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Identification of arid soil inducible genes in Pseudomonas fluorescens strain Pf0-1

Katila Pipitone
University of Nevada, Las Vegas

IDENTIFICATION OF ARID SOIL INDUCIBLE GENES IN *PSEUDOMONAS FLUORESCENS* STRAIN PF0-1

by

Katila Pipitone

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Katila Pipitone

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[redacted]
Examination Committee Chair

[redacted]
Dean of the Graduate College

[redacted]
Examination Committee Member

[redacted]
Examination Committee Member

[redacted]
Graduate College Faculty Representative

ABSTRACT

Identification of Arid Soil Inducible Genes in *Pseudomonas fluorescens* Strain Pf0-1

by

Katila Pipitone

Dr. Eduardo A. Robleto, Examination Committee Chair
Associate Professor in Microbiology
University of Nevada, Las Vegas

This thesis contains three major sections: introduction, literature review and the project entitled: 'Identification of Arid Soil Inducible Genes in *Pseudomonas fluorescens* Strain Pf0-1'. The introduction section describes a general background, the current and potential applications of *P. fluorescens* and the main goal of this dissertation. The literature review chapter discusses two main areas. The first area offers insights about different types of in vivo expression technology (IVET) as a tool in gene identification. This section also describes the limitations of IVET, as well as the benefits over other methods for gene identification. The second part of the literature review is a compendium of previously reported genetic factors involved in soil survival. Finally, the last part of this thesis documents the identification of arid soil inducible gene in *P. fluorescens* strain Pf0-1.

This study, identification of arid soil inducible genes in *Pseudomonas fluorescens*, investigated adaptation mechanisms of *Pseudomonas fluorescens* strain Pf0-1 in arid soil. Auxotrophy-based in vivo expression technology (IVET) was employed to identify 26

arid-soil inducible genes in *P. fluorescens*. Based on analysis of Clusters of Orthologous Groups of proteins (COGs), ten genes are involved in metabolism; four genes are engaged in information storage and processing; three are signaling and regulation cellular processes genes; and nine are poorly characterized or hypothetical. Four genes of different functional groups (Pfl01_2143: glutamine synthetase; Pfl01_2660: GTPase Subunit of Restriction Endonuclease like; Pfl01_5595: hypothetical protein; and Pfl01_3972: putative diguanylate phosphodiesterase EAL domain) were inactivated and tested for their influence in soil colonization. Only two of the four strains carrying defective alleles showed slight but significant decreases in soil colonization. The growth patterns of mutant strains carrying defective alleles on Pfl01_2143 and Pfl01_5595 showed a decline in arid soil persistence, which were partially restored in strain derivatives carrying a complementing plasmid. Overall, these results indicate that adaptation of *P. fluorescens* to soil requires the expression of many genes, perhaps acting cooperatively. We further surmise that nitrogen limitation and metabolism are important factors in soil colonization in arid soils.

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CHAPTER 1

INTRODUCTION

1.1 *Pseudomonas fluorescens*

The genus *Pseudomonas* belongs in the subdivision gamma Proteobacteria, which encompasses a large proportion of known gram negative bacteria. *Pseudomonas spp.* are chemoorganotrophic aerobic rods with a single or multiple polar flagellum(a). Other classic microbiological indicator tests for of *Pseudomonas* identification include oxidase positive, catalase positive, indole-negative, methyl red negative and Voges-Proskauer negative. *Pseudomonas spp.* may exhibit diverse lifestyles which range from endophytic, epiphytic, saprophytic to pathogenic to animals and plants.

Pseudomonas fluorescens is ubiquitous in soil and on plant surfaces. Under iron-limited conditions, *P. fluorescens* produces a mixture of low molecular weight siderophores that contribute to its ecological competence, such as pyoverdine (12, 13, 15, 16), thioquinolobactin (9), ornicorrugatin (8), pyochelin (19), salicylic acid (10, 11), quinolobactin (14), enantio-pyochelin (21). Pyoverdine has been recognized as the main chemophore that imparts a yellow-green characteristic to *P. fluorescens* (2). *P. fluorescens* develops biofilms and produces a variety of secondary metabolites with antimicrobial properties such as 2,4-diacetylphloroglucinol (2,4-DAPG) (20), phenazines (1), pyoluteorin (3) and hydrogen cyanide (17).

1.2 Applications of *Pseudomonas fluorescens*

Pseudomonas fluorescens is a member of the rhizobacteria, a group of bacteria that are found in soil immediately beside and around plants' roots. In this environment, exudation and secretion from roots provides increased ability of nutrients, compared to non-rhizophere soil (7). These nutrients support rhizobacterial communities, which in turn may influence soil characteristics. Root effluxes caused by nutrient absorption can also dramatically influence soil chemistry such as pH and nitrogen transformations. Thus, the rhizosphere is a site where complex interactions among roots, soil and rhizobacteria occur.

As an aggressive rhizosphere colonizer, *Pseudomonas fluorescens* also displays antagonism against other microorganisms, which in some cases are plant pathogens for examples *Gaeumannomyces graminis*, *Thielaviopsis basicola*, and *Fusarium oxysporum*. *P. fluorescens* produces a plant growth factor, pyrroloquinoline, that has been shown to promote tomato plant (*Solanum lycopersicum)* growth, (5). Such properties have resulted in use of *P. fluorescens* as Plant Growth-Promoting Bacteria (PGPB) or for biocontrol of plant diseases (4, 6). Furthermore, *P. fluorescens* is closely related to a human pathogen, *P. aeruginosa,* associated with chronic lung infections and infections in severely burned patients. Thus, understanding genetic factors and processes governing how *P. fluorescens* adapts to soil provides basic knowledge on molecular mechanisms in soil microbiology and lead to the development of strategy to improve beneficial applications or may help in designing better infection preventive measures.

1.3 Questions to be Addressed

The main objective of this dissertation is to genetically dissect microbial processes that contribute to adaptation of *P. fluorescens* Pf0-1 to arid soils from the Mojave Desert. Adaptation is a complex trait that involves many processes. We employ a promoter trapping strategy, In Vivo Expression Technology (IVET), to identify promoters that are active during prolonged exposure to arid soil but become inactive in laboratory culture medium (18). These arid soil-inducible promoters are likely to drive the expression of genes that are important for soil colonization and persistence. This study is aided in two ways. One, the whole genome sequence of *Pseudomonas fluorescens*, available at National Center for Biotechnology Information (NCBI), accommodates our analysis of genes downstream of the 'trapped' promoters. Two, a recent study has identified 22 genetic regions that are expressed by *P. fluorescens* Pf0-1 in response to exposure to agricultural soil. Thus, in addition to examining *P fluorescens* adaptation to arid soil, the hypothesis that *P. fluorescens* occupies its niche in diverse soils by expressing different genes is tested.

We identified 26 genes that are expressed in our soil conditions. Based on analysis of Clusters of Orthologous Groups of proteins (COGs), ten genes are involved in metabolisms; four genes are engaged in information storage and processing; three are cellular (regulation and signaling) processes genes; four are poorly characterized genes. Interestingly, we discover five uncharacterized genes that are not within a distinct COG. Four genes of different functional groups were randomly selected for genetic disruption and complementation analysis. Two, out of four, strains carrying disruptions in arid soil-induced genes showed decreases in their ability to persist in soil. Genetic

complementation of the genes affecting persistence partially restored the wild phenotype. Comparative analysis with those genes expressed in agricultural soil indicated no overlap, suggesting that *P. fluorescens* adapts to diverse soil environments because of its diverse genetic resources.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Two fundamentals principles that pertain to the identification of *Pseudomonas fluorescens* genes that are important for soil persistence are discussed in this chapter. First refers to the methodology used in genetic searches to identify soil factors, which covers the principle of In Vivo Expression Technology (IVET) and its contrast to other methods for identification of genes important for stressful environments. Second will cover genetic elements described previously as important for soil colonization.

2.2 Use of *in vivo* Expression Technology (IVET)

IVET originates from a 'promoter probing technique' that allows positive selection of transcriptionally active DNA regions. It was first used in an experiment in which colonization of *Xanthomonas campestris* of turnip seedlings was studied (74) . After this groups' publication, the technique was extensively modified. In 1993 Mahan and coworkers coined the term IVET and used this technique to study pathogenicity of *Salmonella enterica* serovar typhimurium in mice (56, 63). IVET is the technique of choice for pathogenicity studies in phylogenetically diverse pathogens such as *Pseudomonas aeruginosa* (33), *Yersinia enterocolitica* (18), *Staphylococcus aureus* (55), *Vibrio cholera* (54, 75), *Vibrio vulnificus* (52), *Listeria monocytogenes* (28), and *Shigella*

flexneri (96, 97). The use of IVET has allowed identification of over a hundred virulence factors. Furthermore, results from studies in pathogens and non-pathogens suggest IVET provides a powerful tool not only for identification of epistatic genes that are essential in a complex environment, but also for identification of the genes that are expressed during dynamic ecological succession of a specific niche (35).

IVET is a promoter searching technique that entraps niche-specific DNA regions that are required for growth in a natural habitat, but are inactive in laboratory media (4). These specific promoters, that are induced, drive the expression of genes, and thus assumed to be required for growth, in a specific niche. IVET relies on two factors: the construction of genomic library in a pIVET plasmid and the construction of a knock-out strain that is compromised in the environment of interest. The specific features of the pIVET construct vary depending on the type of selection. However, the main feature of pIVET is that it is non-replicative in the bacterial species under investigation. In our case, the pIVET plasmid possesses a R6K origin of replication, which requires the π protein (*pir* gene). The π protein is a replication factor, engineered into the *E. coli* strains via lambda. Dimeric π protein binds to iteron sequences of *ori-γ*, located in the *oriR6K* region, causing a DNA conformational change and formation of a nucleoprotein structure. This facilitates the binding of required replication initiation factors, DnaA and integration host factor (IHF), to their binding sites. In summary, pIVET plasmid doesn't replicate without protein, and is lost during plasmid partitioning. Another important feature in pIVET is the presence of a multiple cloning site (MCS) immediately upstream of a bicistronic gene arrangement. The first gene of this arrangement is a promoterless marker, which is transcriptionally fused to a reporter gene cassette, *lacZ*. The type of

marker depends on the selection used and includes variations that rely in antibiotic resistance cassette, the synthesis of an essential growth factor, or the activation of a resolvase system. The second gene of the genetic fusion is a promoterless reporter gene; expression of this gene confers a phenotypic change that is easily detected or measured by a simple assay. The *lac* operon and *gfp* (green fluorescent protein) are the two most common reporters used in IVET. The generation of random genomic fragments to be cloned in the vector should be diversified in order to enhance the screening efficiency of IVET. This can be achieved by using multiple restriction enzymes and partial digests of the genome under study.

Upon the construction of the genomic library, constructs are introduced into the strain under study via conjugation. Transconjugants are then selected based on the resistance carried in pIVET. Because pIVET replication is dependent on the presence of *pir*, not present in the recipient strain, selection for the pIVET antibiotic marker indicates that conjugation and chromosomal recombination events have taken place in the recipient. The RecA-dependent event results in a strain with a two-copy chromosomal arrangement of the genomic fragment originally cloned into pIVET. If a region containing a promoter is cloned (fused to the essential growth factor gene), one of the copies activates transcription in its native gene, while the other activates transcription of the bicistronic arrangement in pIVET, which carries a selective feature (Figure 2.1). These integrants have been referred to as "fusions" (39).

Four different IVET systems have been used to study niche-specific genes. The (essential growth factor) auxotrophy-based selection IVET requires the construction of a strain with a deficiency in the synthesis of a growth factor that is absent or limiting in the

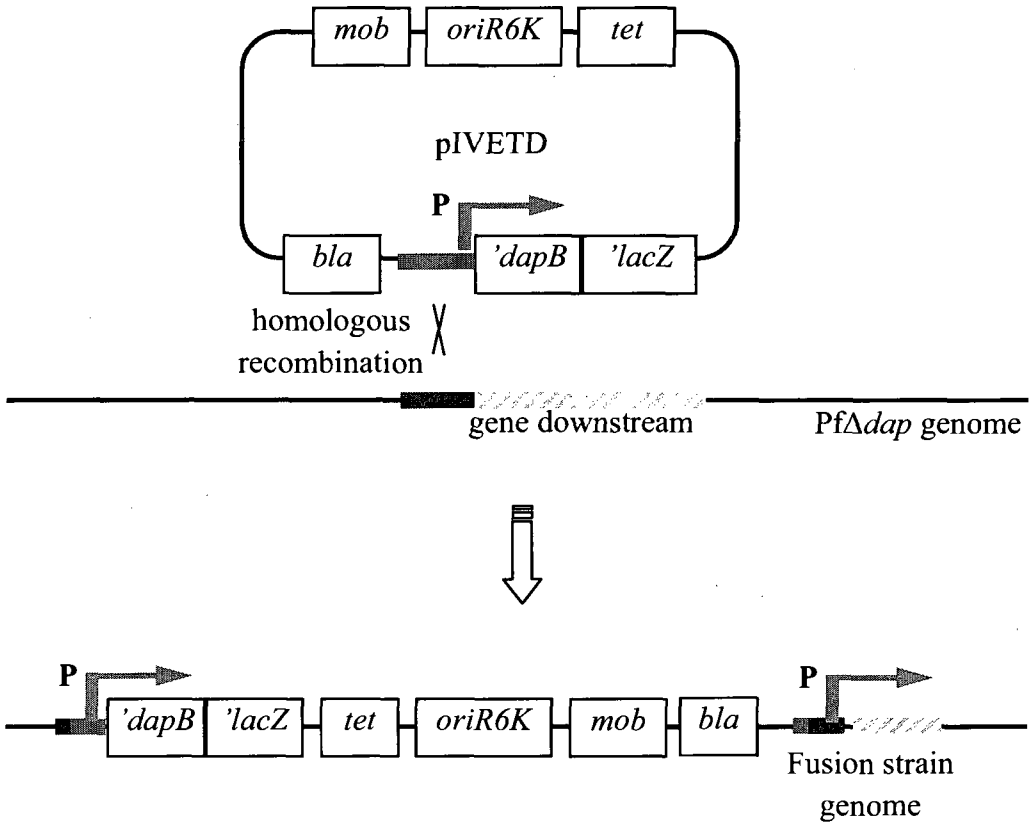

Figure 2.1 Homologous recombination between the genomic library sequence and its homologous sequence on the chromosome. The arrow indicates the presence of a promoter. A single recombination event results in an integration of the plasmid construct into the host chromosome. This results in a two-copy chromosomal arrangement of the genomic fragment originally cloned into pIVET causing a genetic rearrangement and two copies of promoters: one drive the expression of the 'dapB while another activate the transcription of the native gene.

strain with a deficiency in the synthesis of a growth factor that is absent or limiting in the environment of interest. This is usually achieved by constructing an in-frame deletion of an essential growth factor (*egf*) gene by standard molecular methods. Some auxotrophic markers that provide an outstanding IVET selectivity include *purA* (purine synthesis) in *Pseudomonas aeruginosa* (49), *thyA* (nucleotide synthesis) in *Salmonella enterica* (8), *dapB* (lysine synthesis) in *Pseudomonas fluorescens* (10, 85) and *inhA* (mycolic acid

synthesis) in *Mycobacterium* (57). Defects in the gene of choice for IVET and growth in the environment studied can be restored by two events: First, the growth factor may be supplemented exogenously. Second, restoration of growth occurs when a recombinant pIVET*egf* vector contains a genomic fragment with an active promoter, generating transcription of the *egf* coding sequence. Transcriptional activity of the *egf* also results in expression of the reporter gene, which in most cases is *lacZ,* and can be assayed in laboratory culture conditions using 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal). X-Gal is spontaneously oxidized, forming 5,5'-dibromo-4,4'-dichloro-indig, which is an insoluble blue product. If the transcriptional activity of pIVET*egf* fusion is constitutive, colonies display a deep blue phenotype in laboratory culture. In contrast, the fusion strain that contains a niche-specific promoter displays a white colony phenotype on X-gal containing rich medium.

Similarly, the antibiotic-based selection IVET uses a pIVET construct with a promoterless antibiotic resistance gene(Ab^r), instead of '*egf* in the auxotrophic-based IVET (74). Antibiotic selection is simpler than auxotrophic-based selection because it does not require the construction of auxotrophic strain. However, this strategy necessitates that the environment of interest be amended with an antibiotic to which the wild type strain is sensitive. The antibiotic-amended environment selects for the "fusion" strains containing a fragment that activates transcription of the promoterless antibiotic gene.

Recombinase-based IVET (RIVET) is a more sophisticated and sensitive version of IVET used to detect slight increases in promoter activity. There are two versions of RIVET: the original, based on genetic screening, and a modified version, which may be

used with positive selection or screen. Both RIVET versions rely on a site-specific recombinase, an enzyme that recognizes and binds to two specific repeated sequences, forming a cointergrate between the two sites. There are many recombinases but TnpR is the most commonly used in RIVET. Dimeric TnpR is encoded by *tnpR*, from transposonγδ (Tnγδ). It recognizes direct repeat sequences at *res* sites, a 114-bp sequence which contains three direct repeat binding sites: site I, site II and site III (34, 100). TnpR dimers bind to all three sites. DNA cleavage and ligation occurs at site I (43, 90). The recombinase works in conjunction with other key DNA constructs in RIVET.

In the original RIVET, a key construct, located in the chromosome of the bacterium of interest, features two sets or *resI* sites flanking an Ab^r gene, which serves as a genetic marker. The other construct, located in pIVET, consists of a genomic arrangement similar to the one generated with other IVET strategies and results in a random genomic fragment cloned upstream of a promoterless *tnpR*. When mobilized to the test species, the pIVET constructs give rise to a fusion strain through a single recombination between the random sequence in pIVET and its chromosomal homolog. An active promoter in the random sequence drives the expression of *TnpR*, which brings about site-specific recombination between the two sets of *res1*. Expression of *tnpR* mediates the resolution and loss of a fragment that contains the *resI*-Ab^r, leaving behind one complete *resI* sequence in the genome and generating Ab (100). The reporter system in the modified RIVET is the same as that in the original version. However, the marker system in the modified RIVET involves epistasis between two genetic markers, Ab^r and *gfp*. The $Ab^r$is located in the chromosome and is transcriptionally fused to *lacI*, a transcriptional repressor. A pair of *res1* sites flanks the Ab^r-*lacI* fusion, resulting in a

sequence of *res1-Ab*^{r}*–lacI-res1*. Elsewhere in the chromosome, a *gfp* is under the control of the LacI repressor. Therefore, the presence of an active promoter in the genomic fragment drives the expression of *tnpR* and recombination occurs at the *res1* sites, causing a loss of the *Ab*^{r}*–lacI-res1* sequence. Loss of this sequence leads to expression of *gfp*, which can be detected in a spectrophotometer at wavelengths of 395_{nm} or 509_{nm}.

One other IVET system is based on system-specific selection. It includes a promoterless system-specific gene, which is used to identify DNA fragments of interest. The mechanics and protocols used with this IVET system are very similar to the auxotrophy-based IVET (86).

2.2.1 Limitations of IVET

IVET allows niche-specific gene identification but requires a few time-consuming steps. Inactivation of the marker gene requires a genetic allele exchange in the bacterial species of interest. Such requirement may narrow IVET applications to well studied systems with available genetic alteration and mobilization tools. IVET cannot detect niche-specific down-regulated promoters since these promoters would be inactive, causing the loss of function in the marker gene in the environment of interest. Also, it has been shown that approximately 50% of the genetic regions selected by IVET present a 'cryptic' arrangement (98, 99). This arrangement can be explained in two possible ways: An artifact or a false positive fusion may be generated by a situation where a high AT-content locus could function as a 'false' promoter during exposure to stress. Alternatively, it is possible that a cryptic" promoter could be selected. The term "cryptic" refers to an identified region of DNA that has not been previously characterized or annotated. Interestingly, some cryptic regions lie inside and in the opposite orientation

of a previously ascribed coding sequence. It has been hypothesized that these cryptic regions generate anti-sense mRNAs that regulate the annotated gene in which they are embedded (99). Overall, IVET is an excellent strategy to identify and study features of gene regulation in experimentally challenging environments.

2.3 Other Genomic Approaches

DNA microarrays, together with whole-genome sequences, have revolutionized mRNA analysis. Quantification of gene expression by microarray provides a global view of how genes are regulated at the transcriptional level in response to certain stressors or to a certain growth phase. However, biological processes are complex and much more remains to be discovered. Classic DNA microarrays cannot detect antisense RNA (a short RNA molecule silencing gene expression at the post-transcriptional level). The extraction of high quality and quantity mRNA from hard-to-control environments is difficult to achieve due to the short half-life of bacterial mRNA. Application of microarrays to study of bacterial responses toward complex environments such as a rhizosphere is intricate. First, there is no easy technique to discriminate the mRNA of interest from total mRNA in the different bacterial species living in the environment of interest. This means one has to isolate the bacteria of interest from the mixed population, causing a delay in the extraction process. Furthermore, microarray chips are expensive, and due to background noise caused by variable factors such as mRNA quality and dust on the chip, many replicates are needed for statistical analysis (35, 36, 73)

2.4 Genetic Factor in Survival in Soil

Soil is a heterogeneous environment where dynamic interactions between biotic and abiotic factors take place. Bacterial survival in soil is complicated and influenced by adaptation, which is defined as any developmental, physiological or behavioral change that contributes to survival over time. The genes controlling these processes are usually up-regulated during exposure to soil and sometimes down-regulated under other growth conditions. Identification of these genes and understanding their function provides a better understanding of bacterial adaptation. Previously, research studies have identified several *P. fluorescens* genes that contribute to survival in soils. Based on their functions, they can be classified into 4 groups: chemotaxis and motility, nutrient scavenging; adaptation to environmental stress, and secretion of secondary metabolites (86).

2.4.1 Motility and chemotaxis

P. fluorescens possesses a polar flagellum, a complex organelle, whose function and synthesis are encoded by at least 36 genes. A flagellum consists of three main structures: the filament, hook and basal body. The filament is a tail like structure, made of the protein flagellin, which is assembled into a helix with a hollow core. The filament is connected to a hook and anchored to the basal body. The structure of the Gram-negative basal body is similar to those belonging to type III secretion systems (TTSS), suggesting a common evolutionary path between the two structures (31). As early as 1968, direct viable cell counts from a competitive assay between flagellated wild-type *P. fluorescens* and a non-flagellated mutant in TSB media showed a higher CFU/ml count for the wild- type than for the mutant, suggesting that flagellated cells are more competitive than non-flagellated ones (101). Later, field studies revealed that the

presence of flagella contributes not only to motility, but also to rhizosphere attachment, which facilitates rhizosphere colonization (20, 21, 82). Transposon mutagenesis studies showed that mutations in *fliC*, *fliS* and *adnA* genes result in no motility, while a mutation in *fliT* resulted in decreased motility (14). The *fliC* gene encodes flagellin, which is the main protein of the flagellum filament. *FliS* codes for a protein involved in flagellum assembly. *AdnA*, which is 85% identical to *fleQ* in *P. aeruginosa,* encodes a transcriptional factor that has been reported to affect flagellum synthesis, biofilm formation, and attachment to soil and seeds (15, 22). In contrast to *fleQ*, which directly regulates flagellum synthesis in *P. aeruginosa* (6, 19), *adnA* in *P. fluorescens* also affects other cellular processes (88). Interestingly IVET studies showed that *fliF*, which encodes the flagellum M ring protein in *P. fluorescens,* is up-regulated during colonization of sugar beet roots (29). This implies that the presence of the flagellum is important in root colonization. Whether flagellum mediated-motility or attachment provides adaptation of *P. fluorescens* to soil environments remains to be determined.

Flagellum-mediated bacterial motility is a complicated behavior consisting of alternations between runs and tumbles. A run is characterized by a linear motion in a random direction as the distal end of a flagellum rotates in one direction. A tumble is caused by rotation of the flagellum in the direction opposite to the one generating a run, and results in a stop, reorientation and preparation for the next run. Even though seemingly random, the motility of bacteria is influenced by environmental cues, a process called chemotaxis. Chemotaxis is defined as a phenomenon by which a bacterium migrates towards an attractant against the concentration gradient and/or away from a repellent along the concentration gradient. Although little is known about *P. fluorescens*

chemotaxis, the proteins involved in this process (Mcp and Che) are strongly resemble those found in other *Bacteria* and *Archaea*, suggesting that *P. fluorescens* Mcp and Che proteins function in a similar movement (30, 103). In *E. coli*, the chemotaxis response is accomplished by a signal transduction cascade. This signal cascade is initiated by transmembrane chemoreceptors (Mcp), passed onto Che proteins via phosphorylation, and ultimately transferred to the flagellum motor switch (Mot). This cascade modulates the frequency of runs and tumbles. When an attractant triggers the signal, cells exhibit positive chemotaxis, characterized by longer runs and less frequent tumbles; as the attractant concentration increases, the response becomes greater. On the other hand, decreased attractant concentration or sensing of repellents results in negative chemotaxis, outlined by shorter runs and more frequent tumbles. Thus, the net bacterial kinetic is a biased movement towards attractants and repulsion from repellents (11).

Not surprisingly, experiments in which IVET was used to identify niche-specific genes have shown that regulation of chemotaxis is an important process. Up-regulation of Mcps occurred when *Vibrio cholera* was inoculated into mice. Interestingly, Mcp in genes in *Pseudomonas stutzeri* were repressed during rice rhizosphere colonization (85). Further, the *cheR* and *cheY* genes were expressed in *E. chrysanthemi* and *P. aeruginosa* that were inoculated onto spinach and into mouse, respectively (110). Taken together, the reports on chemotaxis suggest that it is a key feature in bacterial adaptation to soil environments.

2.4.2 Desiccation and stress response

Soil can place organisms under several forms of stress. Soil desiccation, which is the most limiting factor for bacterial growth, can significantly impair cellular functions

due to increased proteolysis, increased accumulation of DNA mutations caused by reactive oxygen species, and also alter membrane permeability (83). Desiccated or dry soil has very low water potential. Water potential measures the tendency of a water molecule to move from one place to another and reach equilibrium. Free water has a potential of zero while soil water potential is variable and depends on temperature and rate of evaporation. Capsules and biofilms are structures that generally protect cells from dehydration and other detrimental factors such as antibiotics, phagocytosis, and reactive oxygen species (72). Biofilms are composed of hydrated extracellular polysaccharides (EPS) and may be occupied by single or multiple species. Such matrices retain water in conditions of soil dryness. Several physiological responses contribute to desiccation tolerance, which include regulation of intracellular osmotic pressure and stabilization of membrane lipids and intracellular proteins (83). Under moderate water limitation, bacteria retain cytoplasmic solutes in order to lower osmotic pressure. During extreme water deficit, accumulation of intracellular trehalose and sucrose contributes to membrane protection (17). Although it is not clear how trehalose stabilize proteins, *in vitro* experiments in which cells were subjected to osmotic stress, showed that addition of trehalose improved enzyme stability and activity compared to conditions in which trehalose was absent (5, 23). Furthermore, a mutant strain of *Saccharomyces cerevisiae* with a defective trehalose transport system survived less than the wild-type in a desiccated environment (23). Investigations in trehalose-lipid membrane interactions conducted *in vitro* showed that the polyhydroxyl groups of trehalose replace water molecules and form hydrogen bonds around the polar regions of phospholipids, thereby maintaining phospholipid integrity (78).

Periods of dryness followed by short exposure to rehydrating conditions in soil, in a dry climate region, can cause soil water potential to fluctuate from -20 MPa to 2.8 MPa (50). Such extreme fluctuation may result in damage to cells or cell lysis. In conditions of rapid rehydration, bacteria may use three different strategies to reduce osmotic shock such as secretion of intracellular solutes, catabolism of cytoplasmic organic molecules and polymerization of solutes. *P. fluorescens* under hypo-osmotic shock may release 22%-26% of the cytoplasmic pool of amino acids and up to 11%-21% of low molecular weight neutral sugars into the environment (83).

The genetic elements that initiate the rapid response to desiccation stress in *P. fluorescens* are the alternative sigma factors RpoS (σ^S) and AlgU, also known in *E. coli* as RpoE (σ^{22}) (91). The transcriptional activator AlgU is highly conserved among Gram negative bacteria and positively regulates biosynthesis of EPS (102). Further, a deletion of *algU* causes a significant decrease of EPS production and increased sensitivity to osmotic stress on agar medium. *P. fluorescens algU* is located in the *algU-mucA-mucB* DNA locus. Three factors regulate *algU* expression: AlgU levels, the anti-sigma factor MucA and the two-component regulation system of GacA/GacS. GacA and GacS control many cellular functions that may affect soil adaptation, including biofilm formation, EPS secretion, antibiotic production, response to nutrient starvation and quorum sensing (91, 109).

2.4.3 Nutrient scavenging

Iron, carbon, nitrogen, and phosphate are very well known to influence growth of *P. fluorescens* in soil. In bulk soil, carbon is a limiting factor for growth, while phosphate and nitrogen are limiting in the rhizosphere or soils that support plant growth.

Transmission microscope images of cells subjected to either carbon, nitrogen, phosphate or iron limitation showed aberrant morphology such as formations of cyctoplasmic vacuoles, granules in the nucleoid, plasmolysis, and cell lysis. In soil conditions, bacteria possess multiple scavenging mechanisms to procure adequate levels of nutrients.

a.) Iron acquisition

Iron is a cofactor of enzymes that catalyze many biological redox reactions and therefore is essential for living organisms including most bacteria. In anoxic conditions iron exists in the +2 oxidation state, (ferrous state). Ferrous iron is water soluble and readily used by most living organisms. However, in oxic conditions, oxidation occurs and favors formation of ferric iron, which is in the +3 oxidation state. Ferric iron is water insoluble and cannot be utilized for metabolic functions. Thus, most aerobic microbes require iron acquisition mechanisms in order to grow. One well studied iron acquisition mechanism in bacteria is that of siderophore production. Siderophores are low molecular weight compounds that can tightly bind insoluble iron and transport it into the cell. Once in the cell ferric iron is converted to its soluble form and incorporated into general metabolism (16).

P. fluorescens, an obligate aerobe, produces several siderophores that include pyoverdine (67, 80, 81), thioquinolobactin (59), ornicorrugatin (58), pyochelin (104) and salicylic acid (62, 64). Pyoverdine, also known as pseudobactin, is the siderophore that gives the yellow-green pigmentation to *P. fluorescens*. Pyoverdine is composed of three domains: a dihydroxyquinoline chromophore, a variable peptide chain comprising six to twelve amino acids, and a side-chain containing either dicarboxylic acid or a dicarboxylic acid amide (16). Since the sequence of the peptide chain is strain-specific, diversity of

pyoverdine has been employed to differentiate among *Pseudomonas* strains. This technique is called siderotyping (65, 66). Pyoverdine is optimally produced in a medium containing succinate and elevated levels of Zn^{2+}, Cu^{2+} or Co^{2+} (89, 95). Pyoverdine production contributes to ecological fitness in both bulk soil and rhizosphere soil. Complete synthesis of pyoverdine (and its receptors) requires the extracytoplasmic function (ECF) alternative sigma factor, PbrA (13, 60, 93, 94). Expression of *pbrA* is negatively controlled at the transcriptional level by the Fur (Ferric uptake regulator) repressor. Under high ferric concentrations, +3 iron molecules bind to Fur, forming a Fur-ferric complex. This complex has an increased binding affinity toward a consensus DNA sequence located in the promoter region of *pbrA*. The binding between the complex and the *fur* operator region precludes *pbrA* transcription, which in turn represses transcription of pyoverdine genes, *pvd* (69, 71). PbrA$^-$ cells not only exhibit deficiencies in pyoverdine synthesis but also display a protease-negative phenotype on skim milk agar, suggesting a role in regulation of proteolysis (60). Furthermore, the cytochrome C gene, *ccmC*, is involved in pyoverdine production (7, 27). This concept is supported by studies in which a CcmC$^-$ strain of *Pseudomonas fluorescens* showed a reduction in the production of two siderophores: pyoverdine and quinolobactin (7, 27).

The uptake of pyoverdine in *P. fluorescens* depends on the outer membrane receptor PbuA (known as FpvA in *P. aeruginosa*), and on the TonB-dependent membrane transporter (encoded by *TonB1*, *TonB2* and *PA0695 in P. aeruginosa*). The Twin-Arginine Translocation system mediates membrane localization of PbuA, which attaches to TonB. PbuA can bind to both free pyoverdine and to ferriated pyoverdine but only the latter activates TonB. This results in TonB-mediated transport, which is

powered by proton motive force (24). It is assumed that PbuA is a species-specific receptor that allows the recognition of 'self' pyoverdine-Fe complex and transports it into the cell. However, there are reports indicating that PbuA of *P. fluorescens* and *P. putida* recognizes a broad spectrum heterologous pyoverdine produced by other *Pseudomonas spp.* (16). When cyctoplasmic iron is too high, TonB-dependent iron uptake is inhibited by the iron repressed outer membrane protein (IROMP), which prevents the development of iron cytotoxicity (70). In summary, *P. fluorescens* has several iron acquisition mechanisms that improve adaptation to soils.

b.) Carbon metabolism

Carbon is the backbone of all organic compounds and is used by all organisms to generate cellular components. In addition, catabolism of carbon compounds generates energy. In general, the TCA, the Embden-Meyerhof and pentose phosphate pathways provide energy and the starting substrates for the generation of all building blocks in the cell.

Among heterotrophic bacterial species, *P. fluorescens* is metabolically diverse. In conditions of carbon limitation, *P. fluorescens* may use a wide range of alternative carbon sources, including recalcitrant compounds such as biphenyl, ethylbenzenes (76), naphthalene (108), and styrene (53). In bulk soil, *P. fluorescens* is exposed to carbon deprivation, a major stress (37). Such stress activates the sigma factor σ^s, a global regulator affecting expression of genes important for survival, antibiotic production, and degradation of some organic compounds (46). Note that the source of carbon used for growth has a direct effect on cell energetics, morphology and other traits. For example, glucose-grown *P. fluorescens* has a cell envelope with a high lipopolysaccharide density.

Interestingly, lipopolysaccharide density has been shown to affect resistance to actinomycin D, a compound first isolated from soil microorganisms (105).

c.) Nitrogen metabolism

Cells require nitrogen for the synthesis of compounds such as purines, pyrimidines, imidazole derivatives and amino acids. In *E. coli*, the status of cellular nitrogen is signaled by the cellular ratio of α-ketoglutarate to glutamine. A high ratio of α-ketoglutarate to glutamine indicates insufficient nitrogen and carbon excess. Also, a high C/N ratio suggests high cellular energy levels, whereas a low ratio indicates nitrogen excess as well as carbon and energy deficiency (26).

In nitrogen rich cells, ammonia is transformed into glutamine and glutamate by glutamine synthetase and glutamate dehydrogenase, respectively. During nitrogen deficiency, the synthesis of enzymes that degrade nitrogen-containing compounds, with the exception of ammonia, ammonium or glutamine, is induced. This process has been denoted as nitrogen regulated response (Ntr) and it is responsible for assimilation of any nitrogen containing compound. The central regulators of Ntr are σ^{54} (*rpoN*) and the two-component regulators, NtrC (*glnG*) and NtrB (*glnL*). NtrB is a phosphokinase and NtrC is the transcriptional activator of σ^{54}. Nitrogen limitation triggers NtrB-mediated NtrC phosphorylation, resulting in the activation of NtrC. Activated NtrC induces the expression of *glnALG* operon, which encodes glutamine synthetase, NtrB and NtrC respectively. The raise in the cellular NtrC activates the Ntr regulons (26).

In rhizosphere where nitrogen is a limiting factor for *P. fluorescens* growth, glutamine and glutamate, found in root exudates, are major sources of nitrogen (40). Both amino acids can be transported into the cell via different transportation systems

depending on the availability of ammonia. Once in the cell, intracellular glutamate is assimilated with α-ketoglutarate into glutamine, the central intermediate of nitrogen metabolism (48).

Interestingly, *P. fluorescens* strain NCIMB 11764 can utilize cyanide as nitrogen sole source during nitrogen limiting conditions. Assimilation of cyanide into ammonia of *P. fluorescens* strain NCIMB 11764 occurs through two pathways: cyanide nitrilase (CCN) and cyanide oxygenase (CNO). CCN, which is also known as cyanide dihydratase or cyanidase, catalyzes hydrolysis of cyanide in concerted fashion to form ammonia and formic acid (25). CNO route, which is more sensitive breaks down cyanide into carbon dioxide and ammonia (48).

d.) Phosphate metabolism

Phosphorous is an essential element; it is a molecular component of nucleic acids, membrane lipids, and biological high-energy molecules such as ATP and NADP. *P. fluorescens* primarly uses inorganic phosphate (P_i) as a source of phosphorous, but can also use organophosphates, phosphonates and phosphites. *P. fluorescens* senses and adapts to low phosphorus conditions via the expression of the Pho regulon (68), The response to low extracellular P_i is mediated by PhoB-PhoR, a two-component regulatory system (68, 84). During P_i starvation, the response regulator PhoB is phosphorylated by the sensor kinase PhoR, causing an increase in binding affinity towards the *pho* box, a sequence located in the promoter region of P_i-starvation responsive genes. Binding of PhoB to *pho* boxes facilitates recruitment of σ^{70} and initiates the expression of genes involved in P_i transport and assimilation (68). When P_i is sufficient, PhoB interacts with the Pst (phosphate transporter system), through an unknown process, prevents PhoR-

mediated PhoB phosphorylation, and reduces transcription initiation of *pho* promoters (47). Not surprisingly, a Gfp-based IVET assay detected upregulation of *Erwinia chrysanthemi phoB* during colonization of spinach leaves (85, 110). Furthermore, a *P. fluorescens phoB*$^-$ mutant is sensitive to P_i limitation, suggesting it is important in survival and ecological succession (68).

2.4.4 Secondary metabolites

Unlike primary metabolites, secondary metabolites are organic compounds that are not involved in growth per se, but their production provides other fitness benefits in complex environments. Well-characterized secondary metabolites produced by *P. fluorescens* include siderophores (see iron acquisition), broad spectrum antimicrobials, plant growth promoters and enzymes that are responsible for transport, and catabolism of xenobiotic compounds.

Four well-know broad-spectrum antimicrobial agents of *P. fluorescens* are 2,4-diacetylphloroglucinol (DAPG) (106), pyrrolnitrin (45), pyoluteorin (38), phenazines (41) and hydrogen cyanide, each of which are encoded by the gene clusters *phlABCD* (3), *pltFABCDEFGR* (45), *prnABCD* (32), *phzFABCD* (61),and *hcnABC* (9, 79), respectively. *In vitro* analysis of the protozoa *Vahlkampfia* and *C. steinii* in the presence of *P. fluorescens* supernatants showed growth inhibition of the protozoa. This finding provides support for the concept that *P. fluorescens* secondary metabolites are used as a defense mechanism against protozoa grazing (42). Other studies conducted with 2,4-DAPG$^-$ strains indicate that this compound suppresses take-all disease in wheat, black root rot in tobacco, and tomato wilt, caused by the fungal pathogens *Gaeumannomyces graminis*, *Thielaviopsis basicola*, and *Fusarium oxysporum*, respectively (41). Pyrrolnitrin

antagonized *Bipolaris maydis*, a soil-borne pathogen that causes Southern maize leaf blight, and *Sclerotinia homoecarpa*, which causes Dollar spot in turf grass. Pyoluteorin and phenazines protect plant roots against damping-off disease caused by *Pythium* and take-all disease. The discussion above suggests that production of secondary metabolites is likely to be an adaptation feature in *Pseudomonas spp.* because it confers antibiosis properties against other soil denizens (41).

Regulation of the synthesis of *P. fluorescens* secondary metabolites involves both global and pathway-specific controls. Four global systems of regulation have been described to affect production of secondary metabolites: the two-component regulatory system GacS (global antibiotic and cyanide regulator) and GacA (51), the housekeeping sigma factor RpoD, the alternative stress-respond sigma factor RpoS (107), and the sigma factor RpoN (77). As previously discussed, all four genetic regulators also influence other cellular functions. RpoN, RpoS and RpoD positively control *P. fluorescens* antagonistic activities through their interaction with different promoter sequences and with core RNA polymerase. Although GacA possesses DNA binding properties, its binding locus on the target promoter(s) has not been extensively characterized. It has been hypothesized that GacA and GacS positively control transcription through signal transduction, but the complete signal transduction pathway has not been fully elucidated (12). Recently, it was reported that GacS/A promotes transcription of three small non-coding RNAs: RsmX, RsmY and RsmZ. An *RsmXYZ* triple mutant strain displays an identical phenotype as a *gacS/A* double mutant with respect to microbial antagonistic acivities. Additionally, research in the GacS/A system indicates that additional regulation is mediated by two repressor proteins, RsmA and RsmE. It is speculated that

RsmAE mRNA transcripts are bound by RsmXYZ mRNA, which prevents translation of RsmAE, resulting in up- regulation of synthesis of secondary metabolites (44).

Pathway-specific regulation for *P. fluorescens* antagonistic activities includes genetic autoinduction, as well as transcriptional and post-transcriptional regulation (1). Supernatants extracted from *P. fluorescens* late stationary phase cultures demonstrated increased transcriptional activity of a *phlA-'LacZ* reporter system by 15 to 20 fold compared to the control (87). Studies in which HPLC fractions from culture supernatants, also used in expression studies, indicated that a fraction corresponding to synthetic 2,4-DAPG increased *phlA* expression, suggesting that *phlA* is autoregulated (92). In addition, Tn*5* experiments revealed a genetic region, denoted as *phlF*, that repressed expression of 2,4-DAPG production in *P. fluorescens*. The *phlF* gene is located 363 bp upstream of the *phl* operon. Intergenic region investigations of these two loci, phlA and phlF, showed the presence of several inverted repeats, one of which contains a σ^{70}-30 element, implying σ^{70}-mediated gene expression of *phlABCD*. Further investigations confirmed that PhlF exerts negative control of the *phl* operon at the transcriptional level. It is proposed that PhlF dimerizes and binds to the intervening inverted repeat sequences and prevents σ^{70} from accessing its -30 element (2).

2.5 Conclusion

This chapter has described IVET as an effective technology to identify genes expressed in soil conditions. The Pseudomonads and their close relatives have several mechanisms that allow them to adapt to soil environments. Genetic regulation of

adaptation mechanisms is complex, multilayered and sometimes activated by overlapping or redundant processes.

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CHAPTER 3

IDENTIFICATION OF ARID SOIL INDUCIBLE GENES IN PSEUDOMONAS FLUORESCENS

3.1 Abstract

Adaptation of *Pseudomonas fluorescens* strain Pf0-1 in arid soil was investigated. Auxotrophy-based in vivo expression technology (IVET) was employed to identify 26 arid-soil inducible genes in *P. fluorescens*. Based on analysis of Clusters of Orthologous Groups of proteins (COGs), ten genes are involved in metabolism; four genes are engaged in information storage and processing; three are signaling and regulation cellular processes genes; and nine are poorly characterized or hypothetical. Four genes of different functional groups (Pfl01_2143: glutamine synthetase; Pfl01_2660: GTPase Subunit of Restriction Endonuclease like; Pfl01_5595: hypothetical protein; and Pfl01_3972: putative diguanylate phosphodiesterase EAL domain) were inactivated and tested for their influence in soil colonization. Only two of the four strains carrying defective alleles showed slight but significant decreases in soil colonization. The growth patterns of mutant strains carrying defective alleles on Pfl01_2143 and Pfl01_5595 showed a decline in arid soil persistence, which were partially restored in strain derivatives carrying a complementing plasmid. Overall, these results indicate that adaptation of *P. fluorescens* to soil requires the expression of many genes, perhaps acting

cooperatively. We further surmise that nitrogen limitation and metabolism are important factors in soil colonization in arid soils.

3.2 Introduction

The Pseudomonads are good model systems to examine adaptation to diverse environments. They possess a large complement of genes and numerous two-component signal transduction systems. One interesting environment that presents many abiotic and biotic complexities to bacteria is soil. It has been shown previously that *Pseudomonas fluorescens* has many genes enabling adaptation to agricultural soils (15). Some of the genetic regions involved in adaptation have unusual modes of genetic regulation (16). This study, identification of arid soil inducible genes in *Pseudomonas fluorescens*, describes the genetic compositions of adaptation of *Pseudomonas fluorescens* Pf0-1 in arid soil from the Mojave Desert. A '*dapB* marker in combination with a '*lacZ* reporter system was applied, as part of an auxotrophic-based *in vivo* expression technology (IVET), to 'trap' promoters that are inducible in arid soil, but become inactive in laboratory culture medium. The whole genome sequence of *Pseudomonas fluorescens*, deposited at the National Center for Biotechnology Information (NCBI), accommodates our search of genes downstream from the 'trapped' promoters. Specifically, we hypothesize that adaptation of *P. fluorescens* Pf0-1 to arid soil requires expression of genes that are specific, and thus differ from those previously reported in to be expressed in agricultural soil.

3.3 Material and Methods

3.3.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. Wild type *Pseudomonas fluorescens* (Pf0-1) and Pf0-1Δ*dap* were grown in aerated Luria-Bertani (LB) or Pseudomonas Minimum Medium (PMM) at 27°C stirred at 250 rpm. The reagents and concentrations of PMM were: 35 mM K_2HPO_4 (Potassium Phosphate Dibasic), 22mM KH_2PO_4 (Potassium Phosphate Monobasic), 8 mM $(NH_4)_2SO_4$ (Ammonium Sulfate), 1.2 mM $MgSO_4$ (Magnesium Sulfate), 25mM $C_4H_4Na_2O_4$ (Sodium Succinate). *Escherichia coli* strains were grown in aerated LB medium at 37°C stirred at 250 rpm. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 10 μg/ml, tetracycline, 10 μg/ml or 25 μg/ml, depending on the bacterial strain; carbenicillin, 100 μg/ml, and streptomycin, 20 μg/ml. In addition, agar media used to grow Pf0-1Δ*dap,* or its derivatives, was amended with, 10 μg/ml of diaminopimelic acid (DAP). Media used to detect β-galactosidase activity contained 35 μg/ml of X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside).

3.3.2 Construction of A Pf0-1 genomic library

A Pf0-1 genomic library was constructed in the pIVETdap vector (a gift from Mark W. Silby) (15). The pIVETdap constructs and their generation is illustrated in Figure 3.1. Purified Pf0-1 genomic DNA was extracted from an overnight culture in PMM medium, using the Wizard® Genomic DNA Purification Kit (Promega; Madison, WI). The genomic DNA was partially digested with *Sau*3A1 (New England Biolabs, Beverly, MA) for 18 minutes. The partially digested DNA was electrophoresed in 0.7% agarose at

Table 3.1 Strains and plasmids

Strains, Plasmids or Primers	Genotype or Description	Reference or Source
E. coli		
DH5αλ pir	φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR* _λ*pir*	(7)
P. fluorescens		
Pf0-1	Wild type, Amp^r	(15)
PfΔdap	Pf0-1 Δ*dapB*	This Study
Pf0-1::pKNOCK Fr2	Pf0-1 with partial deletion of *Fr2*	This Study
Pf0-1::pKNOCK Fr4	Pf0-1 with partial deletion of *Fr4*	This Study
Pf0-1::pKNOCK Fr9	Pf0-1 with partial deletion of *Fr9*	This Study
Pf0-1::pKNOCK Fr10	Pf0-1 with partial deletion of *Fr10*	This Study
Pf0-1::pKNOCK Fr2pJB	Pf0-1 with partial deletion of *Fr2* Complemented by pJB866	This Study
Pf0-1::pKNOCK Fr2pJB Fr2	Pf0-1 with partial deletion of *Fr2* Complemented by pJB866::Fr2	This Study
Pf0-1::pKNOCK Fr10pJB	Pf0-1 with partial deletion of *Fr10* Complemented by pJB866	This Study
Pf0-1::pKNOCK Fr10pJB Fr10	Pf0-1 with partial deletion of *Fr10* Complemented by pJB866::Fr10	This Study
Plasmids		
pGEM-T Easy	Ap^r; cloning vector for PCR products	Promega
pIVETdap	*dapB*' cloned in SpeI site of pUIC3	(15)
pJB866	Broad-host-range vector, Tc^r	(2)
pRK2013	Helper plasmid, IncP Tra^+ Mob^+ ColE1, Km^r	(2)
pKNOCK-Km	pBSL63 derivative carrying RP4 oriT and R6K γ-ori, Km^r	(1)
pSR47s	Km^r; *sacB*-containing suicide vector (requiring R6K replication origin)	(11)

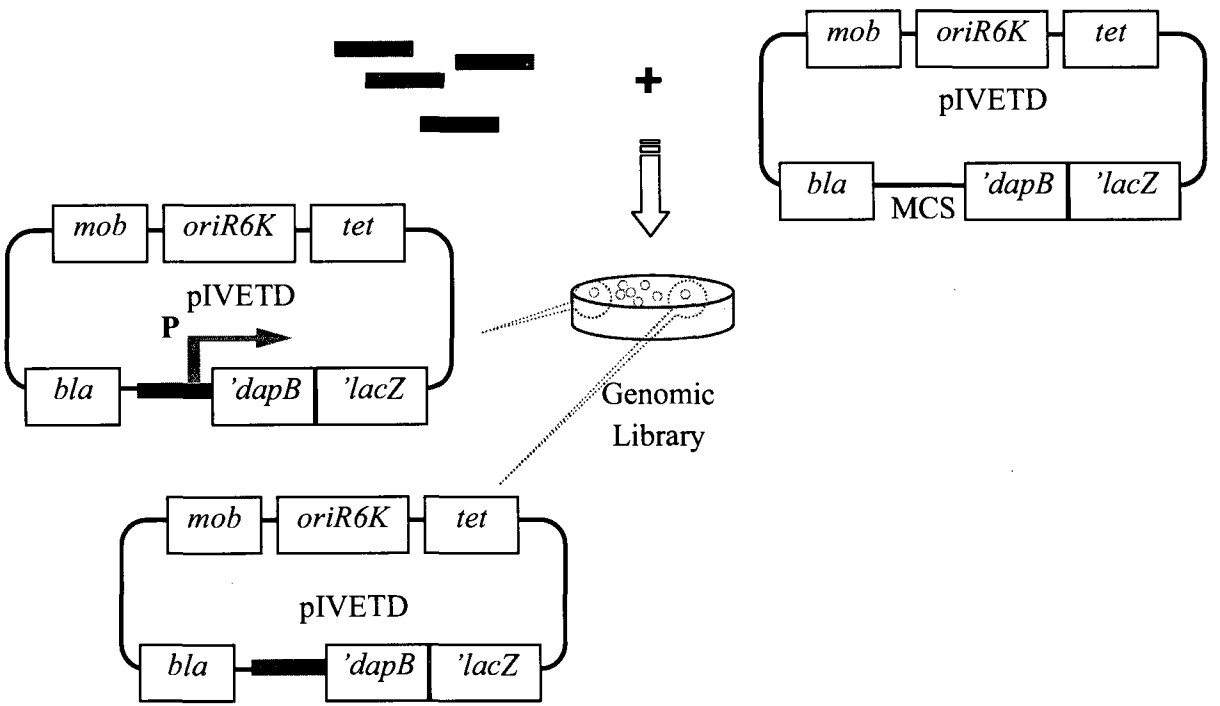


Figure 3.1 Construction of Pf0-1 genomic library in pIVETD. 1-3 kb of Pf0-1 genomic fragments were obtained from Sau3A1 partial digestion of Pf0-1 genomic DNA. pIVETD is a suicide plasmid with a multiple cloning sites immediately upstream a promoterless *dapB*. Pf0-1 genomic library was cloned in DH5αλpir, and were counterselected.on laboratory medium plates. Each colony on this plate represented a clone with a pIVETD construct carrying a fragment with or without a promoter.

98 V for 40 minutes. One to 3 kb fragments were isolated and purified from agarose fragments using a Qiagen gel isolation kit (Qiagen,Valencia,CA) and ligated to dephosphorylated pIVETdap/*Bgl*II, yielding pIVETdap/BglII/genomic. pIVETdap/BglII was previously prepared from pIVETdap extracted from an overnight culture of an *E. coli* strain carrying pIVETdap using QIAprep Spin Miniprep Kit (Qiagen, CA). Purified pIVETdap was digested with *Bgl*II in the presence of Buffer D at 37°C for 24 hours and subsequently inactivated by heat treatment at 80°C for 15 minutes (Promega; Madison, Wisconsin). Dephosphorylated pIVETdap/BglII was prepared by treatment of pIVETdap/BglII with Calf Intestinal Alkaline Phosphatase at 37°C for 24 hours (Promega; Madison, Wisconsin). Ligation reactions were transformed into Strain DH5αλpir and selected on nalidixic acid and tetracycline LB medium. A pool of 9375 clones from several independent ligations was kept at -80°C. Twenty-one random clones were subjected to PCR analysis for fragment variations, using pbla as the forward primer and pdap as the reverse primer (4). Nineteen out of 21 clones show PCR products of various sizes (data is not shown). This suggested that 8482 out of 9375 clones contained insertions at the MCS; as predicted, 8482 clones accounted for approximately 94.09% coverage of the Pf0-1 genome.

3.3.3 Construction of Pf0-1Δdap

A Pf0-1 mutant strain carrying an in-frame deletion of the *dapB* gene was constructed. The primer pairs DapB1/DapB2 and DapB3/DapB4 were used to amplify upstream and downstream regions respectively via PCR under the following conditions: a cycle of primary denaturation at 95°C for 5 minutes; 25 cycles of denaturation at 95°C for 2 minutes, annealing at 62°C for 1 minute and extension at 72°C for 2 minutes; and

one cycle of final extension at 72°C for 5 minutes. The 5' ends of DapB2 and DapB3 contained complementing linker sequences of 5'-AAACCAGCGGCCGCTATACG-3' and 5'-CGTATAGCGGCCGCTGGTTT-3' that were used to anneal both PCR products together. Annealed fragments were ligated into the plasmid pSR47s using the *Sal*I and *Sac*I sites, resulting in pJGΔ101, which was transformed into *E. coli* DH5αλpir. Derivatives of pSR47s do not replicate in Pf0-1 that lack the π replication factor (see chapter 2) (7). Conjugation of pSR47s derivatives into Pf0-1 and kanamycin selection demands recombination between the genomic insert in the plasmid and its counterpart in the chromosome. A second recombination event, brought about by counter-selection based on sensitivity to high levels of sucrose (encoded by *sacB*), results in recombinant strains that contain either the original allele or the plasmid borne version, PCR is later used to discern recombinants. Further screening of recombinants included testing growth in the absence of Dap.

3.3.4 Selection of recombinant strains carrying promoters specific to arid soil

Figure 3.1, 3. 2 and 3.3 shows the genetic scheme used in this study. In figure 3.2, Clones from the Pf0-1 genomic library constructed in pIVETdap were conjugated en masse into Pf0-1Δdap by triparental matings. Because pIVETdap, carrying the Pf0-1 genomic library, is non-replicative in *P. fluorescens*, selection for transcojugants requires the integration of clones into homologous regions of the Pf0-1Δdap chromosome, which are referred to as "fragments" or "fusions". The fusion strains were selected in LB medium, containing antibiotics and DAP. Thus, each transconjugant clone in this step represented a fusion strain bearing transcriptionally active or inactive promoters, or non-promoter fragments upstream of the bicistronic sequence of *dapB-lacZ*.

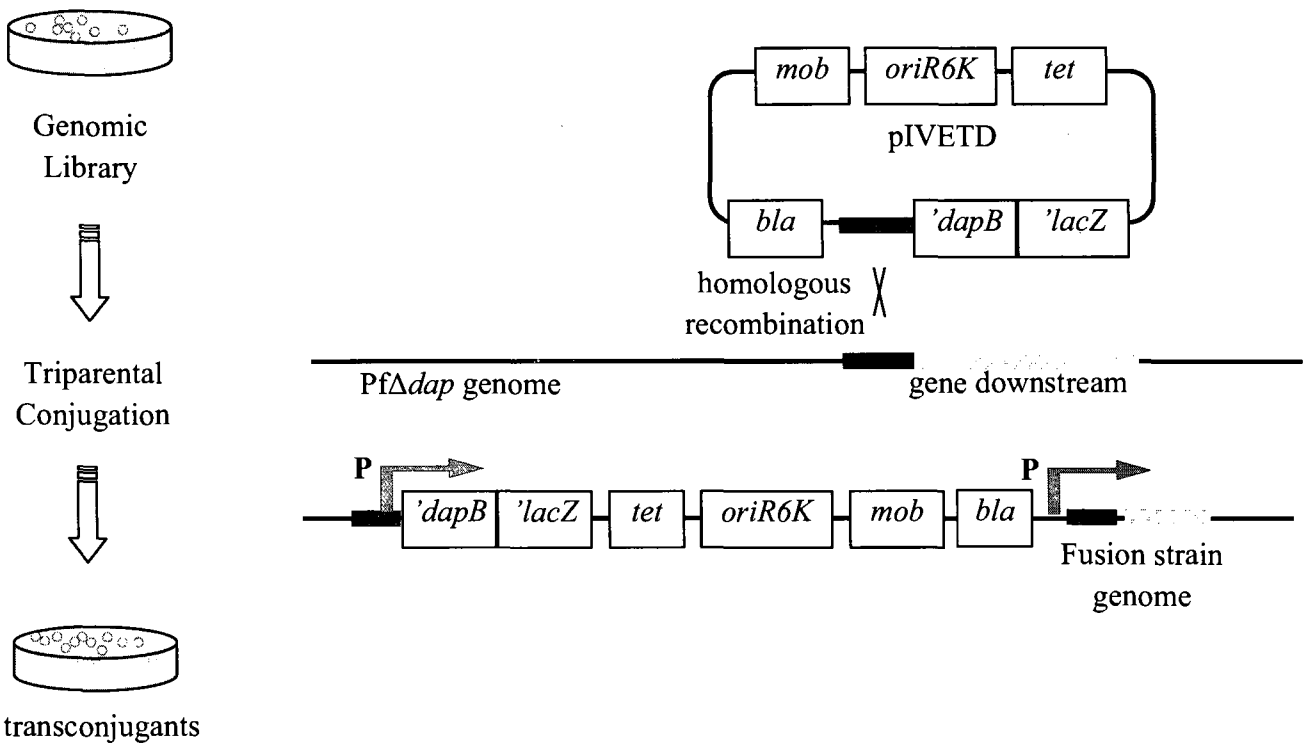


Figure 3.2 The schematic screening of soil-inducible promoters by IVET. Genomic library cloned in pIVETD was transconjugated into PfΔ*dap* via tripartental conjugation. During the conjugation a single homologous recombination occurs between the homologous sequences. Each colony on the counterselected plate represents a fusion strain with or without a promoter upstream the '*dapB*.

Approximately 50-200 fusion strains from a single independent conjugation were pooled and used in our soil assay.

3.3.5 Soil assay

Non-sterile arid soil, obtained from the Nevada Test Site was used for this study. Coarse analysis of the soil used here showed 0.91% organic matter, 89.0% sand, 4.1% silt, and 6.9% clay, with a pH level of 8.3 (for a more detailed description of other soil properties see Titus 2001). A pool of pIVET fusion clones was diluted and adjusted with sterile double distilled water to 0.01 OD_{550}. One ml of the adjusted bacterial suspension, containing approximately 5×10^5 CFU, was inoculated onto a 35 ml Pyrex test tube containing 5 g of soil. Preliminary experiments measuring how the test soil loses water over a period of 8 days show 18%, 14% and 10% moisture content at 0, 3 and 8 days of incubation. A value of 11-19% water content is field capacity, while a water content of 3-10% is considered the permanent wilting point for a soil with a loamy-sand texture.

After a 7-day period of incubation at room temperature, 1 gram of inoculated soil was extracted and combined with 9 ml of sterile double distilled water to make a soil suspension. This solution was sonicated for 30 seconds and 1 ml of the solution was subsequently used to inoculate a 35 ml Pyrex test tube containing 5 grams of soil. After the assay was incubated for another 7 days at room temperature, the procedure to generate a soil suspension was repeated, used for a 10-fold dilution series and plated onto selective media containing X-gal and DAP. Plates were incubated at 27°C for 2 days and counted. Three types of colonies (blue, pale blue and white) appeared on plates. A blue colony indicates that the fusion strain bares a constitutive promoter (Figure 3.3).

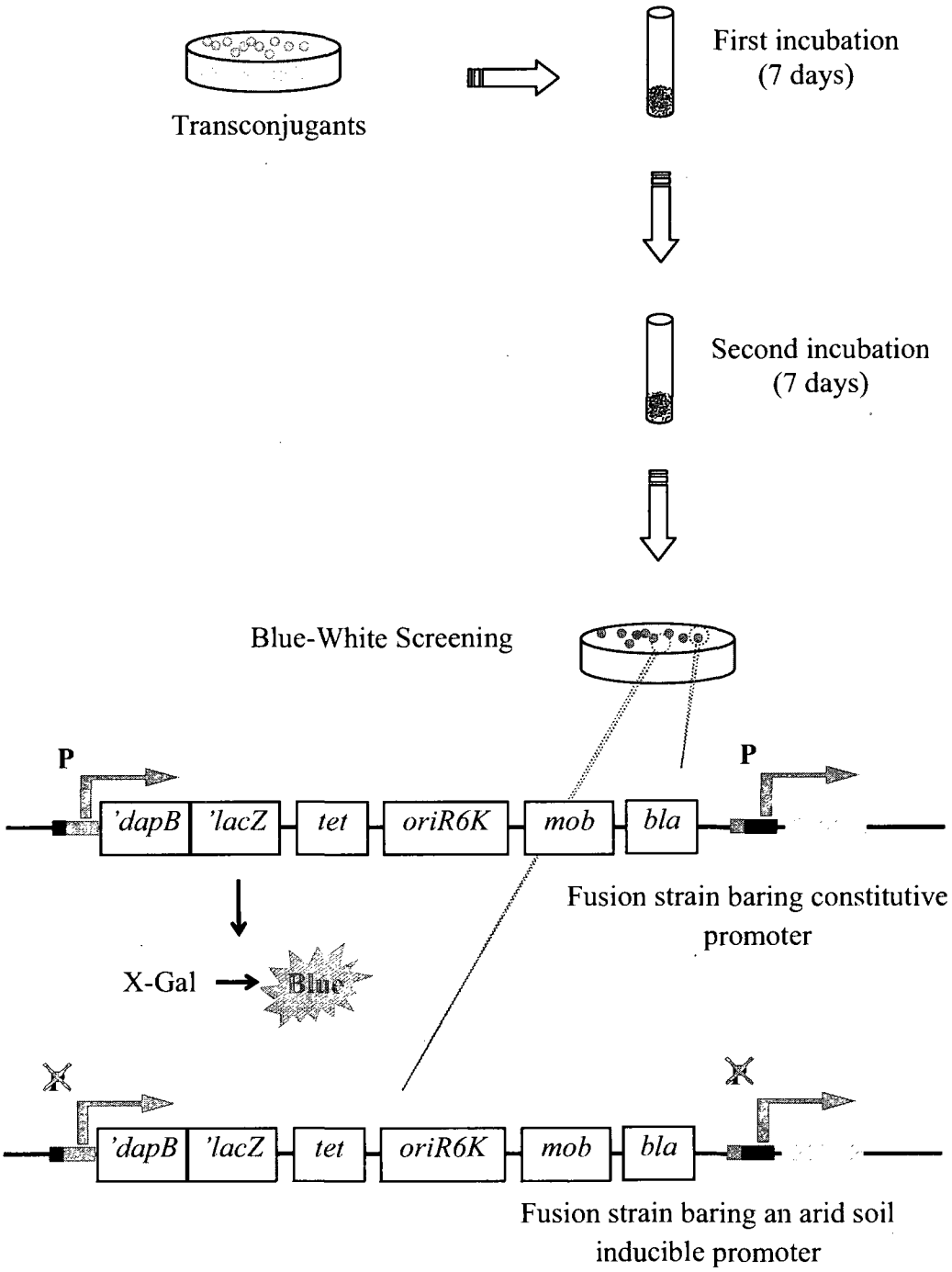


Figure 3.3 Soil assay and white-blue screening. A pool of pIVET fusion clones was diluted and adjusted with sterile double distilled water to 0.01 OD_{550}. 1 ml of diluted culture was inoculated into 5 grams of non-sterile soil and was incubated at room temperature for 7 days. White colonies on X-gal plate were fusion strains carrying arid soil inducible promoters.

Pale blue colonies represent a fusion strain that contains a weakly upregulated promoter during arid soil persistence. White colonies occur when a fusion strains bares a promoter that was active in arid soil but became inactive in the culture medium. From each independent soil assay, approximately 1% of the colonies on the selective plates appeared white, and only one white was selected for further analysis.

3.3.6 Analysis of soil-activated fragments

The white colonies were cultured independently in aerated LB broth containing DAP at a concentration of 10 μg/ml. This procedure was repeated twice before plasmid extraction. This subculture procedure was followed to ensure that a fraction of cells had a recombination event that excise the Campbell construct into its plasmid form (13). The plasmid preparations were then used to transform *E. coli* DH5αλpir. Tetracycline resistant transformants were used to study genomic fragments, containing an arid soil inducible promoter, by PCR, using the primers pdap and pbla. The PCR products were 1-3 kb.

3.3.7 Sequence analysis

All PCR products to be sequenced were sent to the Nevada Genomic Center at the University of Nevada, Reno. Each PCR product was sequenced in both directions using the pbla, and pdap primers. The two sequences represent the 5' and 3' ends of the fragment recovered. Sequences of arid-inducible fragments were then compared to the whole genome sequence of *Pseudomonas fluorescens* at the NCBI database (3).

3.3.8 Construction of knock out strains

Internal sequences of Fr2, Fr4, Fr9 and Fr10 were amplified using Pf0-1 genomic template and primers shown in table 2 under the following conditions: a cycle of primary denaturation at 95°C for 5 minutes; 25 cycles of denaturation at 95°C for 2 minutes, annealing at 49°C for 1 minutes and extension at 72°C for 1 minute; and one cycle of final extension at 72°C for 5 minutes. The 0.5 kb PCR fragements were resolved by electrophoreses in 0.7% agarose at 98 V for 20 minutes and purified (Qiagen, CA). Purified products were cloned in pGEMTeasy (Promega, WI), and cloned plasmids were prepared using QIAprep Spin Miniprep Kit (Qiagen, CA). The pGEMTeasy contructs baring Fr2, Fr4, Fr9 and Fr10 were digested with EcoRI. Digestions were subsequently inactivated by heat treatment at 80°C for 15 minutes (Promega, WI), which yielded Fr2/EcoRI, Fr4/EcoRI, Fr9/EcoRI and Fr10/EcoRI respectively. Digested products were purified by QIAquick PCR Purification Kit and were ligated to linear dephosphorylated pKNOCK/EcoRI (1), by incubating the fragment, the plasmid and ligase at 4°C for 24 hours in the presence of a buffer provided by LigaFast™ Rapid DNA Ligation System (Promega; Madison, Wisconsin). Ligation reaction yielded four derivatives: pKNOCK/EcoRI::Fr2, pKNOCK/EcoRI::Fr4, pKNOCK/EcoRI::Fr9 and pKNOCK/EcoRI::Fr10, which were transformed into *E. coli* DH5αλpir. Linear dephosphorylated pKNOCK/EcoRI was used as control and prepared from an overnight culture of an *E. coli* strain carrying pKNOCK. pKNOCK was extracted using QIAprep Spin Miniprep Kit (Qiagen, CA) and was incubated with *Eco*RI at 37°C for 24 hours. The resulting product was incubated with Calf Intestinal Alkaline Phosphatase at 37°C

Table 3.2 Primers

Primers	Description	Reference or Source
Pbla	5′-CAGGGTTATTGTCTCATGAGCG-3′	(4)
Pdap	5'-CCGCCTCTACCAGCGTCTTGCC-3	(4)
DapB1	5'- GCATGAGAGCTCACCCTTTCCGTCAAAGTGC -3'	This Study
DapB2	5′- AAACCAGCGGCCGCTATACGTCGCATGCCGACTCC -3′	This Study
DapB3	5'- CGTATAGCGGCCGCTGGTTTGTACGACATGCAGG -3	This Study
DapB4	5'- TTACATGTCGACTTGCTCGCTACCAGCGG -3'	This Study
fFr2	5'- GTAACTGTTGGCCTGGAA -3'	This Study
rFr2	5'- GCCAAACGCGATCACA -3'	This Study
fFr4	5'- CCGCGTTATTCGCAGA -3'	This Study
rFr4	5'- TGTAATCATCCGGCCAGA -3'	This Study
fFr9	5'- GAGCCGACTGCACGAA -3'	This Study
rFr9	5'- TGGTCATGAGTTCGCTGA -3'	This Study
fFr10	5'- CGCACGTTCAGGCTGA -3'	This Study
rFr10	5'- CCAACAGCCACGAGCA -3'	This Study
fFr2com	5'- ATTGCGGCCGCTCAGGCTTCGGTCAGATACC-3'	This Study
rFr2com	5'- CGCACTAGTCGATGAAATTCGCAGCCATTGA -3'	This Study
fFr10com	5'- GCGCAATTCTTACTCTTTGTCCAGCATGCCA -3'	This Study
rFr10com	5'- ATTGCGGCCGCTATGAGCACTAGCGCAGCACA -3'	This Study

for 24 hours (Promega; Madison, Wisconsin) and was purified by QIAquick PCR Purification Kit (Qiagen, CA). *E. coli* DH5αλpir derivatives containing pKNOCK/EcoRI::Fr2, pKNOCK/EcoRI::Fr4, pKNOCK/EcoRI::Fr9 and pKNOCK/EcoRI::Fr10 were conjugated with PF0-1 in the presence an *E. coli* pRK2031. Transconjugants from each mating were selected for Ampicillin resistance and kanamycin resistance, which gave rise to Pf0-1::pKNOCK Fr2, Pf0-1::pKNOCK Fr4, Pf0-1::pKNOCK Fr9 and Pf0-1::pKNOCK Fr10 respectively. These four strains were subject to the arid soil assay previously described; their ability to colonize arid soil was measured at 0, 1, 3 and 7 days after inoculation.

3.3.9 Complementation

Two primer pairs fFr2com/rFr2com and fFr10com/rFr10com were used to amplify Pfl_2143 and Pfl_5593 from the Pf0-1 genome, respectively (see table 2). Taq Vent DNA polymerase was used under the following conditions: a cycle of primary denaturation at 95°C for 5 minutes; 25 cycles of denaturation at 95°C for 2 minutes, annealing at 59°C for 1 minutes and extension at 72°C for 2 minutes; and one cycle of final extension at 72°C for 5 minutes. The PCR products were electrophoresed in 0.7% agarose at 98 V for 30 minutes and 1.3kb and 1.4kb fragments were isolated and purified from agarose using a Qiagen gel isolation kit. Purified PCR products were digested with either *Afl*III (Fr2) and *Not*I or *Eco*RI and *Not*I (Fr10) at 37°C for 24 hours (Promega; Madison, Wisconsin), yielding Fr2/AflIII/NotI and Fr10/EcoRI/NotI. Purified Fr2/AflIII/NotI and Fr10/EcoRI/NotI were ligated to linear dephosphorylated pJB866/AflIII/NotI and pJB866/EcoRI/NotI respectively, yielding pJB866Fr2 and pJB866::Fr10. pJB866Fr2 and pJB866::Fr10 were transformed into *E. coli* DH5αλpir

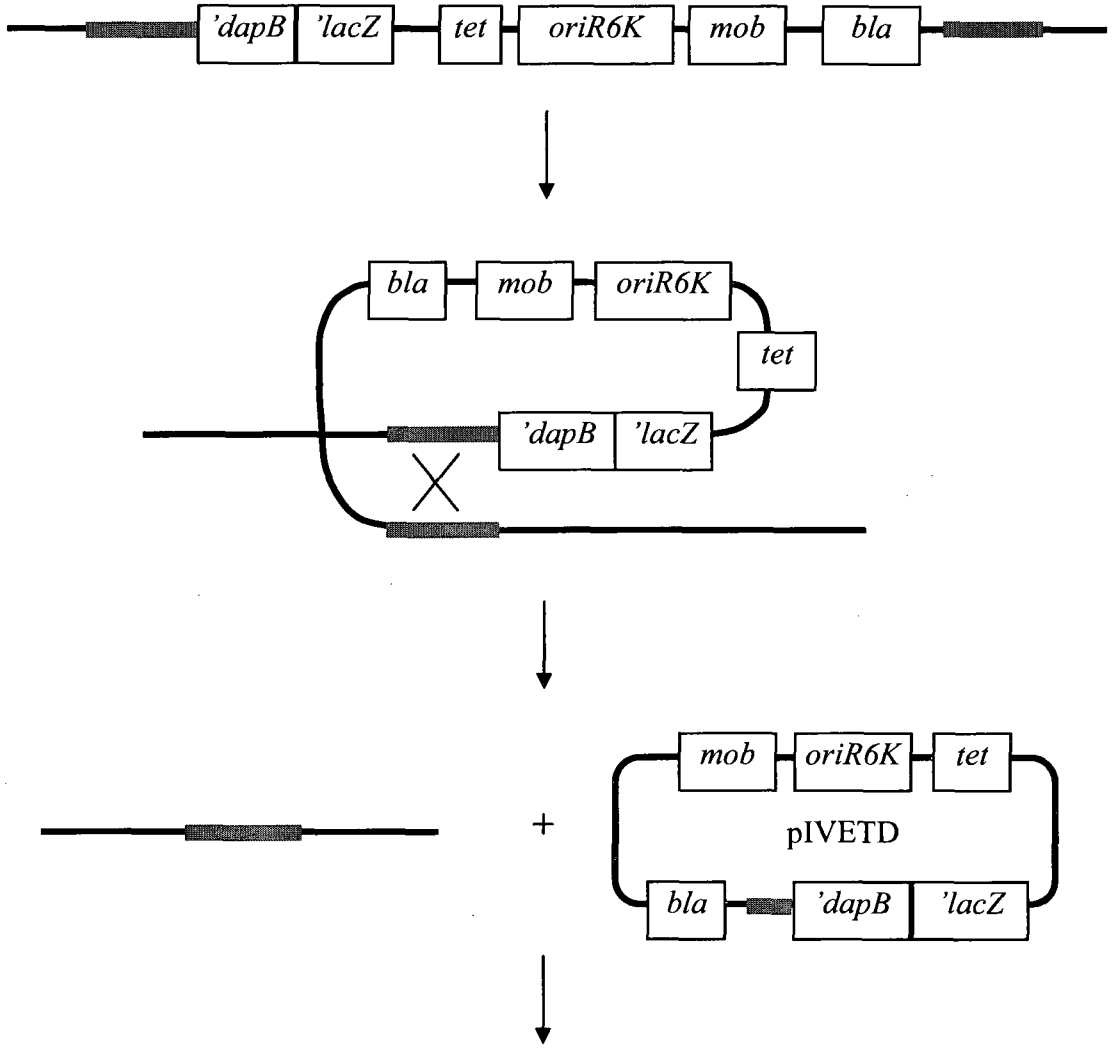
yielding the strains of *E. coli* pJB866*::Fr2* and *E. coli* pJB866Fr10 respectively. The E. coli strains carrying the complementing fragments were selected in LB plates containing 5 μg/ml tetracycline and 10 μg/ml nalidixic acid. The complementing plasmids carried by *E. coil_pJB866::Fr2* and *E. coil_pJB866::Fr10* were conjugated into Pf0-1::pKNOCK Fr2 and Pf0-1::pKNOCK Fr10 via triparental matings, generating Pf0-1::pKNOCK Fr2+Fr2 and Pf0-1::pKNOCK Fr10+Fr10. The two complemented strains were subject to soil colonization of arid soil.

3.4 Results and Discussion

In this experiment, a Pf0-1 genomic library with 95.5% coverage was cloned upstream of a promoterless *dapB-lacZ* gene construct and used in combination with a *dapB*$^-$ mutant, constructed by deleting of 807 bp of the *dapB* gene, to isolate fragments that are transcriptionally active in arid soil. The genomic library, cloned into a non-replicative plasmid was conjugated into the *dapB*$^-$ mutant thereby generating recombinant clones that contained promoterless *dapB* transcriptional fusions placed randomly in the chromosome of *P. fluorescens*. Our assay tested recombinant cells for their ability to synthesize lysine in soil conditions and to repress transcription of *lacZ* on laboratory medium. Thirty independent colonies recovered from soil which showed reduced or no *lacZ* activity on laboratory medium were selected for further analysis, which represented approximately 1% of all the colonies tested on laboratory medium.

4.3.1 Recovery and analysis of soil-activated DNA fragments

Thirty fragments were recovered from the phenotypically white fusions. The strategy to recover the genomic fragments of interest is shown in Figure 4.



Plasmid Extraction

Transformation in *E. coli* DH5αλpir

PCR using pdap and pbla primers

Figure 3.4 Strategy of fragment recovery from a fusion strain

Recombination events in Pf0-1 derivatives carrying the pIVET recombinants yielded plasmid intermediates, generated during subculturing, that were transformed and recovered in *E. coli*. The pIVET clones were then amplified using primers that flanked the cloning site (Figure 2). This approach yielded fragments of approximately 1-3 kb in size.

The sequenced fragments were mapped by matching their sequence to the *Pseudomonas* genome sequence at NCBI. Identification of putative arid soil inducible promoters was performed using the Softberry promoter locator software, which recognized bacterial sigma 70 sequences with 80% accuracy (Table 3.3) (17). However, not all the fragments recovered from the fusion strains contained a promoter that was recognized by the Softberry software, which suggests the presence of promoters recognized by other sigma factors (6). We also employed the PromScan software program to locate promoters recognized by sigma 54 (19). Also, indicated in table 3 is the locus associated (genome position) for each of the soil-activated fragments in soil conditions. Interestingly, our promoter *in silico* analysis did not show putative promoters for fragments 5, 8, 9 and 13. These fragments may be under the control of other sigma factors and the identification of their promoters will require further experimentation. In addition to the *in silico* promoter studies, we conducted further analysis of these fragments (Table 3.4). Based on functional groups, 22 out of 26 sequences could be classified into four major groups: metabolisms, cellular processes, information storage and processing and poorly characterized. Five of the 26 fragments have not been previously characterized. Interestingly, four fragments, which mapped to Pfl_2143, Pfl_2547, Pfl_0250 and Pfl_2186, were recovered twice in independent soil assays.

Table 3.3 Fragment analysis

Fragment	Promoter Locator	promoter	Genomic Position
1	Softberry	Yes	294599
2	Softberry	Yes	2428134
3	Softberry/ PromScan	No	5918226
4	Softberry	Yes	3053004
5	Softberry/ PromScan	No	2470094
7	Softberry	No	5009954
8	Softberry/ PromScan	No	6215466
9	Softberry/ PromScan	Yes	4496043
10	Softberry	Yes	6266901
11	Softberry	Yes	6322555
12	PromScan	Yes	5025784
13	Softberry/ PromScan	No	3773146
14	Softberry	Yes	6175349

Table 3.3 Fragment analysis (continued)

Fragment	Promoter Locator	promoter	Genomic Position
15	Softberry	Yes	1064366
16	Softberry	Yes	1243253
18	Softberry	Yes	845845
19	Softberry	Yes	713425
20	Softberry	Yes	3345214
21	Softberry	Yes	3161874
23	Softberry	Yes	4273273
24	Softberry	Yes	2724286
25	Softberry	Yes	4812587
27	PromScan	Yes	3172705
28	PromScan	Yes	2915005
29	Softberry	Yes	267167
30	PromScan	Yes	4434208

a.) Analysis of metabolism genes

We recovered a total of twenty-six different soil-activated fragments in our colonization assay. Table 3.4 shows ORFs associated with these fragments grouped by ORF number and predicted function in the *P. fluorescens* genome, the corresponding cluster of orthologous group, and the direction of transcription in relation to the associated annotated ORF. Ten of the 26 fell within the broad group of metabolism. Five fragments from this group map to ORFs associated with transport and metabolism of amino acids, lipids and carbohydrates. There are two soil-activated fragments involved in energy generation. Our soil assays also yielded one fragment associated with each of three following groups: cell membrane/cell wall biogenesis, inorganic ion transport and production of secondary metabolites. These results suggest, as expected, that arid soil is an environment with low availability of carbon and nitrogen for *P. fluorescens*. Further analysis of the fragments with metabolic functions indicates that eight fragments are transcribed in the opposite orientation to the corresponding annotated ORF (Table 4). With the exception of fragments 2 (Pfl01_2143, glutamine synthase), 29 (Pfl01_0225, amino acid permease) and 28 (Pfl01_2547, glucotranferase), all other fragments are transcribed in the opposite orientation to those proposed by the annotated genome. This is interesting and suggests a few scenarios. One, the sequences for such fragments may need further curation as they may be coding products in the complementary strand, or may resemble a promoter when fused to a promoterless *dapB* gene. Two, synthesis of gene products that affect energy production, cell wall biosynthesis, secondary metabolite production and carbon metabolism may be repressed in conditions of low availability of carbon and nitrogen and that their repression is mediated by antisense mRNA. Fragments

Table 3.4 Predicted functions of ORFs

Fragment	◊	locus tag	Product Name	COG ID	Grouping
Metabolism					
2[b]	sense	Pfl01_2143	Glutamine Synthetase	COG1629	Amino acid transport and metabolism
29	sense	Pfl01_0225	Amino acid ABC transporter, permease protein	COG0765	Amino acid transport and metabolism
7	anti	Pfl01_4448	Pyruvate Kinase	COG0469	Carbohydrate transport and metabolism
28[ab]	sense	Pfl01_2547	4-alpha-glucanotransferase	COG1640	Carbohydrate transport and metabolism
12[a]	anti	Pfl01_4455	Insecticidal Toxin Protein, putative	COG3209	Cell wall/membrane/envelope biogenesis
25	anti	Pfl01_4265	Cytochrome C	COG2010	Energy production and conversion
30[a]	anti	Pfl01_3916	Luciferase-like	COG2141	Energy production and conversion
1	anti	Pfl01_0250	TonB-dependent receptor	COG1629	Inorganic ion transport and metabolism
21	anti	Pfl01_2744	Thiolase	COG0183	Lipid transport and metabolism
26	anti	Pfl01_0911	Fumarylacetoacetase	COG0179	Secondary metabolites biosynthesis, transport and catabolism

◊ is the orientation relative to annotation; (a) refers to the gene that does not contain a promoter based on sigma 70; (b) refers to the gene that had been recovered twice. Locus tag is NCBI identification number for gene identification.

Table 3.4 Predicted functions of ORFs (Continued)

Fragment	◊	locus tag	Product Name	COG ID	Grouping
Cellular Process (regulation and signaling)					
4	sense	Pfl01_2660	GTPase Subunit of Restriction Endonuclease like	COG1401	Defense Mechanism
11[c]	sense	Pfl01_5642	Transcriptional Regulator, RpiR family	COG1737	Replication, recombination and repair
9[c]	sense	Pfl01_3972	Putative diguanylate phosphodiesterase (EAL domain)	COG2200	Signal transduction mechanisms
Information Storage and Processing					
13[c]	anti	Pfl01_3287	Transcriptional antiterminator, Rof	COG4568	Transcription
18	sense	Pfl01_0719	Transcriptional Regulator, LysR family	COG0583	Transcription
8[c]	anti	Pfl01_5547	Ribonuclease PH	COG0689	Transcription
24	sense	Pfl01_2366	Transcriptional Regulator, XRE family	COG1709	Translation, ribosomal structure and biogenesis

◊ is the orientation relative to annotation; (c) refers to the gene that does not contain a promoter based on sigma 70. Locus tag is NCBI identification number for gene identification.

Table 3.4 Predicted functions of ORFs (Continued)

Fragment	◊	ORF	Product Name	COG ID	Grouping
Poorly Characterized					
16	sense	Pfl01_1075	Extensin-like protein	COG3921	Function unknown
10	sense	Pfl01_5595	Hypothetical protein	COG3516	Function unknown
23	sense	Pfl01_3777	Hypothetical protein	COG0596	General function prediction only
5[a b]	anti	Pfl01_2186	Ribonuclease H	COG0328	Predicted transcriptional regulator
Uncharacterized Genes					
14	anti	Pfl01_5509	Hypothetical protein		
19	sense	Pfl01_0609	Hypothetical protein		
27[a]	sense	Pfl01_2750	Hypothetical protein		
3	anti	Pfl01_5256	Hypothetical protein		
20	sense	Pfl01_2901	Xylose isomerase-like TIM barrel		

◊ = orientation relative to annotation; (a) indicates the absence of a sigma 70 promoter; (b) indicates that the region was recovered twice in independent assays. Locus tag is NCBI identification number for gene identification.

28, 12 and 30 are predicted to contain a σ^N promoter and code for a glucotransferase (carbon transport), antisense mRNA corresponding to a putative insecticidal protein gene, and antisense mRNA that matches the coding sequence of a luciferase gene. This result implies that when *P. fluorescens* experiences Nitrogen starvation pathways for carbon uptake are activated and pathways that consume energy or mediate cell envelope biosynthesis are repressed. However, similar processes or genes have been reported to be activated in soil-influenced environments. For example, elevated transcription of fragment 29 (Pfl01_0225; amino acid ABC transporter permease) have been detected in *P. fluorescens* SBW25 exposed to sugar beet rhizopheres (4). Interestingly, a gene encoding a TonB periplasmic transmembrane barrel that allows passage of inorganic ions via proton-motive force, is transcriptionally activated in sugar beet rhizosphere, however, in our case the expression of this gene may be repressed since Fragment 1 transcribes in the opposite orientation to this ion transporter. Lastly, similar to our results, a homologous gene to Fragment 2 (Pfl01_2143; glutamine synthetase) has been detected to be activated when the sulfate-reducing gamma proteobacterium *Desulfovibrio desulfuricans* is exposed to sediments (10).

b.) Analysis of regulation and signaling genes

Three cellular processing genes were transcriptionally induced in arid soil. Fragment 4 (Pfl01_2660; GTPase Subunit of Restriction Endonuclease), Fragment 11 (Pfl01_5642; Transcriptional Regulator, RpiR family) and Fragment 9 (Pfl01_3972; Putative diguanylate phosphodiesterase). In *E. coli*, the transcriptional regulator, RpiR, negatively controls the expression of ribose phosphate isomerase, RpiB (18). In the pentose pathway, RpiB cooperates with RpiA in catalyzing the formation of ribose 5-

phosphate from ribulose 5-phosphate. Ribose 5-phosphate ribose 5-phosphate is an important compound for cellular anabolism of nucleotides, histidine and tryptophan. Fragment 4, the GTPase subunit of a putative restriction endonuclease protein contains a conserved P-loop NTPase domain. The P-loop NTPases are involved in diverse cellular functions through phosphorylation of nucleosides, nucleotides, sugars, coenzyme precursors, adenosine 5'-phosphosulfate and polynucleotides (9). Fragment 9, diguanylate phosphodiesterase, EAL domain is involved in turnover of an important secondary messenger, cyclic dinucleotide 3, 5-cyclic diguanylic acid (c-di-GMP) (14, 20). Cellular levels of c-di-GMP influence several physiological processes, such as cellulose biosynthesis and biofilm formation in the Gram-negative enteric pathogens. Levels of this signal molecule also affect survival of *Salmonella enterica* serovar typhimurium in mice and the transition between sessile and motile phenotypes in *S. typhimurium*, *P. aeruginosa*, and *E. coli*. Increased cellular concentrations of c-di-GMP favor biofilm formation while a decreased in its concentration induces toxin production in *Vibrio spp*. The activation of these genes indicates that adapting to arid soil requires that *P. fluoresecens* engage in biofilm formation and protection of its genome integrity.

c.) Analysis of information storage and processing genes

The information process and storage genes are involved in transcription and translation, as well as ribosomal structure and biosynthesis. *In silico* promoter analysis of these fragments indicated that Fragment 13 (Pfl01_3287, transcriptional antiterminator Rho) and Fragment 8 (Pfl01_5547, Ribonuclease PH) do not contain canonical σ^{70} or σ^{54} promoters. Further, BLAST analysis against the annotated *P. fluorescens* genome showed that these two fragments are transcribed as antisene to the predicted ORFs found

in these regions. On the other hand, Fragments 18 (Pfl01_0719, LysR type transcriptional regulator) and 24 (Pfl01_2366, XRE transcriptional regulator) appeared to be transcriptionally controlled by σ^{70} and are transcribed as annotated. Fragment 13 contains a member of the Rho-dependent transcription termination (ROF), which inhibits Rho-dependent transcription termination. Pfl01_0719 is a putative transcriptional regulator of the LysR family. The majority of LysR transcription regulator proteins appear to be transcription activators and most are known to negatively regulate their own expression. Interestingly, Pfl01_0719 sequence is highly conserved among *Pseudomonas spp* with 71%-85% identity and has been reported to regulate production of detoxifying gene products in *P. putida* (12). Furthermore, it has been shown that this regulator affects soil survival of *P. putida* (5) and persistence of *P. aeruginosa* persistence in rat lungs (8).

Pfl01_5547 is *rph* gene, which encodes a ribonuclease PH 3' exoribonuclease. The *rph* gene is involved in maturation of tRNA precursors and removes terminal nucleotides near the CCA acceptor arm of mature tRNAs (See http://www.ncbi.nlm.nih.gov/Structure/cdd/). Interestingly, our result detects upregulation of *rph* antisense, suggesting a decrease in tRNA turn-over in soil environments. Pfl01_2366 contains a transcriptional regulator domain of the XRE family. The XRE shows helix-turn-helix properties and regulates genes that respond to the presence of xenobiotic compounds (See http://www.ncbi.nlm.nih.gov/Structure/cdd/). Taken together, the activation of genetic regions coding for storage and process of information suggests that in arid soil *P. fluorescens* represses protein synthesis by

altering message stability and tRNA turnover and it activates genes that increase persistence or that permit the degradation/detoxification of soil compounds.

d.) Poorly characterized and uncharacterized genes

This class contains a total of nine fragments that code for either a hypothetical or poorly characterized proteins. Interestingly, we also detect sense and antisense transcripts in this group. Pfl01_1075, Pfl01_5595, Pfl01_3777, Pfl01_0609, Pfl01_2750 and Pf01_2901 are transcribed as predicted by the genome annotation. However, Pfl01_2186, Pfl01_5509 and Pfl01_5256 are transcribed from the opposite strand from the one predicted by annotation. Altogether, the soil-activated expression of these genes are important for soil survival, however, further studies are necessary to illuminate the processes coded by these fragments.

3.4.2 Soil colonization of mutants and complementation studies

One gene from each of four COG functional groups were chosen to conduct inactivation and complementation analysis. The strains carrying single inactivated alleles were constructed by generating internal deletions using the pKNOCK-km system (1) and tested for their ability to colonize arid soil. The results for the experiments using Fragment 2 and Fragment 10 are shown in Figure 3.5. The genetic inactivations in Fragment 4 (Pfl0_2660; GTPase subunit restriction endonuclease-like protein) and Fragment 9 (Pfl01_3972; putative diguanylate phosphodiesterase) did result in a significant decrease in soil colonization compared to the wild type strain (data not shown). Values for soil colonization at 0 days of incubation suggest that cell size, cell density per OD unit, the ability attach to soil particles, or a combination of these factors varies slightly for the test strains. Nevertheless, at an inoculum density corresponding to

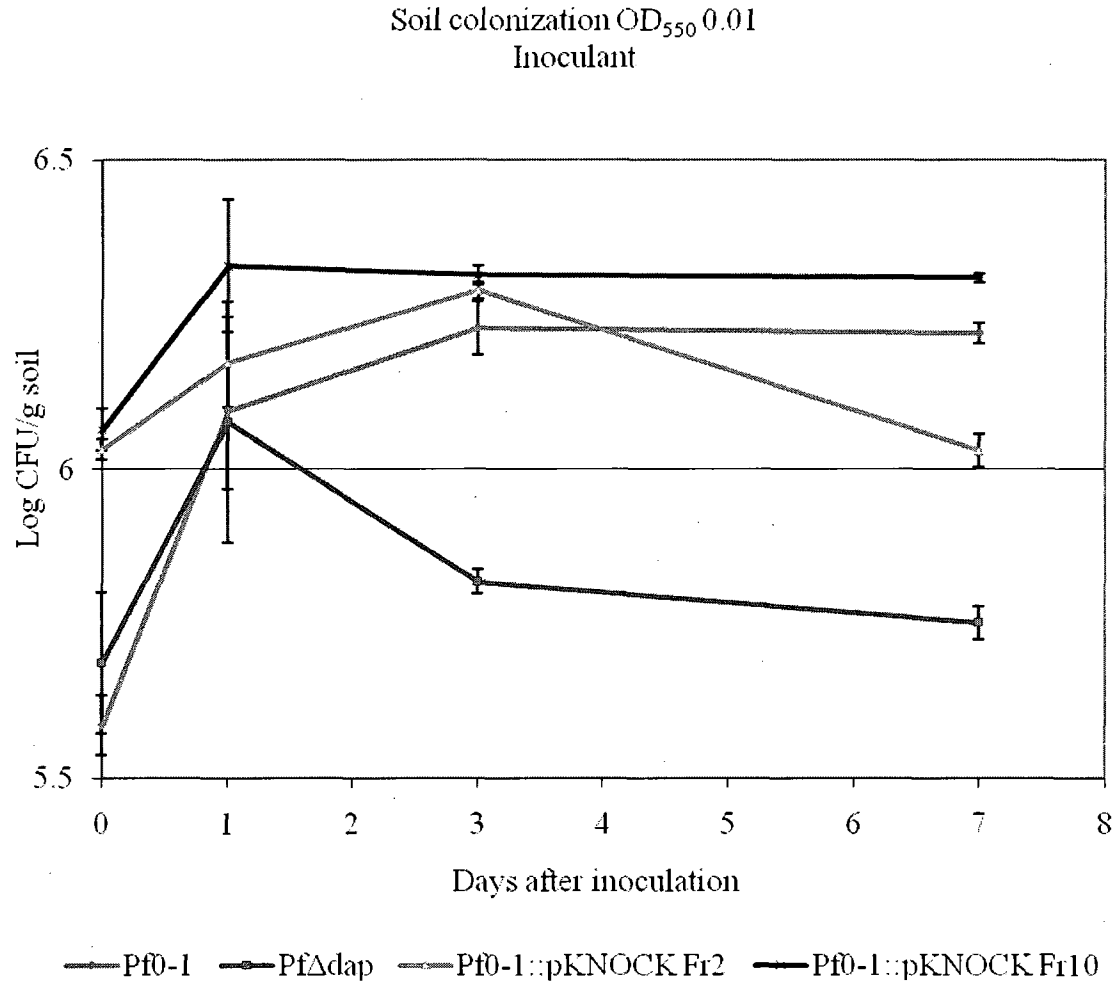


Figure 3.5 Arid soil colonization patterns of knock-out strains and other derivatives. Inoculum suspensions were adjusted to 0.01 OD_{550}. Pf0-1::pKNOCK Fr2 contains a partial deletion of Pfl01_2143, which encodes glutamine synthetase. Pf0-1, wild type; PfΔdap, dap auxotroph. Pf0-1::pKNOCK Fr10 features a partial deletion of Pfl01_5595, which expresses a protein of unknown function. The experiment was repeated three times (*t* test: $P<0.05$).

OD_{550nm} 0.01, approximately 10^5 CFU/ml, Pf0-1::pKNOCK Fr10 exhibited a more pronounced decline in Log CFU/g of soil than either the wild type strain or Pf0-1::pKNOCK Fr2. As expected, PfΔdap showed an even faster decline in Log CFU/g soil values than that observed for Pf0-1::pKNOCK Fr2. We also conducted soil colonization assays using a lower inoculum density adjusted to OD_{550nm} 0.001, approximately 10^4 CFU/g soil. Figure 3.6 shows the growth patterns of the test strains used in this study and indicates that inoculum concentration influences soil colonization differently in the test strains. While genetic inactivation of Fragment 2 showed similar values in Log CFU/g soil to those observed for the wild type at a lower inoculum concentration, it resulted in a significant decline when cells were placed in soil at a higher inoculum concentration. On the other hand, a significant decline in soil colonization was observed in strains that contained genetic defects in Fragment 10 at a low concentration, but not at a high concentration. The decline in soil colonization observed in either Pf0-1::pKNOCK Fr2 or Pf0-1::pKNOCK10 was less striking than that observed in the DapB-deficient strain.

In light of these results, we conducted soil colonization assays for Pf0-1::pKNOCK Fr2 and its derivatives at a high inoculum and for Pf0-1::pKNOCK10 and its derivatives at a low inoculum. Pf0-1::pKNOCK Fr2 and Pf0-1::pKNOCK Fr10 were complemented in *trans* by using the plasmid pJB866. Soil colonization of the mutant strains and their complemented counterparts is shown in figures 3.7 and 3.8. Interestingly, carrying the plasmid used for complementation impaired the ability of the Pf0-1::pKNOCK Fr2 and Pf0-1::pKNOCK Fr10 to colonize soil; these strains showed a significant decrease in Log CFU/g of soil compared to the knockout strains carrying no complementing plasmid.

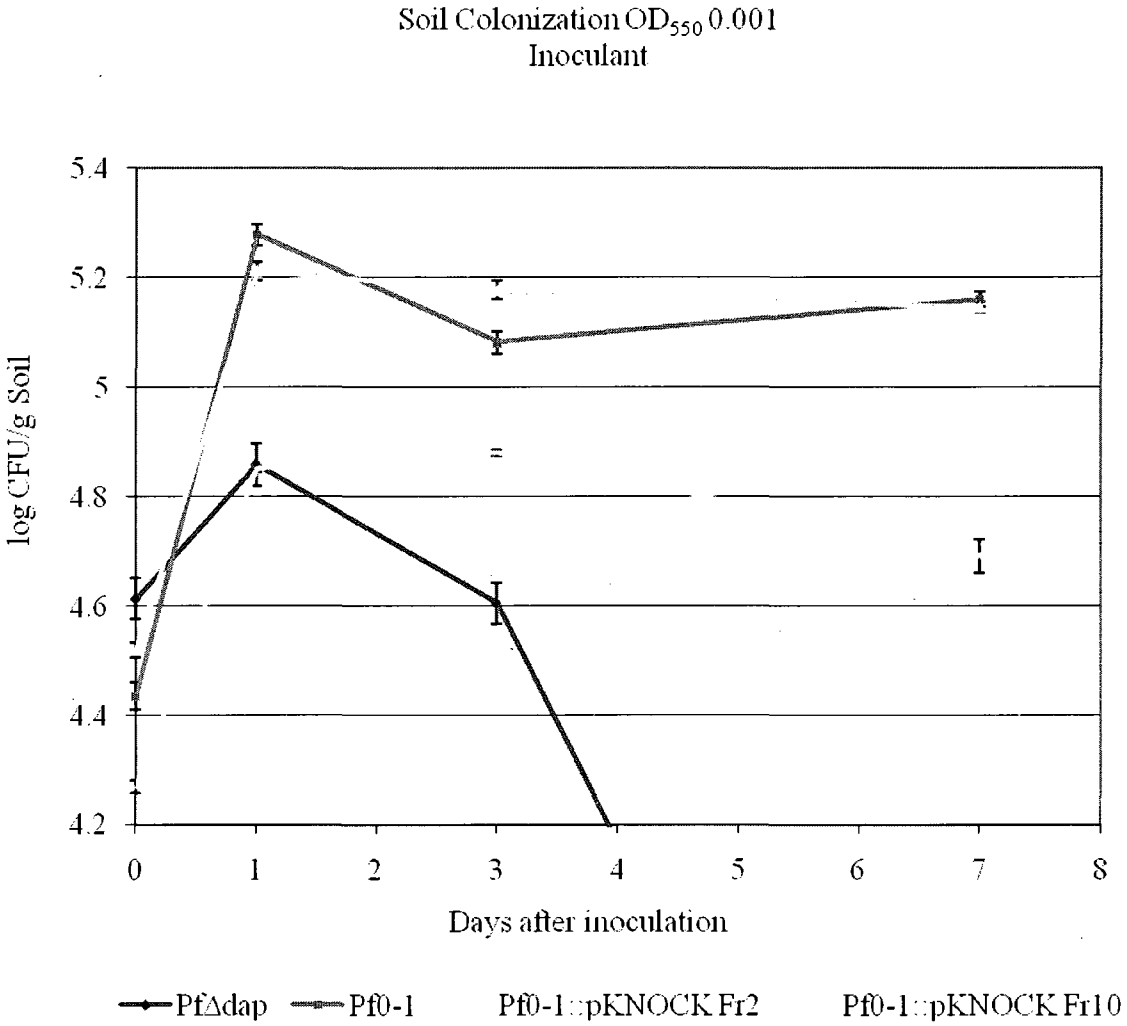


Figure 3.6 Arid soil colonization patterns of knock-out strains and other derivatives. Inoculum suspensions were adjusted to 0.001 OD_{550}. Pf0-1::pKNOCK Fr2 contains a partial deletion of Pfl01_2143, which encode glutamine synthetase. Pf0-1, wild type; PfΔdap, dap auxotroph. Pf0-1::pKNOCK Fr10 features a partial deletion of Pfl01_5595, which expresses a protein of unknown function. The experiment was repeated three times (*t* test: $P<0.05$).

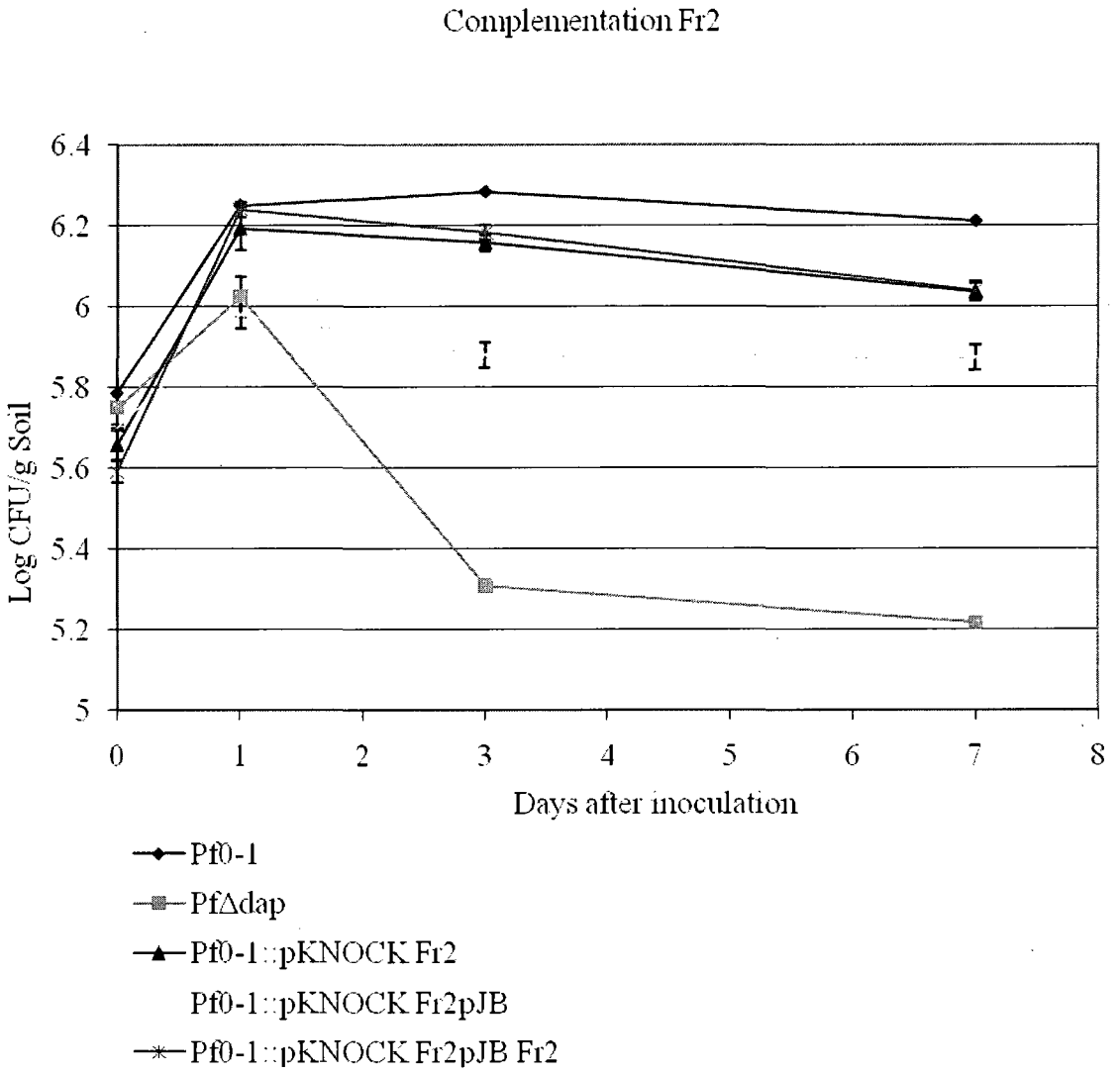


Figure 3.7 Arid soil colonization patterns of knock-out strains and other derivatives. Inoculum suspensions were adjusted to 0.01 OD_{550}. Pf0-1, wild type; PfΔdap, dap auxotroph. The strain Pf0-1::pKNOCK Fr2 contains a partial deletion of Pfl01_2143, which encodes glutamine synthetase. Pf0-1::pKNOCK Fr2pJB is a knock-out strain complemented with the vector pJB866. Pf0-1::pKNOCK Fr2pJB Fr2 is a knock-out strain complemented with the vector pJB866 with a full length Pfl01_2143. The experiment was repeated three times (*t* test: $P<0.05$).

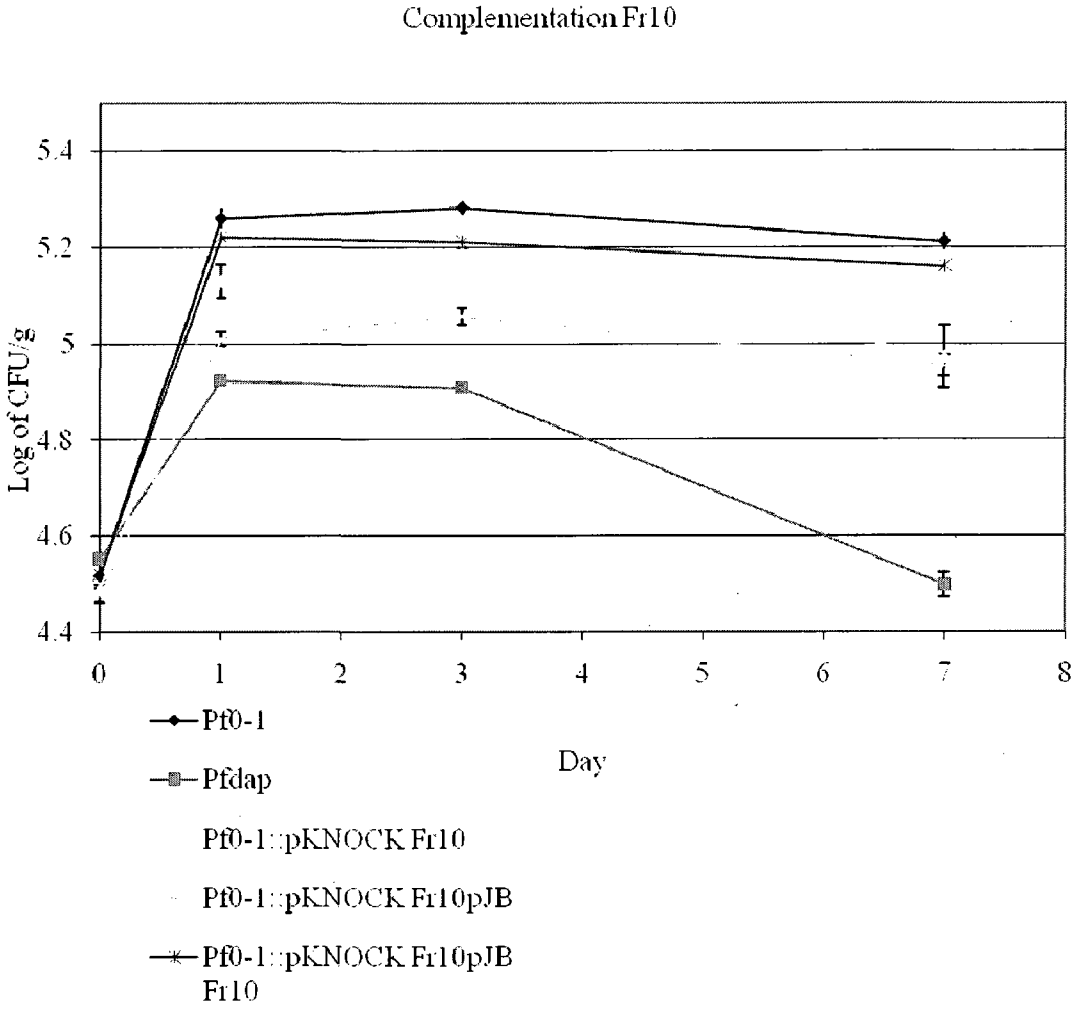


Figure 3.8 Arid soil colonization patterns of knock-out strains and other derivatives. Inoculum suspensions were adjusted to 0.001 OD_{550}. Pf0-1, wild type; PfΔdap, dap auxotroph. The strain Pf0-1::pKNOCK Fr10 contains a partial deletion of Pfl01_5595, which encodes a protein of unknown function. Pf0-1::pKNOCK Fr10pJB is a knock-out strain complemented with the vector pJB866. Pf0-1::pKNOCK Fr10pJB Fr10 carries the vector pJB866 with a full length Pfl01_2143. The experiment was repeated three times (*t* test: $P<0.05$).

Partial complementation was observed when Pf0-1::pKNOCK Fr2 and Pf0-1::pKNOCK Fr10 possessed pJB866 with the corresponding complementing gene. Perhaps there is an associated cost in fitness by carrying pJB866. This was evident by the presence of a subpopulation of cells that lost the plasmid after exposure to soil (data not shown). Our soil colonization assays suggest that nitrogen is limiting in arid soil conditions and that deficiency of such nutrient compromises the ability to attain and maintain high populations in arid soil. Moreover, while nitrogen metabolism, as indicated by defects on Fragment 2, appears important for reaching high populations, defects in Fragment 10 seem to influence maintenance of soil populations at lower levels. Alternatively, it also possible to speculate that defects in the genes encoded by these fragments affect desiccation tolerance of soil populations. Previous studies conducted in the same system indicate that soil moisture limits growth after three days of incubation. This is evident because the observed leveling off of log CFU/g soil after day three of incubation is independent of inoculum levels. This is consistent with the observations on water loss experiments that were conducted in this soil. Future experiments involving different soil moisture and inoculation levels will elucidate the effects of Fragment 2 and Fragment 10 on adaptation of *P. fluorescens* to arid soil.

3.4.3 Comparison of arid and agricultural soil inducible genes

Recently Mark W. Silby and Stuart B. Levy identified 22 *iiv* genes in *P. fluorescens* strain Pf0-1 that are expressed in an agricultural soil from Massachusetts, using a similar IVET approach (15). Similar to our studies, close to 50 percent of the "fusions" reported in that study were found to be transcribed "cryptically". Moreover, only three of the 22 showed an effect on early soil colonization. Further comparison of

the "agricultural soil" genes and those reported here indicate no overlap in gene expression in these two diverse environments. These observations suggest that *P. fluorescens* follows very distinct strategies to establish growing populations and to persist when exposed to soil environments and that antisense regulation is a key concept in adaptation to diverse environments.

3.5 Conclusion

This study identified arid-soil inducible genes that contribute to survival in *P. fluorescens* strain Pf0-1. The genetic regions featured here are expressed either as predicted by the genome annotation or in the opposite orientation, which suggests antisense or mRNA stability as a mean to control gene expression in arid soil. Further examination of a select group of four genes indicated that inactivation of two of these genes negatively affected the ability of *P. fluorescens* to colonize arid soil. Sequence analysis of these two genes revealed that nitrogen metabolism and uncharacterized functions significantly affect adaptation to arid soil. However, the phenotypes conferred by these genes were not as striking as the one observed in a lysine auxotroph strain. Perhaps adaptation to soil environments requires expression of many genes that act additively. This concept may be addressed by conducting future soil colonization studies that involve double knock-outs. Another intriguing area of future studies would be the characterization of those regions that are transcribed opposite to the annotated ORF as it may provide insights into gene regulation in diverse and fluctuating environments.

3.6 References

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VITA

Graduate College
University of Nevada, Las Vegas

Katila Pipitone

Home address:
6728 Divers Loons Street
North Las Vegas, NV 89084

Degree:
Bachelor of Science, School of Biological Sciences, 2003
Mahodol University International College

Thesis Title:
Identification of Arid Soil Inducible Genes in *Pseudomonas fluorescens* Strain Pf0-1

Thesis Examination Committee:
Chairperson, Dr. Eduardo Robleto, Ph.D.
Committee Member, Dr. Ronald Yasbin, Ph.D.
Committee Member, Dr. Helen Wing, Ph.D.
Graduate Faculty Representative, Dr. Ronald Gary, Ph.D.