rendering localization quite precise and reproducible. However, in our experience bacteria expressing GFP in root systems were detectable only with the aid of high power objectives in conjunction with epifluorescent or laser scanning microscopes. This feature rendered GFP less suitable than GUS as a biomarker for examining macroscopic colonization patterns.

Problems with GFP as a Molecular Biomarker

GFP has been used successfully as a biomarker/bioreporter in a wide variety of bacteria (Chapter 5). However, in our hands repeated attempts using several protocols and two different GFP transposons resulted in the tagging of only three bacteria that displayed stable, detectable levels of green fluorescence. There are several possible explanations for the failure to tag most of the bacterial isolates in the "test collection" with GFP.

First, it is possible that the *gfp* gene is not expressed at high enough levels to allow detection of the GFP protein. For example, in the case of pTnPS65-T it is possible that the P*trp* promoter is not active in some of the rice isolates. The P*trp* promoter is active in *S. meliloti* 1021 (Gage et al., 1996; Figure 6-4) However, *E. coli* cells harboring pTnPS65-T are not fluorescent. It is possible that *E. coli* and other bacteria do not express genes controlled by this promoter properly, explaining why non-fluorescent kanamycin resistant transconjugants were found.

Second, the *gfp* gene and the *npt* antibiotic resistance gene contained on pRL765gfp are both under control of the P*psbA* promoter. Therefore kanamycin resistant transconjugants would be expected to also express GFP. However, none of the stable kanamycin resistant transconjugants obtained in this study displayed observable levels of green fluorescence, and, at best, showed faint fluorescence that rapidly faded. This would suggest that although the *gfp* gene is expressed at low levels, the fluorescence emitted from the GFP protein was inefficient.

Alternatively, the GFP protein may not be processed correctly and/or sequestered in non-fluorescent inclusion bodies (Heim et al., 1994).

Difficulties in obtaining stable green fluorescent transconjugants may also be due to minor toxic effects produced by GFP. This hypothesis is supported by other evidence, including the difficulty constructing plasmids with the *gfp* gene under the control of strong constitutive promoters, especially using a medium containing high salt concentrations during the transformation process (Stoltzfus and de Bruijn, unpublished data; Herman Spaink, personal communication).

Finally, it is also possible that the transposons were not properly integrating into the genomes of these bacteria. Plasmid pTnPS65-T carries the origin of replication of R6K and therefore requires the π protein for replication (de Lorenzo et al., 1990). This plasmid should not replicate in most bacteria. Plasmid pRL765gfp carries the pRK2 origin of replication, which replicates in *E. coli* and other Enterobacteriaceae. Three bacteria, *Enterobacter cloacae*, *Klebsiella planticola* and strain R66(40) showed intense green fluorescence following introduction of plasmid pRL765gfp. R66(40) is related to Enterobacteriaceae (Table 4-2). However, when removed from kanamycin selection, the green fluorescence was quickly lost. The most logical explanation is that the pRL765gfp plasmid was replicating autonomously in these bacteria and

that the transposon never integrated into the genome. Because of the rapid loss of fluorescence in the absence of a selectable marker these bacteria could not be used for *in-situ* colonization studies.

Colonization by *S. meliloti* 1021, R33(120) and R100(64)

Derivatives of three types of bacteria, *S. meliloti* 1021, R33(120) and R100(64), were obtained that did express GFP and GUS. No observable differences in colonization patterns were observed between different transconjugants of the same isolates. Nor were major differences in colonization patterns observed when comparing the same isolate expressing GFP or GUS. Bloemberg et al. (1997) observed no difference in growth and motility of *Pseudomonas* strains harboring the *gfp* gene on a plasmid, suggesting that GFP activity did not constitute an excessive metabolic burden. Therefore, the integration of only two genes into the genome of these isolates would not be expected to add significantly to metabolic load and/or alter colonization patterns.

Two to three weeks after inoculation of R33(120) or R100(64) to Lemont rice seedlings grown in the pillow system no endophytic colonization was observed. Colonization was limited to the surface and, on rare occasions, to isolated epidermal cells (Figure 6-7, 6-8, 6-9). This

does not mean that these bacteria are not endophytes. One possible explanation is that they were isolated from Philippino rice varieties at heading stage. It is, therefore, possible that endophytic colonization does not take place until later in rice plant development. It is also possible that Lemont is not amenable to colonization by these isolates or that colonization does not take place due to a shortcoming of the pillow system. For example, colonization may require the mechanical wounding that takes place when a root pushes its way through field soil. Some endophytes may also use fungi as aids for endophytic establishment (Paula et al., 1991). If colonization required the partnership between the tagged bacterial isolate and a fungi or other bacteria, colonization would not be expected to occur in an otherwise gnotobiotic pillow system.

Despite the fact that strains R100(64) and R33(120) could be re-isolated following inoculation, endophytic colonization was not observed using variants of these strains harboring the Gus or GFP biomarkers. These re-isolation studies were carried out on plants grown in the test tube system while observations using biomarkers were carried out using plants grown in the pillow system. Therefore, the re-isolation of bacteria experiment can not be directly correlated to the biomarker experiments.

Spatial Localization Using Three Dimensional Reconstructions

Bacteria expressing GFP could be detected using the laser scanning confocal microscope. This also allowed optical sectioning (Figure 6-8) and three dimensional reconstruction of the images (Figure 6-9). Without endogenous fluorescence of rice tissue/cells it was not possible to gain information on the spatial relationship between the bacteria and the plant tissues (Figure 6-9 A and B). In selected cases, the autofluorescence of plant tissue could be used to orient the viewer (Figure 6-9 C and D). However, in our experience, the yellow fluorescence from rice roots was quite variable and unsuitable for reliable three dimensional reconstruction. Propidium iodide can be used to fluorescently stain plant tissues (Gage et al., 1996). With the proper instrumentation, this adaptation of the protocol allowed real color optical sectioning and three dimensional reconstruction of the images (Figure 6-8, 6-9 E and F).

The fluorescence observed using this stain was very bright and tended to mask weak GFP signals, even after short staining times and long periods of destaining. This may not be a problem for digital imaging, since the relative intensities of the different fluorescence could be altered. However, it created problems for scanning infected tissues using the

oculars of a standard epifluorescent microscope. In addition the staining of the plant tissue was variable, with intense staining in some areas and virtually no staining in others. Therefore, development of better stains or staining techniques would greatly enhance the use of GFP as a biomarker for bacterial colonization of plant tissues.

CONCLUSIONS

GFP and GUS are promising tools for visualizing endophytic colonization of rice and other plants. However, better vectors with suitable promoters and new versions of GFP need to be developed for this biomarker to reach its full potential. GFP is useful for observing microscopic colonization by individual bacterial cell, or clumps of cells, and can be used in conjunction with laser scanning confocal microscopy to determine spatial relationships between bacteria and plant tissues. GUS is more suited for visualization of macroscopic colonization patterns.

The use GFP and GUS as biomarkers revealed the absence of a significant level of endophytic colonization of rice seedlings by two isolates, R100(64) and R33(120). However, due to the limited scope of this study, definitive conclusions on the endophytic nature of these bacteria can not be made. A more detailed investigation including the putative endophytes described at the end of Chapter 4 needs to be carried out. This investigation should involve the study of endophytic colonization using different rice varieties, specifically Philippino varieties from which these bacteria were originally isolated. It should also examine older plants, as endophytic colonization may not occur in young seedlings.

Table 6-1

Strain	Relevant Characteristics	Reference
***E. coli* DH5α**	*supE*44 Δ *lacU* (ϕ80*lac* ZΔM15) *hsdR*17 *recA*1 *endA*1*gyr A*96 *thi*-1 *relA*1	Sambrook et al., 1989
***E. coli* HB101**	*supE*44 *hsdS*20($r_B^-m_B^-$) *recA*13 *ara*-14 *proA*2, *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	Sambrook et al., 1989
***E. coli* S17-1 λpir**	*thi pro hsdR hsdM*$^+$ *recA* RP4 2-TC::Mu-Km::Tn7(Tp^r/Sm^r) λ-pir lysogen	Victor de Lorenzo, Centro de Investigaciones Biologicas, Madrid
***Klebsiella planticola* KpM5A1**	Isolated from the rice rhizosphere	Ladha et al., 1983
Enterobacter cloaca	Isolated from the rice rhizosphere	Ladha et al., 1983
***Azoarcus indigens* LMG 9098**	Endophyte of Kallar Grass	Reinhold-Hurek et al., 1993
***Herbaspirillum seropedicae* Z67**	Endophyte of sugarcane	Baldini et al., 1986
***Sinorhizobium meliloti* 1021**	Symbiont of *Medicago sativa*	Meade et al., 1982
***Azorhizobium caulinodans* ORS571**	Symbiont of *Sesbania rostrata*	Dreyfus and Dommergues, 1981
T105	Isolated from surface-sterilized rice	Stoltzfus et al., 1997
R22(88)	Isolated from surface-sterilized rice	Stoltzfus et al., 1997
R90(8)	Isolated from surface-sterilized rice	Stoltzfus et al., 1997
R100(64)	Isolated from surface-sterilized rice	Stoltzfus et al., 1997
-R6a(126)	Isolated from surface-sterilized rice	Stoltzfus et al., 1997
R33(120)	Isolated from surface-sterilized rice	Stoltzfus et al., 1997
R45(42)	Isolated from surface-sterilized rice	Stoltzfus et al., 1997
R032.S2.3	Isolated from surface-sterilized rice with an in-planta selection step	Stoltzfus et al., 1997
R061.S1.3	Isolated from surface-sterilized rice with an in-planta selection step	Stoltzfus et al., 1997
R66(40)	Isolated from surface-sterilized rice	Chapter 3, this work

Table 6-1 continued

Strain	Relevant Characteristics	Reference
Sinorhizobium meliloti*::*gfp	*Sinorhizobium meliloti* 1021 derivative containing mTnS65-T	This work
Sinorhizobium meliloti*::*gus	*Sinorhizobium meliloti* 1021 derivative containing mTn121TH1	This work
R33::*gfp*	R33(120) derivative containing mTnS65-T	This work
R33::*gus*	R33(120) derivative containing mTn121TH1	This work
R100::*gfp*	R100(64) derivative containing mTnS65-T	This work
R100::*gus*	R100(64) derivative containing mTn121TH1	This work

Table 6-1 Bacterial Strains

List of the strains, relevant characteristics and references for the bacteria used in this study.

Name	Relevant Characteristics	Reference/ Source
pCAM121	Sm/Sp, Ap;mTn*5SSgusA21*(*Paph-gusA-trpA* terminator translational fusion with adjacent unique *Spe*I site) in pUT/mini-Tn*5* Sm/Sp	Wilson et al., 1995
pTB93F	Cm, Sp; Broad host range vector containing P*trp-gfp*S65-T	Gage et al., 1996
pBluescript	Ap; Unique cloning sites	Stratagene, La Jolla, CA, USA
pUC 4K	Km, Ap;Tn*903* aminoglycoside 3'phosphotransferase gene in pUC 4	Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA
pUT/mini-Tn*5*Km	Km, Ap;mTn*5* (Kanamycin cassette with uniqe *Not*I site)	de Lorenzo et al. 1990
pTnPS65-T	Km, Ap; mTn*5*PS65-T (*Ptrp-gfpS65-T* from pTB93F) in pUT/mini -Tn*5* Km	This work
p121TH1	Km, Ap; mTn121TH1(mTn*5SSgusA2*with the Sm/Sp cassette exchanged with the Km cassette from pUC 4K)	This work
pRL765gfp	Km; pRL765:*gfp oriT*(RK2) *oirV*(p15A)	Tombolini et al., 1997
pRK2013	Km; ColE1 *ori*RK2-*mob*$^+$ RK2*tra*$^+$	Figurski and Helinski, 1979

Table 6-2 Plasmids

List of plasmids, relevant information and references for the constructs used in this study.

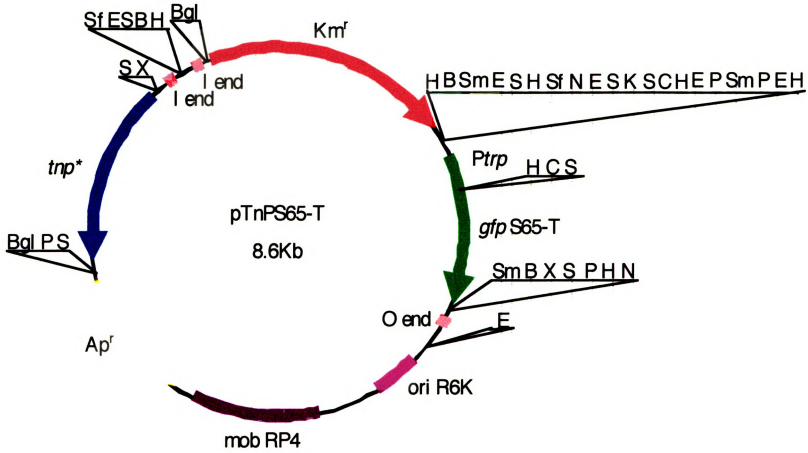


Figure 6-1 Map of pTnPS65-T

Structure of plasmid pTnPS65-T, constructed for the delivery of a mini-Tn*5* transposon containing a kanamycin resistance cassette and the gene for the S65T mutant of GFP controlled by the P*trp* promoter. Restriction sites: B, *Bam*HI; Bgl, *Bgl*I; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sal*I; Sf, *Sfi*I; Sm, *Sma*I; X, *Xba*I.

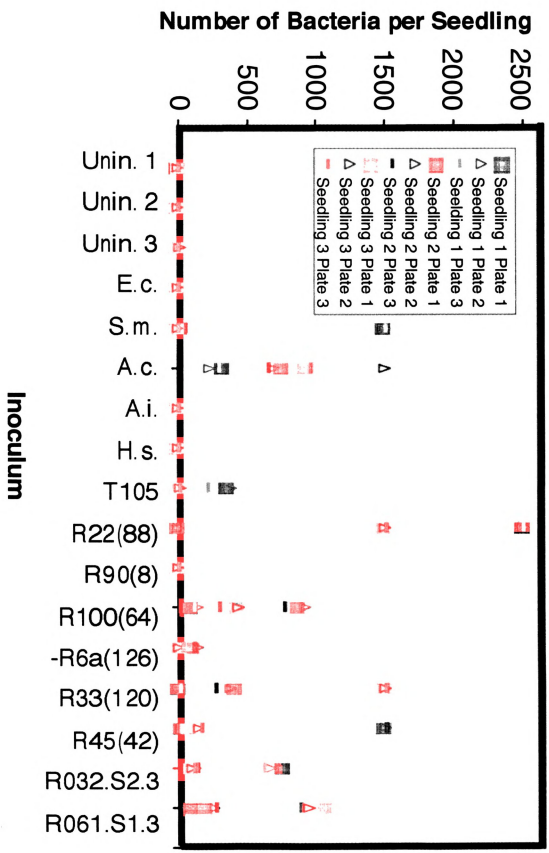

Number of Bacteria per Seedling
0
500
1000
1500
2000
2500
Inoculum
Unin. 1
Unin. 2
Unin. 3
E.c.
S.m.
A.c.
A.i.
H.s.
T105
R22(88)
R90(8)
R100(64)
-R6a(126)
R33(120)
R45(42)
R032.S2.3
R061.S1.3
Seedling 1 Plate 1
Seedling 1 Plate 2
Seedling 1 Plate 3
Seedling 2 Plate 1
Seedling 2 Plate 2
Seedling 2 Plate 3
Seedling 3 Plate 1
Seedling 3 Plate 2
Seedling 3 Plate 3

Figure 6-2 Re-isolation of Bacteria from Sterilized Rice Seedlings

This graph shows the number of bacteria isolated from rice seedlings inoculated with pure culture of the bacteria indicated on the X-axis, grown for three weeks in sterile test tubes. Three seedlings were inoculated with each strain. Following surface sterilization, each seedling was macerated and dilutions of the macerate were spread on three TY plates. Bacteria were counted and the identity of several colonies was verified using rep-PCR genomic fingerprints. "Unin. 1-3" represent seedlings inoculated with sterile TY media.

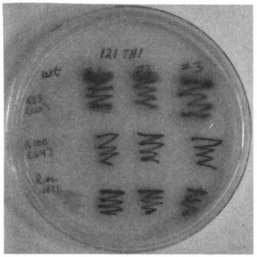


Figure 6-3 Bacteria Marked with GUS

Bacteria grown on a TY plate supplemented with 50 µg/ml X-gluc. The bacteria were tagged with the transposon Tn121TH1. Two of the strains, R33(120) (top) and R100(64) (middle), were isolated from surface-sterilized rice. The third strain, *Sinorhizobium meliloti* 1021(bottom), was used as a control. Parental bacteria are shown on the far left (no color). Three transconjugants for each isolate shown on the right produced a dark blue color, indicative of GUS activity.

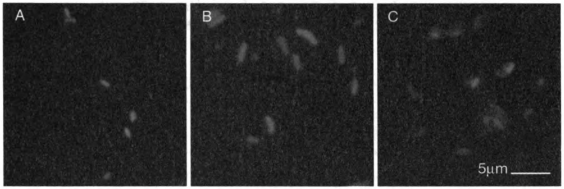


Figure 6-4 Bacteria Marked with GFP

Fluorescent micrographs of three bacterial strains expressing GFP are shown. These strains were used for visualization of colonization of rice seedlings. (A)*Sinorhizobium meliloti*::*gfp*, used as a control. (B) R33::*gfp* and (C) R100::*gfp*, isolated from surface-sterilized rice. The bacteria were tagged with the transposon TnPS65-T. Bacteria were grown in liquid TY media and placed on glass slides in 0.8% agarose. The micrographs were taken using a Kodak Digital Science DC120 digital camera attached to a Zeiss Axiophot microscope equipped with a standard FITC filter set

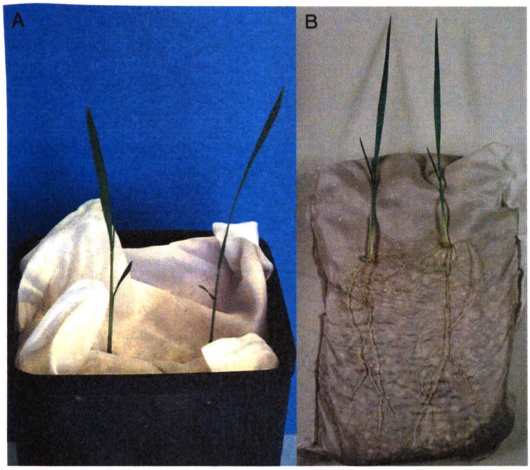


Figure 6-5 Growth of Rice Seedlings in the Pillow System

Pictures of two week old rice seedlings grown in the 'pillow system' are shown. (A) Seedlings grow sandwiched between two pillows. (B) Picture showing the root systems of seedlings associated with one of the two pillows.

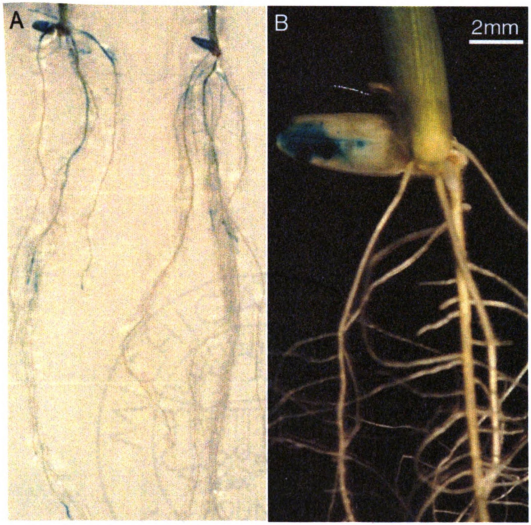


Figure 6-6 Gus Staining of Rice Plants

Pictures of rice seedlings inoculated with bacteria expressing the GUS biomarker incubated in buffer containing X-Gluc are shown. The blue color is indicative of bacterial Gus activity. (A) Colonization patterns of R100::*gus* bacteria on two week old rice roots. (B) Seed colonization pattern of plants inoculated with R33::*gus* bacteria.

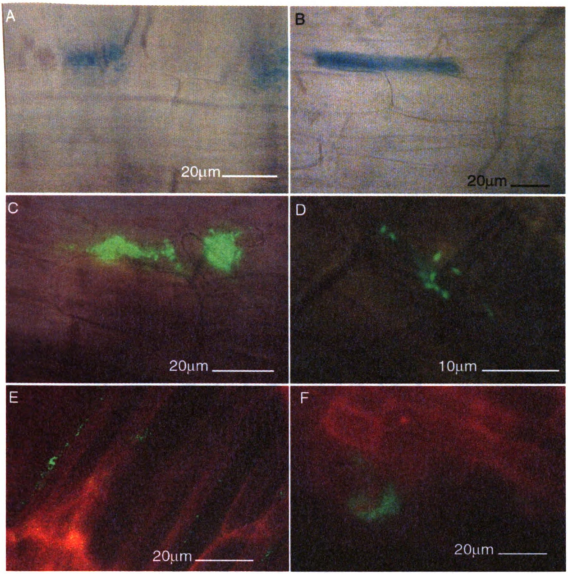


Figure 6-7 Microscopic Observation of Colonization

Micrographs of plants inoculated with bacteria expressing the biomarker GUS (A and B) or GFP (C-F) are shown. Transmitted micrographs take using a Leitz microscope of (A) R100::*gus* bacteria (blue color) in a clump on the surface of a rice root and (B) colonizing an epidermal cell. Overlay of a transmitted and a fluorescent micrograph of (C) *S. meliloti*::*gfp* bacteria (green color) in a clump on the surface of a rice root and (D) individual bacterial cells at the junction between epidermal cells. Fluorescent micrographs of rice roots stained with propidium iodide (red color) and colonized by (E) R33::*gfp* bacteria (green color) at the junctions of epidermal cells and (F) R100::*gfp* bacteria (green color) in clumps on the surface of the root.

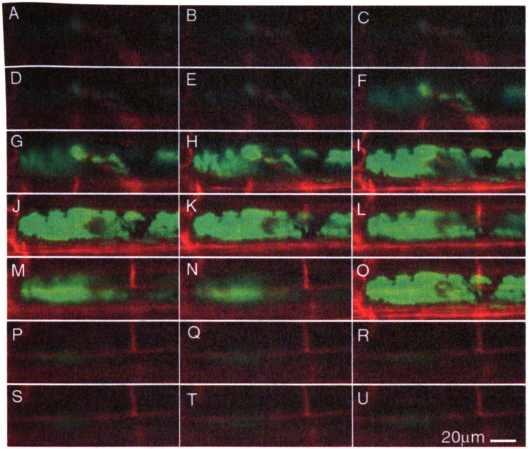


Figure 6-8 Optical Sections of a Rice Cell Colonized by R100(64) Expressing GFP

Optical sections of an epidermal cell colonized by R100::*gfp* bacteria (green color). Each section is 0.3 μm deeper into the sample. The rice root tissue was stained with propidium iodide (red color). Section one starts at the surface of the rice root and section 80 is 24μm below the surface of the root. (A-E) Individual optical sections 1 through 5. (F) Overlay of sections 1-5. (G-N) Overlays containing ten optical sections, starting with sections 1 through 10 and ending with sections 71 through 80. (O) Overlay of all 80 optical sections. (P-T) Individual optical sections 76 through 80. (U) Overlay of optical sections 76 through 80.

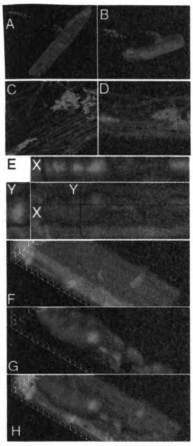


Figure 6-9 Three Dimensional Reconstruction of Optical Sections

Three dimensional computer reconstructions of rice root colonization by bacteria expressing the GFP biomarker. These renderings are constructed from optical sections made using a laser scanning confocal microscope. (A & B) Two views of an epidermal root cell and attached root hair filled with R100::*gfp* bacteria (green color). (C & D) Two views of R33::*gfp* bacteria (green color) colonizing the surface of a rice root (red color). Artificial colorization is based on fluorescent intensity. A low level of yellow autofluorescence from the root tissue is depicted in red. The intense green fluorescence from the tagged bacteria is depicted in green. (E-H) Images created using dual channel detection of R100::*gfp* bacteria colonizing an epidermal cell on a rice root. The red fluorescence is from propidium iodide stained root tissue. The green fluorescence is from R100::*gfp* bacteria. (E) Phi Z sections along the X and Y axis. (F) Oblique view of the 3D reconstruction showing only the root tissue (red channel), (G) only the bacteria (green channel), and (H) the root tissue and bacteria (red and green channels) combined.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

DIVERSITY OF RICE ISOLATES

Bacteria isolated from mechanically-abraded, surface-sterilized rice tissue are genotypically diverse. This may indicate that many types of bacteria can colonize rice plants endophytically, or it may indicate that the surface sterilization used was inadequate to eliminate rhizoplane and rhizosphere bacteria. Bacteria which could be isolated following inoculation to sterile seedlings did not show endophytic colonization when observed using biomarkers. Without further observation it is impossible to determine the endophytic nature of the isolates in this collection .

Many of the bacteria isolated have similar ARDRA fingerprints or SSU rRNA gene sequences to bacteria commonly isolated from the rhizosphere of rice. Some diazotrophic bacteria in this collection have not been commonly isolated from the rhizosphere of rice but show similarities to other putative rice endophytes. These bacteria may represent obligate endophytes and deserve further study as candidates for supplying rice with biologically-fixed nitrogen.

UTILITY OF *nifD*-PCR

The *nifD*-PCR primers presented here are capable of amplifying a specific fragment from most diazotrophic bacteria. These primers identified 20 bacteria from a collection of putative rice endophytes, 19 of which were shown to be authentic diazotrophs. These primers are suitable for identifying nitrogen-fixing bacteria in collections of environmental isolates and for detection of diazotrophs in the environment.

UTILITY OF GUS AND GFP AS COLONIZATION BIOMARKERS

GUS and GFP are both useful for localizing tagged bacteria following inoculation to plant tissues. GUS is better suited for determining overall colonization patterns. GFP in conjunction with optical sectioning and three dimensional reconstruction allows visualization of spatial relationships between the bacteria and plant tissue.

COLONIZATION OF SEEDLINGS BY RICE ISOLATES

Bacteria isolated from mechanically-abraded, surface-sterilized rice can be isolated following inoculation to sterile seedlings grown in test tubes. However, since plants grown in test tubes appear less vigorous than seedlings grown in pots the significance of this is unclear. Biomarkers were used to examine the colonization patterns for two of these bacteria,

R33(120) and R100(64). No endophytic colonization was found. R100(64) was, on very rare occasions, found to colonize individual epidermal cells.

APPENDIX I

Strain	Soil Type	Variety	Isolation Medium
-R11b(59)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R11c(59)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R136(33)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R139(43)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R14(5)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R141(99)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R143(44)	Pangasinan	Pinidua	Culm Extract & Water Agar[1]
-R144(100)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R144(30)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R145(69)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R146(23)	Pangasinan	Pinidua	Culm Extract & Water Agar[1]
-R16(47)	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R3	Bicol	*Oryza minuta*	Culm Extract & Water Agar[1]
-R38(26)	Pangasinan	Pinidua	Culm Extract & Water Agar[1]
-R6a(126)	Bulacan	Bomalasang	Culm Extract & Water Agar[1]
K10	Banaue	Oking Seroni	Congo Red[2]
K100	Pangasinan	Pinidua	Congo Red[2]
K103	Banaue	*Oryza minuta*	Congo Red[2]
K107	Banaue	*Oryza minuta*	Congo Red[2]
K108	Banaue	*Oryza minuta*	Congo Red[2]
K109	Bulacan	*Oryza minuta*	Congo Red[2]
K11	Banaue	Oking Seroni	Congo Red[2]
K110	Bulacan	*Oryza minuta*	Congo Red[2]
K113	Bulacan	*Oryza minuta*	Congo Red[2]
K3	Banaue	Oking Seroni	Congo Red[2]
K34a	Banaue	Bomalasang	Congo Red[2]
K36a	Banaue	Bomalasang	Congo Red[2]
K36c	Banaue	Bomalasang	Congo Red[2]
K37	Banaue	Bomalasang	Congo Red[2]

Appendix I continued

K45	Bulacan	Bomalasang	Congo Red[2]
K46	Bulacan	Bomalasang	Congo Red[2]
K5	Banaue	*Oking Seroni*	Congo Red[2]
K6	Banaue	*Oking Seroni*	Congo Red[2]
K71	Pangasinan	Pinidua	Congo Red[2]
K8	Banaue	*Oking Seroni*	Congo Red[2]
K9	Banaue	*Oking Seroni*	Congo Red[2]
K93	Pangasinan	Pinikitan	Congo Red[2]
K94	Pangasinan	Pinikitan	Congo Red[2]
K95	Pangasinan	Pinikitan	Congo Red[2]
K96	Pangasinan	Pinikitan	Congo Red[2]
L21	Banaue	Bomalasang	LGIP[3]
R100(64)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R103(73)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R103(96)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R104(113)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R104(75)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R10a(32)	Bicol	*Oryza minuta*	Malate Semi Solid Media[4]
R10a(86)	Bicol	*Oryza minuta*	Malate Semi Solid Media[4]
R11b(103)	Bicol	*Oryza minuta*	Malate Semi Solid Media[4]
R11c(74)	Bicol	*Oryza minuta*	Malate Semi Solid Media[4]
R19(122)	Pangasinan	Pinidua	Malate Semi Solid Media[4]
R19(77)	Pangasinan	Pinidua	Malate Semi Solid Media[4]
R1a(35)	Bulacan	Bomalasang	Malate Semi Solid Media[4]
R22(88)	Pangasinan	Pinidua	Malate Semi Solid Media[4]
R29(20)	Bicol	Pinikitan	Malate Semi Solid Media[4]
R29(91)	Bicol	Pinikitan	Malate Semi Solid Media[4]
R32(121)	Bicol	Pinidua	Malate Semi Solid Media[4]
R32(95)	Bicol	Pinidua	Malate Semi Solid Media[4]
R33(120)	Banaue	Pinidua	Malate Semi Solid Media[4]
R33(94)	Banaue	Pinidua	Malate Semi Solid Media[4]

Appendix I continued

R39(129)	Bulacan	IR42	Malate Semi Solid Media[4]
R40(39)	Maahas	Pinidua	Malate Semi Solid Media[4]
R41(16)	Banaue	Pinikitan	Malate Semi Solid Media[4]
R42(21)	Bulacan	IR42	Malate Semi Solid Media[4]
R43(41)	Bulacan	IR42	Malate Semi Solid Media[4]
R44(51)	Bulacan	IR42	Malate Semi Solid Media[4]
R45(42)	Bulacan	IR42	Malate Semi Solid Media[4]
R46(9)	Bicol	Pinidua	Malate Semi Solid Media[4]
R48b	Pangasinan	Pinikitan	Malate Semi Solid Media[4]
R49(56)	Pangasinan	Pinikitan	Malate Semi Solid Media[4]
R4a(118)	Bulacan	Bomalasang	Malate Semi Solid Media[4]
R54(10)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R55(112)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R58(76)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R58b(102)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R59(24)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R59b(13)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R61(12)	Banaue	IR74	Malate Semi Solid Media[4]
R62(15)	Banaue	IR74	Malate Semi Solid Media[4]
R62b(117)	Banaue	IR74	Malate Semi Solid Media[4]
R63b(124)	Banaue	IR74	Malate Semi Solid Media[4]
R65(49)	Banaue	IR74	Malate Semi Solid Media[4]
R66(40)	Banaue	IR74	Malate Semi Solid Media[4]
R68(101)	Banaue	IR74	Malate Semi Solid Media[4]
R69(72)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R70b	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R70b(37)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R70b(68)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R72(104)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R73(4)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R73(6)	Maahas	Oking seroni	Malate Semi Solid Media[4]

Appendix I continued

R73c(36)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R75(50)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R76b(27)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R77(132)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R77(18)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R78(57)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R79(108)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R79(79)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R8(28)	Bulacan	Bomalasang	Malate Semi Solid Media[4]
R8(54)	Bulacan	Bomalasang	Malate Semi Solid Media[4]
R81(1)	Maahas	Pinikitan	Malate Semi Solid Media[4]
R81(90)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R82(128)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R83(71)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R84(22)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R85(92)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R85b(130)	Banaue	IR74	Malate Semi Solid Media[4]
R87b(110)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R87c(111)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R88	Banaue	IR74	Malate Semi Solid Media[4]
R89(66)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R89(87)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R90(8)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R92(60)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R92b(34)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R93(58)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R93b	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R94(17)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R94b	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R95(7)	Maahas	Oking Seroni	Malate Semi Solid Media[4]
R96(115)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]

Appendix I continued

R97(106)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R97b((34)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R98(2)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R98(46)	Pangasinan	Oking Seroni	Malate Semi Solid Media[4]
R99(65)	Maahas	Pinikitan	Malate Semi Solid Media[4]
S2	Bicol	*Oryza minuta*	Malate Semi Solid Media[4]
S489	Maahas	IR74	Malate Semi Solid Media[4]
T10	Banaue	Oking Seroni	0.1% TSA[4]
T105	Banaue	*Oking minuta*	0.1% TSA[4]
T116	Bicol	*Oryza minuta*	0.1% TSA[4]
T12	Banaue	Oking Seroni	0.1% TSA[4]
T14	Banaue	Oking Seroni	0.1% TSA[4]
T60	Banaue	Pinidua	0.1% TSA[4]
T62	Pangasinan	Pinidua	0.1% TSA[4]
T77	Banaue	Pinikitan	0.1% TSA[4]
T90	Pangasinan	Pinikitan	0.1% TSA[4]
T93	Pangasinan	Pinikitan	0.1% TSA[4]
T94	Pangasinan	Pinikitan	0.1% TSA[4]
T96a	Pangasinan	Pinikitan	0.1% TSA[4]
T96b	Pangasinan	Pinikitan	0.1% TSA[4]

[1] Barraquio WL, Revilla L, Ladha JK (1997) Isolation of endophytic diazotrophic bacteria from wetland rice. Plant and Soil 194: 15-24

[2] Congro Red medium (g/L)

K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
Yeast Extract	0.5
$FeCl_3 \cdot 6H_2O$	0.015
DL-Malic acid	5.0
KOH	4.8
H_2O	1000 ml

Adjusted to pH 7.0 with 0.1N KOH

Bacto-Agar	20.00

1:400 ml aqueous solution of congo red 15.0 ml/L was added to media aseptically before plating. (Congro red solution and basal medium were autoclave separately)

[3] Reis VM, Olivares FL, Döbereiner J (1994) Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. Wold J Microbiol Biotechnol 10: 401-405

[4] 0.1% TSA medium (g/l)

Tryptic Soy Agar	1.0
H_2O	1000 ml

Adjusted to pH 7.0 with 0.1N KOH

Bacto-Agar	20.00