PHENOTYPIC, GENOTYPIC AND COLONIZATION PROPERTIES OF 2,4-DIACETYLPHLOROGLUCINOL-PRODUCING *PSEUDOMONAS* SPP. ISOLATED FROM ROOTS OF WHEAT

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of OLGA MAVRODI find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Fluorescent *Pseudomonas* spp. that produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) inhibit a variety of soilborne plant pathogens and play an important role in the suppression of take-all disesase of wheat. Diversity within *phlD*, an essential gene in the biosynthesis of DAPG, was studied by restriction fragment length polymorphism (RFLP) analysis of 123 DAPG-producing isolates from throughout the USA and worldwide. Clusters (genotypes) defined by RFLP analysis of *phlD* correlated closely with clusters defined by rep-PCR of total genomic DNA, validating the utility of *phlD* as a marker of genetic diversity and population structure among DAPG producers. Additional population diversity was revealed by random amplified polymorphic DNA (RAPD) analysis of genomic DNA. Genotypes defined by RFLP analysis of *phlD* were conserved among isolates from the same site and cropping history. Genotypic diversity among 30 strains representing all of the *phlD* RFLP groups did not correlate with production *in vitro* of monoacetylphloroglucinol, DAPG or total phloroglucinol compounds.

Twenty-seven of these 30 strains lacked pyrrolnitrin and pyoluteorin biosynthetic genes as determined by the use of specific primers and probes.

Members of the D genotype of DAPG-producing strains of *P. fluorescens* are exceptionally aggressive root colonizers of wheat and pea. Four genes broadly implicated in microbe-host interactions, *sss*, *dsb*A, *pts*P, and *orf*T, were investigated for their contribution to this aggressive colonization phenotype. These genes influence global processes including phenotypic plasticity, secretion, organic nitrogen utilization, and transmembrane transport, respectively, and were identified in an ordered genomic library of Q8r1-96 by colony hybridization and PCR. All four genes were mapped, sequenced and the corresponding gene replacement mutants were constructed and characterized. Mutants in *dsbA*, *sss,* and *orfT* colonized the roots of wheat grown in natural soil as effectively as did the parental strain when introduced separately, but were less competitive root colonists when introduced together with the parental strain. However, the *ptsP* mutant, colonized wheat roots less effectively than Q8r1-96 whether introduced alone or in combination with the parental strain, indicating that this gene is critical for effective rhizosphere colonization by *P. fluorescens* Q8r1-96.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

CHAPTER 1

CHAPTER 2

CHAPTER 3

Dedication

To my family, Dmitri and Irina Mavrodi and

to my parents Vassily and Tatyana Fisenko.

GENERAL INTRODUCTION

Take-all decline. The soilborne fungus *Gaeumannomyces graminis* var. *tritici* causes take-all disease of wheat and barley worldwide (Asher and Shipton, 1981), with reported yield losses in the United States of over $1 billion annually. At present, methods to control take-all are limited because no resistant cultivars or effective and economical fungicides for chemical seed treatment are available (Cook, 2003). Take-all can best be managed by crop rotation away from wheat, barley, or other susceptible grasses, but the trends in many cereal-based cropping systems are toward several consecutive crops of wheat before a break (for economic reasons) and reduced tillage or direct seeding (for erosion control) (Cook, 2003). These practices exacerbate take-all and other root diseases. In addition, because of the rising public concern about the application of chemical fungicides to control plant diseases and the threat of these chemicals to human health and the environment, biological control is considered to be the best alternative to chemical methods. The most promising approach to a biological control for take-all is based on the natural phenomenon of take-all decline (TAD), the spontaneous decrease in the incidence and severity of take-all that occurs with monoculture wheat or barley in *G. g. tritici*-infested fields following a severe outbreak of the disease (Cook and Weller, 1987). TAD occurs worldwide and is considered a classic example of a suppressive soil – one in which "the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (Baker and Cook, 1974). The suppressiveness of take-all decline soils is transferable by adding a small amount of suppressive soil to a nonsuppressive (conducive) soil and is eliminated by soil fumigation or pasteurization (Cook and Rovira, 1976).

Evidence that DAPG-producing *Pseudomonas* spp. are responsible for TAD. The microbiological basis for the suppressiveness associated with TAD soils was originally thought to be associated with the presence of certain strains of *Pseudomonas fluorescens* (Cook and Weller, 1987; Weller *et al.,* 1988) that produce antifungal metabolites such as phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (DAPG) (Bull *et al.,* 1991; Raaijmakers and Weller, 1998). However, PCA-producing fluorescent *Pseudomonas* spp. were not detected on the roots of wheat grown in suppressive soils at levels required to control take-all (Raaijmakers *et al.,* 1997). In contrast, several lines of evidence indicate that DAPG-producing fluorescent *Pseudomonas* spp. are a key component of the suppressiveness of TAD soils. For example, such *Pseudomonas* spp. are present on the roots of wheat grown in TAD soils at population densities above the threshold (10^5 CFU g^{-1} root; Raaijmakers and Weller, 1998) required for take-all control, but populations of these *Pseudomonas* spp. are below the threshold or not detected on roots from conducive soils (Raaijmakers *et al.,* 1997). There is a strong association between high populations of indigenous DAPG-producing fluorescent *Pseudomonas* spp. and low severity of take-all. TAD soils lose their suppressiveness after pasteurization (60°C, 30 min), and steamed conducive soils gain suppressiveness after introduction of DAPG producers. DAPG was detected on roots of wheat grown in TAD soil at an average concentration of 19 ng g^{-1} root fresh weight, but was not detected on roots grown in a conducive soil (Raaijmakers and Weller, 1999).

TAD and 2,4-diacetylphloroglucinol. Antifungal metabolites produced in situ by plant growth-promoting rhizobacteria (PGPR) play key roles in the control of many soilborne pathogens including *G. g. tritici* (Thomashow and Weller, 1995). Single mutations that abolish the production of such metabolites can simultaneously reduce biocontrol activity, and the active metabolites have

been isolated directly from the spermosphere and rhizosphere (reviewed in Raaijmakers *et al.,* 2002). Strains of *Pseudomonas* that produce pyrrolnitrin, pyoluteorin, phenazine derivatives, and DAPG are a major focus of biocontrol research, and numerous biosynthetic and regulatory genes involved in their synthesis currently are under investigation (reviewed in Haas and Keel, 2003).

The polyketide metabolite DAPG is not only a key determinant in the suppression of take-all but also a variety of other root and seedling diseases including black root rot of tobacco, crown and root rot of tomato, and Pythium damping-off of cucumber and sugar beet (Raaijmakers *et al.*, 2002). The DAPG biosynthetic locus includes six genes that function in regulation, synthesis, and export (Bangera and Thomashow, 1999). Particularly noteworthy is the *phlD* gene product, which functions in the synthesis of the DAPG precursor monoacetylphloroglucinol (MAPG) and has remarkable homology with members of the chalcone/stilbene synthase family of plant enzymes (Bangera and Thomashow, 1999). The *phl* biosynthetic genes are conserved among all known DAPG-producing pseudomonads including isolates from suppressive soils in the US, Europe, and Africa (Keel *et al.,* 1996; McSpadden Gardener *et al.*, 2000). With few exceptions, detection of the genes is correlated perfectly with DAPG production as measured by HPLC.

DAPG producers are genotypically and phenotypically diverse. Because soils suppressive to *G. g. tritici* occur worldwide and *Pseudomonas* spp. that produce DAPG have been isolated from numerous crops grown under a variety of different conditions, the genotypic and phenotypic diversity of DAPG producers has been studied intensively for more than a decade. Phylogenetic comparisons based on either sequencing or amplified ribosomal DNA restriction analysis (ARDRA) of 16S ribosomal DNA genes have revealed three distinct lineages (phylogenetic groups) among 45 DAPG $^{+}$ and 138 DAPG $^{+}$ fluorescent *Pseudomonas* spp.

studied by Keel *et al.* (1996) and later by McSpadden Gardener et al. (2000). These groups, originally designated as ARDRA groups 1, 2, and 3 by Keel *et al.* (1996), correspond to groups A, B, and C as defined by McSpadden Gardener *et al.* (McSpadden Gardener *et al.*, 2000). Group 1 (A) contains all strains that produce DAPG, hydrogen cyanide (HCN), and pyoluteorin (PLT). Strains that produced DAPG and HCN but not PLT are clustered in Group 3 (B) and Group 2 (C). The latter group consists only of two strains, F113 and P12. The two main lineages, 1(A) and 3 (B), are distinguished by their differential capacity to produce pyoluteorin (Keel *et al.*, 1996) and to utilize certain carbon sources for growth (McSpadden Gardener *et al.*, 2000). Results of ARDRA indicate that these lineages do not correlate with the geographical origin of isolates, which were obtained from several crop species and continents (Keel *et al.*, 1996; McSpadden Gardener *et al.*, 2000). An additional degree of variation among isolates has been observed by using finer scale genotyping based on genomic fingerprinting by randomly amplified polymorphic DNA (RAPD) (Keel *et al.*, 1996; Picard *et al.*, 2000; Raaijmakers and Weller, 2001) and rep-PCR (McSpadden Gardener *et al.*, 2000; Landa *et al.*, 2002). To date, these different techniques have distinguished at least 17 different genotypes, designated as A-Q, within strains isolated from different states in the U.S. and different European countries and from different host plants (McSpadden Gardener *et al.*, 2000; Raaijmakers and Weller, 2001; Landa *et al.*, 2002).

Root colonization. It has been widely accepted that, in addition to the ability to produce biologically active compounds, successful biocontrol agents also must establish and maintain themselves in the presence of the large, metabolically active resident microbial population supported by the exudates and other organic metabolites provided by the root. It can be concluded from studies over the past 30 years that many bacterial traits are involved in the

colonization process, with the relative importance of individual traits varying according to the bacterial strain, host plant species and age, soil type, environmental conditions, and type of assay used.

Root colonization is defined as an active process involving the multiplication and survival of the introduced bacteria for several weeks on or around the roots in the presence of the indigenous bacteria (Weller and Thomashow, 1994). This process includes colonization of the rhizosphere, inside of the root, the root surface, and the rhizosphere soil (Weller and Thomashow, 1994). Root colonization is a complex process, and includes interactions among the biological agent, the pathogen, and the resident rhizosphere microflora in a constantly changing environment. Root colonization by introduced bacteria generally is considered essential to the suppression of root pathogens. Bacteria growing in or near infection courts on roots are ideally positioned to limit the establishment and/or spread of pathogens. Root colonization has been the subject of intense research during the past two decades because variable colonization remains one of the major impediments to the widespread use of rhizobacteria in commercial agriculture (Weller and Thomashow, 1994). Inconsistency in field trials from site to site and year to year has been correlated with poor colonization of the rhizosphere by the biocontrol agent (Weller and Thomashow, 1994). In order to perform in the field efficiently, a biocontrol strain needs to successfully establish, proliferate, and persist in the rhizosphere (Weller and Thomashow, 1994). Intensive studies over the last decade have revealed several traits critical for the root colonization by *Pseudomonas* spp. Among these traits are (i) attachment to roots, (ii) motility and chemotaxis, (iii) polysaccharide production, (iv) availability and catabolism of nutritional sources, (v) a two-component signaling system, (vi) a site-specific recombinase, and (vii) NADH dehydrogenase I.

(i) Attachment to the roots. Attachment to the roots is a crucial step in establishing plant-microbe interactions, and flagella and different types of pili play a role in bacterial attachment to diverse surfaces. However, their role in root colonization of surfaces by microorganisms is controversial and differs among species and studies. *Pseudomonas* bacteria produce up to nine polar flagella per cell, and it has been demonstrated that such flagella are necessary for the initial stages of biofilm formation (O'Toole and Kolter, 1998), a process considered comparable to colonization and growth on plant root surfaces. It also has been reported that type 1 pili play a role in adhesion of enteric bacteria to grass roots (Haahtela and Korhonen, 1985) and type 3 pili of nitrogen-fixing *Klebsiella* play a role in adhesion to roots (Korhonen *et al.*, 1983). Similarly, in *P. fluorescens* 2-79, pili are present on only a fraction of the cells present in a population, and populations with a higher percentage of fimbriated cells attach more efficiently to corn roots (Vesper *et al.*, 1987). The role of pili has been studied extensively in different bacteria due to their involvement in pathogenesis. It has been shown that type 4 pili of *P. aeruginosa* are involved in twitching motility (Darzins and Russel, 1997), attachment to the epithelial cells and abiotic surfaces, and biofilm formation (Hahn, 1997). Camacho Carvajal (Camacho Carvajal, 2000) recently reported the presence of type 4 pili genes in *P. fluorescens* strain WCS365 and their involvement in efficient competitive root tip colonization, most likely through twitching motility.

(ii) Motility and chemotaxis. In addition to their role in the attachment of microorganisms to plant roots, functional flagella are important for bacterial motility. The crucial role of motility for successful rhizosphere colonization is somewhat controversial because some studies have indicated that motility of *Pseudomonas* is not required for root colonization in wheat and soybean (Howie *et al.*, 1987, Sher *et al.*, 1988). However, flagella were shown to be

essential for colonization of potato roots (de Weger *et al.*, 1987). Studies confirming the role of motility in the colonization process were performed in the absence of percolating water, and it was assumed that motile or non-motile introduced bacterial strains were transported by the growing roots. However, under more natural conditions the presence of percolating water will affect the dispersal of bacterial strains regardless of their ability to swim. In addition, active swimming in the soil also depends on soil moisture, because if the soil is drier than -50 kPa the water films are too thin for flagella-mediated movement (Griffin and Quail, 1968). Chemotaxis plays a role in the establishment of both deleterious and beneficial plant-microbe associations, and experiments with mutants defective in the general chemotaxis gene *cheA* have revealed that in the absence of percolating water, chemotaxis is crucial for competitive colonization of tomato roots by *P. fluorescens* WCS365 (Dekkers *et al.*, 1998a; Lugtenberg *et al.*, 2001).

(iii) Polysaccharides. Gram-negative bacteria like *E. coli* and *Pseudomonas* spp. have a cell wall consisting of inner and outer membranes that contain phospholipid bilayers. The outer membrane also contains lipopolysaccharides (LPS) (Sigee, 1993) that are embedded in the outer face of the phospholipid bilayer and are composed of both lipid and carbohydrate moieties including lipid A, the R-core region, and the O-side chain. The O-side chain is composed of repeating units of short, branched oligosaccharides and extends out for up to 30 nm from the cell surface (Sigee, 1993). The O-side chain is responsible for the serological properties of the bacterial strain and for initial interactions between bacteria and plant cell surfaces (Sigee, 1993). It has been shown that an intact O-antigen of LPS is required for efficient tomato root colonization by *P. fluorescens* WCS365 (Dekkers *et al.*, 1998a) and it was speculated that an intact outer membrane is necessary for efficient uptake of nutrients, secretion of waste products, and tolerance towards osmotic stress, pH variations, and desiccation (Camacho Carvajal, 2000).

However, the exact mechanism by which the O-antigen mediates root colonization is not known (Lugtenberg *et al.*, 2001).

(iv) Availability and catabolism of nutritional sources. Root exudates are assumed to be the major nutrient source for microorganisms living in the rhizosphere. For example, tomato root exudates are primarily composed of sugars and organic acids (Lugtenberg *et al.*, 1999). However, mutants of WCS365 that are impaired in the utilization of sugars found in root exudates are not impaired in root colonization (Lugtenberg *et al.*, 1999). On the other hand, the ability is to synthesize amino acids and vitamins such as biotin, thiamine, and riboflavin is important for plant root colonization by WCS365 (Simons *et al.*, 1996, Simons *et al.*, 1997).

The gene *pyrR* is involved in the competitive root colonization ability of *P. fluorescens* WCS365, and encodes a positive regulator of the *de novo* pyrimidine biosynthetic pathway (Camacho Carvajal, 2000). Generally Gram-negative bacterial cells are thought to have two types of nucleotide biosynthetic pathways: endogenous (*de novo*), which requires the synthesis of precursors, and exogenous (salvage), where precursors are taken from the environment or from nucleic acid degradation processes. Pyrimidines are present in the rhizosphere and root exudates in limiting amounts, and a functional *de novo* biosynthetic pathway that depends on the activity of PyrR is required for survival of *P. fluorescens* WCS365 under these conditions (Camacho Carvajal, 2000).

(v) Two-component signaling system. Two-component signaling systems are common among Gram-negative bacteria, which use them to sense changes in the environment and appropriately modulate the expression of genes or the activity of certain enzymes (Stock *et al.*, 1989). The mechanism of regulation usually involves two proteins: a sensor and a response regulator (Wanner, 1992). The sensor protein is embedded in the outer membrane and contains

sensor and transmitter domains (Wanner, 1992). If the stimulus is present in the environment, the sensor domain senses it (Stock *et al.*, 1989), which triggers conformational changes in both the sensor and transmitter domains (Wanner, 1992). This causes autophosphorylation of the transmitter domain, which then donates the phosphoryl group to a response regulator which in turn regulates transcription of the target genes (Wanner, 1992). A two-component signaling system, *colS*/*colR,* has been reported to be involved in root colonization by *P. fluorescens* strain WCS365 (Lugtenberg *et al.*, 2001). The *colS* gene product shows high homology to sensor kinases and the *colR* gene encodes the response regulator of the two-component regulatory system (Dekkers *et al.*, 1998b). However, the stimulus which the system is sensing in the rhizosphere, and the genes which are regulated by it, are still not identified (Dekkers *et al.*, 1998b).

(vi) A site-specific recombinase. Phase variation is the phenomenon of diversification of a bacterial population into subpopulations, which leads to adaptation to sudden environmental changes. This presence of a mixed population ensures that a certain percent of the overall population will already express a phenotype necessary for survival in a new environment (Henderson *et al.*, 1999). During phase variation, bacteria frequently and reversibly change phenotypes due to genetic rearrangements in specific 'contingency' loci of their genomes (Hallet *et al.*, 2001). This phenomenon has been extensively studied in bacterial pathogenic systems and includes examples of phase variation of type 1 fimbriae from *Esherichia coli,* flagella of *Salmonella typhimurium,* and surface antigens of *Nesseria gonorrhoeae* (Henderson *et al.*, 1999). The mechanisms of phase variation by site-specific recombinases usually include site-specific DNA inversions or deletions that provide simple or more complex switches in gene expression. Phase variation does not occur randomly, and gene switching is controlled by global

regulators. It has been demonstrated that the *sss* gene, which is highly similar to members of the λ integrase family of site-specific recombinases, has a role in root colonization by *P. fluorescens* WCS365 (Dekkers *et al.*, 1998c). The *sss* gene seems to play a role in phase variation, and it is hypothesized to be involved in DNA rearrangements. However, the exact chromosomal target sites of this site-specific recombinase as well as the affected gene(s) remain unknown (Dekkers *et al.*, 1998c).

Phenotypic variation in *P. fluorescens* F113 was observed during alfalfa root colonization and *sss* recombinase was shown to be responsible for phenotypic diversification (Sanchez-Contreras *et al.*, 2002). Although the variants were not superior colonists compared to the parental strain, they did colonize distal parts of the roots more aggressively, suggesting that the ability to diversify may be more important that the particular resultant phenotype. This study also showed that a point mutation in the global regulatory gene *gacA* was responsible for some of the phenotypic differences in one of the more aggressive variant cell types, revealing a link between phase variation and global gene regulation (Sanchez-Contreras *et al.*, 2002). More recently, it was demonstrated that biocontrol traits of *Pseudomonas* spp. isolated from maize are regulated by phase variation, and that *gacS* and *mutS* are involved in this process (van den Broek *et al.*, 2003). Phenotypic variation affecting colony morphology and exoenzyme production by *P. brassicacerum* NFM421 during rhizosphere colonization of *Arabidopsis thaliana* also has been reported (Achouak *et al.*, 2004).

(vii) NADH dehydrogenase I. Camacho Carvajal reported that the *nuoD* gene of *P. fluorescens* strain WCS365, which encodes NADH dehydrogenase I, is involved in competitive colonization of tomato root tips. NADH dehydrogenase I is a membrane-bound enzyme of the aerobic respiratory chain that functions to generate a proton gradient or proton motive force

across the cytoplasmic membrane (Weidner *et al.*, 1993). It is assumed that under the conditions present in the plant rhizosphere, such as competition for nutrients and low oxygen tensions, mutations in the NADH dehydrogenase I gene result in a decreased proton motive force, which affects energy-dependent processes such as motility and transport across the membrane and results in a competitive disadvantage (Camacho Carvajal, 2000).

Rationale and significance. Strains belonging to different PhlD$^+$ genotypes vary in their ability to produce antibiotics (Keel *et al.*, 1996) and to suppress soilborne pathogens (Sharifi-Tehrani *et al.*, 1998). Raaijmakers and Weller (Raaijmakers and Weller, 2001) studied the relationship between genotype and colonization by indigenous populations of DAPG producers on roots of wheat in a Quincy TAD soil and demonstrated that among 101 isolates representing 16 RAPD groups, one group was dominant. This dominant group corresponds to the D-genotype as defined by BOX-PCR and RFLP analyses, and is exemplified by *P. fluorescens* Q8r1-96. D-genotype isolates also accounted for 92% of the PhlD$^+$ isolates from wheat or pea grown in the field in a pea wilt-suppressive soil in Mt. Vernon, WA (Landa *et al.*, 2002). The ability of Q8r1-96 to establish and maintain a large population size (10^5 CFU g^{-1} root or more), even when introduced at low doses (applied at 100 CFU g^{-1} soil), distinguishes it from other PGPR, and is typical of all D-genotype strains tested to date (Raaijmakers and Weller, 2001). *P. fluorescens* Q8r1-96 represent a new class of "premier" plant growth-promoting bacteria that are minimally affected by soil type and environmental conditions, colonize roots consistently, and are found on wheat, barley, peas, lentils, and chickpeas – crops grown as part of cereal-based production systems in the Pacific Northwest. Through the process of active colonization of plant roots these bacteria provide natural protection of their host plants against a variety of soil pathogens.

The characteristics of premier rhizobacteria suggest that such strains differ inherently from other PGPR either in their growth dynamics or in their interactions with plant hosts, and that novel genes or mechanisms of gene regulation are responsible for their unique properties. The current research was aimed towards understanding the molecular basis of the interactions between the host plant and premier beneficial 2,4-diacetylphloroglucinol-producing bacteria as exemplified by *P. fluorescens* Q8r1-96. The strategy included analyses of genotypic and phenotypic diversity among closely related $PhlD^+$ isolates from wheat or pea grown in the pea wilt-suppressive soil and aimed at identifying possible unique phenotypic traits associated with D-genotype strains. The second approach included identifying and characterizing a limited subset of genes from *P. fluorescence* Q8r1-96 as part of an effort to define the genetic basis for the unique root colonization properties and competitive fitness of premier plant growth-promoting *Pseudomonas* spp. Central to these studies was the construction of a highly redundant genomic library of strain Q8r1-96 and the development of a soil-based assay that enabled evaluation of genetic determinants of competitiveness under conditions predictive of field performance. The results of my research will be used to develop consistently-performing, cost-effective microbial biopesticides and to refine alternative methods of plant pest control.

Chapter one has been published in the journal *Phytopathology* and chapters two and three will be submitted to *Applied and Environmental Microbiology*. The formats for each journal differ; therefore, this dissertation uses different manuscript formats based on journal specifications.

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Specific contributions of the defendant and her collaborators to the presented studies

CHAPTER 1: Genetic Diversity of *phlD* from 2,4-Diacetylphloroglucinol-Producing Fluorescent *Pseudomonas* spp.

Olga Mavrodi performed Southern hybridizations, RAPD PCRs, RFLP assays, computer analyses of data, extractions and HPLC detection of phloroglucinol compounds. She interpreted all of the data and wrote the manuscript. Dr. Brian McSpadden Gardener contributed to interpretation of RFLP and RAPD results and to statistical analyses of data. Dr. Dmitri Mavrodi contributed to primer design and experiment planning. Robert F. Bonsall contributed to the HPLC data analysis. Dr. David Weller and Dr. Linda Thomashow contributed to the experemental design, discussions of experimental results, and preparation of the manuscript.

CHAPTER 2: The Role of *dsbA* in Root-Colonization by *Pseudomonas fluorescens* Q8r1-96.

Olga Mavrodi was responsible for construction and screening of the genomic library, DNA sequencing, construction of mutant strains, phenotype analyses, rhizosphere colonization experiments, statistical analyses, interpretation of all data, and preparation of the manuscript. Dr. Dmitri Mavrodi contributed to the screening of genomic library, analysis of DNA sequence data, and planning and discussion of experiments. Dr. David Weller and Dr. Linda Thomashow contributed to experimental planning, discussion of results, and preparation of the manuscript.

CHAPTER 3: The Role of *ptsP, orfT* and *sss* in Root-Colonizing Activity of *Pseudomonas fluorescens* Q8r1-96.

Olga Mavrodi was responsible for construction and screening of the genomic library, DNA sequencing, construction of mutant strains, phenotype analyses, rhizosphere colonization

experiments, statistical analyses, interpretation of all data, and preparation of the manuscript. Dr. Dmitri Mavrodi contributed to the screening of genomic library, analysis of DNA sequence data, and planning and discussion of experiments. Dr. David Weller and Dr. Linda Thomashow contributed to experimental planning, discussion of results, and preparation of the manuscript.

CHAPTER 1

Genetic Diversity of *phlD* from 2,4-Diacetylphloroglucinol-Producing Fluorescent *Pseudomonas* spp.

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ABSTRACT

Fluorescent *Pseudomonas* spp. that produce 2,4-diacetylphloroglucinol (2,4-DAPG) have biological control activity against damping-off, root rot, and wilt diseases caused by soilborne fungal pathogens, and play a key role in the natural suppression of *Gaeumannomyces graminis* var. *tritici* known as take-all decline (TAD). Diversity within *phlD*, an essential gene in the biosynthesis of 2,4-DAPG, was studied by RFLP analysis of 123 2,4-DAPG-producing isolates from six states in the USA and six other locations worldwide. Clusters defined by RFLP analysis of *phlD* correlated closely with clusters defined previously by BOX-PCR genomic fingerprinting, indicating the usefulness of *phlD* as a marker of genetic diversity and population structure among 2,4-DAPG producers. Genotypes defined by RFLP analysis of *phlD* were conserved among isolates from the same site and cropping history. RAPD analyses of genomic DNA revealed a higher degree of polymorphism than RFLP and BOX-PCR analyses. Genotypic diversity in a subset of 30 strains representing all the *phlD* RFLP groups did not correlate with production in vitro of monoacetylphloroglucinol, 2,4-DAPG, or total phloroglucinol compounds. Twenty-seven of the 30 representative strains lacked pyrrolnitrin and pyoluteorin biosynthetic genes as determined by the use specific primers and probes.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are rhizobacteria that have the ability to promote the growth of plants following inoculation onto seeds or subterranean plant parts (13). PGPR mediate improved plant growth either directly, by stimulation of the plant (8,15) or indirectly, through biological control of pathogens or induction of host defense mechanisms (20, 23,35-37). Fluorescent pseudomonads that produce the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are an important group of PGPR that suppress root and seedling diseases on a variety of crops. Examples include *Pseudomonas fluorescens* CHA0 that suppresses black root rot of tobacco (33), take-all of wheat (10) and Fusarium wilt and crown and root rot of tomato (6,34); *Pseudomonas* sp. F113 that suppresses damping-off of sugar beet (7,31); and *P. fluorescens* Q2-87 (9,22) and Q8r1-96 (26) that suppress take-all of wheat. Strains of *P. fluorescens* that produce 2,4-DAPG also have a key role in the natural biological control of take-all known as take-all decline (25-27). 2,4-DAPG inhibits a wide range of fungi and bacteria, and its importance in biocontrol activity has been demonstrated conclusively by genetic approaches (35) and direct isolation from the rhizosphere environment (4,6,25,26).

Genes required for the synthesis of 2,4-DAPG by *P. fluorescens* Q2-87 have been cloned (2). The biosynthetic locus includes *phlA*, *phlC*, *phlB*, and *phlD*, which are transcribed as an operon from a promoter upstream of *phlA* (3). PhlD is responsible for the production of monoacetylphloroglucinol (MAPG), and PhlA, PhlC, and PhlB are necessary to convert MAPG to 2,4-DAPG. The biosynthetic operon is flanked on either side by *phlE* and *phlF*, which code respectively for putative efflux and repressor proteins. PhlD is especially interesting because of its homology to members of the highly conserved chalcone and stilbene synthase family of plant enzymes, which is suggestive of a common evolutionary origin (3). Probes and primers specific

for sequences in *phlD* have been used in combination with colony hybridization and PCR to quantify population sizes of 2,4-DAPG producers in the rhizosphere environment (21,26,27).

Several distinct groups of 2,4-DAPG-producing fluorescent pseudomonads have been identified (11,17,32). Two major phenotypic groups have been distinguished based on the production of antifungal compounds, with one group synthesizing 2,4-DAPG, hydrogen cyanide, and pyoluteorin and the other, only the first two metabolites (11). Some pyoluteorin-producing strains such as Pf-5 (18,19) and CHA0 (10,11,32) also can synthesize pyrrolnitrin, although whether this ability is widespread among this group is unclear. Pyoluteorin-producing strains are comparatively homogeneous, whereas pyoluteorin-negative strains include eight distinct genotypes differentiated by RAPD analysis. The results of Sharifi-Tehrani et al. (32) suggest that promising biocontrol pseudomonads may be identified functionally, based on the ability to produce 2,4-DAPG, or taxonomically, based on amplified ribosomal DNA restriction analysis (ARDRA) fingerprints. However, ARDRA distinguishes only three (11,17,32) or four (21) groups of 2,4-DAPG producers, and does not reflect the full range of diversity among isolates. McSpadden Gardener et al. (17) identified 13 and 15 genotypes by BOX-PCR and ERIC-PCR, respectively, in a collection of *phlD*-containing strains including reference strains from a previous study (11) and isolates from wheat grown in soils from the U.S. and The Netherlands. In another study, Picard et al. (21) differentiated 64 RAPD genotypes among 150 representatives of a single ARDRA group of *phlD*-containing isolates from the roots and rhizosphere of maize. Such data, which relate population structure to the presence of *phlD,* reveal a high level of genetic diversity among *phlD*-containing strains but provide little insight into whether this diversity is paralleled by diversity within *phlD* itself, or if 2,4-DAPG production and biocontrol efficacy are related to the physical structure of *phlD* or the 2,4-DAPG biosynthetic operon.

Knowledge of the diversity within *phlD* is a necessary prerequisite to assessing the potential or frequency of horizontal transfer of the biosynthetic genes between members of the rhizosphere microbial community, and might provide a basis for developing a rapid genetic screen to identify strains with superior biocontrol activity. The objectives of this research were to define the degree of heterogeneity in *phlD* among members of a large, genetically diverse collection of 2,4-DAPG-producing fluorescent *Pseudomonas* spp.; and to determine whether diversity in *phlD* correlates with whole genome diversity as defined by BOX-PCR and RAPD analyses, or with the amount of 2,4-DAPG produced in vitro. In addition, because 2,4-DAPG producers can be differentiated phenotypically by their ability to produce pyoluteorin and under appropriate culture conditions some also produce pyrrolnitrin, we assessed the frequency of co-occurrence of these biosynthetic genes among the various *phlD* genotypes.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and media. A total of 123 *phlD*$^+$ strains of fluorescent *Pseudomonas* spp. isolated from six states in the USA and six other locations worldwide were included in this study (Table 1). The sources and characteristics of most of the strains were described previously (11,17). Those designated STAD were isolated from the rhizosphere of wheat grown in soil from Stillwater, OK with a history of continuous cropping to wheat. All strains were cultured on Pseudomonas agar F (Difco Laboratories, Detroit, MI) or King's medium B (KMB) broth (20 g of Bacto proteose peptone, 1.2 g of KH_2PO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, and 10 ml of glycerol per liter) at 25^{o}C. Stock cultures were stored frozen in Luria-Bertani broth plus 40% glycerol at –80^{o}C.

Randomly amplified polymorphic DNA (RAPD) analyses. Total genomic DNA was isolated from bacterial strains by using a cetyltrimethylammoniumbromide (CTAB)-based miniprep protocol (1) and amplified with the RAPD primer M13 (5'-GGTGGTCAAG-3'). PCR was conducted in a total volume of 25 μl containing 1 x Stoffel fragment buffer, 80 pmol of M13 primer, 0.08 U of Taq polymerase Stoffel fragment (Perkin-Elmer, Norwalk, CT), 4 mM $MgCl_2$, 200 μmol each of dATP, dTTP, dGTP, and dCTP (Perkin-Elmer), and 40 ng of genomic DNA. The amplification was performed with a PTC-200 thermocycler (MJ Research, Inc., Watertown, MA) and a cycling program that included initial denaturation at 94°C for 1.5 min followed by 2 cycles of 94°C for 30 sec, 36°C for 30 sec, and 72°C for 2 min, 29 cycles of 94°C for 30 sec, 36°C for 15 sec, 45°C for 15 sec and 72°C for 1.5 min, and a final extension at 72°C for 30 sec. All RAPD PCR reactions were run at least twice with similar results. The amplification products were separated on 1.5% agarose gels in 0.5x Tris-borate EDTA buffer for 6 h at 140 V at 10°C, stained with ethidium bromide, and photographed under UV light. Images were recorded with a Kodak DC120 digital imaging system (Eastman Kodak Co., Rochester, NY) and analyzed by GelCompar version 4.0 (Applied Maths, Kortrijk, Belgium).

Restriction fragment length polymorphism (RFLP) analyses. RFLP-PCR analyses were performed with whole or heat-lysed cells from 1-day-old cultures grown on Pseudomonas agar F. To lyse cells, two bacterial colonies (2-mm diameter) were suspended in 100 μl of lysis solution (0.05 M NaOH, 0.25% sodium dodecyl sulfate [SDS]) and incubated for 15 min at 100°C. The suspension was centrifuged for 1 min at 12,000 rpm in a microcentrifuge to remove cell debris, diluted 50-fold in sterile distilled water, and 5 μl was used as template for PCR amplification.

The gene-specific primers Phl2a (5'-GAGGACGTCGAAGACCACCA-3') and Phl2b (5'-ACCGCAGCATCGTGTATGAG-3') (27) were used to amplify a 745-bp fragment of *phlD*. For

most strains the cycling program was as described Raaijmakers et al. (27). For 25 of 40 strains including representatives from Caldwell (CV and CC), Hallock (HT), Ithaca (OC), Lind, (W), and Quincy (QT), a modified cycling program consisting of initial denaturation at 94°C for 90 sec followed by 35 cycles of 94°C for 35 sec, 53°C for 30 sec, and 72°C for 45 sec was used to eliminate additional fragments resulting from nonspecific amplification. The cycling program was followed by final extension at 72°C for 30 sec. For the remaining 15 strains from Caldwell (CC and CV), the 745-bp *phlD* fragment was isolated from 0.8% agarose gels and re-amplified prior to RFLP analysis.

For all strains, amplification products were precipitated with ethanol, resuspended in sterile distilled water, and the DNA concentration was measured spectrophotometrically at 260 nm (29). Equal volumes (6 μl) of the PCR products were digested (29) with *Hae*III or *Rsa*I at 37°C, or *Taq*I at 65°C for 1.5 h and then stored at -20°C. Banding patterns were resolved by electrophoresis in 3.5% MetaPhor agarose gels (FMC Bioproducts, Rockland, ME) at room temperature in 1x Tris-borate-EDTA buffer for 3 h at 98 V. Bands were visualized with ethidium bromide and photographed under UV light. Images were recorded and analyzed as described above. PCR reactions were conducted at least twice, with similar results, for a subset of 38 strains containing all of the distinct genotypes found in different soils (as defined by RAPD analyses; see below), and only once for the rest of the strains.

Banding pattern analysis. The digitized images of RFLP and RAPD bands were converted, normalized, analyzed, and combined with GelCompar version 4.0 software by correlation-based clustering (28, 29). A 20-bp molecular ruler (Bio-Rad Laboratories, Hercules, CA) was included in every fifteenth lane in order to normalize the banding pattern of RFLP profiles. Banding patterns of RAPD profiles were normalized with a mixture of 1-kb and 0.1-kb DNA ladders

(New England Biolabs, Beverly, MA). The minimum similarity coefficient of the replicate RAPD PCR assays of all 123 individual strains was used to define distinct clusters of banding patterns. Based on this analysis, 38 representative strains from distinct clusters were chosen for RFLP analysis, which was conducted at least twice. Clusters of the RFLP patterns for the 38 strains were defined by using the 95th percentile (near-minimum) similarity coefficient of replicate assays (17) and then applied to the complete set of 123 strains, which was analyzed once.

Screening for pyoluteorin and pyrrolnitrin biosynthetic loci. The oligonucleotide primers listed in Table 2 were developed by using Omiga 2.0 Software (Oxford Molecular Ltd., Oxford, UK). Primers PltBf2 and PltBr for the pyoluteorin (Plt) biosynthetic locus were developed from sequences within the module II ketoacyl synthase domain of *pltB* (Genbank accession no. AF003370), which encodes a protein in P. *fluorescens* Pf-5 similar to type I polyketide synthases (18). Primers PrnCf and PrnCr for the pyrrolnitrin (Prn) biosynthetic locus were developed from sequences within *prn*C (GenBank accession no. U74493) of *P. fluorescens* BL915, which encodes a halogenase that catalyzes chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin (12). PCR amplification was carried out in 25-μl reaction mixture containing 1x Taq DNA polymerase buffer, 200 μM each of dATP, dTTP, dGTP, and dCTP, 20 pmol of each primer, 1.5 mM of $MgCl_2$, and 0.06 U of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR cycling program consisted of initial denaturation at 94^{o}C for 2 min followed by 29 cycles of 94^{o}C for 1 min, 58^{o}C for 45 sec, and 72^{o}C for 1 min. The amplification products were electrophoresed in 0.8% agarose gels in 1x Tris-borate EDTA buffer for 1 h at 100 V and room temperature, stained with ethidium bromide and photographed under UV light.

For Southern blotting and hybridization, total DNA samples were digested with *Eco*RI and *Pst*I restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel, and transferred onto a BrightStar-Plus nylon membrane (Ambion, Inc., Austin, TX) in 0.4 M NaOH with subsequent crosslinking of DNA by exposure of membranes to UV (254nm) (1). Membranes were prehybridized for 2 h at 55 to 60°C in a solution containing 4 x SSC (1x SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0) [29]), 4 x Denhardt's solution (29), 0.1% SDS, and 100 μg of denatured salmon sperm DNA (Sigma Chemical, St. Louis, MO) per ml. Prehybridized membranes were incubated with probes overnight under the same conditions and washed with 2 x SSC, 0.1 % SDS at room temperature (twice), 0.2 x SSC, 0.1 % SDS at room temperature (twice), 0.2 x SSC, 0.1 % SDS at 55- to 60°C (twice), and 0.1 x SSC, 0.1 % SDS at 55- to 60°C (once). DNA-DNA hybrids were detected with the BrightStar nonisotopic detection kit (Ambion, Inc.) according to the manufacturer's protocol. The *plt* and *prn* gene probes were amplified from genomic DNA of *P. fluorescens* Pf-5 with the primer pairs Plt1 and Plt2 (which anneal to the 5' end and internally to *pltB*) or PrnCf and PrnCr (Table 2), respectively, and labeled with the Random Primer Biotin Labeling kit (NEN Life Science Products Inc., Boston, MA).

Extraction and detection of 2,4-DAPG and related metabolites. Strains were grown in KMB broth for 72 h at 25°C. Samples (400 μl) were acidified with 4.5 μl of 10% trifluoroacetic acid (TFA) and extracted twice with 1 ml of ethyl acetate (90% extraction efficiency). The organic phase containing phloroglucinol derivatives was evaporated to dryness and suspended in 100 μl of 35% acetonitrile (ACN) containing 1% TFA. Thirty-microliter volumes of the extracts, or dilutions thereof, were fractionated by C_{18} reverse phase high-performance chromatography (HPLC) on a Waters NOVA-PAK C_{18} Radial-PAK cartridge (4 μm, 8 x 100 mm, Waters Corp.,

Milford, MA) (4). Solvent conditions included a flow rate of 1.0 ml/min with a 2-min initial condition at 10% ACN-0.1% TFA followed by a 20-min linear gradient to 100% ACN-0.1% TFA. HPLC gradient profiles were monitored at the spectral peak maxima (270 nm and 330 nm) characteristic of phloroglucinol compounds in the designated solvent system. The Waters HPLC system included a 710B WISP, 510 pumps, and a 680 automated gradient controller with 990 photodiode array detector (Waters Corp.). 2,4-DAPG and MAPG were identified by retention time and ultraviolet spectra (4). Each strain was grown and assayed three separate times.

Statistics. The statistics related to the genotypic diversity (i.e. Pearson's correlation coefficient, UPGMA clustering) were calculated using GelCompar 4.0 (Applied Maths, Kortrijk, Belgium). When analyzing the HPLC data, all statistics were calculated using the JMP software release 3.2 (SAS, Inc., Cary, N.C.). The peak areas corresponding to each of phloroglucinol compounds were compared using the Tukey-Kramer test. Additionally, the correlation between MAPG and 2,4-DAPG production was calculated using Kendall's tau.

RESULTS

RAPD analysis of *phlD*$^+$ strains. Based upon the presence or absence of amplification products ranging in size from 200 to 3,500 bp, 25 different M13 RAPD patterns (Figure 1) were distinguished among the 123 *phlD*$^+$ isolates tested. Isolates from Quincy, Lind, Fargo, and Ithaca, previously described by McSpadden Gardener et al. (17) as BOX group D, were separated into four RAPD groups designated D (Q8r1-96 and W2-4), D1 (FTAD1R33, Q2-5), D2 (OC4-1), and D3 (FFL1R9) (Table 3). Similarly, BOX groups B, E, F, and J formed three, four, two, and five additional subgroups, respectively (Table 3). Eighty-four percent of the

distinct RAPD groups defined in this collection contained isolates from single geographic locations. However, groups A, D, D1, and D3 included isolates from more than one location. Multiple RAPD genotypes were detected among isolates obtained from Caldwell, Fargo, Lind, and Quincy. For example, FTAD1R isolates from long-term wheat soil at Fargo included members of RAPD groups D1 and I. FFL1R isolates from long-term flax soil were distributed among RAPD groups D3, J, J2, and J3. Single genotypes occurred among HT, OC, STAD, and D27B isolates from Hallock, Ithaca, Stillwater and Woensdrecht1, respectively. Comparison of the RAPD and BOX-PCR clustering patterns indicated 7 identical groups (A, C, H, I, K, M, N), with the remaining 6 BOX groups separated into 18 groups by RAPD analysis (Table 3).

RFLP analyses of *phlD*. The results of the RFLP analyses are summarized in Table 3 for a set of 38 *phlD* strains that includes representatives of each of the 25 distinct groups identified by RAPD analysis. Digestion with *Rsa*I and *Taq*I identified only three and six groups, respectively, whereas digestion with *Hae*III distinguished ten groups of *phlD* strains. Group 1 was distinct by all three digests. Group 2, as defined by RFLP-*Rsa*I analysis, was separated into four groups by *Taq*I analysis and into eight groups by RFLP-*Hae*III analysis. Figure 2 shows the restriction fragment length polymorphisms for the 38 strains listed in Table 3, digested with *Hae*III restriction endonuclease. The combined patterns obtained with the three restriction endonucleases defined 14 distinct genomic groups among the reduced set of 38 representative strains (Table 3) and the full set of 123 different isolates (Table 1). Among the 14 groups, only groups A, D, and J included isolates from more than one location. Of these, the D group was the largest and contained 22 isolates (18% of the total) from Quincy, WA, Lind, WA, Ithaca, NY and Fargo, ND. The second largest group, J, included 17 isolates (14% of the total) from two different soils, one from Caldwell, KS, and the other from Fargo, ND. Group A contained strains

CHA0, Pf1, Pf-5, PINR2, PINR3, PGNR1, PGNR2, and PGNR3, isolated from Swiss, Italian, Texas, and Ghanian soils (11). All of these strains were reported to produce 2,4-DAPG and pyoluteorin (11). The other eleven genotypes each contained isolates from a single location. The JMP and D27B isolates, from two different fields in The Netherlands, clustered in groups F and M, respectively. Group B contained isolates from Quincy, WA, with the QX-87 isolates obtained from a growth chamber experiment in 1987 and the Q isolates isolated from the field in 1998. Clusters E1, K, and L each contained a single representative: Q37-87 from Quincy, WA, F113 from Ireland, and W4-4 from Lind, WA, respectively.

Interestingly, the genomic groups identified by the combined RFLP analysis were nearly identical to the groups defined by BOX-PCR analysis. In the reduced set, 35 of the 38 strains (92%) clustered in the same genotypic groups by both methods. The only exceptions were strains Q37-87 and CC3-1, which were separated by RFLP analysis into groups E1 and J1, respectively. A comparison of the RFLP and RAPD fingerprinting techniques showed seven identical groups (A, C, H, J1, K, M, N), with the remaining seven RFLP groups separated by RAPD into 18 groups (Table 3).

Production of phloroglucinol compounds in vitro. Thirty strains representative of 13 RFLP genotypes were analyzed by HPLC for phloroglucinol production in KMB broth, which supported the synthesis of larger amounts of 2,4-DAPG by a greater number of strains than did yeast malt or *Pseudomonas* F broth (data not shown). Among the 30 strains, 24 produced detectable amounts of 2,4-DAPG as well as monoacetylphloroglucinol (MAPG) and three other uncharacterized phloroglucinol derivatives (Table 4). All three of the RFLP-defined group A strains tested (CHA0, Pf1, and Pf-5), and three other strains (CC3-6, HT5-10, and W4-4), failed to produce detectable phloroglucinol compounds under these assay conditions. Among the 24

strains where phloroglucinol compounds were detected, production of DAPG and MAPG were positively correlated (Kendall's tau = 0.46, $P < 0.001$). Strain CV4-3 produced more MAPG and 2,4-DAPG than the other strains, but in general, the 24 isolates did not produce significantly different amounts of either MAPG or 2,4-DAPG under our assay conditions. Because MAPG is a precursor of 2,4-DAPG (3,30), and the uncharacterized phloroglucinol compounds detected in these analyses are likely to include condensation products of either or both compounds (R. F. Bonsall, O. V. Mavrodi, D. V. Mavrodi, and D. M. Weller, unpublished data), we also assayed total phloroglucinol production. The data indicated that except for RFLP genotypes H and J, representatives within an RFLP genotype (i.e., A, B, D, E, and M), did not differ in the amounts of phloroglucinol compounds produced (Table 4). Only two strains, CV4-3 (group H) and CC3-1 (group J1), produced significantly more phloroglucinol-containing compounds than most of the other strains tested (Table 4).

Screening of *phlD Pseudomonas* spp. for pyoluteorin and pyrrolnitrin biosynthetic loci. Thirty strains representative of 13 *phlD* RFLP groups were screened for the presence of pyoluteorin and pyrrolnitrin biosynthetic loci by PCR with the PltBf2 and PltBr or PrnCf and PrnCr primers, respectively (Table 2). Primers PltBf2 and PltBr amplified the predicted 773-bp fragment of *pltB* from the DNA of *P. fluorescens* strains CHA0, Pf-5, and Pf1. All of these strains are members of RFLP genotype A. No PCR product was amplified from any of the other *phlD* strains (data not shown). Similar results were obtained with the PrnCf and PrnCr primers, which amplified the predicted 719-bp fragment of *prnC* from the same three strains (i.e. CHA0, Pf-5, and Pf1) but not from the other twenty-seven strains (data not shown). Southern hybridization of digested total genomic DNA from the same 30 strains with *plt* and *prn* gene probes yielded results identical to those obtained by PCR screening (Fig. 3). Under stringent

conditions the *plt* and *prn* probes hybridized only with genomic DNA obtained from *P. fluorescens* strains CHA0, Pf5, and Pf1 (Fig. 3).

DISCUSSION

Antibiotic-producing PGPR have been studied intensively during the last decade, and special attention has been given to 2,4-DAPG-producing fluorescent *Pseudomonas* spp. because of their ability to control a wide variety of plant diseases (5-7,22,32-34,37). At the same time, the application of molecular techniques has significantly changed our capacity to rapidly characterize PGPR, their mechanisms of pathogen suppression and growth promotion, and to track indigenous and introduced rhizobacteria in the field. For example, probes and primers specific for sequences within *phlD* have been used to monitor the population dynamics of 2,4-DAPG producers in take-all suppressive and conducive soils (26,27), and in the rhizosphere of maize (21). Studies of the genotypic diversity of 2,4-DAPG producers by different techniques, including BOX-PCR, ERIC-PCR, ARDRA, RAPD, and RFLP analyses, have revealed a substantial number of distinct groups (11,17,21,32). Genomic fingerprinting with rep-PCR, which has been used to analyze the diversity of a wide variety of bacterial taxa (14), revealed 13 and 15 distinct groups of *phlD* isolates by using the BOX and ERIC primers, respectively (17). Our present study extended the genotypic analysis of a large collection of 2,4-DAPG producers to include RAPD analysis of the whole genome and RFLP analyses of the *phlD* gene, which encodes a protein necessary for the biosynthesis of phloroglucinol compounds (2,3). It was not unexpected that the diversity among *phlD* strains as detected by RAPD analysis was consistent with that defined by rep-PCR because both are measures of the overall genomic structure. However, we were surprised to find that the genotypic groups identified by RFLP analysis of

phlD correlated nearly perfectly with those identified by BOX-PCR (17) (Table 3). These two types of analysis, one focused on the entire genome and the other on a single gene, strongly suggest that the *phlD* gene evolved in concert with the rest of the bacterial genome. This observation argues against frequent horizontal transfers of the *phl* locus between strains and should lessen concern about the potential for transfer of the locus to other rhizosphere bacteria when 2,4-DAPG producers are applied as biocontrol agents.

RAPD analysis revealed the highest degree of polymorphism among the strains studied. Five of the groups (B, D, E, F, and J) defined by *phlD*$^+$ RFLP and BOX-PCR were divided into 17 groups by RAPD analysis. The larger number of genotypes distinguished by the RAPD assay as compared to the two other assays was somewhat unexpected. However, Pooler et al. (24) observed a similar situation when studying the genetic diversity of 25 isolates of *Xanthomonas fragariae* by RAPD, ERIC-PCR and REP-PCR. In that study RAPD assays also revealed more genotypic diversity than rep-PCR (24). Both methods clearly provide a high degree of discrimination in analyses of population structure, but based on our experience with *phlD* *Pseudomonas* species, we agree with Louws et al. (14) that rep-PCR genomic fingerprinting analyses are more amenable to whole-cell PCR and provide more consistent results than standard RAPD analyses. We anticipate that these genomic fingerprints or the associated *phlD* RFLP profiles will be useful to predict the relative ability of a 2,4-DAPG producer to suppress certain diseases or to colonize certain host crops. By matching the crop or crop variety with the appropriate *phlD*$^+$ genotype, it may be possible to achieve consistent and effective biocontrol or growth promotion at lower inoculum doses than currently applied.

Previous studies (11,32) have differentiated 2,4-DAPG-producing *Pseudomonas* spp. into two groups based on the number of antifungal metabolites produced. The first group, consisting

of strains isolated from tobacco, tomato, cucumber, and cotton, produces 2,4-DAPG, hydrogen cyanide, and pyoluteorin. In contrast, members of the second group produce only 2,4-DAPG and hydrogen cyanide. We screened our collection of *phlD* isolates for the pyoluteorin and pyrrolnitrin biosynthetic genes to determine whether the ability to produce these other metabolites is widely distributed within a diverse spectrum of 2,4-DAPG producers. We used a genetic approach for this purpose, rather than direct analysis of the metabolites themselves, to avoid potential effects of culture conditions on synthesis. We were surprised to find that the distribution of these genes was very limited within our collection of isolates from widely distributed soils and soils with long histories of agricultural cropping. The clear presence of *plt* and *prn* genes only with strains belonging to group A as defined by *phlD*-RFLP, genomic RAPD, BOX-PCR, and ARDRA analyses may indicate that the co-occurrence of these biosynthetic pathways is limited to this specific group of 2,4-DAPG producers.

Another important goal of our study was to determine the relationship between genotype and 2,4-DAPG production. Our HPLC analyses of metabolites from 30 strains representing 13 distinct *phlD*-RFLP genotypes demonstrated that except for strains of group A (which consistently did not produce phloroglucinol compounds at detectable levels under the culture conditions used), there was no clear association between *phlD*-RFLP genotype and 2,4-DAPG production because most strains produced similar amounts of phloroglucinol compounds regardless of genotype. Notable exceptions included strains CV4-3 and CC3-1, which produced significantly more phloroglucinol compounds than other strains. Keel et al. (11) also did not detect production of phloroglucinol compounds when strains CHA0, Pf1 and Pf-5 were grown on KMB agar. This medium may not be optimal for the production phloroglucinol compounds or the compounds may be degraded. In either case, we think that the amounts detected in vitro are

unlikely to be indicative of the biologically active levels produced in nature. For example, in the case of *P. fluorescens* strain CHA0 2,4-DAPG was not produced in our in vitro assays but it has been recovered from the rhizosphere of tomato plants inoculated with CHA0 (6). In our laboratories, studies are in progress to determine the relationship between production of 2,4-DAPG in situ and the biological control activity of different *phlD* genotypes in the rhizosphere of crop plants (B. McSpadden Gardener and D. M. Weller, unpublished results).

Our results, like those of McSpadden Gardener et al. (17), indicate that in most cases a single *phlD* genotype predominated among isolates from each soil. Although multiple genotypes were observed among isolates from the Fargo, Quincy, Caldwell, Lind, and Woensdrecht soils, one genotype was still predominant. The presence of multiple genotypes in some soils can be explained in part by different isolation techniques for strains obtained from the Quincy site, and by different cropping histories for soils obtained from Fargo and Woensdrecht (17). Because differences in the *phlD* gene reflect the overall genetic diversity among 2,4-DAPG-producing strains from soils of different geographical origins and cropping histories, we conclude that *phlD* itself can be used to study the genetic diversity and population structure of such strains. Recently, a rapid PCR-based technique was developed in our laboratory to quantify population sizes of *phlD* strains in rhizosphere samples (16). With this technique it will be possible to rapidly determine the genotype of the most dominant 2,4-DAPG producers in a sample by PCR-RFLP analyses similar to those described here.

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Figure legends

Fig. 1. Cluster analysis of restricted fragment length polymorphism (RFLP) patterns obtained from the digestion of the 745-bp *phlD* fragment with *Hae*III. Patterns for 38 isolates representing all of the unique RAPD-defined genotypes are shown. Two independent amplifications were used for each strain. Using GelCompar 4.0, the UPGMA algorithm was applied to the similarity matrix generated from the tracks of the whole patterns by using Pearson's correlation coefficient. The similarity coefficient used to define distinct groups is noted (*). Strain designations are listed and distinct clusters of RFLP patterns are labeled numerically.

Fig. 2. Random amplified polymorphic DNA cluster analysis of fingerprint patterns generated with the M13 primer from genomic DNA of *phlD*-containing *Pseudomonas* strains. Genomic fingerprints patterns for 38 isolates representing all of the unique genotypes present in the collection of 123 isolates are shown. Two independent amplifications were used for each strain. Using GelCompar 4.0, the UPGMA algorithm was applied to the similarity matrix generated by using Pearson's correlation coefficient from the tracks of the whole patterns. The similarity coefficient used to define distinct genotypic groups is noted (*). Strain designations are listed and distinct clusters of the genomic fingerprints are labeled alphabetically.

Fig. 3. Southern hybridization of DNA from *phlD* strains with probes for pyoluteorin and pyrrolnitrin biosynthetic loci. Total genomic DNA from each strain was digested with *Eco*RI and

*Pst*I restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel, and transferred onto a BrightStar-Plus nylon membrane. The blots were hybridized with biotin-labeled probes targeting **A**, *pltB* or **B**, *prnC*. The *plt* and *prn* probes were prepared, respectively, from the 440-bp PCR amplified with Plt1 and Plt2 or the 719bp PCR product amplified with PrnCf and PrnCr from *P. fluorescens* strain Pf-5.

TABLE 1. Bacterial strains used in the study

Prefix or strain[w]	Isolate numbers	RFLP[x]	Source	Ref.[y]
CV	1-1, 2-3, 2-4, 2-8, 3-3, 3-6, 4-1, 4-3, 4-5, 4-7	H	Caldwell, KS	17
CC	1-1, 1-3, 2-1, 2-8, 3-6, 4-1, 5-1	J	Caldwell, KS	17
CC	3-1	J1	Caldwell, KS	17
FTAD1R	5, 25, 26, 27, 33, 34, 35, 37, 38	D	Fargo, ND	17, TS
FTAD1R	36	J	Fargo, ND	17
FFL1R	8, 10, 13, 14, 17, 18, 21, 22, 25	J	Fargo, ND	17
FFL1R	9	D	Fargo, ND	17
HT	5-1, 5-5, 5-8, 5-10, 5-12, 5-15, 6-2, 6-4, 6-7	N	Hallock, MN	17
OC	4-1, 4-2	D	Ithaca, NY	17
W	2-4, 2-6, 2-9	D	Lind, WA	17, TS
W	4-4	L	Lind, WA	17
QT	1-5, 5-1, 5-2	D	Quincy, WA	17
QT	1-6, 2-1, 2-2, 3-1, 3-2, 4-2, 6-1	E	Quincy, WA	17, TS
Q	1-3, 1-4, 2-1, 2-6, 2-10, 2-12	B	Quincy, WA	17
Q	2-5, 2-18	D	Quincy, WA	17, TS
Q	2-2, 2-19	E	Quincy, WA	17
QX-87[z]	1, 2, 4, 5, 9, 12, 13, 88	B	Quincy, WA	9
QX-87	37	E1	Quincy, WA	10
QX-87	128	D	Quincy, WA	9
Q8rX-96	1	D	Quincy, WA	26
STADX-97	375, 376, 377, 378, 379, 384, 385, 387, 388 389	C	Stillwater, OK	TS

D27B	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	M	Woensdrecht1	17
JMP	6, 7, 9, 10, 11, 12, 16, 17, 18, 22	F	Woensdrecht2	17
CHA0		A	Switzerland	33
Pf1		A	Switzerland	11
F113		K	Ireland	31
Pf-5		A	Texas	10, 19
PGNR1	1, 2, 3	A	Ghana	11
PINR2	2, 3	A	Italy	11

[w] A complete description of the source of each strain is provided in the referenced publications.

[x] Restriction fragment length polymorphism-defined *phlD*$^+$ genotype, based on combined patterns obtained by digestions of 745 bp of *phlD* with *Hae*III, *Rsa*I, and *Taq*I (Table 3).

[y] TS = this study.

[z] To obtain the strain designation, substitute X with the isolate number.

Table 2. Oligonucleotide primers used in the study

Primer	Sequence	Target[x]	GenBank Accession No.	Position[y]	T_m[z]
PltBf	CGG AGC ATG GAC CCC CAG C	*pltB*	AF081920	8160-8178	64.0°C
PltBr	GTG CCC GAT ATT GGT CTT GAC CGA G	*pltB*	AF081920	8927-8951 (complement)	63.8°C
plt1	ACT AAA CAC CCA GTC GAA GG	*pltB*	AF081920	4812-4831	50.2°C
plt2	AGG TAA TCC ATG CCC AGC	*pltB*	AF081920	5234-5251 (complement)	57.9°C
PrnCf	CCA CAA GCC CGG CCA GGA GC	*prnC*	U74493	3478-3497	66.9°C
PrnCr	GAG AAG AGC GGG TCG ATG AAG CC	*prnC*	U74493	4175-4197 (complement)	62.9°C

[x]*pltB* encodes a protein similar to type I polyketide synthase in P. *fluorescens* Pf-5 (17).

prnC encodes a halogenase catalyzing chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin in *P. fluorescens* BL915 (12).

[y]Position of the primer in the database sequence.

[z]T_m, melting temperature calculated using Omiga 2 .0

TABLE 3. Comparison of the genotypic diversity of *phlD*-containing *Pseudomonas* spp. defined by restriction fragment length polymorphism (RFLP) analysis of *phlD* and genomic fingerprints

Strains[x]	RFLP genotype[y]				Genotype[z]	
	Rsa I	*Taq* I	*Hae* III	Combined	RAPD M13	BOX-PCR
Pf1	1	1	1	A	A	A
Pf-5	1	1	1	A	A	A
CHA0	1	1	1	A	A	A
Q1-3	2	6	6	B	B	B
Q2-10	2	6	6	B	B	B
Q1-4	2	6	6	B	B1	B
Q2-87	2	6	6	B	B2	B
STAD375-97	2	2	11	C	C	C*
Q8R1-96	2	2	2	D	D	D
Q128-87	2	2	2	D	D	D
W2-4	2	2	2	D	D	D
QT1-5	2	2	2	D	D	D
QT5-2	2	2	2	D	D	D
FTAD1R33	2	2	2	D	D1	D
Q2-5	2	2	2	D	D1	D
OC4-1	2	2	2	D	D2	D
FFL1R9	2	2	2	D	D3	D
Q37-87	2	4	10	E1	E	E
QT3-2	2	4	4	E	E	E

QT2-2	2	4	4	E	E	nd
Q2-2	2	4	4	E	E1	E
QT6-1	2	4	4	E	E2	E
QT4-2	2	4	4	E	E3	E
JMP7	2	5	5	F	F1	F
JMP12	2	5	5	F	F	F
CV2-4	2	4	8	H	H	H
CV4-3	2	4	8	H	H	H
FTAD1R36	2	2	8	J	I	I
CC3-1	2	2	9	J1	J1	J
FFL1R22	2	2	8	J	J2	J
FFL1R17	2	2	8	J	J3	J
FFL1R14	2	2	8	J	J	J
CC3-6	2	2	8	J	J4	J
F113	2	2	5	K	K	K
W4-4	3	3	9	L	D3	L
D27B4	2	2	7	M	M	M
D27B10	2	2	7	M	M	M
HT5-10	2	5	9	N	N	N
N	3	6	10	14	25	13

[x] Typical representatives from all unique genotypes. N indicates total number of distinct genomic groups.

[y]Genotypes were defined using RFLP patterns of *phlD* gene generated with restrictions enzymes *Rsa*I, *Taq*I, *Hae*III.

[z]Combined = combined patterns obtained with the three restriction enzymes. RAPD = random amplified polymorphic DNA. Genotypes were defined by banding patterns amplified with M13 primer. BOX-PCR = BOX-polymerase chain reaction, result from McSpadden Gardener et al. (17). Asterisk indicates unpublished data and nd = not determined.

TABLE 4. Production of phloroglucinol compounds by fluorescent *Pseudomonas* spp. in King's medium B broth[z]

Strain	Genotype	MAPG	DAPG	MAPG + 2,4-DAPG	PHL compounds
CHA0	A	ND[c]	ND	ND	ND
PF1	A	ND	ND	ND	ND
Pf-5	A	ND	ND	ND	ND
Q1-3	B	0.17(0.05)abc	0.11(0.13)a	0.23(0.13)ab	0.57(0.15)ab
Q1-4	B	0.16(0.05)abc	0.18(0.13)ab	0.34(0.13)ab	0.78(0.15)ab
Q2-87	B	0.09(0.05)ab	0.08(0.13)a	0.17(0.13)ab	0.32(0.15)a
STAD375-97	C	0.10(0.05)ab	0.11(0.13)a	0.21(0.13)ab	0.51(0.15)ab
Q8R1-96	D	0.16(0.05)ab	0.10(0.13)a	0.26(0.13)ab	0.67(0.15)ab
Q128-87	D	0.11(0.05)ab	0.07(0.13)a	0.18(0.13)ab	0.53(0.15)ab
W2-4	D	0.16(0.05)abc	0.05(0.13)a	0.21(0.13)ab	0.75(0.15)ab
QT5-2	D	0.17(0.05)abc	0.41(0.13)abc	0.57(0.13)abc	0.98(0.15)ab
FTAD1R33	D	0.17(0.05)abc	0.07(0.13)a	0.23(0.13)ab	0.71(0.15)ab
OC4-1	D	0.18(0.05)abc	0.08(0.13)a	0.26(0.13)ab	0.78(0.15)ab
Q37-87	E1	0.33(0.05)abc	0.15(0.13)ab	0.48(0.13)abc	0.92(0.15)ab
QT2-2	E	0.12(0.05)ab	0.08(0.13)a	0.21(0.13)ab	0.50(0.15)ab
Q2-2	E	0.11(0.06)ab	0.07(0.16)ab	0.18(0.16)ab	0.47(0.19)ab
JMP12	F	0.44(0.05)bcd	0.43(0.13)abc	0.88(0.13)bc	1.33(0.15)b
CV2-4	H	0.68(0.05)bcd	0.39(0.13)abc	1.07(0.13)bc	1.51(0.15)b
CV4-3	H	1.98(0.05)f	2.16(0.13)d	4.14(0.13)e	4.36(0.15)d
FFL1R25	J	0.25(0.05)abc	0.03(0.13)a	0.28(0.13)ab	0.82(0.15)ab

CC3-6	J	ND	ND	ND	ND
FTAD1R36	J	0.22(0.05)abc	0.04(0.13)a	0.26(0.13)ab	0.57(0.15)ab
FFL1R14	J	0.38(0.06)bcd	0.34(0.16)abc	0.72(0.16)abc	1.01(0.19)ab
FFL1R17	J	0.14(0.05)ab	0.04(0.13)a	0.17(0.13)ab	0.85(0.16)ab
FFL1R22	J	0.06(0.05)a	0.01(0.13)a	0.07(0.13)a	0.51(0.15)ab
CC3-1	J1	1.29(0.05)c	0.82(0.13)bc	2.11(0.13)d	2.65(0.15)c
W4-4	L	ND	ND	ND	ND
D27B4	M	0.15(0.06)abc	1.12(0.16)bc	1.3(0.16)bc	1.27(0.19)b
D27B10	M	0.25(0.05)abc	0.51(0.13)abc	0.76(0.13)abc	0.76(0.16)ab
HT5-10	N	ND	ND	ND	ND

[z] Strains represent all unique genotypes. Prefix designations are the same as those noted in Table 1. Genotype was defined by the combined restriction fragment length polymorphism analyses of *phlD*. All phloroglucinol (PHL) compounds include monoacetylphloroglucinol (MAPG), 2,4-diacetylphloroglucinol (DAPG), and three other uncharacterized PHL derivatives (R. F. Bonsall, O.V. Mavrodi, D.V. Mavrodi, and D.M. Weller, *unpublished data*). Each value is the mean and standard deviation of the peak area (absorbance units at 270 nm) of three replicate assays. ND = not detected. Values followed by the same letter are not significantly different according to the Tukey-Kramer test ($P < 0.05$).

Fig. 1

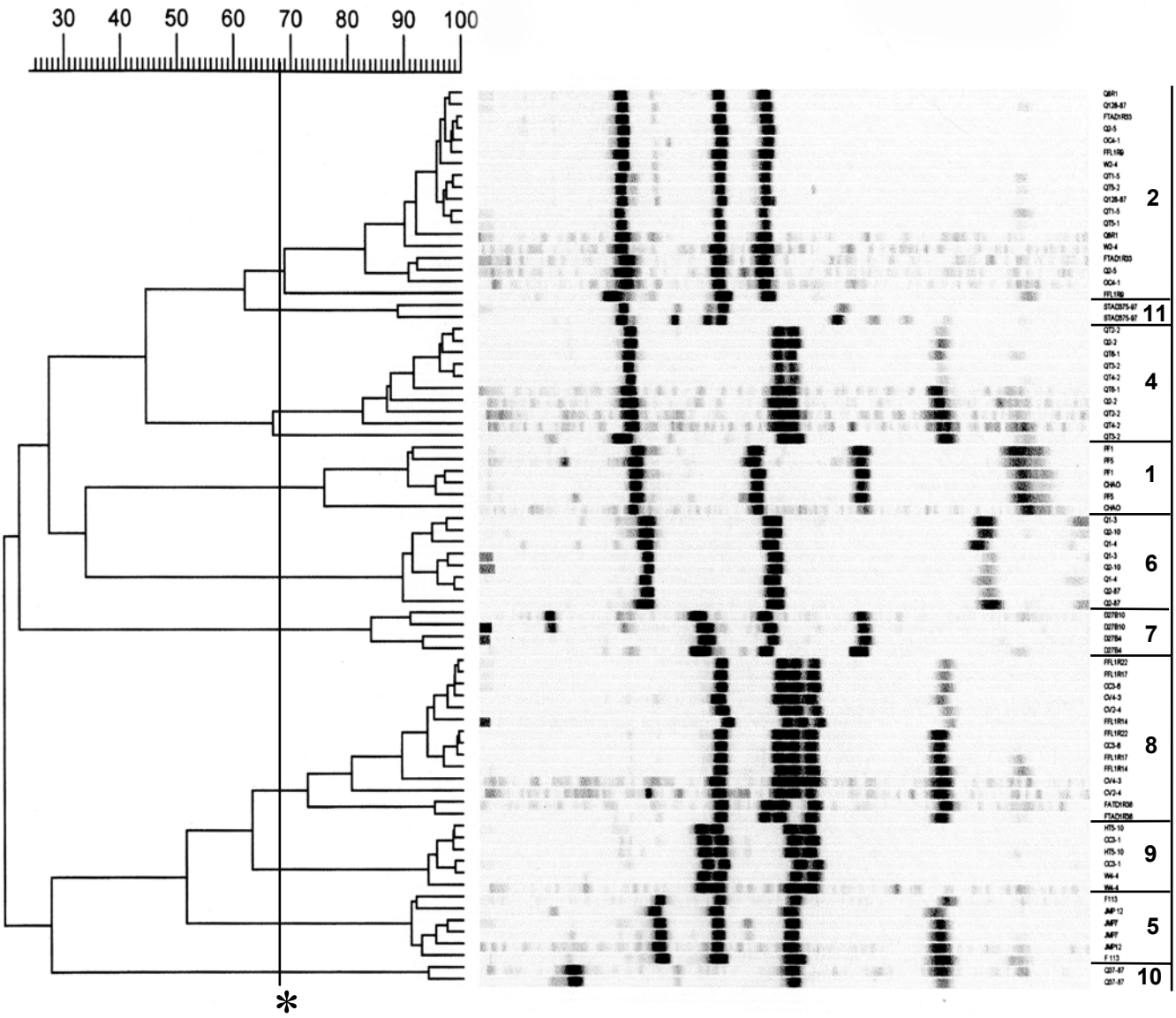


Fig. 2

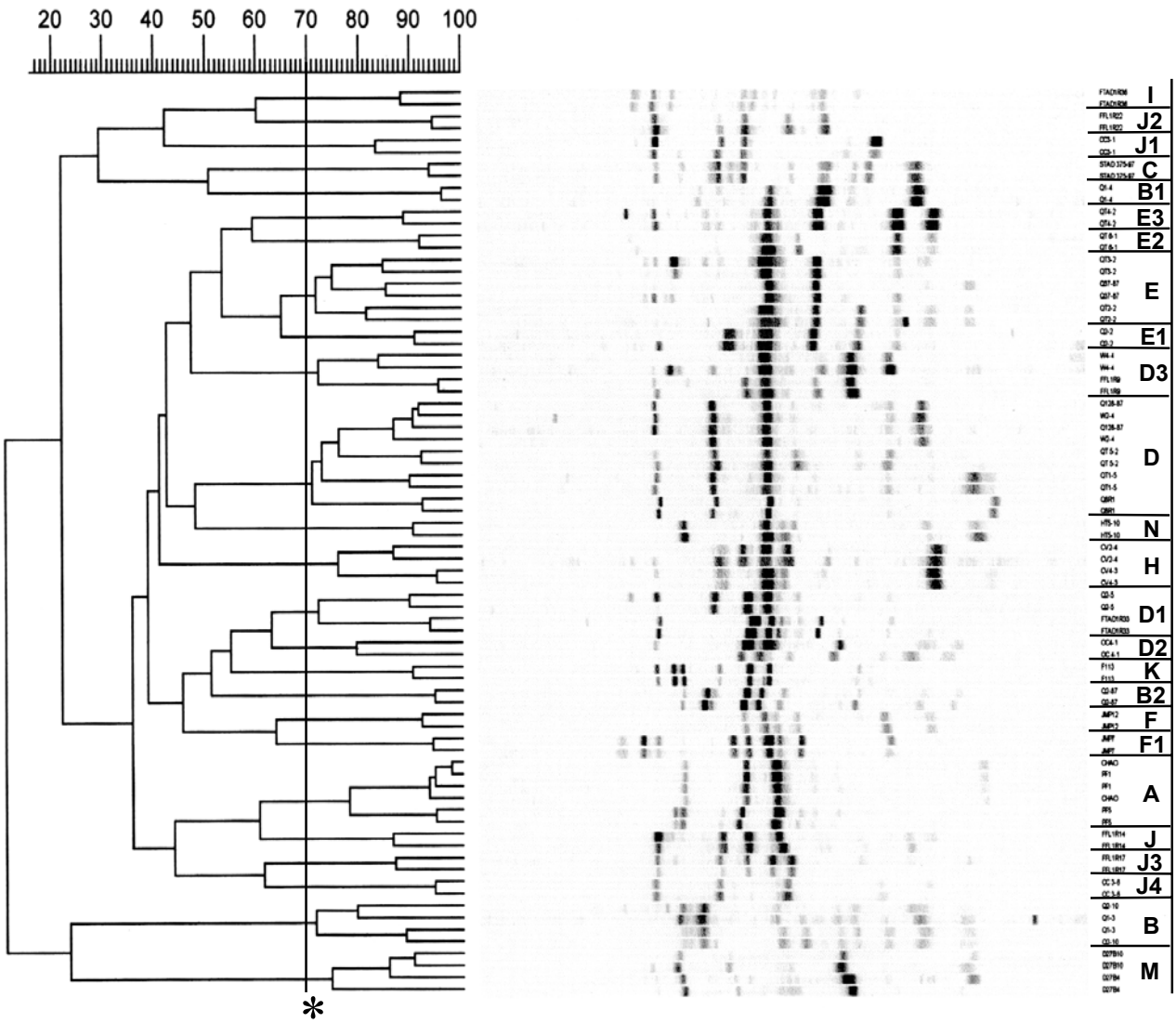
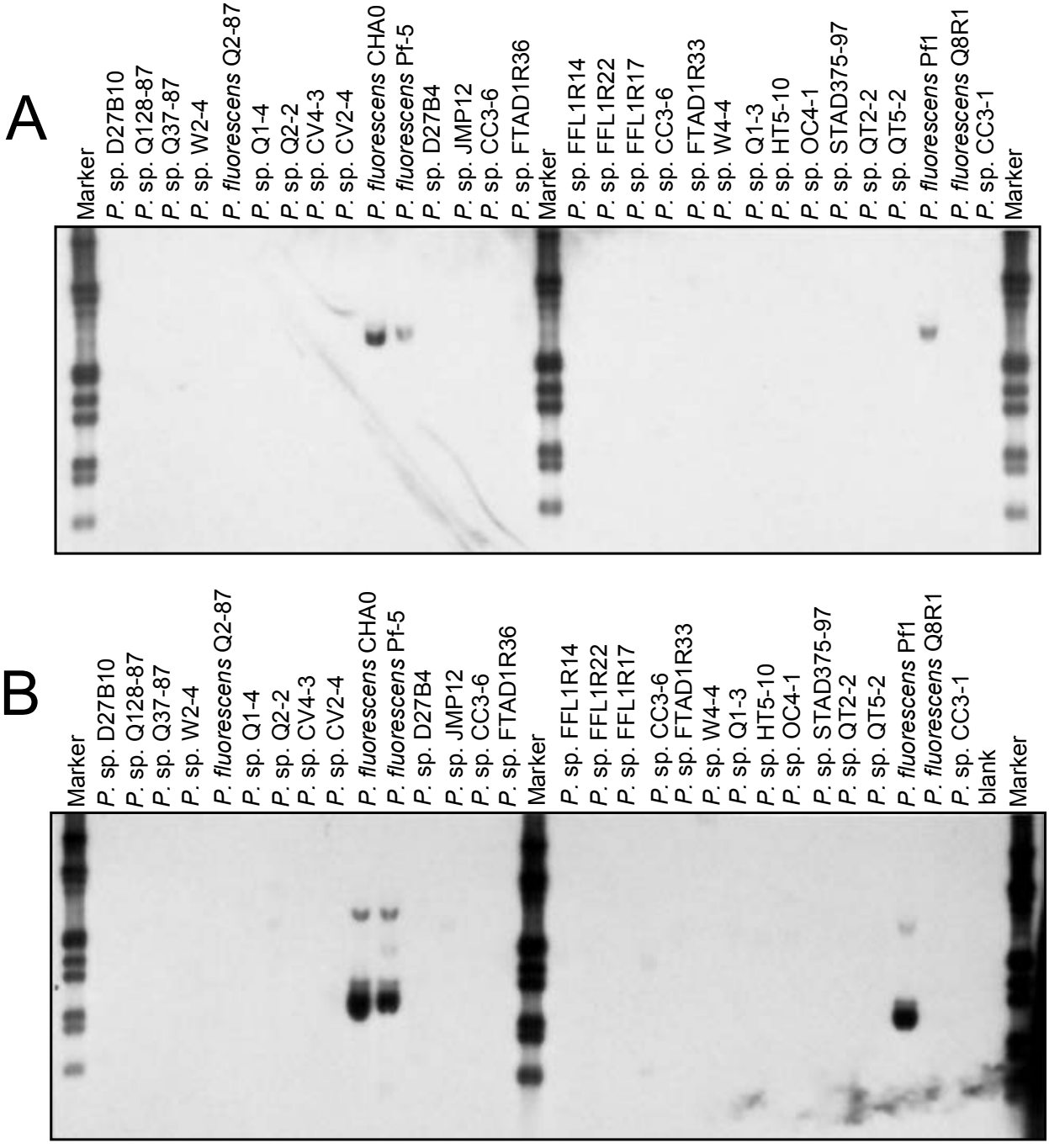

20
30
40
50
60
70
80
90
100
*
I
J2
J1
C
B1
E3
E2
E
E1
D3
D
N
H
D1
D2
K
B2
F
F1
A
J
J3
J4
B
M

Fig. 3


A
Marker
P. sp. D27B10
P. sp. Q128-87
P. sp. Q37-87
P. sp. W2-4
P. fluorescens Q2-87
P. sp. Q1-4
P. sp. Q2-2
P. sp. CV4-3
P. sp. CV2-4
P. fluorescens CHA0
P. fluorescens Pf-5
P. sp. D27B4
P. sp. JMP12
P. sp. CC3-6
P. sp. FTAD1R36
Marker
P. sp. FFL1R14
P. sp. FFL1R22
P. sp. FFL1R17
P. sp. CC3-6
P. sp. FTAD1R33
P. sp. W4-4
P. sp. Q1-3
P. sp. HT5-10
P. sp. OC4-1
P. sp. STAD375-97
P. sp. QT2-2
P. sp. QT5-2
P. fluorescens Pf1
P. fluorescens Q8R1
P. sp. CC3-1
Marker
B
Marker
P. sp. D27B10
P. sp. Q128-87
P. sp. Q37-87
P. sp. W2-4
P. fluorescens Q2-87
P. sp. Q1-4
P. sp. Q2-2
P. sp. CV4-3
P. sp. CV2-4
P. fluorescens CHA0
P. fluorescens Pf-5
P. sp. D27B4
P. sp. JMP12
P. sp. CC3-6
P. sp. FTAD1R36
Marker
P. sp. FFL1R14
P. sp. FFL1R22
P. sp. FFL1R17
P. sp. CC3-6
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P. fluorescens Q8R1
P. sp. CC3-1
blank
Marker

CHAPTER 2

The Role of *dsbA* in Root-Colonization by *Pseudomonas fluorescens* Q8r1-96

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ABSTRACT

Fluorescent *Pseudomonas* spp. that produce 2,4-diacetylphloroglucinol 2,4-(DAPG) suppress a wide range of diseases caused by soilborne plant pathogens and have been divided into 17 genotypes (A-Q) by rep-PCR using the BOXA1R primer (BOX-PCR). The genotype of an isolate is predictive of its ability to colonize the rhizosphere of certain crop species. *Pseudomonas fluorescens* Q8r1-96, a representative of genotype D, is known for its exceptional ability to colonize the roots of wheat and pea as compared to strains of other genotypes. Clones containing the *dsbA* gene, encoding a periplasmic disulfide bond-forming protein, were identified by Southern hybridization in a Q8r1-96 genomic library, sequenced, and used to construct a gene replacement mutant of Q8r1-96 in order to determine the role of this gene in rhizosphere colonization. The *dsbA* mutant exhibited decreased motility and fluorescence, and altered colony morphology; however, it produced more 2,4-DAPG and total phloroglucinol-related compounds and inhibited *Gaeumannomyces graminis* var. *tritici in vitro* better than did the parental strain. When applied individually into a natural soil, Q8r1-96 and the *dsbA* mutant did not differ in ability to colonize the rhizosphere of wheat in greenhouse experiments lasting 12 weeks. However, when the two strains were co-inoculated into the soil, the parental strain consistently outcompeted the *dsbA* mutant. This study demonstrates that *dsbA* does not contribute to the exceptional rhizosphere competence of Q8r1-96, but reduces its competitiveness only when it must compete with itself in the same niche in the rhizosphere.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are root associated bacteria from many different genera that are able to increase the growth of plants when applied to soil, seeds or vegetatively propagated plant parts (Weller and Thomashow, 1994). For the past two decades, an enormous effort to commercially exploit PGPR as biopesticides, biofertilizers or phytostimulants has yielded only a small number of products due in part to the need for application of large doses of inoculum and inconsistent performance of the rhizobacteria among fields and years. Variable root colonization is likely the major impediment to achieving more consistent performance of PGPR strains (Weller and Thomashow, 1994). It is well documented that introduced PGPR must establish and maintain a threshold population density in the rhizosphere environment after application in order to directly stimulate plant growth or prevent or limit infection by soilborne pathogens (Bull *et al.,* 1991; Raaijmakers *et al.,* 1995). However, most introduced rhizobacteria typically establish high population densities in the rhizosphere initially after inoculation, but thereafter the population declines significantly with time and distance from the inoculum source, with the introduced rhizobacteria comprising a progressively smaller proportion of the total rhizosphere microflora. Densities of introduced rhizobacteria also vary considerably from root to root and plant to plant, leaving some plants or roots unprotected (Weller and Thomashow, 1994; Weller, 1988).

Fluorescent *Pseudomonas* spp. that produce the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are among the most effective PGPR that suppress root and crown rots, wilts and damping-off diseases on a variety of crops, and they have a key role in the natural suppressiveness of some soils to certain soilborne pathogens (Duffy and Défago, 1997; Keel *et al.,* 1992; Tamietti *et al.*, 1993). Within collections of 2,4-DAPG-producers isolated

from soil and roots from throughout the world, 17 distinct genotypes (A-Q) have been described by rep-PCR using the BOXA1R primer (BOX-PCR) and restriction fragment length polymorphism (RFLP) of the *phlD* gene (McSpadden Gardener *et al.,* 2000; Mavtodi O.V. *et al.,* 2001; Landa *et al.,* 2002b). Although most of the bacteria from diffrent genotypes are phenotypically very similar (McSpadden Gardener *et al.,* 2000; Raaijmakers and Weller, 2001), they differ considerably in their ability to colonize the rhizosphere of certain crop species (Landa *et al.,* 2002b; Landa *et al.,* 2003; Raaijmakers and Weller, 2001). For example, D-genotype isolates are highly aggressive colonists of wheat and pea and have an affinity for these crops as compared to most other genotypes. D-genotype isolates collected from different locations all display the ability to establish and maintain a large population size in the rhizosphere, above the threshold density required for disease suppression, even when the rhizobacteria are introduced at very low densities (10 to 100 $CFUg^{-1}$ of soil) (Landa *et al.,* 2002b; Landa *et al.,* 2003; Raaijmakers and Weller, 2001). D-genotype isolates are primarily responsible for the suppressiveness of certain soils in Washington state to take-all disease of wheat (Weller *et al.,* 2002), a phenomenon known as take-all decline. D-genotype isolates also are the dominant 2,4-DAPG producers on pea and wheat roots grown in a Fusarium wilt-suppressive soil in Mount Vernon, WA (Landa *et al.,* 2002b)

Root colonization and ecological fitness of PGPR is a complex phenotype affected by many different traits and by environmental factors. Over the last 25 years, considerable research has been conducted to elucidate important colonization traits and genes that can be manipulated to improve colonization (Lugtenberg *et al.,* 2001; Weller and Thomashow, 1994). More recently, the use of molecular tools and highly standardized assays has resulted in the identification of several root colonization determinants involved in the PGPR-plant root interaction: flagella (de

Weger *et al.,* 1987), fimbriae (Camacho Carvajal, 2000.), synthesis of the *O*-antigen of lipopolysaccharide (Dekkers *et al.*, 1998a), and a site-specific *sss* recombinase, which is thought to play a role in DNA rearrangements that regulate the expression of gene(s) involved in the biosynthesis of cell surface components (Dekkers *et al.,* 1998b, Dekkers *et al.*, 2000). Other root colonization traits are associated with the ability of microorganisms to synthesize essential compounds (Dekkers *et al.*, 1998c; Simon *et al.*, 1997), sequester nutrients or tolerate physical stresses, and genes encoding a putrescine transport system (Kuiper *et al.*, 2001) and NADH dehydrogenase NDH-1 (Camacho Carvajal, 2000). A two-component regulatory system ColR/ColS also has been shown to influence root colonization (Dekkers *et al.*, 1998c). Further, the discovery of type III secretion genes in the PGPR strain *P. fluorescens* SBW25 (Preston *et al.*, 2001; Rainey, 1999) suggests that at least some saprophytic rhizobacteria have a more sophisticated relationship with host tissues than previously was thought to occur. Evidence of common determinants involved in the pathogenicity of bacteria such as *Pseudomonas aeruginosa* to plants and animals (Lugtenberg *et al.*, 2001; Rahme *et al.*, 2000) further supports this idea.

Our efforts to understand the molecular basis for the unique root colonizing ability of *P. fluorescens* Q8r1-96, the most completely described of the D-genotype strains, have focused in part on genes common to saprophytic and pathogenic bacteria and implicated in pathogenesis. Because such genes have a role in an intimate and active molecular dialogue between bacteria and their hosts, we hypothesize that they also could provide insight into the unique affinity of D-genotype strains for their plant hosts. Among these genes is *dsbA,* which has been implicated in the interactions of *P. aeruginosa* and *P. syringae* pv. *tomato* with *Arabidopsis* (Rahme *et al.*, 1997; Kloek *et al.*, 2000). DsbA is involved in the assembly of a functional type III secretion

apparatus in *Shigella flexneri* (Watarai *et al.*, 1995) and *Yersinia pestis* (Jakson *et al.*, 1999), in secretion of pectate lyases and cellulase in *Erwinia chrysanthemi* (Shevchik *et al.*, 1995), in formation of toxin-coregulated pili in *Vibrio chlolerae* (Hu *et al.*, 1997), and in stabilization of periplasmic lipase in *P. aeruginosa* (Urban *et al.*, 2001). In this study we identified and characterized the *dsbA* orthologue in *P. fluorescens* Q8r1-96 and evaluated its role in root colonization and the competitiveness of this strain in the wheat rhizosphere. We show that *dsbA* does not contribute to the exceptional rhizosphere competence of Q8r1-96, and that root colonization by mutant of Q8r1-96 defective in *dsbA* is reduced when the mutant is in competition with the wild type, but not in the presence of indigenous rhizosphere microflora.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. A spontaneous rifampicin-resistant derivative of *P. fluorescens* strain Q8r1-96 (Landa *et al.,* 2002b), and Q8r1-96RifGm, a rifampicin-resistant derivative tagged with mini-Tn7-gfp2 (Validov *et al.,* 2004) were used for mutagenesis and competitive colonization experiments, respectively. *Escherichia coli* TOP 10 (Invitrogen Corp., Carlsbad, Calif.) were used for cloning experiments, and *E. coli* S17-1(λ-pir) was used for biparental matings with *P. fluorescens* Q8r1-96. *P. fluorescens* and *E. coli* strains were grown at 28°C and 37°C, respectively, in Luria-Bertani (LB) medium (Ausubel *et al.*, 1995), *Pseudomonas* agar F (PsF) (Difco Laboratories, Detroit, Mich.), *Pseudomonas* agar P (PsP) (Difco) or King's medium B (KMB) (King *et al.,* 1954). Densities of total culturable heterotrophic bacteria (TCB) were determined in one-tenth-strength Tryptic Soy (TS) broth (Difco). Antibiotic supplements (Sigma Chemical Co., St Louis, Mo) were used at the following concentrations: ampicillin, 100

or 40 μg ml^{-1}; rifampicin, 100 or 90 μg ml^{-1}; tetracycline, 10 or 12.5 μg ml^{-1}; gentamycin, 2 μg ml^{-1}; cycloheximide, 100 μg ml^{-1}, chloramphenicol, 13 or 35 μg ml^{-1}; and kanamycin, 25 or 50 μg ml^{-1}.

DNA manipulations. Standard methods were used for plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Ausubel *et al.,* 1995). PCR amplifications were carried out with Taq (Promega, Madison, Wisc.) or KOD Hot Start (Novagen, Inc., Madison, Wisc.) DNA polymerases according to the manufacturers' recommendations. Total DNA of *P. fluorescens* Q8r1-96 was isolated by using the Marmur procedure (P. Gerhardt *et al.*, 1994). The oligonucleotide primers listed in Table 1 were developed by using Oligo 6.65 Software (Molecular Biology Insights, West Cascade, Colo.).

Construction of a cosmid library of *Pseudomonas fluorescens* Q8r1-96. The broad host-range cosmid vector pCPP47 (Bauer and Collmer, 1997) was used for construction of a genomic library of Q8r1-96. Total DNA of *P. fluorescens* Q8r1-96 was partially digested with *Sau*3AI and size-fractionated on a 0.3% agarose gel. The 25- to 35-kb fraction was extracted by using a QIAEX II agarose gel extraction kit (Qiagen, Santa Clarita, Calif.) and ligated with vector arms that were prepared by digesting pCPP47 with *Bam*HI and *Sca*I. The ligation was carried out in the presence of 5 mM ATP. The ligated DNA was packaged into λ particles with a Gigapack Gold kit (Stratagene, La Jolla, Calif.), and cosmids were transduced into *E. coli* XL1-Blue MR and selected on LB supplemented with tetracycline. Amplified and ordered copies of the genomic library were stored in a freezing medium at –80°C.

Screening of the genomic library by hybridization. The library was arrayed manually on 7.4 x 11.4-cm BrightStar-Plus nylon membranes (Ambion, Inc., Austin, Tex.) by replicating clones from the glycerol stocks with a 96-pin Multi-Blot Replicator (1.58 mm diameter solid

pins, 0.2 μl delivery per sample) (V&P Scientific, Inc., San Diego, Calif.) and a library copier that permits arraying in a 386-sample format. After arraying, the clones were grown overnight at 37°C and lysed in 0.4 M NaOH with subsequent UV crosslinking as described elsewhere (Birren *et al.*, 1999).

The probe for hybridization was amplified by PCR with primers DSBA_UP and DSBA_LOW (Table 1), which were developed based on the *dsbA* sequence from *P. fluorescens* SBW25 strain (URL http:www.sanger.ac.uk/Projects/P-fluorescens). Cycling included a 2-min initial denaturation at 94°C, followed by 29 cycles of 94°C for 15 sec, 61°C for 30 sec, and 68°C for 1.2 min, and a final extension at 68°C for 5 min. The PCR product was labeled with [^{32}P] using the *redi*prime™ II Random Primer Labeling System (Amersham Pharmacia Biotech Inc, Piscataway, N.J.) and purified with a Qiagen Nucleotide Removal Kit (Qiagen). Membranes were pre-hybridized for 2 h at 58° C in a solution containing 4 x SSC (Sambrook *et al.,*1989), 4 x Denhardt's solution (Sambrook *et al.,*1989), 0.1% SDS, and 300 μg of denatured salmon sperm DNA (Sigma) per ml. Pre-hybridized membranes were incubated overnight with ~1 x 10^6 cpm of ^{32}P-labeled probe under the same conditions and washed with 2 x SSC, 0.1 % SDS at room temperature (twice), 0.2 x SSC, 0.1 % SDS at room temperature (twice), 0.2 x SSC, 0.1 % SDS at 58°C (twice), and 0.1 x SSC, 0.1 % SDS at 58°C (once).

To localize *dsbA* within positive clones, cosmid DNA was digested with restriction endonucleases *Eco*RI, *Kpn*I and *Sac*I, and the fragments were resolved on an agarose gel, blotted, and hybridized to a biotinylated *dsbA* probe prepared with a Random Primer Biotin Labeling kit (NEN Life Science Products Inc., Boston, Mass.). Gel blotting was performed as described elsewhere (Ausubel *et al*., 1995), with the hybridization conditions as described above,

except that DNA-DNA hybrids were detected with the BrightStar non-isotopic detection kit (Ambion).

Transposon mutagenesis, shotgun sequencing and sequence analysis. To tag *dsbA* and adjacent regions of cosmid clones for DNA sequence analysis, cosmid DNA purified by using an alkaline lysis miniprep protocol (Ausubel *et al.*, 1995) was mutagenized in vitro by using the EZ::TN™ <Kan-2> transposition system (Epicentre Technologies, Madison, Wisc.). Cosmids bearing EZ::TN™ <Kan-2> insertions within the DNA fragment known to contain *dsbA* were identified by restriction mapping and shotgun-sequenced by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) according to manufacturers recommendations. The sequence data were compiled and analyzed with the OMIGA 2.0 software package (Accelrys, San Diego, Calif.). Database searches for similar protein sequences were performed using the NCBI's BLAST network service (URL http://www.ncbi.nlm.nih.gov/BLAST). Databases searches against PROSITE, Profile, HAMAP, and Pfam collections of protein motifs and domains were carried out using the MyHits Internet engine (Pagni *et al.*, 2004), and signal peptide cleavage sites were predicted with SignalP v. 3.0 (Durlov Bendtsen *et al.*, 2004).

Allelic replacement in Q8r1-96. To construct a gene replacement mutant, the *dsbA* gene containing an EZ::TN™<Kan-2> insert was amplified with DSBA_UP and DSBA_LOW primers by using KOD Hot Start DNA polymerase. The cycling program included a 2-min initial denaturation at 94°C followed by 29 cycles of 94°C for 15 sec, 61°C for 30 sec, and 68°C for 1.2 min, and a final extension at 68°C for 5 min. The amplification product was cloned into the *Sma*I site of the gene replacement vector pNOT19 (Schweizer, 1992). The resultant Kan^r plasmids were digested with *Not*I and ligated with a 5-kb fragment of pMOB3 cassette (Schweizer, 1992)

linearized with *Not*I and containing *sacB* and *cat* genes. The resultant plasmid was electroporated into *E.coli* S17-1 (λ-pir) and selected on LB medium supplemented with chloramphenicol and kanamycin. In order to obtain mutants, the pNOT19-EZ::TN+pMOB3 plasmid was mobilized from *E. coli* S17 (λ-pir) into *P. fluorescens* Q8r1-96Rif^r by using a biparental mating technique. Mutant clones were first selected on LB supplemented with rifampicin, kanamycin, and 5% sucrose. Sucrose- and kanamycin-resistant clones were screened for the absence of plasmid-borne *sacB*, *bla,* and *cat* genes by PCR with primers SAC1 and SAC2 (Mavrodi D. V. *et al.*, 2001), BLA1 and BLA2 (Mavrodi D. V. *et al.*, 2001), and Cm_UP and Cm_LOW (Table 1), respectively. The primer pair KAN_UP and KAN_LOW (Table 1) was used to detect the kanamycin resistance gene in *dsbA* mutants, which also were screened by PCR with DSBA_UP and DSBA_LOW primers to confirm the absence of the wild-type *dsbA* allele. All mutant clones were isogenic and only one clone was chosen for further experiments.

Carbon and nitrogen utilization profiles. Carbon substrate utilization profiles were generated by using Biolog SF-N2 MicroPlates (Biolog, Inc., Hayward, Calif.). Fresh colonies grown on KMB agar were scraped and washed in sterile distilled water, and the concentration of cells was adjusted to an OD_{600} of 0.01. One hundred microliter aliquots of bacterial cultures were inoculated into wells of SF-N2 MicroPlates. The plates were incubated at room temperature in the dark, and bacterial growth was detected by measuring OD_{600} 3 and 7 days after inoculation. Four independent repetitions were performed with each strain.

Nitrogen utilization profiles were determined by using Biolog PM3 MicroPlates according to manufacturers' protocol (Biolog, Inc.). Briefly, bacterial cultures grown overnight on R2A agar were scraped and suspended in IF-0 inoculating fluid supplemented with sodium succinate and ferric citrate at 5.4 mg ml^{-1} and 0.49 μg ml^{-1}, respectively. The turbidity of the

bacterial cultures was adjusted to 85%T (Biolog turbidity standard), and PM3 nitrogen utilization MicroPlates were inoculated with 100 µl of bacterial suspension per well. The inoculated plates were incubated in a humidified container at 28°C for 4 days, and examined daily for the formation of purple color. Four independent repetitions were performed for each strain.

Extraction and detection of 2,4-DAPG and related metabolites. Samples (4 ml) of bacterial cultures grown with shaking for 48 h at 27°C in PsF broth were spiked with 2 µg of phenazine as an internal standard, acidified with 200 µl of 10% trifluoroacetic acid (TFA), and extracted twice with 10 ml of ethyl acetate (90% extraction efficiency). The organic phase containing phloroglucinol derivatives was evaporated to dryness and suspended in 1 ml of 35% acetonitrile (ACN) containing 1% TFA. Five-microliter volumes of the extracts or dilutions thereof were fractionated on a Waters NOVA-PAK C_{18} Radial-PAK cartridge (4 µm, 8 x 100 mm, Waters Corp., Milford, Mass.) (Bonsall *et al.,* 1997). Solvent conditions included a flow rate of 1.0 ml min^{-1} with a 2-min initial condition at 10% ACN-0.1% TFA followed by a 20-min linear gradient to 100% ACN-0.1% TFA. HPLC gradient profiles were monitored at the spectral peak maxima (270 nm and 330 nm for DAPG; 284 nm and 330 nm for MAPG) characteristic of phloroglucinol compounds in the designated solvent system. The HPLC gradient profile for phenazine was monitored at spectral peak maxima of 247 nm and 363 nm. The Waters HPLC system included a 717 Plus autosampler, 600E solvent delivery system, 600 controller with 996 photodiode array detector. 2,4-DAPG and MAPG were identified by retention time and ultraviolet spectra (Bonsall *et al.,* 1997). Five independent experiments with two replications were performed.

Production of additional extracellular metabolites. Polysaccharide production was scored visually after 3 days of growth on PsP agar on a scale of 0 to 5, where 0 indicates a non-

mucoid isolate and 5 indicates a moderately mucoid culture. Fluorescence was was scored visually after 3 days of growth at 28°C on PsF agar plates. Fluorescence was assessed on a scale of 0 to 9, where 0 indicates no fluorescence and 9 indicate the greatest fluorescence observed in a sample set. Experiments were repeated twice with six replicates for each medium. Exoprotease production was detected by spotting 5 µl of an exponentially growing bacterial culture adjusted to an OD_{600} of 0.1 on skim milk agar (Sacherer *et al.,* 1994). A clearing zone surrounding the bacterial growth that is indicative of protease production was measured after 48 and 72 hrs of incubation at 28°C. Production of hydrogen cyanide was monitored visually by using cyanide detection paper placed on Petri dish lids (Bakker and Shippers, 1987). The indicator paper was prepared by dipping Whatman No. 3 filter paper in the solution containing 0.5% picric acid and 2 % sodium carbonate. Bacterial cultures were grown for 4 days at 28°C on KMB agar amended with 0.44% glycine. Observations were made every 24 hrs, and all cultures were screened twice.

Motility assays. Logarithmically growing bacterial cultures were adjusted to an OD_{600} of 0.1, and 0.5 µl of the cell suspension was inoculated onto LB medium solidified with 0.3%, 0.5%, 1.0% or 1.5% agar. Plates were incubated right-side-up at 28°C and the diameter of outward expansion was measured at 24, 48 and 72 h after inoculation.

Fungal inhibition in vitro. Inhibition of *G. graminis* var. *tritici* by *P. fluorescens* Q8r1-96$Rif^{r}Gm^{r}$ and the *dsbA* mutant was assayed *in vitro* on PsF plates. A 5-mm-diameter agar plug of a 7-day-old culture of *G. graminis* var. *tritici* grown on one-fifth PDA media was transferred to the center of a PsF agar plate and incubated at room temperature in the dark. After 24 h, 5 µl of an exponentially growing bacterial culture adjusted to an OD_{600} of 0.1 was spotted 1 cm from the edge the agar plate. Plates were incubated in the dark for up to 7 days at room temperature and were assayed at three different time points. The distance from the center of the plug to the

leading edge of the fungus (x) and the distance from edge of the bacterial growth to the growing edge of the fungus (y) were measured. The inhibition index (i) was calculated for each replicate i= y/(x+y). Assays were conducted twice, with each strain replicated 6 times.

Rhizosphere colonization assays. Rhizosphere colonization assays were performed with the *dsbA* mutant and Q8r1-96Rif^{r}Gm^r, a gentamycin-resistant derivative of the parental strain tagged with mini-Tn7-*gfp2* (Validov *et al.*, 2004) in order to distinguish it from mutant strain in mixed inoculation studies. Bacterial inocula were grown overnight on LB agar supplemented with appropriate antibiotics. The biomass was scraped from the plates and added to sieved Quincy virgin soil in a 1% methylcellulose suspension to give approx. 1 x 10^4 CFU g^{-1} (fresh weight) of soil when strains were introduced alone. Mixed inoculation treatments contained a 1:1 mixture of Q8r1-96Rif^{r}Gm^r (~ 0.5 x 10^4 CFU g^{-1} of soil for each strain) and mutant. The actual density of each strain was determined by assaying 0.5 g of inoculated soil as described by Landa *et al*. (2002a). The control treatment consisted of soil amended with a 1% methylcellulose suspension. Each treatment was replicated six times with one pot serving as replicate.

Spring wheat (*Triticum aestivum* L.) cv. Penawawa was pre-germinated on moistened sterile filter paper in Petri dishes for 24 h in the dark. Six pre-germinated seeds were sown in square pots (6.5 cm high x 7 cm wide) containing 200 g of Quincy virgin soil (Landa *et al*., 2003) inoculated with one or both bacterial strains as described above. The soil was watered with 50 ml of metalaxyl solution (2.5 mg/ml) (Novartis, Greensboro, N.C.) to control Pythium root rot and seeds were covered with a 1-cm-thick layer of inoculated soil. Wheat was grown for six successive 2-week cycles in a controlled-environment chamber at 15°C with a 12-h photoperiod. Pots were watered on alternate days and after the first week each pot received 40 ml of Miracle-Gro solution 15-30-15 (Scotts Miracle-Gro Products, Port Washington, N.Y.) once weekly. After

2 weeks of growth (one cycle), one randomly selected plant was harvested from each replicate pot and root samples were prepared to determine the population size of the introduced bacteria. The shoots of the remaining plants were excised, and the soil and associated root systems were decanted into a plastic bag, shaken vigorously to aerate and mix, and then introduced into new pots, which were replanted with six wheat seeds. This process of plant growth, harvesting, and determination of population sizes of inoculated bacteria was repeated for six consecutive cycles.

Population densities of introduced and indigenous rhizosphere bacteria. To enumerate population densities of introduced bacteria, 0.5 g of either soil or roots with tightly adhering rhizosphere soil was placed in 50-ml centrifuge tubes with 10 ml of sterile distilled water. Tubes were vortexed and sonicated in an ultrasonic cleaner (Bransonic 521; Branson, Shelton, Conn.) for 1 minute. A 100-μl sample of the wash solution was serially diluted in a 96-well microtiter plate pre-filled with 200 μl of sterile distilled water per well, and then 50 μl of each dilution was transferred to a well of a 96-well microtiter plate containing 200 μl of one-third-strength KMB broth supplemented with rifampicin, cycloheximide, chloramphenicol and ampicillin (1/3 x KMB^{+++}Rif). The optical density at 600 nm was measured after incubation at room temperature in the dark for 72 h, and bacteria from each microplate were transferred with a replicator tool into two fresh 96-well microtiter plates filled with 200 μl of 1/3 x KMB^{+++}Rif broth amended with either kanamycin or gentamycin. This two-step incubation process was necessary in order to distinguish growth of *P. fluorescens* Q8r1-96$Rif^{r}Gm^{r}$ and the kanamycin-marked *dsbA* mutant in mixed inoculations. If gentamycin and kanamycin were included in the selective media initially, bacterial growth was inhibited. Bacterial growth was assessed after 72 h, and an OD_{600} of ≥ 0.07 was scored as positive (McSpadden Gardener *et al*., 2001). The results

were occasionally verified by PCR with KAN_UP and KAN_LOW, and Gm_UP and Gm_LOW primers. Root colonization experiments were conducted twice.

Densities of total culturable heterotrophic bacteria (TCB) were determined by the terminal dilution endpoint assay in one-tenth-strength tryptic soy broth supplemented with cycloheximide.

Data analysis. All treatments in competitive colonization experiments were arranged in a complete randomized design. Statistical analyses were performed by using appropriate parametric and nonparametric procedures with the STATISTIX 8.0 software (Analytical Software, St. Paul, Minn.). All population data were converted to log CFU g^{-1} (fresh weight) of soil or root. Differences in population densities among treatments were determined by standard analysis of variance, and mean comparisons among treatments were performed by using Fisher's protected least significant difference test (P=0.05). Alternatively, nonparametric population data were analyzed by the Kruskal-Wallis test (P=0.05) and mean comparison among treatments were determined using a Kruskal-Wallis all pairwise comparisons (P=0.05). Production of phloroglucinol compounds, exoprotease production and motility were compared by using a two-sample t test or Wilcoxon rank sum test (P=0.05).

Nucleotide sequence accession numbers. The *dsbA* sequence of *P. fluorescens* Q8r1-96 has been deposited in the GenBank nucleotide sequence database with accession number AY171618.

RESULTS

Identification, cloning, and characterization of the *dsbA* gene. The *dsbA* gene from *P. fluorescens* Q8r1-96 was identified in an ordered genomic library of 1,536 clones by colony

hybridization. By arraying the library in 386-sample format on four 7.4 x 11.4-cm nylon membranes, the entire library was screened in a single hybridization step. This initial round of hybridization yielded eight positive clones from which cosmid DNA was isolated, digested with restriction endonucleases *Eco*RI, *Kpn*I or *Sac*I, resolved in an agarose gel, blotted, and again hybridized with a *dsbA* probe in order to localize the gene within the positive cosmids. One recombinant clone, 3B12, in which *dsbA* mapped to a 4.6-kb *Eco*RI fragment, was selected for further analysis. This cosmid was mutagenized in vitro with EZ::Tn<Kan2> and cosmid DNA from 200 randomly selected kanamycin-resistant clones was digested with *Eco*RI. Cosmids that showed shifts in the electrophoretic mobility of the target 4.6-kb fragment then were sequenced with transposon-based primers.

The sequence data were assembled into a 2,962-bp contig containing five potential open reading frames, one of which encoded a product similar to bacterial thiol:disulfide exchange proteins. This gene, designated *dsbA,* was preceded by a well-conserved ribosome-binding site, AGGAG, and encodes a predicted 213-amino-acid protein of molecular mass 23,080 Da. The contig also contained two putative genes encoding precursors of cytochromes C5 and C4, and two genes for conserved hypothetical proteins, one of which was located immediately downstream of *dsbA* (Fig. 1).

The deduced protein encoded by *dsbA* is highly similar to thiol:disulfide interchange DsbA proteins from other fluorescent pseudomonads including *P. fluorescens* Pf01 (NCBI accession number ZP_00266803; 91% identity), *P. syringae* pv. *syringae* DC3000 (NCBI accession number AA053886; 79% identity), *P. putida* KT2440 (NCBI accession number NP_742297; 77% identity), and *P. aeruginosa* PA01 (NCBI accession number NP_25176; 72% identity). The consensus DsbA catalytic site, Cys-Pro-His-Cys (residues 56-60), a conserved

PROSITE pattern associated with the thoiredoxin family active site (residues 47-65), and a Pfam DsbA-like thioredoxin domain (residues 46-181) also are present in the DsbA protein from *P. fluorescens* Q8r1-96. Results of SignalP-HMM (Dyrlov Bendtsen *et al*., 2004) predictions also revealed the presence of a well-conserved signal peptide and a potential cleavage site between residues 22 and 23.

Phenotypic effects of the *dsbA* mutation. The *dsbA* mutation in *P. fluorescens* Q8r1-96 was pleiotrophic, with mutants exhibiting reduced motility, reduced fluorescence, and changes in colony morphology. Differences in motility were most apparent on 0.3% agar, on which the migration diameter of the *dsbA* mutant was on average 2.2 and 2.0 times less than that of the wild type after 24 and 48 hrs, respectively (Table 2). When higher agar concentrations were used, neither the mutant nor the wild type exhibited swarming behavior.

P. fluorescens produces water-soluble siderophores (pseudobactin/pyoverdin) that strongly bind ferric iron ions under iron limiting conditions. The siderophore/Fe^{3+} complex subsequently is taken up by the bacterial cell via a specific receptor system located in the bacterial outer membrane (Leong, 1986). When grown on a siderophore-inducing PsF plates, the *dsbA* mutant exhibited reduced fluorescence as compared to wild type Q8r1-96 (Table 2). Differences also were observed in the appearance of colonies of the parental and mutant strains: those of the *dsbA* mutant were less mucoid on PsP agar and more orange-tan when grown on LB media supplemented with glucose (Table 2) than those of the wild-type.

The *dsbA* mutant was not impaired in the accumulation of extracellular protease (Table 2) or the production of hydrogen cyanide (data not shown).

Effect of the *dsbA* mutation on carbon and nitrogen utilization. The parental and *dsbA* mutant strains were grown in Biolog SF-N2 and PM3 MicroPlates in order to determine the

effect of the mutation on patterns of utilization of carbon substrates and sources of nitrogen, respectively. Compared to the wild type, the *dsbA* mutant was defective in the utilization of D-galactose as a source of carbon and parabanic acid, biuret, and D-serine as sources of nitrogen. However, these tests also revealed that, unlike the *dsbA* mutant, strain Q8r1-96 did not utilize nitrite, methylalanine, alloxan, glycuronamide, guanine, Gly-Glu and agmatine as sources of nitrogen.

Effect of the *dsbA* mutation on phloroglucinol production and Ggt inhibition in vitro. To determine whether the mutation in *dsbA* affected phloroglucinol production, the relative amounts of phloroglucinol compounds produced by wild-type *P. fluorescens* Q8r1-96 and the *dsbA* mutant were analyzed by HPLC. Both strains produced detectable amounts of 2,4-DAPG as well as monoacetylphloroglucinol (MAPG) and three other uncharacterized phloroglucinol-related compounds (data not shown). Since MAPG is a precursor for 2,4-DAPG (Bangera and Thomashow, 1999; Shanahan *et al*., 1992) and the uncharacterized phloroglucinols detected in these analyses are likely to include condensation products of either or both compounds (R. F. Bonsall, O. V. Mavrodi, D. V. Mavrodi, and D. M. Weller, unpublished data), total phloroglucinol-related compounds also were quantified (Table 2). Results of these analyses indicated that the *dsbA* mutant produced approximately 50% more 2,4-DAPG and total phloroglucinol-related compounds than did wild-type Q8r1-96.

These data correlated with the results of fungal inhibition assays, which showed that the *dsbA* mutant was more inhibitory in vitro to *G. graminis* var. *tritici* than was the wild type. The calculated hyphal inhibition indices for the *dsbA* mutant and the wild-type strain were 0.41, and 0.36, respectively, 4 days after inoculation. After 6 and 7 days, the inhibition indices were 0.22 and 0.13 for the mutant and 0.16 and 0.07 for the parental strain.

Effect of the *dsbA* mutation on the rhizosphere colonization properties of Q8r1-96.

Studies of rhizosphere colonization were conducted by using Q8r1-96Rif^{r}Gm^r, tagged with a mini-Tn7-gfp2 in the *att*Tn7 site of Q8r1-96 (Validov *et al.,* 2004) to confer resistance to gentamycin, and the kanamycin-resistant *dsbA* mutant. These differential selectable markers allowed the two strains to be distinguished in competitive colonization experiments. The kinetics of growth in vitro of strains Q8r1-96Rif^r and Q8r1-96Rif^{r}Gm^r in 1/3x KMB and in MMP minimal media did not differ (data not shown).

The rhizosphere competence of the *dsbA* mutant was compared to that of Q8r1-96Rif^{r}Gm^r in single and mixed (1:1 ratio) inoculation studies on roots of wheat grown in a natural Quincy virgin soil. Figure 2 shows the population dynamics for the two strains in the rhizosphere during six two-week growth cycles. The population densities established in the soil were equivalent for both strains at the beginning of each experiment (cycle 0). In both the individual and the mixed inoculations, the population sizes of the wild-type and mutant strains had increased by four orders of magnitude at the end of cycle 1 and then the densities slowly declined over the next 5 cycles.

When introduced individually, the population dynamics of Q8r1-96Rif^{r}Gm^r and the *dsbA* mutant did not differ consistently in the wheat rhizosphere. In experiment 1, the population sizes of the two strains were comparable through cycle 4, but in cycles 5 and 6 the population size of Q8r1-96Rif^{r}Gm^r was significantly (P = 0.05) greater than that of the mutant. However in experiment 2, the population sizes of the two strains did not differ in cycles 1, 2, 4, and 6; the population size of Q8r1-96Rif^{r}Gm^r was greater than that of the mutant in cycle 5; and the population size of the mutant was greater than that of the wild-type strain in cycle 3 (Fig. 2). In addition, values for mean colonization (Table 3) did not differ for the two strains.

When introduced together into the soil, the *dsbA* mutant consistently colonized the wheat rhizosphere to a lesser extent than did Q8r1-96Rif^{r}Gm^r. In experiment 1, the population sizes of both strains were equivalent in cycles throughout cycles 1, 2 and 3, but the population size of Q8r1-96Rif^{r}Gm^r was significantly greater than that of the mutant. These differences occurred again and were even greater in experiment 2, in which the population size of the mutant was significantly less than that of the wild-type in all cycles (Fig. 2). In experiment 2, mean colonization values were significantly less for the mutant than for the wild type.

Population densities of total culturable aerobic bacteria in the wheat rhizosphere in all four bacterial treatments and in the control were above log 8.6 CFU g^{-1} of root in all six cycles, and population sizes did not differ (data not shown). Likewise, mean colonization values for total culturable aerobic bacteria did not differ (Table 3).

DISCUSSION

Successful root colonizing rhizobacteria must establish and maintain themselves in the presence of a large, metabolically active resident microbial population supported by the exudates and other organic metabolites provided by the root. Given the complexity of these interactions, it is not surprising that studies over the past 30 years have implicated diverse bacterial traits in root colonization by PGPR of different genera and species. These traits range from motility (de Weger *et al.*, 1987), chemotaxis (Lugtenberg *et al.*, 2001) and the presence of cell surface polysaccharides (Dekkers *et al.*, 1998a), which may facilitate effective interactions with root surfaces; to prototrophy (Simons *et al.*, 1997; de Weert *et al.*, 2002), required for growth in the absence of preformed cellular building blocks; and the ability to sequester limiting resources such as iron and oxygen (Camacho Carvajal, 2000), which may provide a competitive advantage

over other rhizosphere microorganisms. More recently, evidence of Type III secretion genes in *P. fluorescens* SBW25 (Preston *et al.*, 2001; Rainey, 1999) and the preferential colonization of wheat and pea by different strains of DAPG-producing *P. fluorescens* (Landa *et al.*, 2002b), has led us to speculate that certain PGPR may participate in more specialized interactions with the host, and that genes involved in these interactions may be among those broadly implicated in pathogen-host interactions. One such gene is *dsbA*, which influences interactions between *P. aeruginosa* and human, animal, and plant hosts (Rahme *et al.*,1997, Rahme *et al.*, 2000) and contributes to pathogenicity in *P. syringae* pv. *tomato* and *Erwinia chrysanthemi* (Kloek *et al.*, 2000; Shevchik *et al.*, 1995). DsbA catalyzes disulfide bond formation in the periplasm of Gram-negative bacteria, and mutants in *dsbA* exhibit a pleiotropic phenotype because the correct folding of many proteins is affected (Collet and Bardwell, 2002). Among the effects of *dsbA* mutations in pathogenic bacteria are deficiencies in pathogenicity and competitiveness associated with loss of motility and the inability to produce fimbriae and secrete multiple exoproducts (Kloek *et al.*, 2000; Shevchik *et al.*, 1995; Dailey and Berg, 1993). The fact that some of these traits also are associated with the ability of saprophytic pseudomonads to colonize and persist in the plant rhizosphere prompted us to investigate the role of *dsbA* in root colonization by *P. fluorescens* Q8r1-96.

We used *dsbA* from another *P. fluorescens* strain, SBW25, as a source probe to identify a *dsbA*-containing clone in the Q8r1-96 gene library. DNA sequence analysis revealed that the putative *dsbA* gene from *P. fluorescens* Q8r1-96 encodes a member of the thioredoxin superfamily containing the conserved dithiol-active site consensus motif C-P-H-C. The enzyme has a molecular mass similar to that of bacterial DsbA (approximately 20 kDa) and contains a well-conserved leader peptide for secretion to the periplasm via the type II Sec pathway. Finally,

the gene is highly conserved (> 70% identity at the amino acid level) in other fluorescent *Pseudomonas* species including *P. syringae*, *P. putida*, and *P. aeruginosa.* Taken collectively, these results strongly suggest that the gene identified in the *P. fluorescens* Q8r1-96 genome is a true orthologue of *dsbA* of *Esherichia coli.*

The inactivation of *dsbA* in *P. fluorescens* Q8r1-96 resulted in a pleiotrophic phenotype similar to that described for similar mutants in other bacterial species. Like the corresponding mutants of *E. coli* (Dailey and Berg 1993) and *P. syringae* pv. *tomato* **(**Kloek *et al.*, 2000), the *dsbA* mutant of Q8r1-96 exhibited reduced motility and did not swarm. This probably is due to defective disulfide bond formation in FlgI, a component of the flagellar motor (Collet and Bardwell, 2002; Dailey and Berg, 1993). Like the *dsbA* mutant of *P. syringae* pv. *tomato* **(**Kloek *et al.*, 2000), that of Q8r1-96 was also less fluorescent than the wild type strain on siderophore-inducing PsF agar (Table 2). In contrast to the *dsbA* mutant of *P. aeruginosa*, which produced reduced clearing zones on skim milk agar (Malhotra *et al.*, 2000), the Q8r1-96 mutant was unimpaired in the accumulation of extracellular protease and hydrogen cyanide. The latter indicates that the GacA/GacS regulatory circuitry, which coordinately regulates the production of secondary metabolites and exoprotease (Whistler *et al.*, 1998), has not been disturbed in the *dsbA* mutant. However, the Q8r1-96 *dsbA* mutant also produced elevated amounts of 2,4-DAPG and total phloroglucinol-related compounds and, as a result, was more inhibitory in vitro to *G. graminis* pv. *tritici* than the wild-type strain. These and other changes, including those affecting colony morphology and nutrient utilization, presumably resulted from inactivation or functional alterations in periplasmic enzymes involved in secretion, nutrient uptake, and the synthesis of cell envelope constituents.

We performed rhizosphere cycling experiments to evaluate the contribution of *dsbA* to the unique root colonizing ability of strain Q8r1-96. Most greenhouse or growth chamber studies of root colonization have utilized assays that last only a few days or weeks, and in recent years molecular analyses of genes functioning in root colonization have increasingly depended on the use of gnotobiotic systems in order to achieve a greater level of sensitivity and reproducibility than usually can be achieved by studies conducted in natural soil. We think that gnotobiotic systems are important tools in identifying genes that may function in colonization, but the results obtained in such systems must be validated in natural soil. Therefore, we performed our cycling experiments in a natural field soil to which bacteria were added only once, after which a succession of wheat crops was grown to allow colonization to be evaluated for months under controlled conditions in the presence of indigenous microbial populations. Cycling does not fully simulate field conditions, but allows a much more extended analysis of rhizosphere competence than short-term or gnotobiotic assays. Cycling experiments have been used to demonstrate that phenazines (Mazzola *et al.*, 1992) contribute to the ecological fitness of the producer strains and to elucidate the differences in rhizosphere competence among BOX-PCR genotypes of 2,4-DAPG-producing isolates of *P. fluorescens* (Landa *et al.*, 2002b).

We performed cycling experiments in which *P. fluorescens* Q8r1-96 and the *dsbA* mutant strain were introduced into the soil either alone or in combination. The mutant performed differently depending on whether the wild type was present. When introduced individually, Q8r1-96 and the *dsbA* mutant colonized the rhizosphere similarily, whereas when the two strains were mixed, the mutant colonized the rhizosphere significantly less. We subscribe to the definition of root colonization proposed by Weller and Thomashow (1994): “the process whereby rhizobacteria introduced on seeds, vegetatively propagated plant parts or into the soil

become distributed along roots growing in raw soil, multiply, and then survive for several weeks in the presence of indigenous soil microflora". On the basis of this definition and these findings, we conclude that DsbA does not significantly contribute to the unique root colonizing ability of Q8r1-96 even though the mutant was less fit in co-inoculation studies. Indeed, it is not surprising that the mutant was less competitive in the presence of the wild type because the two are expected to share tightly overlapping niches in the rhizosphere. One would expect that almost any change would cause some disadvantage to the mutant because the wild type is so finely adapted to the rhizosphere environment, and over many generations the wild type ultimately would be expected to become dominant. Thus, although the reduction in competitiveness seen when the mutant was co-inoculated with the wild type in natural soil reflects reduced fitness, the magnitude of the loss was not great enough to impact on the ability of the mutant to compete with the indigenous microflora.

Over the past 25 years, studies of the role of chemotaxis in root colonization and the importance of active motility in soil have provided conflicting results. De Weger *et al.*, (1987) reported a requirement for flagella in *P. fluorescens* WCS374 for colonization of potato roots and Catlow *et al.* (1990) demonstrated that the distribution of *Rhizobium trifolii* TA on clover roots depends on active motility. It also has been reported that in the absence of percolating water, motility contributed to survival in soil and the attachment of *P. fluorescens* to wheat roots (Turnbull *et al.*, 2001). On the other hand, the importance of active motility in soil is questionable since flagella-mediated movement requires the presence of water films that become too thin to support motility when the soil is drier than -50 kPa (Griffin and Quail, 1968). Passive movement of bacteria on elongating roots was described by Howie *et al.* (1987), but downward percolation of water was shown to be more important for bacterial dispersal through the

rhizosphere (Bahme *et al.*, 1987). The fact that the *dsbA* mutant in this study was not impaired in root colonization can be explained by our experimental conditions, which included regular watering of the plants. The water flow presumably contributed to the distribution of the bacteria along growing wheat roots, thus neutralizing the absence of motility.

In conclusion, the data presented in this study suggests a novel function for the *dsbA* gene, which previously has been described as pathogenesis-related in *P. aeruginosa*. The *dsbA* mutant of Q8r1-96 does not have non-specific growth defects *in vitro* and the effect of the mutation became apparent only when the mutant was tested in the plant rhizosphere in competition with the parental strain. This study demonstrates that *dsbA* does not contribute to the exceptional rhizosphere competence of Q8r1-96, but reduces its competitiveness only when it must compete with the wild type strain in the same niche in the rhizosphere.

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Figure legends

FIG. 1. Restriction map and location of the individual genes in the region of the *P. fluorescens* Q8r1-96 genome containing *dsbA*. Inverted solid triangle indicates position of EZ::TN<Kan-2> insertion, and small horizontal arrows indicate PCR primers used in the study. The shaded arrows indicate the positions of genes and open reading frames (ORFs) that were not relevant to the present study. RBS, ribosome binding site.

FIG. 2. Population dynamics of *P. fluorescencs* Q8r1-96 and its *dsbA* mutant on the roots of wheat (cv. Penawawa) grown in Quincy virgin soil for six consecutive two-week-long cycles. Results of two independent experiments, experiment 1 (A) and experiment 2 (B), are presented. Each strain was introduced into the soil to give a final density of approximately log 4 CFU per g of soil (cycle 0) in single inoculations and ~ 0.5×10^4 CFU per g of soil of each strain in mixed inoculations as described in Materials and Methods. Mean values and standard deviations are presented. Means followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test or the Kruskal-Wallis test (P=0.05) (marked by asterisk).

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain	Relevant characteristics[a]	Reference of origin
Pseudomonas fluorescens		
Q8r1-96 Rif	DAPG$^+$Rif^r	Landa *et al.* 2002
Q8r1-96 RifGm	Q8r1-96 tagged with mini-Tn7-*gfp*2; DAPG$^+$Rif^{r}Gm^r	Validov *et al.,* 2004
Q8r1-*96 dsbA*$^-$RifKm	*dsbA* mutant of Q8r1-96; DAPG$^+$Rif^{r}Km^r	This study
Escherichia coli		
S17-1(λ-pir)	*thi pro hsdM recA rpsL* RP4-2 (Tet^r::Mu) (Kan^r::Tn7)	Lab collection
Top 10	F$^-$ *mcr*A *Δ* (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z *Δ* M15 *Δ lac*X74 *rec*A1 *ara Δ* 139 *Δ* (*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
XL1-Blue MR	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1lac*	Strategene
Plasmids		
pCPP47	Broad-host-range cosmid derived from pCPP34, tandem *cos*$^+$, *par*$^+$, Tet^r	Bauer, 1997
pMOB3	Kan^r *cat oriT sacB*	Schweizer, 1992
pNOT19	ColE1 *bla*; accessory plasmid	Schweizer, 1992

pNOT19-*dsbA* EZ::TN <Kan-2>	pNOT 19 containing the 2.2-kb DNA fragment with *dsbA* EZ::Tn<Kan2>	This study
pNOT19-*dsbA* EZ::TN <Kan-2> + pMOB3	pNOT19-*dsbA* EZ::TN <Kan-2> ligated with 5.8-kb *Not*I fragment from pMOB3	This study
Oligonucleotides		
DSBA_UP	5' GAT ACC AAG CCG ATG CAG A 3'; upper primer for amplification of a 994-bp fragment of *dsbA*; T_m 63.6°C	This study
DSBA_LOW	5' GGA CAT GAC ATG ATG CTC GT 3'; lower primer for amplification of a 994-bp fragment of *dsbA*; T_m 62.8°C	This study
KAN_UP	5' TGG CAA GAT CCT GGT ATC GGT 3'; upper primer for amplification of a 509-bp fragment of Kan^r gene from EZ::TN <Kan-2>; T_m 68.9°C	This study
KAN_LOW	5' GAA ACA TGG CAA AGG TAG CGT 3'; lower primer for amplification of a 509-bp fragment of Kan^r gene from EZ::TN <Kan-2>; T_m 66.8°C	This study
Cm_UP	5' ATC CCA ATG GCA TCG TAA AGA 3; upper primer for amplification of a 560-bp fragment of *cat* gene from pMOB3; T_m 66.9°C	This study

Cm_LOW	5' AAG CAT TCT GCC GAC AT 3'; lower primer for amplification of a 560-bp fragment of *cat* gene from pMOB3; T_m 57.9°C	This study

[a]DAPG, strain produce 2,4-diacetylphloroglucinol; Rif^r, rifampicine resistance; Gm^r, gentamycin resistance; Tet^r, tetracycline resistance; Kan^r, kanamycin resistance; Cm^r, chloramphenicol resistance; T_m, melting temperature calculated by Oligo 6.65 Primer Analysis Software.

TABLE 2. Phenotypic effects of the *dsbA* mutation in *P. fluorescens* Q8r1-96

Test	Bacterial strain	
	Q8r1-96	Q8r1-96 *dsbA*$^-$
Exoprotease production [a]	6.7a; 9.8a	6a; 10a
Motility [b]	20.2a; 32.3a; 38.7a	9.33b; 16.3b; 19.3b
Phloroglucinol production [c]		
MAPG	2.1×10^6 a* (100%) [d]	3.0×10^6 a* (140%)
2,4-DAPG	9.4×10^6 b (100%)	13.9×10^6 a (147.5%)
Total PHL	12.0×10^6 b (100%)	17.6×10^6 a (146.6%)
Morphology on:		
Pseudomonas agar F[e]	Bigger colony size. Fluorescence score of 4	Smaller colony size and cell are slightly more orange than wild type. Fluorescence score of 1
Pseudomonas agar P[f]	Cell material variegated dark and light in dense area. Mucoid score of 2.	Smooth, consistent cell material texture and color. Mucoid score of 0.

LB + 2% glucose[g]	Grayish-brownish tan cell material is in denser areas, isolated colonies are beige, some with darker center.	Cell material smooth consistent orange-tan color, no grey and no color changes between dense and isolated colony areas.

[a] Zone of casein degradation on milk agar plates in mm. Mean values of 3 replicates after 48 and 72 hrs of bacterial growth, respectively. Values followed by the same letter are not significantly different according to two-sample t test.

[b] Diameter of spread in mm on 0.3% LB. Mean values of 6 replicate plates after 24, 48 and 72 hrs of bacterial growth, respectively. Values followed by the same letter are not significantly different according to two-sample t test.

[c] Absorbance units/OD. Values in brackets represent percentages. Mean values of 2 replicates are presented.

[d] Values followed by the same letter are not significantly different according to two-sample *t* test or Wilcoxon rank sum test (marked by asterisk), $\alpha = 0$.

[e] Growth of Q8r1-96 and its *dsbA* mutant on PsF media after 3 days. Fluorescence was assessed on 0 to 9 scale where 0 indicates no fluorescence and 9 indicates the strongest fluorescence observed in a sample set. Values are means of 6 replicate plates.

[f] Growth of Q8r1-96 and its *dsbA* mutant on PsP media after 3 days. Strains were scored on a scale ranging from 0 to 5 where 0 indicates not mucoid at all, 1 indicates slightly mucoid and 5 indicates moderately mucoid. Values are means of 6 replicate plates.

[g] Morphological differences of Q8r1-96 and *dsbA* mutant on LB + 2% glucose media after 7 days.

TABLE 3. Population densities of indigenous and introduced strains on the roots of wheat grown in Quincy virgin soil.

Mean population density[b] of :	Treatment[a]			
	Single inoculation		Mixed inoculation	Control[c]
	Q8r1-96	Q8r1-96 *dsbA*$^-$	Q8r1-96 and Q8r1-96*dsbA*$^-$	
Experiment 1				
Indigenous culturable bacteria	8.8 a	8.7 a	8.7 a	8.6 a
Q8r1-96	7.8 *a[d]	nd	7.2 *a	nd
Q8r1-96 *dsbA*$^-$	nd	7.4 *a	7.1 *a	nd
Experiment 2				
Indigenous culturable bacteria	8.7 a	8.6 a	8.7 a	8.7 a
Q8r1-96	6.8 *a	nd	7.0 *a	nd
Q8r1-96 *dsbA*$^-$	nd	6.8 *a	5.3 *b	nd

[a] Raw Quincy virgin soil was treated with ~10^4 CFU per g of soil of Q8r1-96Rif^{r}Gm^r and/or Q8r1-96dsbA$^-$. Mixed inoculation treatments contained a 1:1 mixture of competing strains (~0.5 x 10^4 CFU per g of soil of each strain). Rhizosphere population

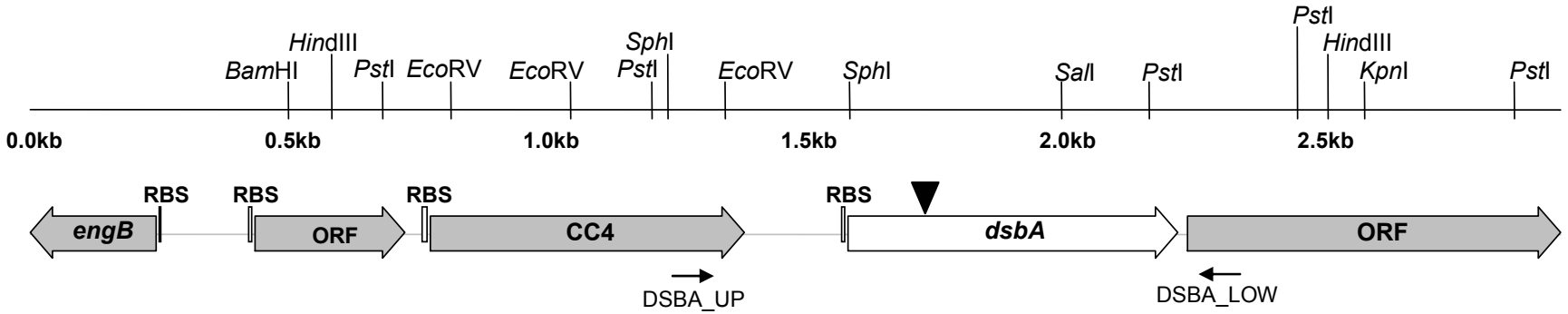
densities of bacteria were determined after 6 cycles (two weeks per cycle) by a PCR based end-point dilution method as described in Materials and Methods.

[b] Mean population densities of introduced bacteria in log CFU per g of root (fresh weight) across six cycles except cycle 0 are presented; nd, none detected.

[c] Treatment without bacterial inoculations.

[d] Different lowercase letters indicate a statistically significant difference according to Fisher's LSD (P=0.05) or Kruskal-Wallis all-pairwise comparisons test (P=0.05) (marked with asterisk).

Fig. 1


BamHI
HindIII
PstI
EcoRV
EcoRV
SphI
PstI
EcoRV
SphI
SalI
PstI
PstI
HindIII
KpnI
PstI
0.0kb
0.5kb
1.0kb
1.5kb
2.0kb
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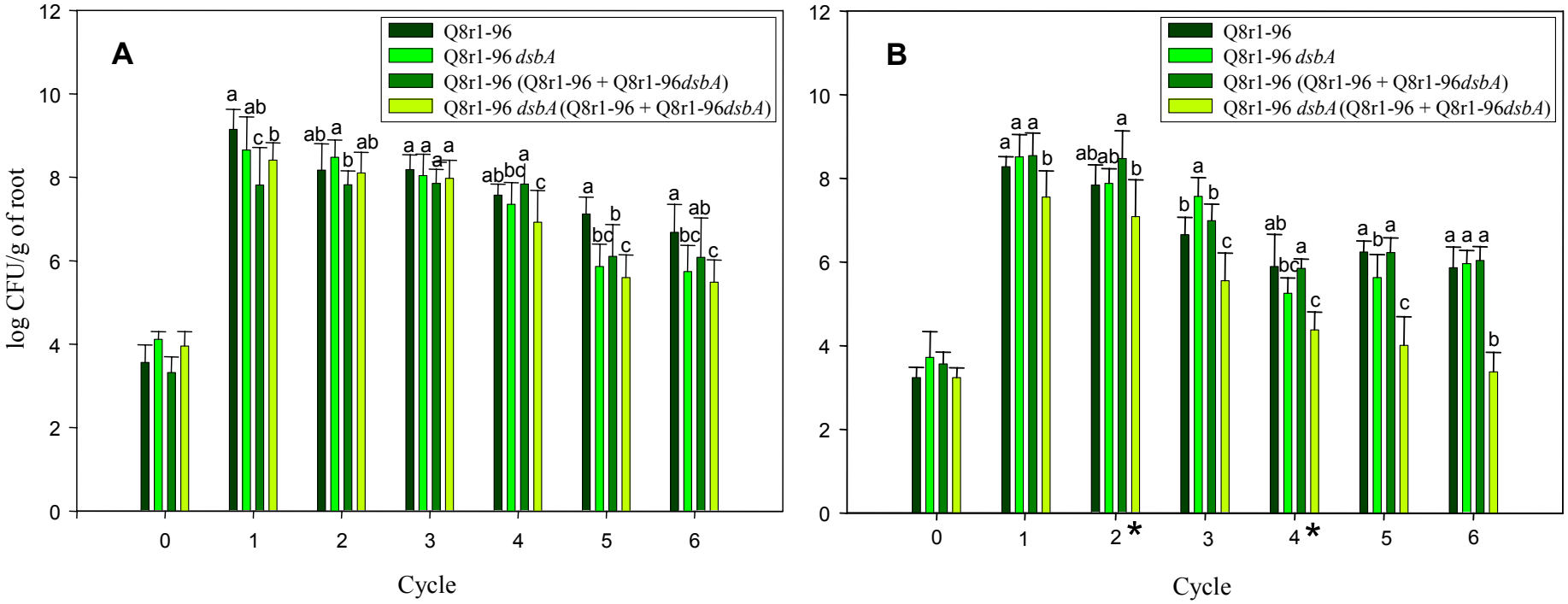
RBS
RBS
RBS
RBS
engB
ORF
CC4
dsbA
ORF
DSBA_UP
DSBA_LOW

Fig. 2


A
B
Q8r1-96
Q8r1-96 dsbA
Q8r1-96 (Q8r1-96 + Q8r1-96dsbA)
Q8r1-96 dsbA (Q8r1-96 + Q8r1-96dsbA)
log CFU/g of root
Cycle
0
2
4
6
8
10
12
0
1
2
3
4
5
6
*
*

CHAPTER 3

The Role of *ptsP, orfT,* and *sss* recombinase in Root Colonization by *Pseudomonas fluorescens* Q8r1-96

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ABSTRACT

Pseudomonas fluorescens Q8r1-96 produces 2,4-diacetylphloroglucinol (2,4-DAPG) and suppresses take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Strain Q8r1-96 is representative of the D-genotype of DAPG producers, which aggressively colonize and maintain large population sizes on the roots of wheat and pea. In order to study the role of *sss* recombinase, *ptsP* and *orfT* in colonization of the wheat rhizosphere, clones containing these genes were identified in a Q8r1-96 genomic library, sequenced, and used to construct gene replacement mutants of Q8r1-96. The *sss* recombinase, *ptsP* and *orfT* genes influence global processes including phenotypic plasticity, organic nitrogen utilization, and transmembrane transport, respectively. Mutants in these genes were characterized phenotypically for 2,4-DAPG production, motility, fluorescence, colony morphology, exoprotease and hydrogen cyanide production, carbon and nitrogen utilization, and the ability to colonize the rhizosphere of wheat grown in a natural soil. The *sss* recombinase and *orfT* mutants produced increased amounts and the *ptsP* mutant produced decreased amounts of 2,4-DAPG and total phloroglucinol-related compounds as compared to the wild type. The *ptsP* mutant was impaired in wheat root colonization, whereas mutants in *sss* recombinase and *orfT* were not impaired. However, all three mutants were less competitive than *P. fluorescens* Q8r1-96 in the wheat rhizosphere when introduced into the soil in mixed inoculation with the parental strain.

INTRODUCTION

Interest in biological control agents has increased recently in response to public concern and recognition of the need for alternatives to chemical pesticides for plant disease control (Whipps *et al.*, 2001). However, despite significant efforts to commercialize plant growth-promoting rhizobacteria (PGPR) for use as biopesticides, biofertilizers or phytostimulants, only a few products have been marketed. A major reason for the slow development of commercial products is the inconsistent performance of introduced PGPR among fields and years. Variable root colonization can contribute significantly to this inconsistency, because the introduced bacteria must attain threshold population sizes in the rhizosphere in order to be effective. Considerable research during the past twenty years has focused on understanding the biotic and abiotic factors that contribute to successful root colonization.

Our studies of root colonization in the wheat rhizosphere have focused on *Pseudomonas fluorescens* Q8r1-96, which produces the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG). Fluorescent pseudomonads that produce 2,4-DAPG are an important group of PGPR that suppress root and seedling diseases on a variety of crops and play a key role in the natural biological control of take-all disease of wheat known as take-all decline (Weller *et al.,* 1997; Raaijmakers *et al.,* 1997; Raaijmakers and Weller, 1998; Duffy and Défago, 1997; Keel *et al.,* 1992; Tamietti *et al.*, 1993). Strain Q8r1-96 is representative of the D genotype of 2,4-DAPG producers as defined by restriction fragment length polymorphism (RFLP) analysis of the *phlD* gene (*phlD*$^+$) (Mavrodi O. V. *et al.*, 2001) and by rep-PCR using the BOXA1R primer (BOX-PCR) (McSpadden Gardener *et al.,* 2000). D-genotype strains account for the majority of *phlD*$^+$ isolates from wheat or pea grown in Washington state soils that are naturally suppressive to take-all, caused by *Gaeumannomyces graminis* var. *tritici,* and Fusarium wilt of pea, caused by

Fusarium oxysporum f. sp. *pisi.* Strain Q8r1-96 is able to establish and maintain large population sizes (up to 10^7 CFU/g of root) on the roots of wheat and pea (Raaijmaker and Weller, 2001) even when introduced at low doses. This ability distinguishes it from other PGPR, and is typical of all D-genotype strains tested to date (Landa *et al.,* 2002b; Landa *et al.,* 2003; Raaijmakers and Weller, 2001).

Root colonization by introduced PGPR is a complex process that includes interactions among the introduced strain, the pathogen, and the indigenous rhizosphere microflora. All of these microorganisms interact with and influence each other within the context of a complex environment. Root colonization has been the subject of intense research during the past two decades because it is one of the major limitations to the widespread use of rhizobacteria in commercial agriculture. Colonization traits such as flagella (de Weger *et al.,* 1987), fimbriae (Camacho Carvajal, 2000), and the synthesis of the *O*-antigen of lipopolysaccharide (Dekkers *et al.*, 1998a), have been shown to be involved in the attachment of *Pseudomonas* cells to plant roots. Genes responsible for the biosynthesis of amino acids, vitamin B1 (Simon *et al.*, 1997), a putrescine transport system (Kuiper *et al.*, 2001), the NADH dehydrogenase NDH-1 (Camacho Carvajal, 2000), and ColR/ColS, a two-component regulatory system (Dekkers *et al.,* 1998c), can influence the efficiency with which PGPR colonize roots. A site-specific *sss* recombinase gene, originally identified in *P. aeruginosa* 7NSK2 as an orthologue of the *Escherichia coli* site-specific recombinase gene *xerC* (Hofte *et al.,* 1994), has a role in root colonization in several strains (Dekkers *et al.*, 1998b; Sanchez-Contrearas *et al.*, 2002; Achouak *et al.*, 2004), and even has been proposed as a potential target for improved colonization through genetic engineering (Dekkers *et al.*, 2000). The protein encoded by *sss,* also known as *xerC*, belongs to the λ integrase family and plays a role in DNA rearrangement and phase variation (Dekkers *et al.,*

1998b, Sanchez-Contreras *et al.*, 2002). One goal of our research was to determine if *sss* plays a significant a role in root colonization *P. fluorescens* Q8r1-96.

Recent findings that some bacterial genes are determinants of pathogenicity in such different hosts as plants and animals (Lugtenberg *et al.,*2001; Rahme *et al.,* 2000) have revealed unexpected commonalities in the ways that bacteria interact with their hosts, and even more fundamental similarities have become apparent with evidence of type III secretion genes in the PGPR strain SBW25 (Rainey and Preston, 2000; Rainey, 1999) and related genes in many other PGPR (Mazurier *et al*., 2004; Preston *et al.,* 2001; Rezzonico *et al*., 2004). These reports have led us to hypothesize that at least some PGPR may have a more intimate relationship with the plant than previously was recognized, and that genes broadly implicated in interactions between bacterial pathogens and their hosts might contribute to the exceptional ability of strains such as Q8r1-96 to colonize and persist in the rhizosphere. A second goal of this work therefore was to assess the role in root colonization by Q8r1-96 of *orfT* and *ptsP*, two genes that influence global processes including organic nitrogen utilization and transmembrane transport, respectively. These genes contribute to the pathogenicity of *Pseudomonas aeruginosa* in both plant and animal systems (Rahme *et al.,* 1997; Rahme *et al.,* 2000) and are highly conserved (identity more than 70%) in the genomes of saprophytic rhizosphere pseudomonads.

In this study we identified and characterized the *sss, orfT,* and *ptsP* orthologues in *P. fluorescens* Q8r1-96. Mutants in each gene were generated and used to evaluate the role of these genes in colonization and strain competitiveness on the roots of wheat grown in nonpasteurized soil, in the presence of indigenous rhizosphere microflora.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. A spontaneous rifampicin-resistant derivative of *P. fluorescens* strain Q8r1-96 (Landa *et al.* 2002b), and Q8r1-96RifGm, a rifampicin-resistant derivative tagged with mini-Tn7-gfp2 (Validov *et al.,* 2004) were used for mutagenesis and competitive colonization experiments, respectively. *Escherichia coli* TOP 10 (Invitrogen Corp., Carlsbad, Calif.) was used for cloning experiments, and *E. coli* S17-1(λ-pir) was used for biparental matings with *P. fluorescens* Q8r1-96. *P. fluorescens* and *E. coli* strains were grown at 28°C and 37°C, respectively, in Luria-Bertani (LB) medium (Ausubel *et al.,* 1995), *Pseudomonas* agar F (PsF) (Difco Laboratories, Detroit, Mich.), *Pseudomonas* agar P (PsP) (Difco) or King's medium B (KMB) (King *et al.,* 1954). Densities of total culturable heterotrophic bacteria (TCB) were determined in one-tenth-strength tryptic soy (TS) broth (Difco). Antibiotic supplements (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations: ampicillin, 100 or 40 μg ml^{-1}; rifampicin, 100 or 90 μg ml^{-1}; tetracycline, 10 or 12.5 μg ml^{-1}; gentamycin, 2 μg ml^{-1}; cycloheximide, 100 μg ml^{-1}, chloramphenicol, 13 or 35 μg ml^{-1}; and kanamycin, 25 or 50 μg ml^{-1}.

DNA manipulations. Standard methods were used for plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Ausubel *et al.,* 1995). PCR amplifications were carried out with Taq (Promega, Madison, Wisc.) or KOD Hot Start (Novagen, Inc., Madison, Wisc.) DNA polymerases according to the manufacturers' recommendations. The oligonucleotide primers listed in Table 1 were developed by using Oligo 6.65 Software (Molecular Biology Insights, West Cascade, Colo.).

Screening of genomic library of *P. fluorescens* Q8r1-96 by hybridization and PCR. A genomic library of *P. fluorescens* Q8r1-96 (Mavrodi *et al.*, submitted for publication) constructed in the broad host-range vector pCPP47 (Bauer and Collmer, 1997) was arrayed manually on 7.4 x 11.4-cm BrightStar-Plus nylon membranes (Ambion, Inc., Austin, Tex.) in a 386-sample format and screened by colony hybridization with gene-specific probes as described elsewhere (Birren *et al.,* 1999).

The *sss* and the *orfT* probes for hybridization were amplified by PCR with primers SSS_UP and SSS_LOW and ORFT_UP and ORFT_LOW (Table 1), respectively. These oligonucleotide primers were developed based on the *sss* recombinase sequence from *P. fluorescens* F113 (GenBank accession number AF416734) and the *orfT* sequence from the unfinished genome of *P. fluorescens* SBW25 (URL http://www.sanger.ac.uk/Projects/P-fluorescens), respectively. The cycling program used with SSS_UP and SSS_LOW primers included a 1.5-min initial denaturation at 94^{o}C followed by 30 cycles of 94°C for 30 sec, 61°C for 20 sec, and 72°C for 1.2 min, and a final extension at 68°C for 5 min. PCR amplification with ORFT_UP and ORFT_LOW primers was run with a similar program except that annealing was performed at 62°C for 30 sec, and extension at 72°C for 1 min.

The PCR products were labeled with [α-^{32}P] dATP using the *redi*prime™ II Random Primer Labeling System (Amersham Pharmacia Biotech. Inc, Piscataway, N.J.) and purified with a Qiagen Nucleotide Removal Kit (Qiagen, Santa Clarita, Calif.). The membranes on which the library was arrayed were pre-hybridized for 2 h at 60^{o}C in a solution containing 3 x SSC (Sambrook *et al.,*1989), 4 x Denhardt's solution (Sambrook *et al.,*1989), 0.1% SDS, and 300 µg of denatured salmon sperm DNA (Sigma) per ml. Pre-hybridized membranes were incubated overnight with ~1 x 10^{6} cpm of ^{32}P-labeled probe under the same conditions and washed with 2 x

SSC, 0.1 % SDS at room temperature (twice), 0.2 x SSC, 0.1 % SDS at room temperature (twice), 0.2 x SSC, 0.1 % SDS at 60°C (twice), and 0.1 x SSC, 0.1 % SDS at 60°C (once). Conditions of Southern hybridization with the *orfT* probe were as described above, except that 4 x SSC was used for pre-hybridization and high stringency washes were performed at 58°C.

Clones containing *ptsP* were identified by PCR with primers PTSP3 and PTSP4 (Table 1), which were developed based on the unfinished genome sequence of *P. fluorescens* SBW25. For PCR screening, the library was divided into "primary" pools (i.e. clones within each individual 96-well plate pooled together) that were further subdivided into series of "secondary" pools (containing clones from each individual 96-well plate pooled either by rows or by columns). The purified cosmid DNA from "primary" and then "secondary" pools was screened by PCR with PTSP3 and PTSP4 using a program that included a 1-min initial denaturation at 94°C followed by 30 cycles of 94°C for 25 sec, 60°C for 15 sec, and 72°C for 20 sec.

Transposon mutagenesis, shotgun sequencing and sequence analysis. To tag *sss*, *ptsP, orfT*, and adjacent regions of cosmid clones for DNA sequence analysis, cosmid DNA purified by using an alkaline lysis miniprep protocol (Ausubel *et al.,* 1995) was mutagenized in vitro by using the EZ::TN™ <Kan-2> transposition system (Epicentre Technologies, Madison, Wisc.). Cosmids bearing EZ::TN™ <Kan-2> insertions within the DNA fragment known to contain genes of interest were identified by restriction mapping and shotgun-sequenced by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) according to manufactures recommendations. The sequence data were compiled and analyzed with the OMIGA 2.0 software package (Accelrys, San Diego, Calif.). Database searches for similar protein sequences were performed using the NCBI's BLAST network service (URL http://www.ncbi.nlm.nih.gov/BLAST). Databases searches against PROSITE,

Profile, HAMAP, and Pfam collections of protein motifs and domains were carried out by using the MyHits Internet engine (Pagni *et al.,* 2004).

Allelic replacement in Q8r1-96. To construct a gene replacement mutant of *sss*, a cosmid clone containing the gene bearing an EZ::TN^{TM}<Kan-2> insert was amplified with SSS_UP and SSS_LOW primers by using KOD Hot Start DNA polymerase. The cycling program included a 2-min initial denaturation at 94^{o}C followed by 30 cycles of 94°C for 15 sec, 61°C for 30 sec, and 68°C for 1.2 min, with a final extension at 68°C for 5 min. The amplification product was cloned into the *Sma*I site of the gene replacement vector pNOT19 (Schweizer *et al.,* 1992). The resultant Kan^{r} plasmids were digested with *Not*I and ligated with a 5-kb fragment carrying a pMOB3 cassette (Schweizer *et al.,* 1992) linearized with *Not*I and containing *sacB* and *cat* genes. The resultant plasmid was electroporated into *E. coli* S17-1 (λ-pir) and selected on LB-medium supplemented with chloramphenicol and kanamycin. In order to obtain mutants, the pNOT19-EZ::TN+pMOB3 plasmid was mobilized from *E. coli* S17 (λ-pir) into *P. fluorescens* Q8r1-96Rif^{r} by using a biparental mating technique. Mutant clones were first selected on LB supplemented with rifampicin, kanamycin, and 5% sucrose. Sucrose- and kanamycin-resistant clones were screened for the absence of plasmid-borne *sacB*, *bla,* and *cat* genes by PCR with primers SAC1 and SAC2 (Mavrodi D. V. *et al.,* 2001), BLA1 and BLA2 (Mavrodi D. V. *et al.,* 2001), and Cm_UP and Cm_LOW (Table 1), respectively. The primer pair KAN_UP and KAN_LOW (Table 1) was used to detect the presence of a kanamycin resistance gene in *sss* recombinase mutants, which also were screened by PCR with SSS_UP and SSS_LOW primers to confirm the absence of the wild-type *sss* allele. All mutant clones were isogenic and only one clone was chosen for further experiments.

A similar strategy was used for construction of a *ptsP* mutant. Briefly, the full-length *ptsP* gene was amplified from *P. fluorescens* Q8r1-96 with PTSP5 and PTSP13 primers (Table 1) and Expand Long PCR polymerase (Roche Applied Science, Indianopolis, Ind.).The cycling program consisted of a 2-min initial denaturation at 94^{o}C followed by 30 cycles of 94^{o}C for 10 sec, 60^{o}C for 30 sec, and 68^{o}C for 3 min 30 sec, with a final extension at 68^{o}C for 5 min. The amplification product was treated with T4 DNA polymerase and cloned into the *Sma*I site of pNOT19. The *ptsP* gene was interrupted by using the EZ::TN™ <Kan-2> transposon system (Epicentre Technologies), and two plasmids containing transposon insertions in different orientations within *ptsP* were digested with *Not*I and ligated with a 5-kb fragment carrying a pMOB3 cassette (Schweizer *et al.*, 1992) as described above. The resultant plasmids were electroporated into *E. coli* S17-1 (λ-pir) and used for gene replacement essentially as described above except that the mutants were screened by PCR with PTSP5 and PTSP13 primers to confirm the absence of the wild-type allele. All mutant clones were isogenic and only one clone was chosen for further experiments.

To construct the *orfT* mutant, the 1,386-bp-long 5' part of *orfT* was amplified by using KOD Hot Start DNA polymerase (Novagen, Inc.) and oligonucleotides orfT_KpnI and orfT_Bam1 (Table 1). The cycling program included a 2-min initial denaturation at 94^{o}C followed by 35 cycles of 94^{o}C for 15 sec, 65^{o}C for 30 sec, and 68^{o}C for 1 min, and a final extension at 68^{o}C for 5 min. The PCR product was digested with *Kpn*I and *Bam*HI, gel-purified, and cloned into the *Kpn*I and the *Bam*HI sites of the gene replacement vector pNOT19. Next, a 1,246-bp fragment containing the 3' part of *orfT* was amplified with primers orfT_Pst and orfT_Bam2 (Table 1) and KOD Hot Start DNA polymerase by using the same cycling program. The PCR product was digested with *Bam*HI and *Pst*I, gel-purified, and cloned into pNOT19

containing the upper shoulder of the *orfT* gene. These manipulations resulted in introduction of a unique *Bam*HI site in *orfT*. The site was then used to clone the EZ::TN™ <Kan-2> transposon (Epicentre Technologies), yielding pNOT19-orfTEZ::TN<Kan-2>. The resultant Kan^r plasmids were digested with *Not*I, ligated as above with a 5-kb fragment of pMOB3 containing *sacB* and *cat* genes, and electroporated into *E.coli* S17-1 (λ-pir). Gene replacement mutagenesis was carried out essentially as described above except that mutants were screened by PCR with primers orfT_SEQ1 and orfT_SEQ2 to confirm the absence of the wild-type *orfT* allele. All mutant clones were isogenic and only one clone was chosen for further experiments.

Motility assays, exoprotease and HCN production, and colony morphology. All assays were performed essentially as described earlier (Mavrodi *et al.,* submitted for publication). For motility assays, the diameter of outward expansion of colonies was measured at 24 and 48 h after inoculation. Exoprotease production was assessed on skim milk agar (Sacherer *et al,*, 1994) as a clearing zone surrounding bacterial growth, and was measured after 72 hrs of incubation at 28°C. Hydrogen cyanide production was monitored visually by using cyanide detection paper placed on Petri dish lids (Bakker and Shippers, 1987). Observations were made every 24 hrs, and all cultures were screened twice. Polysaccharide production was scored visually after 3 days of growth on PsP agar on a scale of 0-5, where 0 indicates a non-mucoid isolate and 5 indicates a moderately mucoid culture. Fluorescence was was scored visually after 3 days of growth at 28°C on PsF agar plates. Fluorescence was assessed on a 0-9 scale, where 0 indicates no fluorescence and 9 indicates the greatest fluorescence observed in a sample set. Experiments were repeated twice with six replicates for each different medium.

Carbon and nitrogen utilization. Carbon substrate utilization and nitrogen utilization profiles were generated by using Biolog SF-N2 and PM3 MicroPlates (Biolog, Inc., Hayward,

Calif.), respectively, as described earlier (Mavrodi *et al.,* submitted for publication). Four independent repetitions were performed with each strain.

Extraction and detection of 2,4-DAPG and related metabolites. Phloroglucinol compounds were extracted with ethyl acetate from bacterial cultures grown for 48 h at 27°C in PsF broth, and fractionated on a Waters NOVA-PAK C_{18} Radial-PAK cartridge (4 μm, 8 x 100 mm, Waters Corp., Milford, Mass.) (Bonsall *et al.,* 1997) as described earlier (Mavrodi *et al.,* submitted for publication). Five independent experiments with two replications were performed.

Fungal inhibition in vitro. Inhibition of *G. graminis* var. *tritici* by *P. fluorescens* Q8r1-96Rif^{r}Gm^r and its mutants was assayed *in vitro* on PsF plates as described previously (Mavrodi *et al.,* submitted for publication). Assays were repeated twice, with 6 replicates per strain.

Rhizosphere colonization assays. Rhizosphere colonization assays were performed with the *sss* recombinase*, ptsP* and *orfT* mutants and Q8r1-96Rif^{r}Gm^r, a gentamycin-resistant derivative of the parental strain tagged with mini-Tn7-*gfp2* (Validov *et al.*, 2004) in order to distinguish it from mutant strains in mixed inoculation studies. Bacterial inocula were grown overnight on LB agar supplemented with appropriate antibiotics. The biomass was scraped from the plates and added to sieved Quincy virgin soil (Landa *et al.,* 2003) in a 1% methylcellulose suspension to give approximately $1x10^4$ CFU g^{-1} (fresh weight) of soil when strains were introduced alone. Mixed inoculation treatments contained a 1:1 mixture of Q8r1-96Rif^{r}Gm^r (~ 0.5×10^4 CFU g^{-1} of soil for each strain). The actual density of each strain was determined by assaying 0.5 g of inoculated soil as described by Landa *et al*. (2002a). The control treatment consisted of soil amended with a 1% methylcellulose suspension. Each treatment was replicated 6 times with one pot serving as replicate.

Spring wheat (*Triticum aestivum* L.) cv. Penawawa was pre-germinated on moistened sterile filter paper in Petri dishes for 24 h. in the dark. Six pre-germinated seeds were sown in square pots (6.5 cm high x 7 cm wide) containing 200 g of Quincy virgin soil (Landa *et al.,* 2003) inoculated with one or both bacterial strains. The soil was watered with 50 ml of metalaxyl solution (2.5 mg/ml) (Novartis, Greensboro, N.C.) to control Pythium root rot and seeds were covered with a 1-cm-thick layer of inoculated soil. Wheat was grown for six successive 2-week cycles in a controlled-environment chamber at 15°C with a 12-h photoperiod. Pots were watered on alternate days and after the first week each pot received 40 ml of Miracle-Gro solution 15-30-15 (Scotts Miracle-Gro Products, Port Washington, N.Y.) once weekly. After 2 weeks of growth (one cycle), one randomly selected plant was harvested from each replicate pot and root samples were prepared to determine the population size of the introduced bacteria. The shoots of the remaining plants were excised, and the soil and associated root systems were decanted into a plastic bag, shaken vigorously to aerate and mix, and then introduced into new pots, which were replanted with six wheat seeds. This process of plant growth, harvesting, and determination of population sizes of inoculated bacteria was repeated for six consecutive cycles.

Population densities of introduced and indigenous rhizosphere bacteria. Samples (0.5 g) of either soil or roots with tightly adhering rhizosphere soil were placed in 50-ml centrifuge tubes containing 10 ml of sterile distilled water, vortexed, and sonicated in an ultrasonic cleaner (Bransonic 521; Branson, Shelton, Conn.) for 1 min. A 100-µl sample of the wash solution was serially diluted in a 96-well microtiter plate pre-filled with 200 µl of sterile distilled water per well, and then 50 µl of each dilution was transferred to a well of a 96-well microtiter plate containing 200 µl of one-third-strength KMB broth supplemented with rifampicin, cycloheximide, chloramphenicol and ampicillin (1/3 x KMB^{+++}Rif). The optical

density at 600 nm was measured after incubation at room temperature in the dark for 72 h, and bacteria from each microplate were transferred with a replicator tool into two fresh 96-well microtiter plates filled with 200 μl of 1/3xKMB^{+++}Rif broth amended with either kanamycin or gentamycin. This two-step incubation process was necessary in order to distinguish growth of *P. fluorescens* Q8r1-96Rif^{r}Gm^{r} and the kanamycin-marked mutants in mixed inoculations. If gentamycin and kanamycin were included in the selective media initially, bacterial growth was inhibited. Bacterial growth was assessed after 72 h, and an OD_{600} of ≥ 0.07 was scored as positive (McSpadden Gardener *et al*., 2001). The results were occasionally verified by PCR with KAN_UP and KAN_LOW, and Gm_UP and Gm_LOW primers. Root colonization experiments were conducted twice with similar results, and data from a representative experiment are reported here.

Densities of total culturable heterotrophic bacteria (TCB) were determined by the terminal dilution endpoint assay in one-tenth-strength tryptic soy broth supplemented with cycloheximide.

Data analysis. All treatments in competitive colonization experiments were arranged in a complete randomized design. Statistical analyses were performed by using appropriate parametric and nonparametric procedures with the STATISTIX V. 8.0 software (Analytical Software, St. Paul, Minn.). All population data were converted to log CFU g^{-1} (fresh weight) of soil or root. Differences in population densities among treatments were determined by standard analysis of variance, and mean comparisons among treatments were performed by using Fisher's protected least significant difference test (P=0.05). Alternatively, nonparametric population data were analyzed by the Kruskal-Wallis test (P=0.05) and mean comparisons among treatments were determined by Kruskal-Wallis all pairwise comparisons (P=0.05). The area under the

rhizosphere colonization progress curve (AUCPC), which represented the total rhizosphere colonization for all six cycles (Landa, 2002b), was calculated with SigmaPlot V. 8.0 (SYSTAT Software Inc., Richmond, Calif.). Motility and production of phloroglucinol compounds and exoprotease were compared by using a two-sample t test or Wilcoxon rank sum test (P=0.05).

Nucleotide sequence accession numbers. The *sss*, *ptsP* and *orfT* sequences of *P. fluorescens* Q8r1-96 have been deposited in the GenBank nucleotide sequence database with accession numbers AY172655, AY816321, and AY816322, respectively.

RESULTS

Identification, cloning, and characterization of *sss, ptsP,* and *orfT*. In order to identify *sss* and *orfT*, an ordered genomic library of *P. fluorescens* Q8r1-96 was screened by colony hybridization or, in case of *ptsP*, by PCR. The initial round of screening yielded eight *sss* and eight *orfT*-positive clones, all of which were further mapped in a second Southern hybridization experiment. Briefly, purified cosmid DNA from each clone was digested with restriction endonucleases *Eco*RI, *Kpn*I and *Sac*I, resolved on an agarose gel, blotted, and hybridized with the corresponding biotin-labeled probe. As a result, one cosmid clone containing each gene of interest was selected for shotgun sequencing by using the EZ::TN™ <Kan-2> transposition system.

The EZ::TN™ <Kan-2>-mediated shotgun sequencing of *sss*-positive cosmid clone 7D10 yielded a 1,851-bp contig that contained a predicted open reading frame with similarity to numerous bacterial and phage site-specific recombinases (Fig. 1a). The gene encodes a predicted 299-amino acid protein of molecular mass 33,772 Da, and is flanked by two open reading frames encoding for a conserved hypothetical protein and a putative hydrolase, YigB.

The deduced Sss protein is highly similar to putative recombinases from *P. fluorescens* WCS365 (NCBI accession number CAA72946; 100 % identity), *P. fluorescens* F113 (NCBI accession number AAL73390; 98 % identity), *P. syringae* pv. *syringae* DC3000 (NCBI accession number AAO53768; 83 % identity), *P. putida* KT2440 (NCBI accession number NP_747331; 76 % identity), *Azotobacter vinelandii* (NCBI accession number ZP_00092285; 73 % identity), and *P. aeruginosa* PA01 (NCBI accession number Q51566; 71 % identity). Further analyses revealed the presence of a XerC profile listed in the HAMAP database of orthologous microbial protein families (Gattiker *et al*., 2003), and two Pfam domains (residues 5-89 and 111-282) associated with phage integrases. Searches against the database of Cluster of Orthologous Groups of proteins (COG) (URL http://www.ncbi.nlm.nih.gov/COG) revealed that the protein belongs to COGs 4973 and 0582, which contain XerC-like site-specific recombinases (E value of 3e-94). Finally, the protein structure predicted by the fold recognition server 3D-PSSM (Kelley *et al*., 2000) closely resembled that of the site-specific recombinase XerD from *E. coli* (Subramanya *et al*., 1997).

Shotgun sequencing of cosmid clone 7D2, which hybridized to the *orfT* probe, yielded a 5,176-bp contig (Fig. 1b) that contained a predicted open reading frame with similarity to Orf338 from *P. aeruginosa* PA14 (Tan *et al*., 1999). The gene, referred to in this paper as *orfT*, is preceded by a putative ribosome-binding site, GGAGA, and encodes a predicted 341-amino-acid protein of molecular mass 38,655 Da. The contig contains a number of other ORFs, two of which are located immediately downstream of *orfT* and probably are co-transcribed with it. These genes encode a conserved hypothetical protein and a putative DnaJ-like protein, respectively.

Database searches revealed that *orfT* is highly conserved in sequenced bacterial genomes (blastp search against the non-redundant GenBank dataset returned >40 hits with E values below

1e-50). The deduced OrfT protein is most similar to its counterparts from *P. fluorescens* Pf01 (NCBI accession number ZP_00262363; 90% identity), *P. syringae* pv. *syringae* DC3000 (NCBI accession number AAO54097; 82% identity), *P. putida* KT2440 (NCBI accession number NP_742571; 74% identity), and *P. aeruginosa* PA14 (NCBI accession number AAD22455; 67% identity). Searches against the COG database revealed that OrfT is a member of COG3178, which contains predicted phosphotransferases (E value of 1e-90), and further analyses revealed the presence of a Pfam domain associated with the aminoglycoside phosphotransferase (APH) enzyme family (residues 152-234). The OrfT structure predicted by 3D-PSSM (Kelley *et al*., 2000) is related to aminoglycoside 3'-phosphotransferases from *Enterococcus faecalis* (Burk *et al*., 2001) and *Klebsiella pneumoniae* (Nurizzo *et al*., 2003).

Shotgun sequencing of the *ptsP*-positive cosmid clone 5F9 yielded a 2,586-bp contig that contained a predicted open reading frame with similarity to numerous bacterial *pstP* genes (Fig. 1c). The gene encodes a predicted 759-amino acid protein of molecular mass 83,208 Da, and is flanked in Q8r1-96 by a well-conserved ribosome-binding site, GGAG, and a putative transcriptional terminator comprised of a 93-bp-long region of imperfect dyad symmetry.

The deduced PtsP protein is highly similar to its orthologues from *P. fluorescens* Pf01 (NCBI accession number ZP_00264741; 95 % identity), *P. syringae* pv. *syringae* DC3000 (NCBI accession number AAO58710; 92 % identity), *P. putida* KT2440 (NCBI accession number NP_747246; 91 % identity), *Azotobacter vinelandii* (NCBI accession number CAA74995; 87 % identity), and *P. aeruginosa* PA01 (NCBI accession number NP_249028; 86 % identity). Results of domain searches revealed the presence of a conserved PROSITE phosphoenolpyruvate (PEP)-utilizing enzyme signature (residues 620-638), and Pfam GAF (residues 17-154), N-terminal (residues 178-302), mobile (residues 318-399), and TIM barrel

(residues 424-715) domains associated with PEP-utilizing enzymes. Finally, searches against the COG database indicated that the protein belongs to COG3605 containing bacterial PtsP proteins (E value of 0.0), and the protein structure predicted by 3D-PSSM (Kelley *et al*., 2000) was related to pyruvate phosphate dikinases from *Clostridium symbiosum* (Herzberg *et al*., 2002) and *Trypanosoma brucei* (Cosenza *et al*., 2001).

Effects of the *sss, ptsP,* and *orfT* mutations on motility, fluorescence, morphology, and exoprotease and hydrogen cyanide production. The motility and swarming behavior of wild-type Q8r1-96 and its *sss* recombinase*, ptsP,* and *orfT* mutants were compared on LB medium solidified with different agar concentrations (0.3%, 0.5%, 1.0% and 1.5%). The motility of the wild type strain was significantly ($P = 0.05$) greater than that of the *ptsP* and *orfT* mutants on 0.3% agar. As shown in Table 2, the migration diameter of the *ptsP* and *orfT* mutants was on average 1.1 and 1.2 times less, respectively, than that of the wild type. In contrast, the *sss* mutant did not differ from Q8r1 in motility. When higher agar concentrations were used, neither the mutants nor the wild type showed swarming ability.

When grown on siderophore-inducing PsF plates, the *sss* and *orfT* mutants did not differ from the wild type in the level of fluorescence. However, the *ptsP* mutant demonstrated increased fluorescence when compared to Q8r1-96 (Table 2). We also observed differences in colony morphology among the wild type and mutant strains. The *sss* and *orfT* mutants were more mucoid, and the *ptsP* mutant was less mucoid on PsP medium than the parental strain. Isolated colonies of the *ptsP* mutant appeared more yellowish than the parental strain when grown on LB medium supplemented with glucose, whereas the *sss* and *orfT* mutants were indistinguishable from Q8r1-96 (Table 2).

We also examined whether the *sss, ptsP,* and *orfT* mutations in *P. fluorescence* Q8r1-96 affected the secretion of exoprotease or the production of hydrogen cyanide. Results of the assays indicated that extracellular protease production was significantly greater in the *sss* mutant, unaltered in the *orfT* mutant, and significantly lower in the *ptsP* mutant, than in the parental strain (Table 2). None of the mutants was impaired in the production of hydrogen cyanide.

Effects of the *sss, ptsP,* and *orfT* mutations on carbon and nitrogen utilization. Carbon substrate utilization profiling in Biolog SF-N2 MicroPlates revealed that the *orfT* and *ptsP* mutants were defective in utilization of D-galactose, and D-galactose and uridine, respectively. In contrast, the carbon substrate utilization pattern of the *sss* mutant did not differ from that of wild-type Q8r1-96. On the other hand, nitrogen utilization profiling on Biolog PM3 MicroPlates indicated that the *sss* mutant utilized L-methionine, L-cysteine, and glucuronamide as nitrogen sources whereas the parental type did not (Table 3). These tests also revealed that wild-type *P. fluorescens* Q8r1-96, unlike its *ptsP* mutant, did not utilize L-cysteine, ethylenediamine, agmatine, glucuronamide, guanine, Gly-Glu, and alloxan as sources of nitrogen (Table 3). Finally, the *orfT* mutant was defective in utilization of biuret and formamide.

Phloroglucinol production and fungal inhibition in vitro. *P. fluorescens* Q8r1-96 and the *sss, orfT,* and *ptsP* mutants all produced detectable quantities of 2,4-DAPG, as well as MAPG and three other uncharacterized phloroglucinol-related compounds (Table 2). Since MAPG is thought to be a breakdown product of 2,4-DAPG (Bangera and Thomashow, 1999; Shanahan, *et al.,* 1992), and the uncharacterized phloroglucinols detected in these analyses are likely to include condensation products of either or both compounds (R. F. Bonsall, O. V. Mavrodi, D. V. Mavrodi, and D. M. Weller, unpublished data), we also assayed strains for the production of total phloroglucinol-related compounds (Table 2). Results of the analyses indicated that *sss* and *orfT*

mutants produced greater amounts of MAPG, 2,4-DAPG and total phloroglucinol-related compounds than wild-type Q8r1-96 [in the *sss* mutant only production of 2,4-DAPG was significantly greater (P = 0.05)], whereas the *ptsP* mutant produced significantly smaller amounts of MAPG and 2,4-DAPG.

Analyses of the fungal inhibition data indicated that 6 days postinoculation, the calculated hyphal inhibition indexes for *ptsP* and *orfT* mutants were 0.0, and 0.08, respectively, compared with an index of 0.16 for the wild-type strain. After 7 days, inhibition indexes were 0.0 for both the *ptsP* and *orfT* mutants, and 0.07 for the parental strain. In contrast, inhibition indexes for the *sss* mutant were 0.12 and 0.02 at six and seven days postinoculation, respectively.

Impact of the *sss, ptsP,* and *orfT* mutations on rhizosphere colonization by Q8r1-96. Studies of rhizosphere colonization were conducted using Q8r1-96Rif^{r}Gm^r tagged with a mini-Tn*7*-gfp2 (Validov *et al.*, 2004) as the wild type strain and kanamycin-resistant *sss, ptsP,* and *orfT* mutants. This allowed for the wild-type and mutant strains to be distinguished in competitive colonization experiments. Prior to colonization assays, the growth kinetics of *P. fluorescens* Q8r1-96Rif^{r}Gm^r tagged with mini-Tn*7*-gfp2 was checked in vitro in 1/3x KMB and MMP minimal media. No differences were found in the growth kinetics of the mini-Tn*7*-tagged derivatives as compared to the parental strain (data not shown).

To test the rhizosphere competence of *sss, ptsP,* and *orfT* mutants, the strains were inoculated individually or in pairwise combinations (1:1 ratio) with Q8r1-96Rif^{r}Gm^r into Quincy virgin soil. Figures 2a, 2b and 2c show the population dynamics of the strains in the rhizosphere of wheat over six growth cycles. The values for cycle 0 indicate that the population densities of all introduced strains were roughly equivalent at the beginning of all experiments. In both single and mixed inoculations, population sizes of the wild-type and the *sss* recombinase and *orfT* mutant

strains increased by four orders of magnitude by the end of cycle 1 and then slowly declined over the following six cycles.

Although populations of both Q8r1-96 Rif^{r}Gm^r and its *sss* mutant fluctuated in the wheat rhizosphere when the strains were introduced individually, they did not differ significantly as indicated by AUCPC and mean colonization values (Table 4a). The population densities of both strains were similar in cycles 3 and 4, and the density of the *sss* mutant was less than that of the wild-type strain in cycles 5 and 6 (Fig. 2a). However, when introduced in combination with the parental strain, the *sss* mutant colonized the wheat rhizosphere to a lesser extent than did Q8r1-96 Rif^{r}Gm^r in cycle 2 and thereafter, and the AUCPC and mean colonization values of the mutant were significantly lower than those of the wild type (Table 4a).

As in the case of the *sss* mutant, in single inoculation treatments the population dynamics of the *orfT* mutant fluctuated during the six growth cycles of wheat (Fig. 2b). Overall, however, the AUCPC and mean colonization values (Table 4b) did not differ from those of Q8r1-96Rif^{r}Gm^r. On the other hand, when introduced together into the soil, Q8r1-96Rif^{r}Gm^r consistently outcompeted the *orfT* mutant. The population size of Q8r1-96Rif^{r}Gm^r was significantly greater than that of the mutant in cycles 1 through 6 (Fig 1b), and the AUCPC and mean colonization values of the mutant were significantly lower than those of the wild type in the mixed inoculations (Table 4b).

In contrast to the *sss* recombinase and *orfT* mutants, the population size of the *ptsP* mutant when introduced into soil alone was significantly smaller (P=0.05) than that of Q8r1-96Rif^{r}Gm^r in cycles 1 and 4 (Fig. 2c). The population densities of both strains were similar in all other cycles, but the AUCPC and mean colonization values for Q8r1-96 Rif^{r}Gm^r were significantly higher than that of the *ptsP* mutant (Table 4c). In mixed treatments, the *ptsP* mutant

consistently colonized the wheat rhizosphere to a lesser extent than Q8r1-96Rif^{r}Gm^r throughout all 6 cycles (Fig 1c), and AUCPC and mean colonization values for the mutant were significantly lower (P=0.05) when compared to the wild type (Table 4c).

Population densities of total culturable aerobic bacteria in the wheat rhizosphere for all experiments were above log 8.4 CFU/g of root and also fluctuated among different treatments. Mean colonization values and AUCPC values of indigenous bacteria are presented in Tables 4a, 4b and 4c.

DISCUSSION

The *P. fluorescens* Q8r1-96 genes studied in this work were chosen for analysis based on the important roles they play in other bacterial systems. Among these three genes, *sss* probably is the best-studied, and encodes a subunit of a site-specific tyrosine recombinase that is involved in proper segregation of circular bacterial chromosomes during cell division and in the stable maintenance of some plasmids (Blakely *et al.,* 2000). In *E. coli,* the orthologue XerC, together with XerD, forms a heterotetrameric enzyme complex that catalyzes two consecutive pairs of strand exchanges at a 28-bp *dif* site in the chromosome. In the complex, XerC specifically cleaves and exchanges the top DNA strand. However, XerC and XerD are similar to each other and act in a cooperative fashion. The Xer recombinase is highly conserved in most species of Enteroacteriaceae as well as in many other bacterial genera including *Pseudomonas* (Villion and Szatmari 1998). In fluorescent pseudomonads, *xerC* (called *sss*) initially was identified in *P. aeruginosa* as a gene that complemented a deficiency in pyoverdin production (Höfte *et al.,* 1994), but further functional studies revealed that *sss* encodes a site-specific recombinase homologous to XerC from *E. coli* (Blakely *et al.,* 2000). Later studies established that this *sss*

recombinase plays an important role in competitive rhizosphere colonization by *P. fluorescens* WCS365, where its activity is linked to phenotypic variation and high mutation frequencies in the global two-component regulatory system GacA/GacS (Dekkers *et al.,* 1998b; Sanchez-Contreras *et al.,* 2002).

Results of sequence analyses (see Results) suggest that the *sss*-like gene that we cloned from *P. fluorescens* Q8r1-96 indeed encodes a true *xerC* homologue. We included this gene in our studies of root colonization as an internal control, since *sss* previously had been shown to contribute to rhizosphere competence and phenotypic variation in fluorescent *Pseudomonas* spp. on a variety of crops. (Achouak et al., 2004; Dekkers *et al.,* 1998b; Sanches-Contreras *et al.,* 2002). For example, under gnotobiotic conditions the *sss* mutant of *P. aeruginosa* 7NSK2 colonized potato root tips 10 to 1,000-fold less than the wild type in mixed inoculations, and the corresponding mutant of *P. fluorescens* WCS365 was impaired in root tip colonization of tomato in potting soil, and of radish and wheat under gnotobiotic conditions (Dekkers *et al.,* 1998b). These gnotobiotic systems are important in identifying genes that may function in colonization, but the results obtained under such artificial conditions must be validated in natural soil. In our studies, the impact of *sss* on the rhizosphere competence of Q8r1-96 was tested in an experimental system involving wheat sown in nonsterile natural soil. The mutant was tested in cycling experiments in which bacteria were added to a natural field soil that was successively cropped to wheat, allowing rhizosphere colonization to proceed for months under controlled conditions in the presence of indigenous microflora and percolating water. Under these conditions, the *sss* mutant of Q8r1-96 colonized the wheat rhizosphere equivalent to its wild type parent, and a deficiency in rhizosphere competence of the mutant became apparent only when plants were inoculated with a mixture containing both strains (Table 4a).

Inactivation of *sss* in *P. fluorescens* Q8r1-96 resulted in a phenotype similar to that of the *sss* mutant of *P. fluorescens* WCS365 (Dekkers *et al.,* 1998b). Q8r1-96 *sss* did not differ from the wild-type parent in its carbon source utilization profile, growth rate, motility, swarming behavior, or siderophore production (Table 2). Among the minor differences observed were the production of elevated amounts of exoprotease and phloroglucinol–related compounds (Table 2). Profiling in Biolog PM3 plates revealed that, in contrast to the wild type, Q8r1-96 *sss* was able to utilize L-cysteine, L-methionine and glucoronamide as nitrogen sources (Table 3). The fact that the *sss* mutant was not impaired in the accumulation of extracellular protease or the production of hydrogen cyanide suggests that the GacA/GacS regulatory circuitry, which coordinately regulates the production of secondary metabolites and exoprotease (Whistler *et al.,* 1998), has not been disturbed in Q8r1-96 *sss*. We have no immediate explanation for the phenotypic changes observed in the *sss* mutant, and we attribute them to the pleiotropic nature of the *sss* mutation.

The second gene studied in this work, *orfT*, was chosen for analysis based on its role in interactions between *P. aeruginosa* PA14 and *Arabidopsi*s (Rahme *et al.,* 1997). Like the *sss* mutant of Q8r1-96, the *orfT* mutant did not differ from the wild type in its ability to colonize the rhizosphere of wheat when it was introduced into soil alone, but it colonized significantly less well than the parental strain when the two strains were introduced together (Table 4b). Inactivation of *orfT* resulted in a phenotype with reduced motility, deficiency in the utilization of D-galactose, and minor alterations in the nitrogen utilization profile (Table 3). On the other hand, the *orfT* mutant did not differ from the wild type in the production of exoprotease, hydrogen cyanide, siderophores, and phloroglucinol-related compounds. Further, the mutant was

indistinguishable from *P. fluorescens* Q8r1-96 on PsF and LB media supplemented with 2% glucose, but was slightly more mucoid on PsP medium.

Results of computer analyses of OrfT indicate that its orthologues are found in all *Pseudomonas* genomes sequenced to date. The predicted proteins share a HRDxxxN motif with eukaryotic and some prokaryotic Ser/Thr and Tyr protein kinases (Shi *et al.,* 1998) and some aminoglycoside phosphotransferases that are responsible for bacterial resistance to aminoglycoside antibiotics such as streptomycin and kanamycin (Nurizzo *et al.,* 2003). The motif represents part of an active center where the conserved aspartate residue acts as a catalytic base (Nurizzo *et al.,* 2003). Despite these findings, this eukaryote-like protein kinase motif is too generic to provide any clues to the specificity of OrfT, and similarity to aminoglycoside phosphotransferases is partial and limited to about 40% of the polypeptide chain. Thus, the exact function of *orfT* in the rhizosphere competence of *P. fluorescens* Q8r1-96 at this point remains unclear.

Similarly to *orfT,* the third gene studied in this work, *ptsP*, was chosen for analysis based on its role in interactions between *P. aeruginosa* PA14 and *Arabidopsi*s (Rahme *et al.,* 1997). In contrast to the *sss* recombinase and *orfT* mutants, the rhizosphere competence of Q8r1-96 *ptsP* was strongly impaired, and its rhizosphere population densities were significantly lower than those of the wild type not only in mixed but also in single inoculations (Table 4c; Fig. 2c). The *ptsP* mutant exhibited altered morphology, reduced motility and exoprotease production, and an increased level of fluorescence. It produced significantly lower amounts (ca. 54% and 60% of the wild type level) of MAPG and 2,4–DAPG, and differed from Q8r1-96 in the ability to utilize L-cysteine, ethylenediamine, agmatine, glucuronamide, guanine, alloxan, and Gly-Glu as a

nitrogen sources (Table 3). The carbon substrate utilization profiling revealed that Q8r1-96 *ptsP* is defective in utilization of D-galactose and uridine (Table 3).

The pleiotropic phenotype exhibited by Q8r1-96 *ptsP* appears to reflect the proposed global regulatory function of this gene, which is highly conserved in the unfinished genomes of *P. fluorescens* Pf01, SBW25 and Pf-5 (data not shown). The product of *ptsP* forms part of an alternative phosphoenolpyruvate (PEP):carbohydrate phosphotransferase (PTS) system. PTSs are found in a wide range of Gram-positive and Gram-negative microorganisms and have been best studied in enteric bacteria, where they are involved in sensing, transport and metabolism of carbohydrates, as well as in catabolite repression and inducer exclusion. The first component of a typical PTS is a carbohydrate-specific multisubunit (or multidomain) Enzyme II (EII) that forms a membrane translocation channel and phosphorylates the incoming sugar (Postma *et al.,* 1996). The second part of PTS is involved in phosphorylation of all PTS carbohydrates. It lacks specificity and is comprised of cytoplasmic Enzyme I (EI) and histidine protein (HPr).

In *E. coli, ptsP* encodes a nitrogen-specific EI paralogue called EI^{Ntr} that, together with EII^{Ntr} and NPr (paralogues of EII^{Fru} and HPr, respectively), forms a regulatory PTS phosphoryl transfer chain (Rabus *et al.,* 1999). Functional analyses have shown that this chain is involved in sugar-dependent utilization of certain amino acids and somehow links together the metabolism of carbon and nitrogen. In *P. putida*, EII^{Ntr} either positively or negatively (and largely in a glucose-independent fashion) controls expression of over 100 genes, some of which are members of the σ^{54} regulon (Cases *et al.,* 2001). Based on the results of that study, it was proposed that EII^{Ntr}, which together with EI^{Ntr} and NPr presumably forms an alternative PTS system in *P. putida*, functions as a global rather than a promoter-specific regulatory factor. Genes encoding for EI^{Ntr} also were described in *A. vinelandii* (Segura & Espin, 1998), *Bradyrhizobium japonicum* (King

& O'Brian, 2001), *P. aeruginosa* (Tan *et al.,* 1999), and *Legionella pneumophila* (Higa & Edelstein, 2001). In these microorganisms, as in *E. coli*, *ptsP* primarily plays a regulatory role and does not directly participate in phosphorylation or the utilization of carbohydrates.

In conclusion, the data presented in this study suggest novel functions for two genes, *ptsP* and *orfT*, that previously were described as pathogenesis-related in *P. aeruginosa*. The *ptsP* and *orfT* mutants of Q8r1-96 do not have nonspecific growth defects in vitro, and the effect of mutations became apparent only when mutants were tested in the rhizosphere in competition with the parental strain. In this respect, both genes fulfill the criteria for "true" rhizosphere colonization determinants (Lugtenberg *et al.,* 2001). To our knowledge, this is the first report to provide evidence for the involvement of *ptsP* in rhizosphere colonization by fluorescent pseudomonads. Results of sequence analyses suggest that in *P. fluorescens* Q8r1-96, as in *P. aeruginosa* (Reizer *et al.,* 1999) and *P. putida* (Cases *et al.,* 2001), PtsP(EI^{Ntr}) forms a part of regulatory phosphoryl transfer chain that may coordinate the metabolism of carbon and nitrogen. Unfortunately, despite the fact that EI^{Ntr}, EII^{Ntr} and NPr have been purified and characterized biochemically, the exact function of this phosphoryl transfer chain remains poorly understood even in *E. coli* (Tchieu *et al.,* 2001). Based on observations made in other bacteria, we speculate that PtsP may be involved in the regulation of amino acid metabolism in *P. fluorescens* Q8r1-96. This could explain the crucial role of this gene in rhizosphere competence, because amino acids represent one of the major components of root exudates and are thought to be among major sources of nitrogen, carbon, and energy for rhizophere microorganisms (Lugtenberg *et al.,* 2001).

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Figure legends

FIG. 1. Restriction maps and locations of individual genes in regions of the *P. fluorescens* Q8r1-96 genome containing *sss* (A), *orfT* (B), and *ptsP* (C). Inverted solid triangles indicate positions of EZ::TN<Kan-2>insertions, and small horizontal arrows indicate PCR primers used in the study. The shaded arrows indicate the positions of genes and open reading frames (ORF) that were not relevant to the present study. RBS, ribosome binding site.

FIG. 2. Population dynamics of *Pseudomonas* strains Q8r1-96 and *sss* (A), *orfT* (B), and *ptsP* (C) mutants on the roots of wheat cv. Penawawa grown in Quincy virgin soil for six consecutive two weeks-long cycles as described in Materials and Methods. Each strain was introduced into the soil to give a final density of approximately log 4 CFU per g of soil (cycle 0) in single inoculations and ~ 0.5×10^4 CFU per g of soil of each strain in mixed inoculations. Mean values and standard deviations are presented. Means followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test or Kruskal-Wallis test (P=0.05) (marked by asterisk).

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain	Relevant characteristics[a]	Reference of origin
Pseudomonas fluorescens		
Q8r1-96 Rif	DAPG$^+$ Rif^r	Landa *et al.* 2002
Q8r1-96 Rif^r Gm^r	Q8r1-96 tagged with mini-Tn*7*-*gfp2*; DAPG$^+$ Rif^r Gm^r	Validov *et al.,* 2004
Q8r1-96 *sss*	*sss*:: EZ::TN<Kan2>; DAPG$^+$ Rif^r Kan^r	This study
Q8r1-96 *ptsP*	*ptsP*:: EZ::TN<Kan2>; DAPG$^+$ Rif^r Kan^r	This study
Q8r1-96 *orfT*	*orfT*:: EZ::TN<Kan2>; DAPG$^+$ Rif^r Kan^r	This study
Esherichia coli		
S17-1(λ-pir)	*thi pro hsdM recA rpsL* RP4-2 (Tet^r::Mu) (Kan^r::Tn7)	Simon *et al.,1983*
Top 10	F$^-$ *mcr*A *Δ* (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z *Δ* M15 *Δ lac*X74 *rec*A1 *ara Δ* 139 *Δ* (*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen Corp.
Plasmids		
pCPP47	Broad-host-range cosmid derived from pCPP34, tandem *cos*$^+$, *par*$^+$, Tet^r	Bauer and Collmer, 1997

pMOB3	Kan^r *cat oriT sacB*	Schweizer, 1992
pNOT19	ColE1 *bla*; accessory plasmid	Schweizer, 1992
pNOT19-*sss* EZ::TN <Kan-2>	pNOT19 containing the 2.57-kb DNA *Sma*I fragment with *sss* EZ::TN<Kan2>	This study
pNOT19-*ptsP* EZ::TN <Kan-2>	pNOT 19 containing the 3.8-kb DNA *Sma*I fragment with *ptsP* EZ::TN<Kan2>	This study
pNOT19-*orfT_ Kpn*I-*orfT_ Bam*HI	pNOT 19 containing the 1.2-kb DNA *Kpn*I+*Bam*HI fragment with *orfT_ Kpn*I- *orfT_ Bam*HI	This study
pNOT19-*orfT_ Bam*HI - *orfT_ Pst*I	pNOT 19 containing the 1.1-kb DNA *Bam*HI+ *Pst*I fragment with *orfT_ Bam*H- *orfT_ Pst*I	This study
pNOT19-*orfT* EZ::TN <Kan-2>	pNOT 19 containing the 2.3-kb DNA *Sma*I fragment with *orfT* EZ::TN<Kan2>	This study
pNOT19-*sss* EZ::TN <Kan-2> +pMOB3	pNOT19-*sss* EZ::TN <Kan-2> ligated with 5.8-kb *Not*I fragment from pMOB3	This study
pNOT19-*ptsP* EZ::TN <Kan-2> +pMOB3	pNOT19-*sss* EZ::TN <Kan-2> ligated with 5.8-kb *Not*I fragment from pMOB3	This study

pNOT19-*orfT* EZ::TN <Kan-2> +pMOB3	pNOT19-*sss* EZ::TN <Kan-2> ligated with 5.8-kb *Not*I fragment from pMOB3	This study
Oligonucleotides		
SSS_UP	5' CGT CTT TCG CAC CTG ATG GA 3'; upper primer for amplification of a 994-bp fragment of *sss* from *P. fluorescens* F113; T_m 63.6°C	This study
SSS_LOW	5' CCT GGG ATG GGC ACT GTC A 3'; lower primer for amplification of a 994-bp fragment of *sss* from *P. fluorescens* F113; T_m 62.8°C	This study
ORFT_UP	5’ CTA CGT TTA CAA CAG CTG GA 3’; upper primer for amplification of a 969 -bp fragment of *orfT* from *P. fluorescens* SBW25; T_m 62.6°C	This study
ORFT_LOW	5’ TTC ACC CAG TTC GCT CAG T 3’; lower primer for amplification of a 969-bp fragment of *orfT* from *P. fluorescens* SBW25; T_m 67.9°C	This study
ORFT_KpnI	5' AAG TAG TAC GGG GTG ACC AG 3'; upper primer for amplification of a 1386-bp 5’ part of *orfT* from *P. fluorescens* Q8r1-96; T_m 61.2°C	This study
ORFT_Bam1	5' TTT TGG ATC CAC GAA GGG TTT GCA 3'; lower primer for amplification of a 1386-bp 5’ part of *orfT* from *P. fluorescens* Q8r1-96; T_m 76.8°C	This study

ORFT_Bam2	5' GCT GGA TCC TCA GGA TGC GGT C 3'; upper primer for amplification of a 1274-bp 3' part of *orfT* from *P. fluorescens* Q8r1-96; T_m 74.8°C	This study
ORFT_Pst	5' GCC CCA GCA ATA CAA ACA AC 3'; lower primer for amplification of a 1274-bp 3' part of *orfT* from *P. fluorescens* Q8r1-96; T_m 65.2°C	This study
ORFTseq1	5' GAC GAT TTG TTC CGC GAT GC 3'; upper primer for amplification of a 994-bp fragment of *orfT* from *P. fluorescens* Q8r1-96; T_m 70.8°C	This study
ORFTseq2	5' TCC AGC CAG CCG CGC ACA C 3'; lower primer for amplification of a 994-bp fragment of *orfT* from *P. fluorescens* Q8r1-96; T_m 77.7°C	This study
ptsP3	5' TCCAACGACCTCACCCAGTA 3'; upper primer for amplification of a 257-bp fragment of *ptsP* from *P. fluorescens* SBW25; T_m 65.4°C	This study
ptsP4	5' CGCAGCATCCACTTCACCTT 3'; lower primer for amplification of a 257-bp fragment of *ptsP* from *P. fluorescens* SBW25; T_m 68.0°C	This study
ptsP5	5' GGT GGT GAC ATT CAA GCG CGA 3'; upper primer for amplification of a 2.6-kb fragment of *ptsP* from *P. fluorescens* Q8r1-96; T_m 73.5°C	This study
ptsP13	5' GAG TGT GTT CAT CGC CAG CC 3'; lower primer for amplification of a 2.6-kb fragment of *ptsP* from *P. fluorescens* Q8r1-96; T_m 68.1°C	This study

KAN_UP	5' TGG CAA GAT CCT GGT ATC GGT 3'; upper primer for amplification of a 509-bp fragment of Kan^r gene from EZ::TN <Kan-2>; T_m 68.9°C	This study
KAN_LOW	5' GAA ACA TGG CAA AGG TAG CGT 3'; lower primer for amplification of a 509-bp fragment of Kan^r gene from EZ::TN <Kan-2>; T_m 66.8°C	This study
Cm_UP	5' ATC CCA ATG GCA TCG TAA AGA 3; upper primer for amplification of a 560-bp fragment of *cat* gene from pMOB3; T_m 66.9°C	This study
Cm_LOW	5' AAG CAT TCT GCC GAC AT 3'; lower primer for amplification of a 560-bp fragment of *cat* gene from pMOB3; T_m 57.9°C	This study

[a]DAPG, strain produce 2,4-diacetylphloroglucinol; Rif^r, rifampicine resistance; Gm^r, gentamycin resistance; Tet^r, tetracycline resistance; Kan^r, kanamycin resistance; T_m, melting temperature calculated by Oligo 6.65 Primer Analysis Software.

TABLE 2. Phenotypic effects of *sss*, *ptsP*, and *orfT* mutations in *P. fluorescens* Q8r1-96.

Test	Bacterial strain			
	Q8r1-96	Q8r1-96 *sss*	Q8r1-96 *ptsP*	Q8r1-96 *orfT*
Exoprotease production [a]	6.7a; 9.8a	7.5b; 11.2b	4.5b; 8.7b	6.8a; 10.7a
Motility [b]	20.2a; 32.3a; 38.7a	19.2a; 30.8a; 36.5a	17b; 29.6b; 35.1b	17.2b; 27.8b; 32.8b
MAPG production	2.1×10^6 a (100%) [c]	2.8×10^6 a (132%)	1.1×10^6 *b (54%)	2.5×10^6 a (120%)
2,4-DAPG production	9.4×10^6 a (100%)	12.7×10^6 b (134%)	5.7×10^6 b (60%)	11.5×10^6 a (122%)
Total phloroglucinol-related compounds	12.0×10^6 b (100%)	16.1×10^6 a (133%)	7.6×10^6 *a (63%)	14.6×10^6 a (121%)
Morphology on:				
Pseudomonas agar F [d]	Yellow-orange tinted cell material. Fluorescence score 4	Similar to wild type Fluorescence score 4	Similar to wild type Fluorescence score 6	Similar to wild type Fluorescence score 4
Pseudomonas agar P [e]	Cell material variegated dark and light in dense area Mucoid score 2	Similar to wild type Mucoid score 3	Similar to wild type Mucoid score 1	Smooth, consistent cell material texture and color Mucoid score 3.

LB + 2% glucose[f]	Grayish-brownish isolated colonies, some with darker center.	Similar to wild type	Dark grey-purple cell material; isolated colonies are yellow-tan.	Similar to wild type

[a] Zone of casein degradation on milk agar plates in mm, after 48 and 72 hrs of bacterial growth, respectively. Values are means of 3 replicate plates. Values followed by the same letter are not significantly different according to two-sample *t* test.

[b] Diameter of spread in mm on 0.3% LB. Mean values of 6 replicate plates after 24, 48 and 72 hrs of bacterial growth, respectively. Values followed by the same letter are not significantly different according to two-sample *t* test.

[c] Absorbance units/OD. Values in brackets represent percentages. Values are means of 2 replicate extractions.

[d] Values followed by the same letter are not significantly different according to two-sample *t* test or Wilcoxon rank sum test (marked by asterisk), $\alpha = 0$.

[e] Growth of Q8r1-96 and its mutants on PsF media after 3 days. Fluorescence was assessed on 0 to 9 scale where 0 indicates no fluorescence and 9 indicates the strongest fluorescence observed in a sample set. Values are means of 6 replicate plates.

[f] Growth of Q8r1-96 and its mutants on PsP media after 3 days. Strains were scored on a scale ranging from 0 to 5 where 0 indicates not mucoid at all, 1 indicates slightly mucoid and 5 indicates moderately mucoid. Values are means of 6 replicate plates.

[g] Morphological differences of Q8r1-96 and its mutants on LB + 2% glucose media after 7 days.

TABLE 3. Nitrogen sources from Biolog PM3 MicroArray that were differentially utilized by *P. fluorescens* Q8r1-96 and its *sss*, *ptsP,* and *orfT* mutants.

Nitrogen sources [a]	Bacterial strain			
	Q8r1-96	Q8r1-96 *sss*	Q8r1-96 *ptsP*	Q8r1-96 *orfT*
Biuret	+	+	+	-
L-Cysteine	-	+	+	-
L-Methionine	-	+	-	-
Ethylenediamine	-	-	+	-
Agmatine	-	-	+	+
Formamide	+	+	+	-
Glucoronamide	-	+	+	-
Guanine	-	-	+	-
Thymidine	+	+	-	+
Alloxan	-	-	+	-
Gly-Glu	-	-	+	+

[a] Nitrogen utilization profiles with four independent repetitions per strain were determined by using Biolog PM3 MicroPlates as described in Materials and Methods.

TABLE 4A. Population densities of indigenous and introduced strains on the roots of wheat grown in Quincy virgin soil.

Mean population density[b] of :	Treatment[a]			
	Single inoculation		Mixed inoculation	Control [c]
	Q8r1-96	Q8r1-96 *sss*	Q8r1-96 and Q8r1-96 *sss*	
Indigenous culturable bacteria	8.75 bc	9.02 a	8.92 ab	8.59 c
Q8r1-96	7.8 *a (45.3 a) [d]	nd	7.8 *a (45.0 a)	nd
Q8r1-96 *sss*$^-$	nd	7.6 *a (44.5 a)	7.0 *b (41.4 b)	nd

[a] Raw Quincy virgin soil was treated with ~10^4 CFU per g of soil of Q8r1-96Rif^{r}Gm^r and/or Q8r1-96 *sss*. Mixed inoculation treatments contained a 1:1 mixture of competing strains (~0.5x10^4 CFU per g of soil of each strain). Rhizosphere population densities of bacteria were determined after 6 cycles (two weeks per cycle) by dilution plating as described in Materials and Methods.

[b] Mean population densities in log CFU per g of root (fresh weight) across six cycles except cycle 0 are presented; nd, none detected.

[c] Treatment without bacterial inoculations.

[d] Different lowercase letters indicate a statistically significant difference according to Fisher's protected least significant difference test (P=0.05) or * Kruskal-Wallis all-pairwise comparisons test (P=0.05). Values in brackets represent area under the rhizosphere colonization progress curve (AUCPC) of introduced bacteria for six cycles.

TABLE 4B. Population densities of indigenous and introduced strains on the roots of wheat grown in Quincy virgin soil.

Mean population density [b] of :	Treatment[a]			
	Single inoculation		Mixed inoculation	Control [c]
	Q8r1-96	Q8r1-96 *orfT*	Q8r1-96 and Q8r1-96 *orfT*	
Indigenous culturable bacteria	8.68 *b	8.96 *a	8.79 *ab	8.66 *ab
Q8r1-96	6.8 a (39.5 b) [d]	nd	7.1 a (41.3 a)	nd
Q8r1-96 *orfT*⁻	nd	6.9 a (39.9 b)	5.4 b (32.0 c)	nd

[a] Raw Quincy virgin soil was treated with ~10^4 CFU per g of soil of Q8r1-96Rif^{r}Gm^r and/or Q8r1-96 *orfT*. Mixed inoculation treatments contained a 1:1 mixture of competing strains (~0.5x10^4 CFU per g of soil of each strain). Rhizosphere population densities of bacteria were determined after 6 cycles (two weeks per cycle) by dilution plating as described in Materials and Methods.

[b] Mean population densities in log CFU per g of root (fresh weight) across six cycles except cycle 0 are presented; nd, none detected.

[c] Treatment without bacterial inoculations.

[c] Different lowercase letters indicate a statistically significant difference according to Fisher's protected least significant difference test (P=0.05) or * Kruskal-Wallis all-pairwise comparisons test (P=0.05). Values in brackets represent area under the rhizosphere colonization progress curve (AUCPC) of introduced bacteria for six cycles.

TABLE 4C. Population densities of indigenous and introduced strains on the roots of wheat grown in Quincy virgin soil.

Mean population density[b] of :	Treatment[a]			
	Single inoculation		Mixed inoculation	Control [c]
	Q8r1-96	Q8r1-96 *ptsP*	Q8r1-96 and Q8r1-96 *ptsP*	
Indigenous culturable bacteria	8.27 b	8.51 a	8.54 a	8.46 ab
Q8r1-96	6.8 a (39.8 a)[d]	nd	6.9 a (40.3 a)	nd
Q8r1-96 *ptsP*$^-$	nd	5.7 b (34.3 b)	4.0 c (24.3 c)	nd

[a] Raw Quincy virgin soil was treated with ~10^4 CFU per g of soil of Q8r1-96Rif^{r}Gm^r and/or Q8r1-96 *ptsP*. Mixed inoculation treatments contained a 1:1 mixture of competing strains (~0.5x10^4 CFU per g of soil of each strain). Rhizosphere population densities of bacteria were determined after 6 cycles (two weeks per cycle) by dilution plating as described in Materials and Methods.

[b] Mean population densities in log CFU per g of root (fresh weight) across six cycles except cycle 0 are presented; nd, none detected.

[c] Treatment without bacterial inoculations.

[d] Different lowercase letters indicate a statistically significant difference according to Fisher's protected least significant difference test (P=0.05). Values in brackets represent area under the rhizosphere colonization progress curve (AUCPC) of introduced bacteria for six cycles.

Fig.1

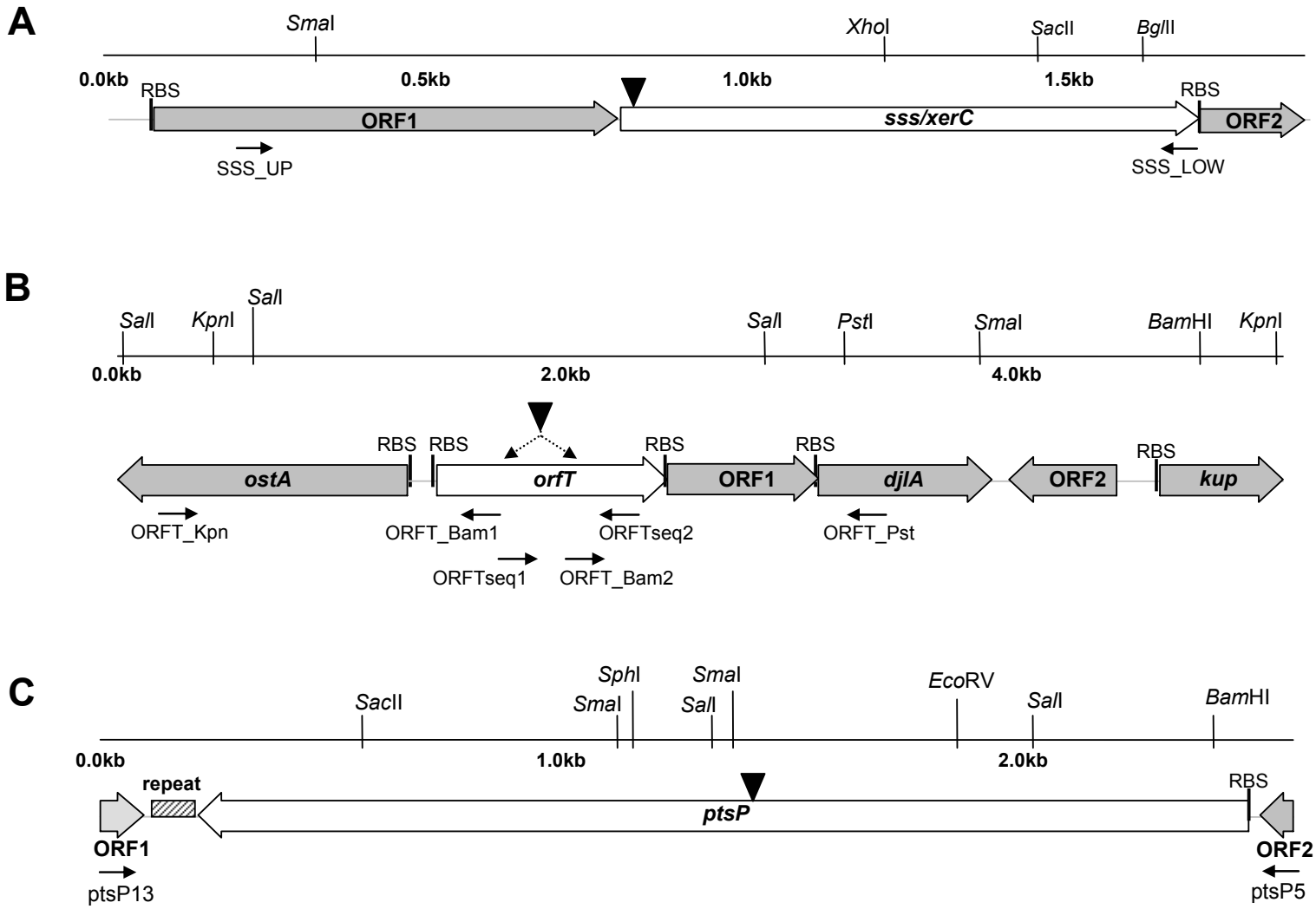

A
SmaI
XhoI
SacII
BglII
0.0kb
0.5kb
1.0kb
1.5kb
RBS
ORF1
sss/xerC
RBS
ORF2
SSS_UP
SSS_LOW
B
SalI
KpnI
SalI
SalI
PstI
SmaI
BamHI
KpnI
0.0kb
2.0kb
4.0kb
RBS
RBS
RBS
RBS
RBS
ostA
orfT
ORF1
djlA
ORF2
kup
ORFT_Kpn
ORFT_Bam1
ORFTseq2
ORFT_Pst
ORFTseq1
ORFT_Bam2
C
SacII
SphI
SmaI
SmaI
SalI
EcoRV
SalI
BamHI
0.0kb
1.0kb
2.0kb
repeat
ptsP
RBS
ORF1
ORF2
ptsP13
ptsP5

Fig.2A

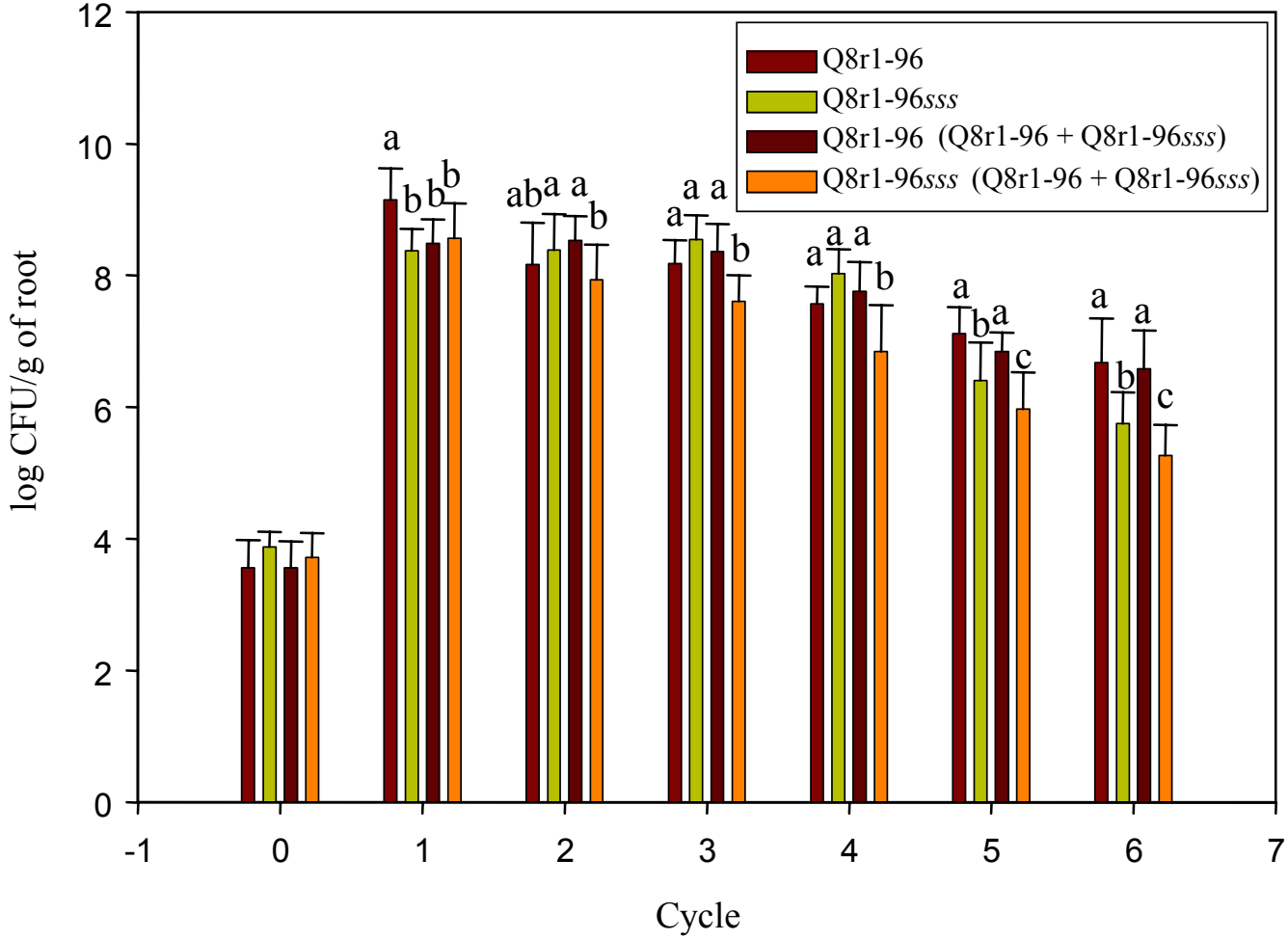

log CFU/g of root
Cycle
Q8r1-96
Q8r1-96sss
Q8r1-96 (Q8r1-96 + Q8r1-96sss)
Q8r1-96sss (Q8r1-96 + Q8r1-96sss)
0
2
4
6
8
10
12
-1
0
1
2
3
4
5
6
7
a
b b b
ab a a b
a a b
a a a b
a b a c
a b a c

Fig.2B

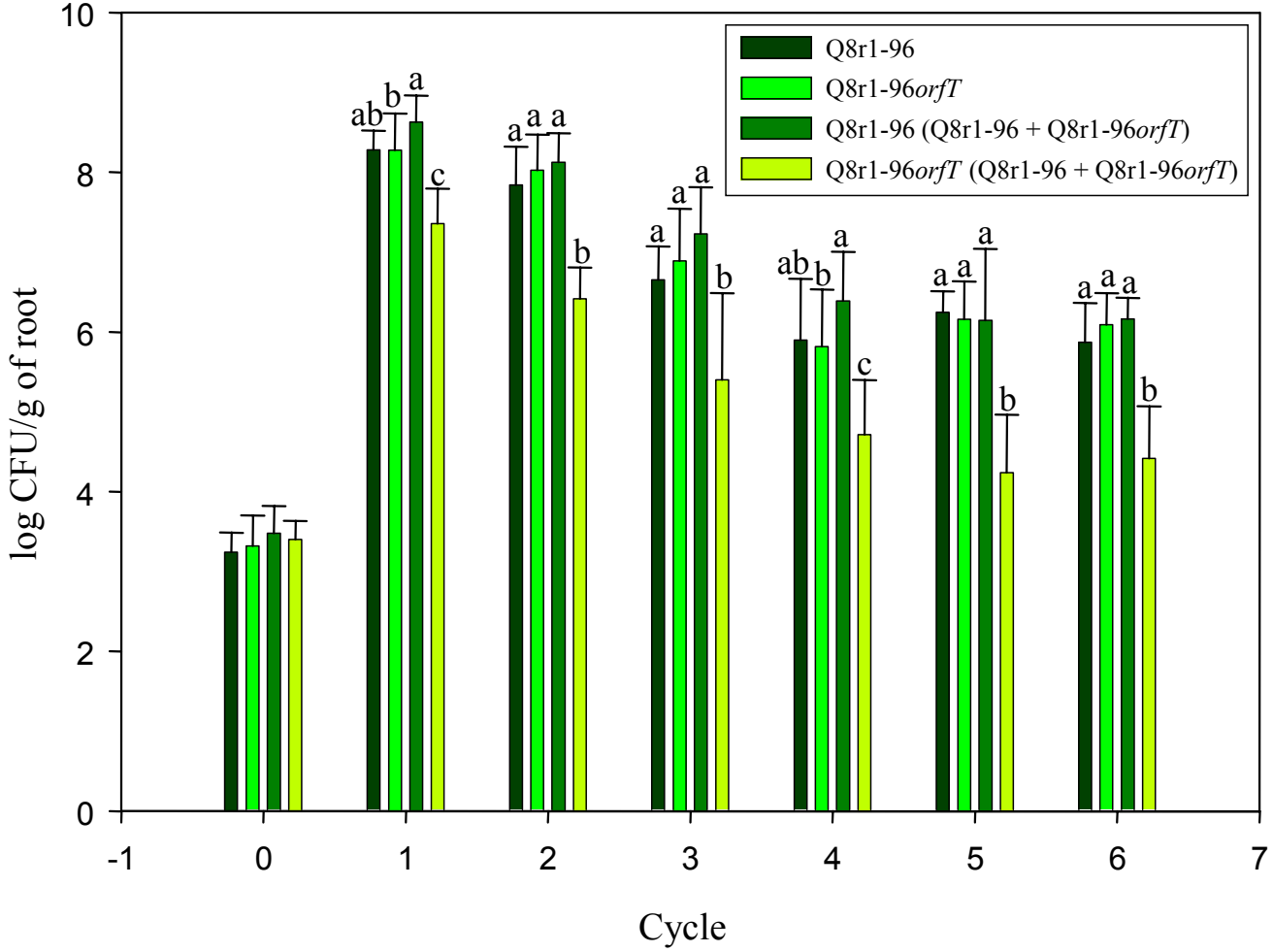
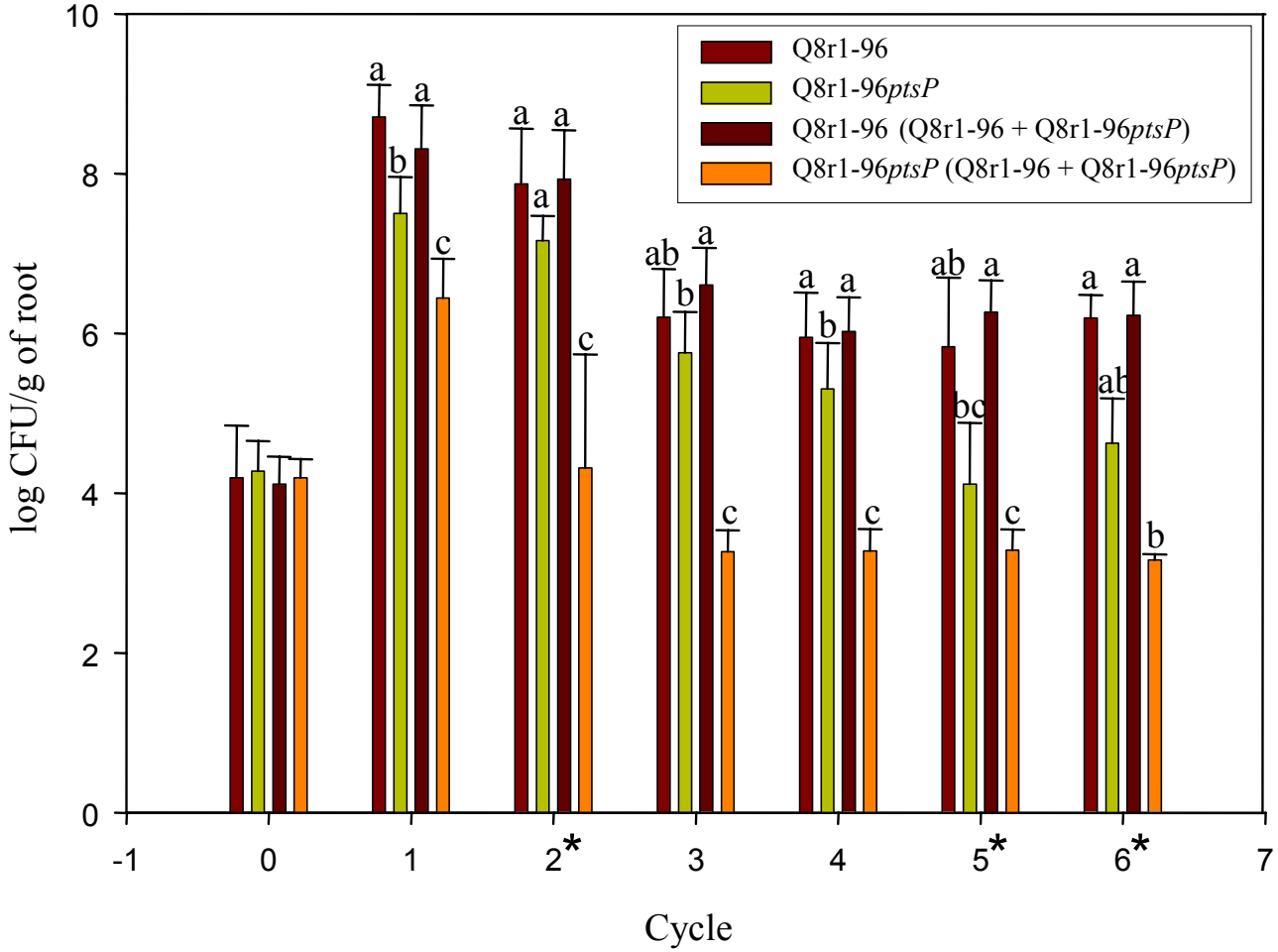

Q8r1-96
Q8r1-96orfT
Q8r1-96 (Q8r1-96 + Q8r1-96orfT)
Q8r1-96orfT (Q8r1-96 + Q8r1-96orfT)
log CFU/g of root
Cycle
0
2
4
6
8
10
-1
0
1
2
3
4
5
6
7
ab b a c
a a a b
a a a b
ab b a c
a a a b
a a a b

Fig.2C


Q8r1-96
Q8r1-96ptsP
Q8r1-96 (Q8r1-96 + Q8r1-96ptsP)
Q8r1-96ptsP (Q8r1-96 + Q8r1-96ptsP)
log CFU/g of root
Cycle
0
2
4
6
8
10
-1
0
1
2*
3
4
5*
6*
7

CONCLUSIONS

BOX-PCR and RAPD analyses revealed a high degree of genetic diversity among 123 Phl^+ *Pseudomonas* spp. strains isolated from different geographical locations worldwide. The BOX-PCR clustering correlated with RFLP clusters within *phlD,* a gene responsible for the production of MAPG and DAPG. Based on these results, 14 genotypes designated A-N were identified among the 123 bacterial isolates, and it was concluded that the *phlD* gene alone can be used as a genetic marker to study diversity and population structure among 2,4-DAPG-producing strains. Results of Southern hybridization and PCR-based screening of a subset of 30 strains representing all *phlD* RFLP clusters revealed that the pyoluteorin and pyrrolnitrin biosynthetic loci are found exclusively among isolates of the BOX-PCR A genotype. Genotypic diversity within a subset of 30 strains representing all *phlD* RFLP clusters did not correlate with the production of the phloroglucinol compounds *in vitro* under the conditions tested.

A genomic library of *P. fluorescens* Q8r1-96 consisting of 1536 clones was screened by PCR and Southern hybridization to identify clones containing the genes *sss*, *dsbA*, *ptsP*, and *orfT*. These genes influence global processes including phenotypic plasticity, secretion, organic nitrogen utilization, and transmembrane transport, respectively. DNA sequence analysis of the cloned genes revealed that i) the product of *dsbA* is highly similar to thiol:disulfide interchange proteins from other fluorescent pseudomonads; ii) *sss* recombinase is similar to *sss* genes from *P. aeruginosa*, *P. fluorescens* WCS365, and F113, and encodes a site-specific tyrosine recombinase; iii) PtsP most likely forms part of a regulatory PTS phosphoryl transfer chain that is thought to link the metabolism of carbon and nitrogen; iv) the deduced product of *orfT* is similar to prokaryotic Ser/Thr and Tyr protein kinases and some aminoglycoside phosphotransferases.

Gene replacement mutants in all four genes were constructed and phenotypically characterized. The *dsbA* mutant exhibited altered colony morphology and decreased motility and fluorescence. It produced elevated amounts of 2,4-DAPG and inhibited *Gaeumannomyces graminis* var. *tritici in vitro* more than did the parental strain. The *sss* mutant was phenotypically similar to the wild type parent, and inactivation of *orfT* resulted in a phenotype with reduced motility, deficiency in utilization of D-galactose, and minor alterations in the nitrogen utilization profile. The *ptsP* mutant exhibited altered morphology, reduced motility and exoprotease production, and an increased level of fluorescence. It produced significantly lower amounts (ca. 63% of the wild type level) of MAPG, 2,4–DAPG, and total phloroglucinol-related compounds, and differed from Q8r1-96 in the ability to utilize six compounds as a nitrogen sources and D-galactose and uridine as carbon sources.

When applied individually into a natural soil, the *dsbA, sss*, and *orf*T mutants did not differ from Q8r1-96 in the ability to colonize the rhizosphere of wheat. However, when the wild type and mutant strains were co-inoculated into the soil, the parental strain consistently outcompeted the mutants, indicating that *dsbA, sss*, and *orf*T contribute to the rhizosphere competitiveness of Q8r1-96. The *ptsP* mutant consistently colonized the wheat roots to a lesser extent than the wild type both in single and mixed inoculations, thus suggesting that the gene is critical for the rhizosphere colonization by *P. fluorescens* Q8r1-96.