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CARBON, NITROGEN, AND OXYGEN LIMITATION-INDUCED LOCI OF RHIZOBIUM MELILOTI ISOLATED BY TN5-LUXAB MUTAGENESIS AND THEIR ROLE IN COMPETITION AND SURVIVAL

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Daniel Martin Ragatz

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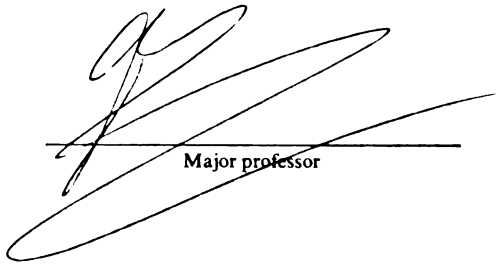

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By

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ABSTRACT

CARBON, NITROGEN, AND OXYGEN LIMITATION-INDUCED LOCI OF *RHIZOBIUM MELILOTI* ISOLATED BY TN*5-LUXAB* MUTAGENESIS AND THEIR ROLE IN COMPETITION AND SURVIVAL

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Soil bacteria, such as *Rhizobium meliloti,* live in environment where nutrients are scarce, and slow growth or no growth of bacteria over time is the rule, rather than the exception. Competition for available resources is very high, and changing environmental conditions, such as exposure to microaerobiosis (or anoxia) due to oxygen gradients within the soil pore matrix, requires the bacteria to sense and respond in a timely manner in order to survive. In addition, the processes of microbial competition and colonization of plant roots are of great importance when contemplating the use of beneficial microbes in agriculture or for bioremediation purposes. In order to examine this problem, a Tn*5-luxAB* transposon was used to generate insertions in *R. meliloti,* which could then be examined for induction by environmental conditions.

A large collection of genes (69) was isolated in response to three conditions: nitrogen and carbon limitation, and microaerobiosis at 1% O_2 concentration. Many of the tagged loci were similar to known genes, and some of these genes were even known to be regulated by the stress of interest. However, most of the tagged loci were novel, and thus seem to be specific in *R. meliloti* to the starvation conditions (or to microaerobiosis). This supports the hypothesis that soil microorganisms, such as *R. meliloti,* regulate gene expression in respon se to soil and rhizosphere conditions differently than non-soil bacteria such as *Escherichia coli.*

The importance of these tagged loci in competition and persistence experiments was tested and several *R. meliloti* strains harboring nutrient regulated fusions were significantly reduced in numbers when coinoculated with the wild-type 1021 in nutritionally poor soil. However, competition for nodulation experiments with these same strains yielded the surprising finding that none of the Tn*5*-1063 tagged loci were necessary for nodule competitiveness versus the wild-type strain 1021.

It's harder to believe than not to.

- Steve Taylor

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CHAPTER 1

INTRODUCTION

Parts of this chapter have been submitted for publication in *Molecular Microbiology* (Ragatz *et al., 1997).*

INTRODUCTION

Over the last two decades, interest in microbial ecology has increased dramatically as new molecular tools have become available for detecting and tracking microbial populations in natural environments, and as interest has grown in the use of genetically engineered microorganisms (GEMs) for a variety of purposes, such as biodegradation. Of particular interest has been an understanding of how bacteria sense and respond to changing environmental conditions. In the first part of this chapter I will give an overview of one such natural environment, the bacterial soil system, and in the second part I will discuss two stresses to which microorganisms are subjected in such an environment. This chapter will not be an attempt to give a comprehensive review, but to highlight findings that are definitive and to provide an overview of soil microbial ecology.

NOTE: The word 'starvation' has been widely used in the literature, but as this implies an understanding of the physiological response of the bacterium, I have chosen to use the word 'deprivation' in this thesis.

BACTERIA IN THE SOIL

Soil bacteria in the environment are frequently exposed to nutrient deprivation conditions. In fact the majority of non-rhizosphere soil is so oligotrophic that it has been called a "nutritional desert" (Metting, 1985). Consequently, bacterial growth is extremely limited and non-growth, or very low growth, may be

considered the norm (Kjelleberg *et al.*, 1987). The mean generation times of bacteria in the soil have been estimated to be from less than 1 to 80 per year (Shields *et al.*, 1973; Gray, 1976; Lynch, 1988; Matin, 1991), for bacteria whose generation times may be hours or less in a laboratory setting.

Not only is the soil environment largely oligotrophic, it is an extremely complex mixture of variously sized organic and mineral particles, living organisms, and their remains. It has been described as the most complex microbiological habitat (Stotzky, 1972; Metting, 1993). Bacteria inhabit soil pores of varying sizes and compositions, and the analysis of the effects of soil factors should actually be judged at the level of the soil pore rather than soil as a whole (Smiles, 1988). Figure 1-1 illustrates microbial microcommunities in the soil pores of a complex soil matrix. Microbial microcommunities in soil pores are dynamic due to the rapid flux of water, solutes, and other environmental cues, as well as the particular make-up of the microbial communities. Environmental factors will vary greatly both in time and across short distances within microhabitats (Metting, 1993).

Bacteria are not randomly scattered among or in soil pores, but are generally adsorbed to soil particles (Hattori and Hattori, 1976), often preferentially to organic or organic/clay complexes (Hisset and Gray, 1976). Paul and Clark (1989) showed that bacteria occupy less than 1% of the soil pore space; therefore, bacteria are more numerous within microhabitats such as soil pores than total soil counts would suggest. Competition for available resources, as well as the ability to sense and respond to environmental changes, are therefore very important to the survival of a given species of microorganism.

The types of bacteria present in bulk soil vary according to the conditions of the soil and environment, but are largely gram negative rods (Alexander, 1977;

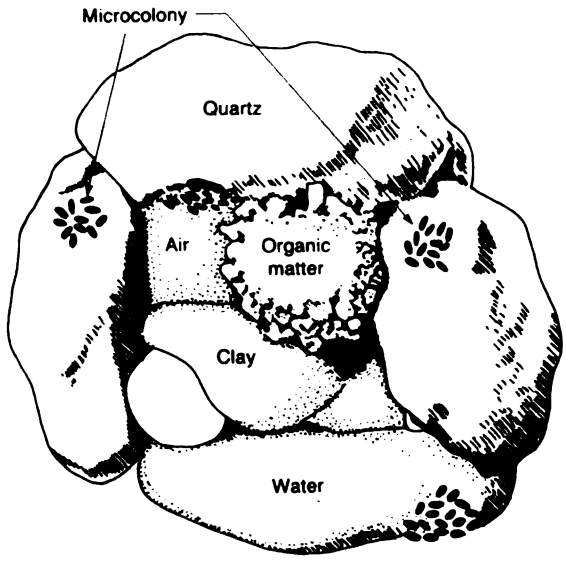

Figure 1-1. Section through a soil matrix showing microhabitats and patchy distribution of bacterial microcolonies. Air and water pores are also indicated (Brock, 1979).

Atlas and Bartha, 1989). *Bacillus* sp. (a gram-positive species) are sometimes present in large numbers (Alexander, 1977), however, their population size is misleading since viable counts do not distinguish between colonies developed from a spore or a vegetative unit. Frequently 60 to 100 percent of *Bacillus* cells in the soil exist as spores (Mishustin and Mirsoeva, 1967; Siala *et al.*, 1974).

Understanding how bacteria survive and persist under oligotrophic conditions is important both in terms of increasing our fundamental knowledge of microbial ecology, as well as in applied contexts, such as the release of genetically engineered microorganisms (GEMs) into the environment. In addition, isolation of environmentally regulated promoters will aid in designing GEMs to be used in

specific environmental situations, such as in bioremediation experiments, where nutrient regulated promoters can act as switches to provide a greater level of regulation of gene expression.

BACTERIA IN THE RHIZOSPHERE

In contrast to bacterial numbers in bulk soil, bacterial numbers in the rhizosphere—an area encompassing the surface of the root (the rhizoplane) to a few millimeters away from it—are much higher. This rhizosphere effect is often expressed as a ratio of the microbial population in the rhizosphere to that in the bulk soil (the R/S ratio). Both bacterial numbers and diversity are increased in the rhizosphere (Foster, 1986; Foster and Rovira, 1978) with R/S ratios in the tens to hundreds (Bolton *et al.*, 1993). This effect is due to an increase in soluble carbon and nutrients found close to the plant roots.

These compounds encompass a wide variety of substances from low-molecular weight compounds such as sugars and amino-acids, to mucilage and lysates, and become available through root leaching, as well as active secretion by the plant root (Bolton *et al.*, 1993). In fact, the presence of microorganisms in the rhizosphere has been shown to increase active root exudation (Barber and Martin, 1976). Many other factors affect root exudation including temperature, irradiance, soil moisture content, plant age and nutrient status, and stresses (Rovira, 1959; Vancura, 1988).

Although more nutrients are available, the higher number of microorganisms in the rhizosphere makes competition for these nutrients even more important. This is exacerbated by the fact that bacteria are not randomly

distributed on the root surface, but limited to microcolonies covering only 4-10% of the root surface (Rovira *et al,* 1974; Rovira, 1979). The position of these microcolonies correlates with the presence of soil organic matter on the root surface and often coincides with epidermal cell junctions (Bowen and Rovira, 1976; van Vuurde and Schippers, 1980). In addition, the higher concentration and diversity of microorganisms can increase the level of deleterious compounds, such as antibiotics, produced by competing microorganisms. Also, oxygen levels can be severely depleted due to root and microbial respiration leading to conditions favorable for anaerobic or microaerobic growth (Alexander, 1977). Therefore, the growth of microorganisms even in the relatively nutrient-rich rhizosphere can still be limited (van Elsas and van Overbeek, 1993).

As in bulk soil in general, rhizosphere microorganisms are predominantly short, gram negative rods (Alexander, 1977). In fact, the percentage occurrence of short, gram-positive rods, so called "coccoid" rods, and spore-forming bacteria declines (Alexander, 1977). The reason for this observation is not clear; there does not appear to be a selective stimulation or inhibition of gram-variable rods. *Pseudomonas, Flavobacterium, Alcaligenes,* and *Agrobacterium* species are especially common (Alexander, 1977). In contrast to the rhizosphere effect on bacteria, total counts of fungi in the rhizosphere are not significantly altered compared to bulk soil, however the predominance of specific fungal genera may be stimulated (Alexander, 1977; Sivasithamparam *et al.*, 1979).

BACTERIAL RESPONSE TO ENVIRONMENTAL STRESSES

Bacteria in the soil are subjected to a large number of stresses which affect their physiological state. The intensities and interactions among these stresses vary over time, as well as over very small distances from one microcommunity to the next. Stress factors which are most important include nutrient deprivation, oxygen availability, water limitation, temperature and pH extremes, and UV irradiation. This chapter will only discuss the two stresses that are relevent to the research described in later chapters, i.e., nutrient deprivation and oxygen availability. For an in-depth consideration of the soil environment, the reader is referred to texts by Alexander (1977), Lynch (1988), van Elsas and van Overbeek (1993), and Metting (1993).

Nutrient deprivation

The manner in which bacteria deal with the problem of nutrient deprivation varies between classes of microorganisms. Some bacteria, such as *Bacillus* sp. and *Myxococcus* sp., can differentiate into stress resistant endospores and myxospores, respectively, when confronted with nutrient deprivation (Losick *et al.*, 1986; Kaiser, 1986). This process has been well studied and involves a complicated cascade of gene expression, temporally regulated by an alternative sigma factor (sigma B), which in turn is post-translationally regulated by a multi-component network of regulatory proteins (Hecker and Völker, 1990; Antelmann *et al.*, 1996; Akbar *et al.*, 1997). However, the majority of bulk soil bacteria are actinomycetes and gram negative, non-sporulating rods (Rovira, 1956; Alexander, 1977; Lynch and Wood,

1988; Atlas and Bartha, 1989), most of which are not known to differentiate into protective forms, and thus, must deal with nutrient deprivation by other means. These bacteria include *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Erwinia*, *Pseudomonas*, *Rhizobium*, and *Xanthomonas* species (Miller and Wood, 1996).

Matin *et al.* (1989) have described two fundamental ways in which gram negative bacteria confront nutrient deprivation, both of which involve the synthesis of new proteins in response to the deprivation condition: (i) by altering the bacterium to make it a more efficient scavenger of scarce nutrients, or (ii) by making it more resistant to stresses in general. Thus, understanding which genes are regulated before, during, and after the onset of deprivation conditions is important for the elucidation of the mechanisms by which bacteria survive and compete under oligotrophic conditions.

Contrary to earlier assumptions, so-called non-differentiating bacteria undergo an elaborate process of molecular realignment when they are exposed to deprivation conditions, which lead to the development of a resistant cellular state. For example, when *E. coli* is starved it forms small, nearly spherical cells and develops enhanced resistance to a variety of stresses including oxidation, hyperosmosis, heat, low pH, and disinfectant agents (Matin, 1990). Similar states of enhanced resistance have been observed in *Salmonella typhimurium* (Spector and Cubit, 1992), *Pseudomonas putida* (Givskov *et al.*, 1994), and *Rhizobium leguminosarum* (Crockford *et al.*, 1996). In *Vibrio* sp., starved cells form ultramicrocells and develop resistance against growth inhibiting agents and conditions (Holmquist *et al.*, 1994). Interestingly, it has been found that carbon, rather than nitrogen or phosphorus, deprivation is the determinant in forming this resistant state (Holmquist and Kjelleberg, 1993; Nyström *et al.*, 1992). This is in

contrast to *E. coli* where cells will form their protective state regardless of the specific nature of the deprivation condition (Matin, 1990).

Most of the knowledge regarding deprivation responses in non-differentiating bacteria derives from work in *E. coli, S. typhimurium,* and *Vibrio* sp. In *E. coli,* over 70 polypeptides that are initiated or increased during carbon, phosphorus, or nitrogen deprivation have been visualized using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Matin, 1991). Many of these polypeptides are uniquely induced by deprivation of a single nutrient; however, several of them are induced by two deprivation conditions and a group of fifteen proteins constitutes a core set which is induced under all deprivation conditions examined. Similar results have been observed using 2D-PAGE protein analysis of *S. typhimurium* (Spector and Cubitt, 1992), where six polypeptides are induced by three or more conditions, and *Vibrio* sp. (Nyström *et al.*, 1990) where three proteins are commonly induced during nutrient deprivation conditions.

Although much is known about bacterial gene expression patterns from laboratory studies involving growth of bacterial cultures under ideal conditions in terms of temperature, pH, and nutrients, comparatively little is understood about their behavior under unfavorable conditions, such as those found in the soil environment. In fact, few investigations on nutrient deprivation-induced gene expression have been carried out on common soil isolates, which may have different strategies for dealing with nutrient deprivation than enteric or marine bacteria. Only recently, the effect of nutrient deprivation on *P. putida,* a common gram negative soil bacterium, has been reported (Givskov *et al.*, 1994). A large number of proteins induced by carbon and nitrogen deprivation have been detected using 2D-PAGE protein analysis. These proteins are temporally regulated,

as in the case of *E. coli* and *Vibrio* sp., and include a group of eight proteins induced by multiple stresses. Another common soil isolate, *Anabaena* sp., has been studied under nitrogen deprivation conditions using a *luxAB* reporter (Cai and Wolk, 1997). Several genes were isolated and some were similar to genes required for nitrite and nitrate uptake and utilization.

To date, only a few roles have been assigned to the large collection of deprivation-induced proteins in any of the bacterial species studied (Givskov *et al.*, 1994). However, some progress has been made in identifying the core proteins induced under multiple conditions. In *E. coli*, for instance, three of the 15 core proteins have been identified: DnaK, GroEL, and HtpG. These proteins had first been identified in response to heat shock, but they are also increased during deprivation (Matin, 1991). These proteins appear to have a role in protein folding and macromolecular assembly (chaperone function).

Regulation of the genes encoding these proteins has not been examined in most of the organisms studied, except for *E. coli* where the regulatory mechanisms have been at least partially elucidated. Many of the core proteins appear to be regulated by the heat-shock alternative sigma factor σ^{32}, but are not dependent on cyclic AMP/CRP. In contrast, most of the carbon-deprivation induction specific proteins are regulated by cAMP/CRP. The putative sigma factor, KatF, has been found to regulate most of the carbon deprivation-induced proteins, as well as six of the core proteins (McCann *et al.*, 1991). The core proteins appear to have a distinct role in the development of cellular resistance, since the deletion of *rpoH* (encoding σ^{32}) in *E. coli* results in an approximately 50% reduction in strain viability under carbon deprivation conditions (Jenkins *et al.*, 1991). Recently, two carbon deprivation regulated genes in *P. putida* have been isolated using mini-Tn*5*

transposon mutagenesis (Kim *et al.*, 1995). These genes were found to be regulated by another sigma factor, σ^{54}. This sigma factor has been shown to be required for the expression of many genes such as flagellin in *Pseudomonas* sp. (Totten *et al.*, 1990). However, this is the first time it has been implicated in the regulation of deprivation-induced genes (Kim *et al.*, 1995).

A few other deprivation enhanced (induced) proteins have been elucidated: a protease (Reeve *et al.*, 1984), the ribosomal modulation factor Rmf (Wada *et al.*, 1990; Yamagishi *et al.*, 1993), and the product of the *uspA* gene, the universal stress protein (Nyström and Neidhardt, 1992, 1993). The latter protein is induced by the cessation of cell growth, regardless of the condition inhibiting growth, and its regulation is independent of all global regulators tested thus far including RpoS, PhoB, RelA, SpoT, AppY, OmpR, H-ns, Lrp, and RpoH (Nyström and Neidhardt, 1992). Deletions in UspA result in mutants which are highly susceptible to a variety of stresses including osmotic shock and carbon deprivation (Nyström and Neidhardt, 1994). UspA is thought to modulate the flow of carbon in central metabolic pathways, however its regulation and the subsequent pleiotrophic phenotype diplayed in *E. coli* is not understood. There is no evidence to indicate that UspA is a global regulator of gene expression; however, overproduction of UspA seems to affect the pI of other proteins, which may indicate a possible role of UspA in post-translational modification (Nyström and Neidhardt, 1994).

Oxygen limitation

The availability of oxygen in natural environments, such as soil, is one of the most important parameters affecting growth and competition of microorganisms.

Soils are frequently subjected to varying oxygen conditions due to rainfall and drying. When soils are flooded a unique oxidation-reduction profile is created with a thin, oxidized soil surface layer, and an underlying thicker, reduced layer deficient in oxygen. Even within soil particles, steep oxygen gradients exist within a few millimeters of the soil surface. Soils with a sufficient supply of decomposable organic matter may be depleted of oxygen within one day, due to growth of heterotrophic microorganisms that scavenge the available oxygen (Foth, 1984). Thus, in order to survive and compete, soil microorganisms must be able to adapt to changing oxygen conditions by, (i) altering their ability to bind oxygen as terminal electron acceptor via alternative cytochromes, (ii) by using alternative electron acceptors such as nitrate, or (iii) changing to anaerobic metabolism.

Genes regulated in response to low oxygen or anaerobiosis have been described in many systems. Among the most well studied organisms are the facultative anaerobes, such as *E. coli*, *Bacillus* sp., *S. typhimurium*, *Pseudomonas aeruginosa*, *Vibrio fischeri*, and *Rhodobacter sphaeroides*. In *E. coli*, two global regulatory systems have been described for sensing and responding directly to molecular oxygen: a two-component sensor/regulator system comprised of ArcB/ArcA (Iuchi and Lin, 1988, 1992, 1993), and a transcriptional sensor-regulator protein, FNR (Lambden and Guest, 1976; Chippaux *et al.*, 1978; Spiro and Guest, 1990; Lin and Iuchi, 1991). Genes responding to these regulators are part of a group of four global regulatory systems required during anaerobic metabolism, and a significant overlap occurs among the regulatory systems. Many other bacterial systems have been identified with FNR homologues (Fischer, 1994; Spiro, 1994).

In addition to genes responding to the absence of oxygen, specific microaerobic (microoxic) respiratory chains, utilizing a set of genes which are different than those expressed during anaerobic conditions, are induced during microoxic conditions in *E. coli* (Lin and Iuchi, 1991; Gunsalus, 1992). These microoxic conditions also induce gene expression by aerobic rhizobia. The well-studied two-component FixL/FixJ system (Figure 1-2) senses and responds to low oxygen levels and initiates a complex cascade of gene regulation events (Agron *et al.*, 1994; Fischer, 1994). The FixL/FixJ regulatory cascade is extremely complex, and varies among the rhizobial genera (Figure 1-2). This system is responsible for the microaerobically-regulated gene expression patterns described in rhizobia to date, including the genes required for nitrogen-fixation (*nif & fix;* David *et al.*, 1988), as well as genes necessary for rhizopine synthesis (*mos;* Murphy and Saint, 1991), nodulation competitiveness (*nfe;* Sanjuan and Olivares, 1989, 1991; Soto *et al.*, 1993, 1994), and melanin production (*melA and melC;* Hawkins and Johnston, 1988; Hawkins *et al.*, 1991). In *Bradyrhizobium japonicum*, it has also been shown to control genes required for anaerobic nitrate respiration (Anthamatten and Hennecke, 1991; Fischer, 1994). Recently, however, Zhulin *et al.* (1995) demonstrated that FixL and FixJ were not involved in oxygen response and regulation of *R. meliloti* behavior (chemokinesis and aerotaxis) in oxygen gradients, indicating that at least one other oxygen sensing mechanism must exist in rhizobia.

Although much has been learned about the nature of the genes, promoters, and transcriptional control of these regulons, the nature of the sensing systems involved in responding to fluctuation in concentration of molecular oxygen is still poorly understood. The mechanism of O_2-sensing appears to be different in different systems. The FixL mediated pathway is a little better understood since the

FixL protein has been found to contain a heme moiety which is thought to interact directly with oxygen, resulting in an alteration of the Fe oxidation state, which in turn results in the autophosphorylation of FixL (Gilles-Gonzalez *et al.*, 1991). However, it is not known whether the regulation occurs intramolecularly through conformational changes or intermolecularly by promoting or preventing dimerization (Agron *et al.*, 1994). FNR also contains a metal cofactor, Fe(II) (Spiro, *et al.*, 1989) which is required for FNR to act as a transcriptional activator (Green *et al.*, 1991, 1993). It is thought that FNR senses oxygen by participating in a redox reaction that results in the conversion of FNR to a transcriptionally inactive state (Unden *et al.*, 1994). Unlike FixL and FNR, ArcB lacks O_2-reactive sites such as hemes, iron-sulfur clusters or metal ions which could interact directly with oxygen (Iuchi *et al.*, 1990). Spiro and Guest (1991) have suggested that the source of the signal to ArcB is in the electron transport chain and that ArcB detects changes in the ratio of the oxidized to reduced forms of an electron carrier such as a flavin, heme, quinone, or iron-containing component. However, there are no indications that redox mediators, such as quinones or redox enzymes, interact with ArcB (Unden *et al.*, 1994).

SCOPE OF THIS THESIS

In this thesis, I am interested in answering several questions related to the background presented in this chapter.

1) How many loci are induced in *R. meliloti* during deprivation of nutrients and oxygen, and does this compare to genes induced in non-soil genera?

2) What is the nature of these genes and are they different from those found in non-soil genera?

3) Are genes induced by nutrient deprivation and oxygen limitation important for survival and competition in the soil and for competition for nodulation?

Except for recent analysis of *Pseudomonas putida* (Kragelund *et al.,* 1995) and *Anabaena* Sp. (Cai and Wolk, 1997), little work has been done with common soil isolates using molecularly tagged genes, making it difficult to hypothesize how this method would compare to 2D-PAGE analysis. Nevertheless, based on the *P. putida* data, I propose that the number of genes isolated will be similar to those found in non-soil genera. The nature of these genes, however, I propose to be different for soil isolates versus non-soil isolates. Further, I propose that these genes will be very important for competition in the soil, as well as for competition for nodulation.

Since few soil isolates have been studied to date, I have chosen to use *R. meliloti* for the research described in this thesis. *R. meliloti* is a common soil isolate with many advantages for studying environmentally regulated gene expression. First, It is a common gram negative soil bacterium. Second, it has been extensively analyzed using molecular genetic techniques, and both physical and genetic maps are available (Honeycutt *et al.,* 1993). Third, it is amenable to transposon Tn*5* mutagenesis. Finally, it has two distinct niches, free-living in the soil and in a highly specific symbiotic interaction with its plant-host (alfalfa), which allows a comparative study of the role of nutrient deprivation-induced genes in bulk soil, in the rhizosphere, in the infection process, and within the infected cells (nodules) of the plants.

Chapter 2 of this thesis describes the isolation and characterization of 22 genes tagged by Tn*5-lux* mutagenesis which are induced under nitrogen deprivation. Some of the cloned and sequenced genes are identical or similar to genes in bacteria known to be regulated by nitrogen deprivation. However, most of the *R. meliloti* genes induced by nitrogen deprivation appear to be novel. Chapter 3 describes the isolation and characterization of 12 genes tagged by Tn*5-lux* mutagenesis which are induced under carbon deprivation. One of the cloned and sequenced genes is similar to a gene in bacteria known to be regulated by carbon deprivation. Most of the *R. meliloti* genes induced by carbon deprivation also appear to be novel. Chapter 4 describes the isolation and characterization of 34 genes tagged by Tn*5-lux* mutagenesis which are induced under oxygen limitation. One of the cloned and sequenced genes is identical to a gene in rhizobia known to be microaerobically regulated. Most of the *R. meliloti* genes induced by microaerobiosis again appear to be novel.

Chapters 5 and 6 describe experiments designed to examine the importance of tagged loci for the survival and competition in soil and nodulation, respectively, of selected strains. The results of chapter 5 support the hypothesis that these genes are important in competition; however, the results of chapter 6 do not support the hypothesis that these genes are important for competition for nodule occupancy.

Chapter 7 discusses the overall collection of stress induced loci in *R. meliloti* from this study compared to stress induced loci from other bacteria (particularly *E. coli*), as well as other conclusions drawn from this study. In addition, directions for further research are discussed.

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CHAPTER 2

Isolation and Characterization of *Rhizobium meliloti* Genes Whose Promoters are Induced by Nitrogen Deprivation.

Parts of this chapter have been presented at the 6th International Microbial Ecology Symposium (Lim *et al.*, 1993) and the 10th International Nitrogen Fixation Symposium (de Bruijn *et al.*, 1995), and have been submitted for publication in *Molecular Microbiology* (Ragatz *et al.*, 1997).

NOTE: Parts of this chapter were contributed by PyungOK Lim (the generation of the mutant collection, Southern blot analysis of auxotrophs, and screening for nitrogen mutants), Michael Renner (the screening and characterization of auxotrophs), and Anne Milcamps (the gene-replacement of strain N5).

ABSTRACT

Soil bacteria are subject to constantly changing environmental conditions, and the ability of bacteria to sense these changing conditions and respond accordingly is of vital importance to their survival and persistence in the soil and rhizosphere. Nitrogen is particularly limited in the soil and rhizosphere, and consequently it has been of interest to understand how *Rhizobium meliloti*, a common soil isolate, senses and responds to this nutrient deprivation condition. To investigate this problem, *R. meliloti* 1021 was mutagenized using a derivative of Tn*5* which creates transcriptional fusions to promoterless *luxAB* genes. Subsequently, 5000 insertion mutants were screened for gene fusions induced by nitrogen deprivation. The isolation of twenty-two gene fusions induced by nitrogen deprivation is described. These fusions were found to be temporally regulated and induced to a range of intensities. The strains harboring Tn*5-luxAB* fusions were tested for nodulation and nitrogen-fixation phenotypes and two strains were found to be Fix$^-$. Cloning and partial DNA sequence analysis of the transposon tagged loci revealed a variety of novel genes, as well as *R. meliloti* genes with sequence similarity to known bacterial loci. Genes identical to already described genes for exopolysaccharide synthesis (*exoY* and *exoF*) were found, as well as genes with significant similarity to assimilatory nitrate and nitrite reductases.

INTRODUCTION

Bacteria in the soil and rhizosphere are subject to constantly changing environmental conditions to which they must adapt or be out-competed. Rhizobia in particular, must compete for a niche both in the soil as well as the rhizosphere of their host plant in order to be successful in nodulation. As mentioned in Chapter 1, the soil is very oligotrophic, and consequently nutrient deprivation is a common environmental factor. Available nitrogen is particularly limited in the soil and rhizosphere, and consequently it has been of interest to understand how *Rhizobium meliloti*, a common soil isolate, senses and responds to this nutrient deprivation condition.

Some genes have already been isolated in rhizobia and other bacteria that respond to nitrogen deprivation, including the nitrate and nitrite reductase genes (Goldman *et al.*, 1994; Cai and Wolk, 1997) and genes involved in formation of exopolysaccharides (Gray *et al.*, 1990; Ozga *et al*, 1994). In addition, rhizobia have been found to contain a two component nitrogen-regulatory system, consisting of *ntrB* and *ntrC* that is responsible for controlling nitrogen metabolism genes (Dusha and Kondorosi, 1993; Arcondeguy *et al.*, 1997), as well as participating in the regulation of nitrogen fixation genes (although this varies between rhizobial genera; Fischer, 1994; see also chapter 4, Figure 4-2). This *ntr* system regulates two of the three distinct loci involved in glutamine sythesis (*glnII* and *glnT*) in *R. meliloti* which are differentially regulated in response to varying ammonia levels (Szeto *et al.*, 1987; de Bruijn *et al.*, 1989).

In most systems described thus far, identification of nitrogen deprivation-induced proteins has been accomplished via 2D-PAGE analysis. This analysis has

some advantages, since it generates an overall view of the deprivation response at the protein level. However, it has disadvantages as well, since it fails to identify the regulated genes/promoters and corresponding mutations to ascertain the role of the genes.

Therefore, I have taken a different approach for investigating nitrogen deprivation-induced loci, by mutagenizing the genome of *R. meliloti* with a Tn*5* derivative containing the promoterless *luxAB* genes (Wolk *et al.*, 1991), encoding luciferase as gene reporter. This transposon creates transcriptional fusions, bringing the *luxAB* genes under the control of the tagged gene promoter. Under appropriate conditions, these gene fusions will express luciferase, resulting in bacterial bioluminescence (Meighen and Dunlap, 1993). The use of Tn*5* has been shown to be an extremely useful tool for random mutagenesis in many gram negative bacterial systems including *R. meliloti* (Meade *et al.*, 1982; De Vos *et al.*, 1986; Ditta, 1986; de Bruijn, 1987; Simon *et al.*, 1989; Sharma and Signer, 1990; de Bruijn and Rossbach, 1994). The use of luciferase as a reporter has proven to be useful in studies of bacterial gene expression (Carmi *et al.*, 1987; Heitzer *et al.*, 1994). In fact, recently we have used Tn*5*-1063 successfully in *Pseudomonas fluorescens* to isolate gene fusions whose bioluminescence were induced under nitrogen and phosphate deprivation (Kragelund *et al.*, 1995). And it has also been used in *Anabaena* sp. to isolate gene fusions induced by nitrogen deprivation (Cai and Wolk, 1997). In addition, *lux* or *luc* expression has been a useful marker in studies involving the tracking of microorganisms in the soil and rhizosphere (De Weger *et al.*, 1991; Beauchamp *et al.*, 1993; Boelens *et al.*, 1993; Möller *et al.*, 1994; Jansson, 1995).

In this chapter, the generation and characterization of a collection of 22 *R. meliloti* mutants generated by random Tn*5*-1063 mutagenesis, carrying gene fusions induced by nitrogen deprivation, is described. These fusions were found to be temporally regulated and induced to different levels of intensity. The strains harboring Tn*5-luxAB* fusions were tested for nodulation and nitrogen-fixation phenotypes and two strains were found to be Fix$^-$. Cloning and partial DNA sequence analysis of the transposon tagged loci revealed a variety of novel genes, as well as *R. meliloti* genes with sequence similarity to known bacterial loci, including the exopolysaccharide genes *exoY* and *exoF,* and assimilatory nitrate and nitrite reductases.

RESULTS

Tn*5*-1063 mutagenesis of *R. meliloti* 1021

Plasmid pRL1063a, carrying the promoterless *luxAB* transposon Tn*5*-1063 (Figure 2-1A), was used to generate a collection of 5000 *R. meliloti* 1021 insertion mutants. Insertion of Tn*5*-1063 into a *R. meliloti* gene can result in the creation of a transcriptional fusion, whereby the *luxAB* genes become controlled by the resident *R. meliloti* promoter (Figure 2-1B). Due to the presence of an *E. coli* origin of replication in Tn*5*-1063, the interrupted gene can be easily excised from the genome, ligated to form a self-replicating plasmid, and recovered by electroporation or calcium chloride transformation into *E. coli* (Figure 2-1C).

The insertional specificity of the Tn*5*-1063 transposon in *R. meliloti* 1021 was examined by screening the collection of 5000 for auxotrophs and determining the nature of the auxotrophies. Out of 5000 insertion mutants, 62 were found to be auxotrophic, a frequency of 1.2%, distributed among thirteen phenotypic groups (Table 2-1).

The insertional specificity of Tn*5*-1063 was also examined by Southern blot analysis. Total DNA of 69 *R. meliloti* strains, including auxotrophs and selected prototrophs, was isolated, digested with *Eco*RI or *Cla*I (neither enzyme cuts Tn*5*-1063), blotted, and hybridized with pRL1063a plasmid DNA as a probe. The analysis of the Southern blots revealed different sized bands for each isolate (not shown), suggesting random insertion of Tn*5*-1063 in *R. meliloti* 1021. In addition, the number of hybridizing fragments was examined, to determine whether the Tn*5*-1063 insertion occurred via a single transpositional event, or involved other

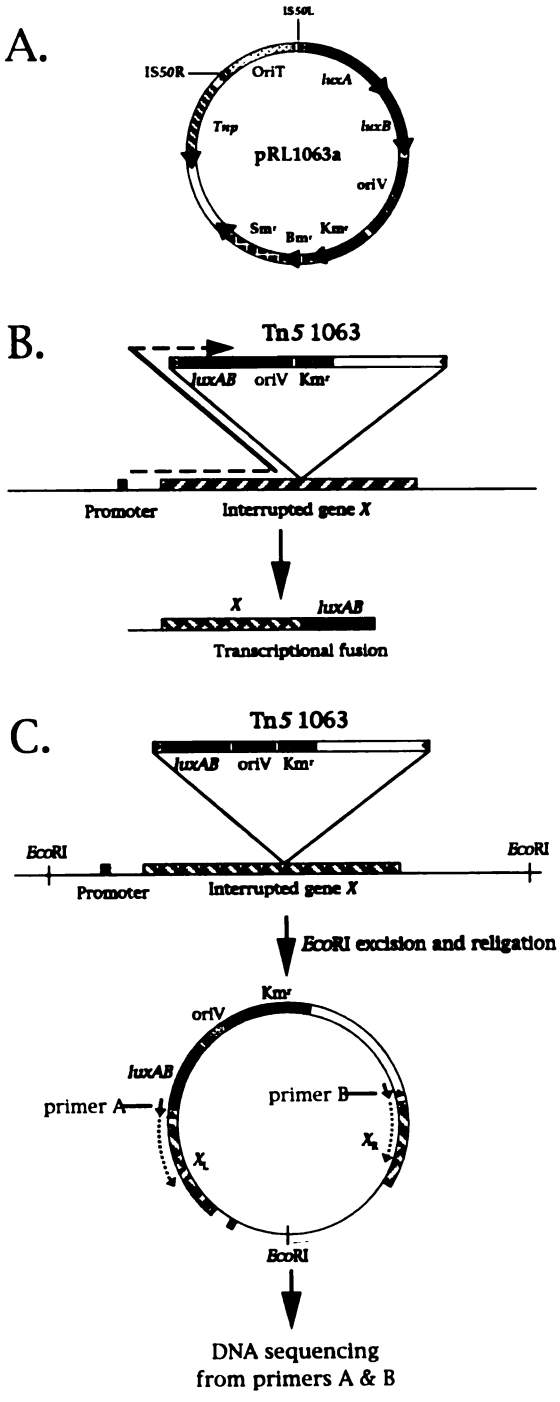


Figure 2-1. Tn5-1063 structure, mutagenesis protocol, and target junction DNA sequencing. The structure of the suicide plasmid carrying Tn*5*-1063 (Wolk et al., 1991) is shown in Panel A. In Panel B, the transcriptional fusions generated by Tn*5*-1063 in target genes is schematically diagrammed. In Panel C, the cloning of Tn*5*-1063 tagged loci is diagrammed and the position of the DNA sequencing primers (A, B), as well as the direction of the sequencing reactions are indicated.

Table 2-1. Phenotypes of selected auxotrophic Tn *5*-1063 insertion mutants of *R. meliloti* 1021.

Strains	Nutritional [a] Requirements	Hybridizing [b] Fragments	Symbiotic Phenotype	
			Nod [c]	Fix [d]
A1	Arginine	1	+	+
A2	Hypoxanthine	1	+	–
A3	Tryptophan	1	+	+
A4	Cytosine or Uracil	1	+	+
A5	Cytosine or Uracil	1	+	+
A7	Leucine	1	–	–
A8	Methionine or Cysteine	1	+	+
A10	Serine or Glycine	1	n.d.	n.d.
A11	Methionine	1	+	+
A12	Leucine	1	–	–
A13	Methionine	n.d.	+	–
A15	Serine or Glycine	n.d.	+	+
A16	Tryptophan	1	+	+
A17	Histidine	1	n.d.	n.d.
A18	Arginine	1	+	+
A19	Cytosine or Uracil	1	+	+
A20	Serine or Glycine	1	+	+
A21	Tryptophan	2	+	+
A23	Methionine or Cysteine	1	+	+
A26	Methionine	1	+	+
A29	Thiamin	1	+	+
A30	Tryptophan	1	+	+
A31	Adenine or Hypoxanthine	1	+	–
A33	Tryptophan	1	+	+
A35	Tryptophan	1	+	+
A36	Leucine	1	+	–
A37	Tryptophan	2	+	+
A39	Cytosine or Uracil	1	+	n.d.
A40	Tryptophan	1	+	+
A42	Tryptophan	1	+	n.d.
A44	Thiamin	1	+	n.d.
A46	Hypoxanthine	3	+	n.d.
A47	Tryptophan	1	+	+
A49	Tyrosine	1	+	+
A50	Cytosine or Uracil	1	+	–
A51	Adenine or Hypoxanthine	2	+	–
A54	Methionine or Cysteine	1	+	+
A56	Thiamin	2	+	+
A57	Methionine	1	+	+
A61	Methionine or Cysteine	1	+	+
A63	Methionine	1	+	+
A64	Methionine	1	+	+
A65	Cytosine or Uracil	1	n.d.	n.d.
A69	Histidine	2	+	+
A71	Phenylalanine	1	+	+

a) As determined by the Holliday (1956) method.
b) Number of genomic *Eco*RI fragments hybridizing with pRL1063a sequences.
c) Ability to nodulate alfalfa plants (de Bruijn *et al.*, 1989). n.d. = not determined.
d) Nitrogen fixation ability of alfalfa nodules as determined by the acetylene reduction assay (de Bruijn *et al.*, 1989)

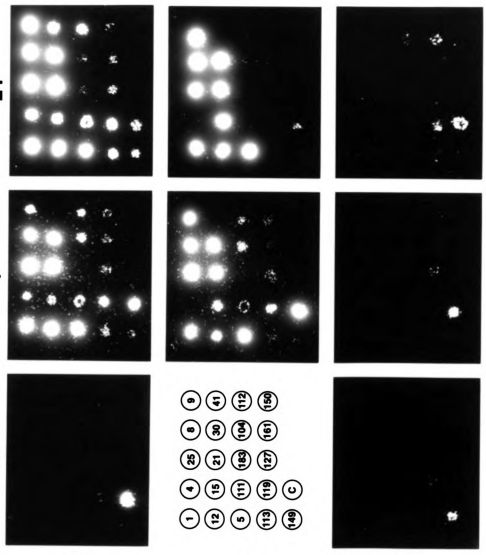
mechanisms, such as multiple transposition or cointegration of the plasmid pRL1063a. A single hybridizing fragment was found in 61 of the 69 (88%) strains tested (not shown), including those which were further characterized (Table 2-2b), indicating a single transposition event in most cases.

The auxotrophic mutants were examined for their symbiotic phenotype by testing their ability to form effective nodules on alfalfa seedlings. Nitrogenase activity of plant nodules was determined by the acetylene reduction assay (see Materials and Methods). All auxotrophic strains, except for two leucine (Leu^-) requiring strains, induced nodules on alfalfa (Nod^+). All leucine mutants induced nodule-like structures which were ineffective in nitrogen fixation (Fix^-). In addition, all adenine or hypoxanthine requiring (Pur^-) strains, one methionine mutant, and one cytosine or uracil (Pyr^-) requiring strain, failed to produce effective nodules (Nod^+ Fix^-). However, three of the six Pyr^- auxotrophs did produce effective nodules (Nod^+ Fix^+; see Table 2-1).

Isolation of *R. meliloti* mutant strains carrying Tn5-1063 fusions induced by nitrogen deprivation

The collection of 5000 random Tn*5*-1063 insertion mutants was screened for *luxAB* expression induced by nitrogen deprivation, as described in Materials and Methods. The bioluminescent images of colonies on filters before and after the transfer to nitrogen deprivation conditions were compared. Twenty-two mutants were isolated whose bioluminescence was consistently activated under nitrogen deprivation conditions (Figure 2-2A).

Figure 2-2. Luciferase activity determination of fusions induced by nitrogen deprivation. A screen of nitrogen deprivation-induced *lux* gene fusions by using the Hamamatsu camera is shown. In Row A, the analysis of 21 nitrogen deprivation-induced fusions is depicted 0, 7, and 24 hours after transfer to nitrogen-free medium, respectively. The strain designations are the same in all panels and are outlined in Row B (first block). The induction patterns of the fusions on 0.2% nitrate, and 0.2% glutamine as sole nitrogen sources are depicted in Rows B & C, respectively. For further details, see text.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C

The strains were examined for growth on nitrate (KNO_3) and glutamine, a poor nitrogen source and a good nitrogen source for *R. meliloti*, respectively. All of the strains grew well on glutamine; however, five of them failed to grow on KNO_3 (Table 2-2b). In addition, the strains were examined for induction and repression of their gene fusions by KNO_3 and glutamine. Ten of the gene fusions were activated in the presence of KNO_3, but none of the gene fusions were induced in the presence of glutamine as the sole nitrogen source (Table 2-2a; Figure 2-2B). Two of the gene fusions (N119 and N150) appeared to be expressed at a low level in the presence of glutamine (Figure 2-2C). This appears to be a background expression level, since a similar level of bioluminescence was observed on GTS (minimal media) plates lacking glutamine (not shown).

The nitrogen deprivation-induced gene fusions were examined for temporal expression patterns, as described in the Materials and Methods. The level of expression in response to nitrogen deprivation (Figure 2-3) ranged from very high (A), to medium (B), to low (C). In addition, the temporal regulation of the expression of the fusions varied from a very rapid induction pattern within a few hours (strains N21, N110, N3, N104, and N12), to a late induction pattern after 10 to 15 hours (strains N119, N161, N113, and N4). Some fusions (strains N161 and N183) were induced only after 18 to 24 hours of nitrogen deprivation conditions (data not shown). These fusions were induced at such comparatively low levels that they could not be graphically displayed with the other fusions.

The varied temporal patterns of nutrient deprivation regulated loci are similar to what has been seen in *E. coli* (Matin, 1991), *S. typhimurium* (Givskov, *et al.*, 1994), and *P. fluorescens* (Kragelund *et al.*, 1995). These data suggest that the fusions are under the control of different promoters.

Table 2-2. Phenotypes of Tn *5*-1063 insertion mutants of *R. meliloti* 1021 induced by nitrogen deprivation.

Strains	*Lux* Fusion Expression [a] in the Presence of:			Growth on: [b]		Symbiotic [c] Phenotype:		Hybridizing [d] Fragments	Genome [e] Location	Gene [f] Similarity
	-N	$+NO_3^-$	+Gln	$+NO_3^-$	+Gln	Nod	Fix			
Rm N1	+	+	–	+	+	+	+	1	n.d.	NS
Rm N3	+	–	–	+	+	+	+	1	chr	NS
Rm N4	+	–	–	+	+	+	+	1	chr	NS
Rm N5	+	+	–	–	+	+	+	1	pSym-b	*nas*B
Rm N8	+	+	–	–	+	+	+	1	n.d.	NS
Rm N9	+	+	–	–	+	+	+	1	n.d.	*nas*B
Rm N12	+	–	–	+	+	+	+	1	pSym-b*	NS
Rm N15	+	+	–	–	+	+	+	1	n.d.	NS
Rm N21	+	+	–	+	+	+	+	2	n.d.	n.d.
Rm N25	+	+	–	+	+	+	+	1	chr	NS
Rm N30	+	+	–	–	+	+	+	1	n.d.	*nas*A
Rm N110	+	+	–	+	+	+	+	1	pSym-b*	NS
Rm N111	+	–	–	+	+	+	+	1	n.d.	*glt*
Rm N112	+	–	–	+	+	+	–	1	pSym-b	*exo*Y
Rm N113	+	–	–	+	+	+	+	1	pSym-a	NS
Rm N119	+	+	–	+	+	+	+	2	n.d.	n.d.
Rm N127	+	–	–	+	+	+	+	1	n.d.	NS
Rm N149	+	–	–	+	+	+	–	1	pSym-b	*exo*F
Rm N150	+	–	–	+	+	+	+	1	n.d.	NS
Rm N161	+	–	–	+	+	+	+	1	n.d.	NS
Rm N183	+	–	–	+	+	+	+	1	chr	NS

a) Induction of Tn*5*-1063 fusions by growth of mutants on GTS without nitrogen (-N), with 0.2% nitrate ($+NO_3^-$), and with 0.2% glutamine (+Gln).

b) Growth of mutants on GTS with 0.2% nitrate or 0.2% glutamine as a sole nitrogen source.

c) Symbiotic phenotype of alfalfa roots inoculated with mutant strains for presence of nodules on roots (Nod $^+$) and presence of nitrogenase as determined by acetylene reduction (Fix $^+$).

d) Number of fragments of genomic DNA cut with *Eco*RI which hybridize to a probe of whole pRL1063a.

e) Genomic location of Tn*5*-1063 insertions (Honeycutt, 1993; *from personal correspondence with R. Honeycutt).

f) Genes sharing significant similarity with *R. meliloti* ORFs: *nasAB* (Lin *et al.*, 1993); *exoFY* (Müller *et al.*, 1993); n.d. = not determined; NS = no significant similarity to known genes in GenBank.

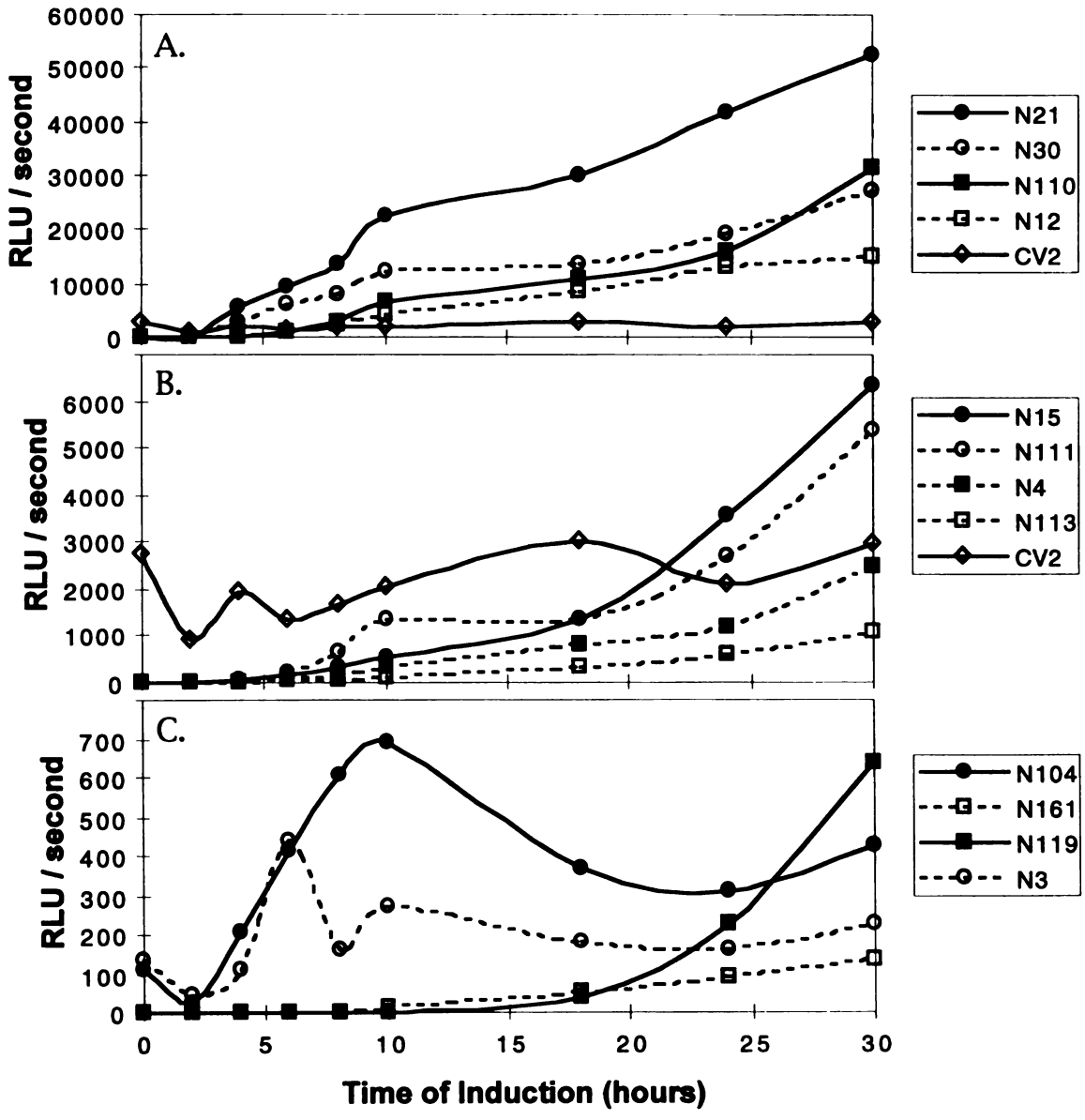

Figure 2-3. Temporal *lux* expression pattern of selected nitrogen deprivation-induced gene fusions. The temporal *lux* expression patterns of 14 Tn*5*-1063 induced gene fusions after transfer to nitrogen free medium is shown in Panels A-C. On the Y-axis, relative light units per second are indicated (RLU/sec). On the X-axis, five hour intervals are indicated (see bottom of Panel C). CV2 is included as a control.

In order to examine the physical structure of the transposon tagged strains, total DNA of the Tn*5*-1063 containing strains was isolated, digested with either *Eco*RI or *Cla*I (neither enzyme cuts the transposon), blotted, and hybridized with plasmid pRL1063a DNA as a probe. Two of the 22 strains displayed two hybridizing fragments for *Eco*RI and *Cla*I restricted DNA (Table 2-2d), indicating that either an integration of the plasmid pRL1063a or a multiple transposition event had occurred. These two strains were not analyzed further except to test for nodulation and nitrogen-fixation ability (see Table 2-2).

The strains carrying fusions induced by nitrogen deprivation were examined for their symbiotic phenotype by testing their ability to form effective nodules on alfalfa seedlings. Nitrogenase activity of plant nodules was determined by the acetylene reduction assay (see Materials and Methods). All strains except for two (strains N112 and N149; described below), induced nodules (Nod^+) on alfalfa that were nitrogen-fixation competent (Fix^+) (Table 2-2).

In order to identify the nitrogen deprivation-induced genes, Tn*5*-1063 mutated loci were cloned from the *R. meliloti* genome. Total genomic DNA of strains carrying Tn*5*-1063 fusions was digested with *Eco*RI or *Cla*I restriction enzymes which do not cut the transposon (Figure 2-1C). The resulting fragments were self-ligated and transferred into *E. coli* DH5α or HB101 by electroporation or $CaCl_2$ transformation. The DNA sequence of the *R. meliloti* DNA fragments flanking the Tn*5*-1063 inverted repeats was determined by using unique primers (Figure 2-1C) homologous to the left and right ends of the Tn*5*-1063 (see Materials and Methods). A nine base-pair duplication of the target site of Tn*5*-1063 was observed in each case, and the DNA sequences flanking the Tn*5*-1063 were fused by omitting the duplicated target sequence.

Significant open reading frames (ORFs) were found for all of the isolated gene fusions, as determined by analyzing the DNA sequence with a codon preference program based on the codon usage of *R. meliloti* (Figure 2-4). This program also indicates start and stop codons, as well as potential non-coding regions (Figure 2-4A). In addition, potential frame-shifts (Figure 2-4B) caused by compressions of *R. meliloti* 1021 GC-rich stretches during DNA sequencing could be recognized and resolved by further sequencing (Figure 2-4C).

The partial DNA sequences of the Tn*5*-1063 tagged loci from strains N4 and N41 were found to be identical, thus indicating that the two strains are clonal.

Sequence similarities of *R. meliloti* Tn*5*-1063 tagged loci induced by nitrogen deprivation

The amino-acid sequences deduced from the primary DNA sequence were compared to sequences in the non-redundant protein databases of GenBank (Table 2-2f). The sequence data indicated that the Tn*5*-1063 tagged locus in strain N30 possessed significant similarity (Figure 2-5A) to assimilatory nitrate reductases of *Klebsiella pneumoniae* (NasA; Lin *et al.*, 1993), *Oscillatoria chalybea* (NarB; Unthan *et al.*, 1996), and *Synechococcus* sp. (NarB; Omata *et al.*, 1993), and to the formate dehydrogenases FdnG (Berg *et al.*, 1991) and FdoG (Plunkett *et al.*, 1993) of *E. coli.* Since this particular mutant strain is unable to grow with KNO_3 as sole nitrogen source (Table 2-2d), it is likely that the gene tagged encodes the assimilatory nitrate reductase of *R. meliloti.* In addition, this gene fusion is induced in the presence of its substrate, KNO_3 (Figure 2-2B; Table 2-2b), which has also been observed for the *K. pneumoniae nasA* gene (Goldman *et al.*, 1994). The alignment

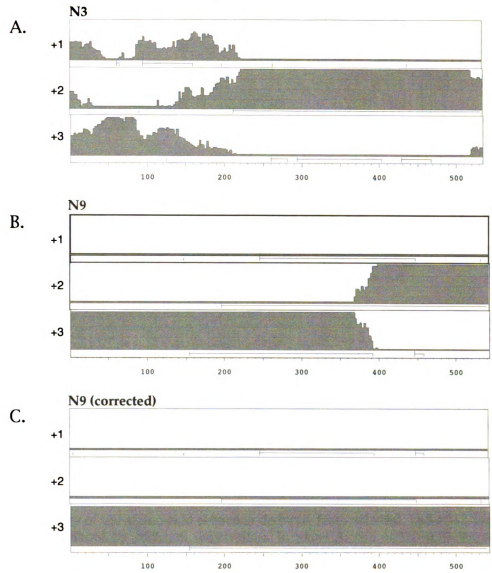

Figure 2-4. Codon-usage analysis of Tn*5*-1063 tagged target gene DNA sequences. In Panel A, the codon-usage analysis of the Tn*5*-1063 tagged strain N3 is shown. The three open reading frames (ORFs) in the direction of *lux* reporter gene transcription are indicated by +1, +2, and +3, according to C. Halling (see Materials and Methods). The DNA coordinates (in intervals of 100 bp) are indicated on the horizontal axes at the bottom of Panels A-C. In Panels B & C, the codon-usage analysis of mutant strain N9 is shown, before (B) and after (C) correcting for a frameshift in the DNA sequence (see text). The significance of the codon usage is indicated by the shaded areas. Start and stop codons are indicated by long and short vertical lines, respectively, at the bottom of the panels. Horizontal lines connecting the start & stop codons indicate potential ORFs.

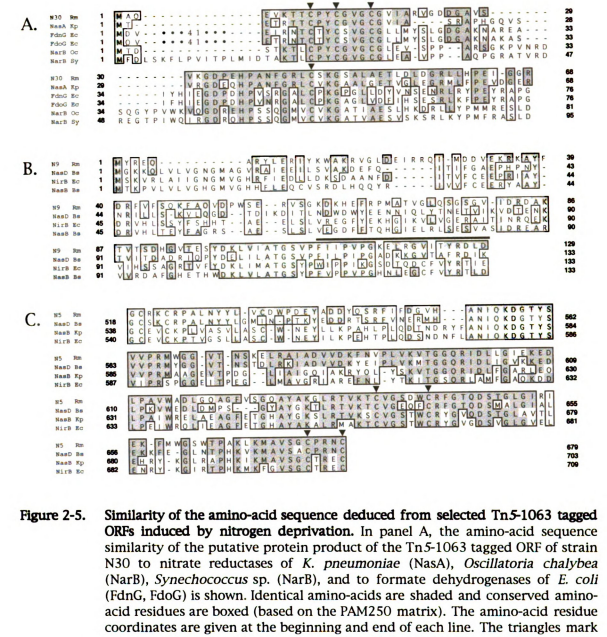

Figure 2-5. **Similarity of the amino-acid sequence deduced from selected Tn*5*-1063 tagged ORFs induced by nitrogen deprivation.** In panel A, the amino-acid sequence similarity of the putative protein product of the Tn*5*-1063 tagged ORF of strain N30 to nitrate reductases of *K. pneumoniae* (NasA), *Oscillatoria chalybea* (NarB), *Synechococcus* sp. (NarB), and to formate dehydrogenases of *E. coli* (FdnG, FdoG) is shown. Identical amino-acids are shaded and conserved amino-acid residues are boxed (based on the PAM250 matrix). The amino-acid residue coordinates are given at the beginning and end of each line. The triangles mark a conserved four cysteine consensus [4Fe-4S] cluster-binding motif. In panel B, similarity of the putative protein products from the Tn*5*-1063 tagged ORF of strain N9 to nitrite reductases from *Bacillus subtilis* (NasD), *A. vinelandii* (NasA), and *K. pneumoniae* (NasB) is shown. In panel C similarity of the putative protein products from the Tn*5*-1063 tagged ORF of strain N5 to nitrite reductases from *Bacillus subtilis* (NasD), *K. pneumoniae* (NasB) and *E. coli* (NirB) is shown. The Tn*5*-1063 of strains N9 and N5 is located within the same ORF. The solid bar in panel B indicates the start of an NAD-binding domain. In panel C, the conserved cysteines of the 4Fe-4S / sirohem domain found in nitrite reductases and sulfite reductases are indicated with triangles.

of the deduced amino-acid sequence of the Tn*5*-1063 tagged locus of strain N30, with the nitrate reductases and formate dehydrogenases is shown in Figure 2-5A. A conserved four cysteine consensus [4Fe-4s] cluster-binding motif (Breton *et al.*, 1994), marked by triangles in the amino-terminal region, has been postulated to contribute to iron-sulfur binding (Berg *et al.*, 1991).

The Tn*5*-1063 tagged loci in strains N5 and N9 share significant similarity with the *Bacillus subtilis* assimilatory nitrite reductase NasD (Ogawa *et al.*, 1995), the *K. pneumoniae* assimilatory nitrite reductase NasB (Lin *et al.*, 1993), and the *E. coli* NADH-dependent nitrite reductase NirB enzymes (Peakman *et al.*, 1990) (Figure 2-5B). These isolates did not grow on minimal medium with nitrate as the sole nitrogen source (Table 2-2d), confirming that their assimilatory nitrite reduction pathway was inactivated. As in the case of strain N30, the gene fusions were induced in the presence of KNO_3 (Figure 2-2B; Table 2-2b). In mutant strain N5, it was observed that in addition to assimilatory nitrite reductase inactivation, a non-motile phenotype was present. Gene replacement of the wild-type *R. meliloti* gene with the mutated analogous gene restored the motility, but growth with nitrate as sole nitrogen source was still abolished. This confirmed that the tagged gene was indeed in the assimilatory nitrite reductase locus, and that the loss of motility in the original strain was not linked to the Tn*5*-1063 mutation.

The Tn*5*-1063 tagged loci of strains N112 and N149 were identified as the *exoY* and *exoF* genes, respectively, due to a 100% homology with the sequences obtained from GenBank (Müller *et al.*, 1993). These genes are involved in exopolysaccharide production, and are organized as contiguous genes in an operon in *R. meliloti* (Glucksmann *et al.*, 1993). The phenotype of *exoY* and *exoF* mutants includes an absence of exopolysaccharide production, as well as the presence of

nodule-like structures which are unable to fix nitrogen (Gray *et al.*, 1990; Leigh and Walker, 1994). Mutant strains N112 and N149 were indeed found to lack exopolysaccharide production under nitrogen deprivation conditions, based on visual inspection of the plates for mucoid colonies typical for the N-starved wild-type strain (data not shown). When these mutants were inoculated on alfalfa roots (Table 2-2c), nodule-like structures were formed which did not fix nitrogen (Fix^-), confirming the phenotype described for these mutants (Gray *et al.*, 1990; Leigh and Walker, 1994).

Even though significant ORFs were identified in each of the remaining nitrogen deprivation-induced loci (data not shown), no significant similarity of these loci to sequences in the non-redundant protein databases at GenBank was found, suggesting that they represent novel genes (Table 2-2f).

DISCUSSION

Insertional specificity of Tn*5*-1063: Characterization of auxotrophic mutants

In the collection of 5000 *R. meliloti* Tn*5*-1063 mutants examined, auxotrophic mutants were isolated with a frequency of 1.2%. This frequency is higher than has been observed with Tn*5* derivatives in *R. meliloti* thus far (Meade *et al.*, 1982). However, it is lower than frequency observed with nitrous acid mutagenesis (Kerppola and Kahn, 1988). With regard to the type of auxotrophies, Meade *et al.* (1982) also found a high number of auxotrophs which could be supplemented by methionine (9 of 20), but none supplemented by tryptophan. In *Rhizobium leguminosarum*, however, a high number of tryptophan and methionine auxotrophs was also observed (Pain, 1979). The high number of tryptophan and methionine requiring auxotrophs may indicate non-randomness of Tn*5*-1063 insertion, or a high number of genes involved in tryptophan and methionine synthesis.

At least eight genes are needed for tryptophan synthesis from the precursor chorismate, which are tightly arranged in an operon in *E. coli* (Yanofsky *et al.*, 1981) and *S. typhimurium* (Yanofsky and van Cleemput, 1982). At least nine genes, involved in the biosynthesis and transport of general aromatic amino-acids, are necessary for tryptophan synthesis in *E. coli* and *S. typhimurium* (Pittard and Wallace, 1966; Gollub *et al.*, 1967). Four of the genes from the tryptophan auxotrophs in this study (aux 35, 37, 42, and 47) were mapped by Honeycutt *et al.* (1993), and reside within a single cluster, on linkage group Pme #3 (1040 kb),

indicating that the *R. meliloti* genes are also tightly linked, similar to *E. coli* and *S. typhimurium*, and may be similarly arranged.

Methionine metabolism also requires a large number of genes for its synthesis and transport. In most bacteria examined, at least eight genes are involved in methionine metabolism, and nine genes in *E. coli* (Rowbury, 1983).

The inability of leucine, methionine, purine, and pyrimidine auxotrophs of *R. meliloti* to form effective nodules has been reported previously (Dénarié *et al.*, 1976; Meade *et al.*, 1982; Kerppola and Kahn, 1988). Kerppola and Kahn (1988) also found that methionine auxotrophs failed to produce effective nodules; however, *all* of their methionine auxotrophs formed ineffective nodules, unlike what was observed in this thesis (Table 2-1). In addition, they found that purine and pyrimidine requiring mutants formed ineffective nodules. I also observed that purine requiring mutants formed ineffective nodules; however, many of the pyrimidine auxotrophs *were* able to form effective nodules.

These results suggest that the ability of specific host plants to provide the necessary nutrients to their endosymbionts varies. Many studies have shown that a large number of factors affect the types and quantities of root exudates released by plants including: the species of plants used, the age of the plants, and environmental factors such as temperature, moisture, irradiance, and other plant stresses (Rovira, 1956 and 1959; Vancura, 1988). It is my belief that a combination of these factors accounts for the variation of the types of auxotrophies which lead to deficient (Fix^-) nodules.

Characterization of Tn*5*-1063 tagged loci induced by nitrogen deprivation.

In this study, the isolation of 22 *R. meliloti* genes involved in nitrogen-deprivation was reported. Sequence analysis of the tagged loci induced by nitrogen-deprivation revealed genes involved in nitrate and nitrite assimilation (*nasA* and *nasB*) and exopolysaccharide synthesis (*exoY* and *exoP*). It is known that bacterial nitrate and nitrite assimilation genes are induced by nitrogen limiting conditions (Peakman *et al.*, 1990; Lin *et al.*, 1993; Goldman *et al.*, 1994). In addition, it has been observed that exopolysaccharide synthesis decreases in the presence of ammonia, although no specific regulator has been described (Sutherland, 1979; Ozga *et al.*, 1994). Thus, the methods used to isolate and screen for nitrogen deprivation-induced genes did generate a diverse set of loci with predicted functions, as well as a number of novel genes, and suggests that the conditions for nitrogen deprivation applied were sufficient and adequate for the isolation of nitrogen deprivation-induced loci.

The number of genes isolated by nitrogen deprivation is similar to what has been observed in other bacteria (Matin, 1990; Givskov *et al.*, 1994; Kragelund *et al.*, 1995), as was hypothesized (chapter 1); however, this does not imply that saturation was reached, only that at least as many loci exist in *R. meliloti* induced by nitrogen deprivation as exist in *E. coli*. In addition, the large number of novel genes (fourteen), supports the hypothesis that soil bacteria may have different genes for dealing with stress than enteric or marine bacteria. More work needs to be carried out to characterize the novel loci before this hypothesis can be fully tested. This important hypothesis will be examined in greater detail following the

analysis of genes induced by carbon deprivation (chapter 3) and oxygen limitation (chapter 4).

The nitrate and nitrite reductase genes have not yet been isolated in *R. meliloti*. The partial sequence analysis of both loci indicates a high similarity with the *K. pneumoniae nas* genes. In this organism, the *nas* genes are organized in an operon and their regulation occurs through the *ntr* system responding to nitrogen availability, and the *nasR* gene responding to nitrate and nitrite availability (Goldman *et al.*, 1994). The limited sequence data does not warrant a prediction about the organization of these genes in *R. meliloti*, but their regulation clearly appears to be similar.

MATERIALS AND METHODS

Bacterial strains and plasmids

R. meliloti 1021 (Sm^r) has been described by Meade *et al.*, (1982), *E. coli* DH5α by Hanahan (1983), and *E. coli* HB101 by Boyer and Roullaud-Dussoix (1969). Plasmid pRL1063a (Wolk *et al.*, 1991) was kindly provided by P. Wolk (Michigan State University). Plasmid pRK2013 has been described by Ditta *et al.* (1980).

Media and growth conditions

R. meliloti was grown at 28°C in TY (Beringer, 1974) or in GTS medium (Kiss *et al.*, 1979). *E. coli* strains were grown at 37°C in LB medium (Silhavy *et al.*, 1984). Antibiotics were used at the following final concentrations: 250 μg/ml streptomycin (Sm) for *R. meliloti*; 200 μg/ml kanamycin (Km) for *R. meliloti*; 20 μg/ml Km for *E. coli.*

Transposon mutagenesis

The following procedure was used to mobilize plasmid pRL1063a, carrying transposon Tn*5*-1063, to *R. meliloti* 1021 using the helper plasmid pRK2013. *E. coli* DH5α, carrying pRL1063a or pRK2013, and *R. meliloti* 1021 were grown in LB-Km^{20} and TY-Sm^{250} respectively, washed twice with TY, and concentrated five-fold in TY medium. Equal amounts of donor, helper, and recipient cells were spotted on TY

plates. After one day at 28°C, the mating mixtures were resuspended in sterile distilled water and plated on selective plates. Five thousand (5000) Km^r colonies were isolated, purified, grown in liquid TY medium, and stored in microtiter plates at -70°C.

Identification of auxotrophic mutants

Auxotrophic mutants were identified by screening the transconjugants for their inability to grow on GTS minimal medium. They were purified and tested for their auxotrophic requirements using the supplementation test described by Holliday (1956). Amino-acids were added at a final concentration of 20–80 mg/ml, nucleotides at 10 mg/ml, and vitamins at 5 mg/ml (see Holliday, 1956).

Screening of the Tn*5*-1063 insertion mutants for nitrogen deprivation-induced gene fusions

The *R. meliloti* strains carrying Tn*5*-1063 were spotted on membrane filters (Nucleopore, Cambridge, MA) and incubated on solid GTS media for 48 hours. Luminescent colonies on the plate were visualized by spreading 50 µl of N-decanal inside the top of a glass petri-dish, placing the glass petri-dish over the plate, exposing the cells to N-decanal for 60 seconds, and measuring light emission using the Hamamatsu photonic system model C1966–20, as described by Wolk *et al.* (1991).

In order to screen the mutants for nitrogen deprivation-induced gene fusions, the membrane filters bearing the cells were transferred to both GTS

medium and GTS medium lacking nitrogen, and incubated for an additional 7 or 24 hours. Strains carrying Tn*5*-1063 whose luminescence was weak or absent before transfer, but showed strong luminescence afterwards, were selected for further analysis and rescreened.

DNA isolation and manipulation

Plasmid DNA was prepared by the alkaline method as described in Kragelund *et al.* (1995). Total DNA was isolated from *R. meliloti* strains according to de Bruijn *et al.* (1989).

All restriction enzyme digestion, ligation, and Southern blotting experiments were carried out as described in Sambrook *et al.* (1989). Labeling of probes and DNA hybridizations was performed using a non-radioactive DNA labeling and detection kit (Boehringer Mannheim, Mannheim, FRG), according to the manufacturer's recommendations.

DNA Sequence analysis

Sequencing of double-stranded plasmid DNA was performed using the di-deoxy method of Sanger *et al.* (1977) with sequenase kits (U.S. Biochemicals, Cleveland, OH). To determine the *R. meliloti* DNA sequence on both sides of the transposon insertion, two Tn*5*-1063 derived oligonucleotides were synthesized by the Macromolecular Synthesis Facility at Michigan State University and used as sequencing primers. One primer (corresponding to position 110–86 of the Tn*5*-1063 DNA sequence: 5'-TACTAGATTCAATGCTATCAATGAG-3') was designed to

determine the upstream sequence from the Tn*5*-target site in the antisense direction. The other primer (corresponding to positions 7758–7781 of the Tn*5*-1063 DNA sequence: 5'-AGGAGGTCACATGGAATATCAGAT-3') was designed for determining the downstream sequence from the Tn*5*-target site in the sense direction. These primers were modified from previously described sequencing primers for Tn*5*-1063 and other Tn*5* derivatives (Black *et al.*, 1993; Fernandez-Pinas *et al.*, 1994). DNA sequences were analyzed using the software packages of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984), and DNASIS (Hitachi Software Engineering Co., Ltd.). Deduced amino-acid sequences were analyzed using PROSIS (Hitachi Software Engineering Co., Ltd.). Database searches were carried out using the program Blastx (Gish and States, 1993), by screening the non-redundant GenBank database Release 1.4.9MP (8/17/97). The codon preference profiles were determined by the program CodonUse 3.1 (Codon window size = 33, logarithmic range = 3), kindly provided by Conrad Halling (U. of Chicago, Chicago, IL 60637).

Nodulation and nitrogen-fixation assays

R. meliloti strains carrying Tn*5*-1063 insertions were screened for their symbiotic phenotype by inoculation on alfalfa (*Medicago sativa*) seedling roots. Alfalfa seeds were sterilized by soaking for three minutes in 95% ethanol, followed by three minutes in 0.1% $HgCl_2$ and rinsed thoroughly with sterile distilled water. The seeds were placed on a piece of sterile Whatman 3MM filter paper in test tubes containing 20 ml of sterile nitrogen-free B+D liquid medium (Broughton and

Dilworth, 1971). Saturated cultures (late logarithmic stage) of strains carrying Tn*5*-1063 were diluted with sterile H_2O (1:5) and one ml aliquots were added to tubes containing one week old germinated seedlings. Inoculated plants were grown for six to seven weeks in a growth chamber (16 hrs light, 28°C), and examined for the presence or absence of nodules (Nod phenotype).

Nitrogenase activity was measured in glass test tubes containing the nodulated plants by capping each tube with a stopper, injecting acetylene (1/10 volume), and withdrawing a one ml sample from the tube after 30 minutes, followed by a measurement of acetylene reduction to ethylene by gas chromatographic analysis.

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CHAPTER 3

Isolation and Characterization of *Rhizobium meliloti* Genes Whose Promoters are Induced by Carbon Deprivation.

Parts of this chapter have been presented at the 6th International Microbial Ecology Symposium (Lim *et al.*, 1993) and the 10th International Nitrogen Fixation Symposium (de Bruijn *et al.*, 1995), and have been submitted for publication in *Molecular Microbiology* (Ragatz *et al.*, 1997).

ABSTRACT

Carbon availability is an important condition for life, but is severely limited in most natural systems. Limitation is particularly pronounced in the soil, where bacteria grow so slowly they may only divide a few times per year. Consequently, it has been of interest to understand how *Rhizobium meliloti*, a common soil isolate, senses and responds to this nutrient deprivation condition. To investigate this problem, the 5000 *R. meliloti* 1021 Tn*5* insertion mutants that were screened for nitrogen deprivation (see chapter 2) were utilized to screen for gene fusions induced by carbon deprivation. The isolation of twelve gene fusions induced by carbon deprivation is described. The strains harboring Tn*5-luxAB* fusions were tested for nodulation and nitrogen-fixation phenotypes. No strains were found to be Fix$^-$. Cloning and partial DNA sequence analysis of the transposon tagged loci revealed a variety of novel genes, as well as *R. meliloti* genes with sequence similarity to known bacterial loci. Genes with significant similarity to ferredoxin reductases, and to ribose transport/chemotaxis were found.

INTRODUCTION

As is the case for combined nitrogen, carbon is also severely limited in natural environments. Organic nutrient concentration can range from between 0.4 to 10% in temperate soils (Atlas and Bartha, 1987). However, much of this carbon is inaccessible to microbial populations, and the rest is quickly scavenged by indigenous populations (Alexander, 1977; Atlas and Bartha, 1987). Thus the soil environment, though richer in organic carbon than the marine environment, remains largely oligotrophic.

Even in the rhizosphere, where carbon is relatively more abundant, the higher numbers of microorganisms present necessitates the ability of rhizosphere microorganisms to adapt to varying carbon conditions in order to compete successfully (see Chapter 1 for an overview). Therefore, it was of interest to understand how *Rhizobium meliloti*, a common soil and rhizosphere isolate, senses and responds to carbon deprivation.

Many carbon deprivation-induced proteins have been observed to be induced in bacteria ranging from *E. coli* to *Vibrio* (see chapter 1). It has been one of the most studied of the environmental stresses. Some proteins have even been identified; particularly those from *E. coli,* such as the heat-shock proteins DnaK, GroEL, and HtpG (Matin, 1991) and the product of the *uspA* gene, the universal stress protein (Nyström and Neidhardt, 1993; see Chapter 1 for examples in other bacteria). However, the sensing of, and regulation in response to, limiting carbon is still poorly understood, especially in common soil isolates.

Few investigations on nutrient deprivation-induced gene expression have been carried out with common soil isolates, which may have different strategies for

dealing with nutrient deprivation than enteric or marine bacteria. This could be due to differences in the types of carbon available (e.g., enteric vs. soil environments) as well as to the total levels of organic carbon available, which is higher for enterics and is available in regular cycles, and is lower in marine environments. Only recently, the effect of nutrient deprivation on *Pseudomonas putida,* a common gram negative soil bacterium, has been reported (Givskov *et al.*, 1994). Many proteins induced by carbon and nitrogen deprivation have been detected using 2D-PAGE protein analysis. These proteins are temporally regulated—as in the case of *E. coli* and *Vibrio* sp., and the nitrogen deprivation-induced fusions described in chapter 2—and include a group of eight proteins induced by multiple stresses. To date, only a few roles have been assigned to the large collection of deprivation-induced proteins in any of the bacterial species identified by 2D-PAGE analysis (Givskov *et al.*, 1994; see Chapter 1).

Therefore, as described in chapter 2 for nitrogen deprivation-induced fusions, the Tn*5*-1063 mutagenesis approach was taken to generate and characterize a collection of 12 *R. meliloti* mutants carrying gene fusions induced by carbon deprivation.

RESULTS

Isolation of strains carrying Tn*5*-1063 gene fusions induced by carbon deprivation

A screening procedure, modified from the one described in Chapter 2, was used to isolate carbon deprivation-induced genes, since the bioluminescence of the fusions was too weak to detect when cells were incubated without a carbon source for several hours (see Materials and Methods). This is due to the fact that light expression via luciferase requires $FMNH_2$ (Meighan, 1991), the generation of which is closely associated with carbon metabolism. With the modified screening procedure, we isolated 12 gene fusions whose bioluminescence was consistently increased by carbon deprivation (Table 3-1).

Strains carrying fusions induced by carbon deprivation were tested for growth and luciferase expression in the presence of a number of alternative carbon sources (Table 3-1b). All of the strains were able to grow on the carbon sources tested, in liquid and on solid media, with no discernable difference (data not shown); however, growth rates were not determined. After incubation on filters placed on media containing the selected carbon source for 48 hours, the induction of the *luxAB* fusions they carried was examined under the photonic camera. None of the gene fusions were induced when the bacteria that harbored them were incubated only on the citric acid cycle derivatives succinate or fumarate (Table 3-1b). One strain (C47) carried a fusion that was induced in the presence of malate as sole carbon source. Many of the gene fusions were induced in the presence of C_6

Table 3-1. Phenotypes of Tn5-1063 insertion mutants of *R. meliloti* 1021 induced by carbon deprivation.

Strains	Relative Increase in *Lux* Fusion Expression After Inoculation in the Presence of:									Hybridization[c] Fragments	Gene[d] Similarity
	4°C[a]	succinate	malate	glucose	sucrose	fructose	maltose	trehalose	myo-inositol[b]		
Rm C1	–	–	–	+	–	+	–	–	–	1	NS
Rm C4	–	–	–	–	–	–	–	–	–	1	NS
Rm C18	–	–	–	–	+	–	+	+	–	1	*thcD*
Rm C19	–	–	–	–	–	+	–	–	–	1	NS
Rm C22	–	–	–	–	–	–	–	–	–	1	NS
Rm C27	–	–	–	++	–	–	–	–	–	1	*rbsA*
Rm C35	–	–	–	+	–	–	+	–	+	1	NS
Rm C37	–	–	–	+	–	+	+	+	+	1	NS
Rm C47	–	–	+	++	++	+++	++	++	++	1	*xox*F
Rm C55	–	–	–	++	–	–	–	–	–	2	n.d.
Rm C67	–	–	–	–	–	–	–	–	–	1	NS
Rm C101	–	–	–	–	–	–	–	–	–	1	NS

a) Strains were incubated at 4°C for 6 hours.
b) Expression of Tn5-1063 fusions was determined by growing strains on minimal media with 0.2% of the carbon source described. Number of plusses indicates relative strength of induction compared to induction without a carbon source as determined by photon counts.
c) Number of bands of genomic DNA digested with *Eco*RI or *Cla*I hybridizing to the pRL1063a probe.
d) Genes sharing significant similarity with *R. meliloti* ORFs. n.d = not determined. NS = no significant similarity to known genes in GenBank.

carbon sources, including glucose which is not a preferred substrate for *R. meliloti* (based on glucose versus succinate chemotactic data, D.M. Ragatz and F.J. de Bruijn, unpublished; see also Appleby, 1984; Finan *et al.*, 1988; McKay *et al.*, 1988; Day and Copeland, 1991). The gene fusion in strain C47 was particularly sensitive, responding strongly to incubation on all of the carbon sources, except for succinate and fumarate. The induction patterns varied quantitatively and temporally for each strain tested, suggesting that the insertions were in distinct and/or distinctly regulated loci. Strains C4, C22, C67, and C101 carried fusions that were not induced in the presence of any of the carbon sources tested, and therefore seem to be induced by a general carbon deprivation condition, or are too weakly induced to be observed under the conditions used.

Cold- and heat-shock treatments were applied to the Tn*5*-1063 tagged strains in order to determine if other stresses could activate the carbon deprivation-induced fusions. None of the fusions were induced by incubation on filters at 4°C for 6 hours (Table 3-1). However, the data obtained after heat-shock incubation were inconclusive, as the bacterial luciferase encoded by *luxAB* (derived from *Vibrio fischeri*) proved to be exceedingly sensitive to higher temperatures, becoming completely inactivated at 37°C within five to ten minutes of exposure (data not shown).

In order to test the structural nature of the transposition events generating the Tn*5*-1063 tagged strains, total DNA was isolated, digested with *Eco*RI or *Cla*I which do not cleave Tn5-1063 (Wolk *et al.*, 1991), and hybridized with plasmid pRL1063a DNA as a probe. Only one of the twelve strains (C55) harbored more than one hybridizing fragment (Table 3-1c; Figure 3-1), indicating that a single,

Figure 3-1. Similarity of the amino-acid sequence deduced from selected Tn*5*-1063 tagged ORFs induced by carbon deprivation. In panel A, the amino-acid sequence similarity of the putative protein product of the Tn*5*-1063 tagged ORF of mutant strain C18 to rhodocoxin (ThcD) of *Rhodococcus sp.,* and a reductase (MopA) of *Burholderia cepacia* is shown. The solid bar above the sequence indicates a conserved FAD-binding domain. The amino-acid residue coordinates are given at the right of each line. In panel B, similarity of the putative protein product of the Tn*5*-1063 tagged ORF of mutant strain C27 to ribose transport proteins from *E. coli* (RbsA) and *B. subtilus* (RbsA), and to an *E. coli* arabinose transport protein (AraG) are shown. In panel C, similarity of the putative protein product of the Tn*5*-1063 tagged ORF of mutant strain C47 to PQQ-dependant dehydrogenase protein from *P. denitrificans* (XoxF) and methanol dehydrogenases from *P. denitrificans* (MoxF) and *Methylophilis* W3a1(MedH) are shown.

simple transposition event had occurred in the majority of the strains isolated (see also chapter 2).

The strains harboring gene fusions induced by carbon deprivation were examined for their symbiotic phenotype by testing their ability to form effective nodules on alfalfa seedlings. Nitrogenase activity of plant nodules was determined by the acetylene reduction assay (see chapter 2, Materials and Methods). All of the Tn*5*-1063 tagged strains were capable of forming nitrogen-fixation competent nodules (Nod^+ Fix^+) comparable to those induced by the wild-type *R. meliloti* 1021 (summarized in Table 3-1d).

Sequence similarities of carbon deprivation-induced Tn*5*-1063 tagged *R. meliloti* loci

The carbon deprivation-induced *luxAB* gene fusions were cloned from the genome as described in chapter 2 (Figure 2-2), and a partial DNA sequence of the tagged locus was determined. The amino-acid sequences deduced from the primary DNA sequence were compared to sequences in the non-redundant protein databases at GenBank. By analyzing the codon usage in the primary sequence (see chapter 2, Figure 2-5) significant ORFs were identified in all of the tagged loci (not shown). Three of the Tn*5*-1063 tagged loci were found to share significant similarity with known genes (Table 3-1c). In addition, one of the Tn*5*-1063 tagged loci (C37) shared a degree of similarity with an unknown ORF from *E. coli* K-12 (ORF_f375; GenBank Accession# U28377), which maps between approximately 65 and 68 minutes on the chromosome (Table 3-1c). DNA sequences derived from the

Tn5-1063 tagged loci in strains C4 and C22 were found to overlap, indicating that the transposons had inserted within the same locus. In addition, the locus tagged by strains C4 and C22 was found to be identical to the nitrogen deprivation-induced locus tagged in strain N104 (see chapter 2), and the locus tagged in strain C32 (not shown) was found to be identical to the nitrogen deprivation-induced locus tagged in strain N4 (see chapter 2).

The partial DNA sequence of the Tn*5*-1063 tagged locus of strain C18 was found to share a high degree of similarity with several ferredoxin reductase genes. The highest similarity was observed with the rhodocoxin reductase gene *thcD* from *Rhodococcus* sp. (Nagy *et al.*, 1995), and the *mopA* reductase gene of *Burkholderia cepacia* (Saint and Romas, 1996). The similarity was particularly high in the carboxy-terminal region due to the presence of a highly conserved FAD-binding amino acid consensus sequence, **T**XXXX**(I/V)(F/Y)A(A/V/I)GD** (Figure 3-1A). This sequence is uniquely characteristic of FAD-binding oxidoreductases, such as those mentioned here (Eggink *et al.*, 1990). Therefore, it is likely that the locus tagged in strain C18 encodes an FAD-binding oxidoreductase, possibly involved in energy scavenging during carbon deprivation.

The Tn*5*-1063 tagged locus of strain C27 was found to share significant similariy with the *E. coli* ribose transport gene *rbsA* (Bell *et al.*, 1986), the *Bacillus subtilis* ribose transport gene *rbsA* (GenBank Accession# Z25798), and the arabinose tranport gene *araG* (Scripture *et al.*, 1987). The highest similarity was found to occur in the NH_2 -terminal region (Figure 3-1B), which includes an ATP-binding motif, as well as membrane-spanning regions of the *rbsA* and *araG* gene products.

The Tn*5*-1063 tagged locus of strain C47 was found to be surprisingly similar to the predicted amino-acid sequence of *xoxF, located* in the *Paracoccus denitrificans* operon encoding cytochrome c553i (Ras *et al.*, 1991; Figure 3-1C). This ORF corresponds to a gene induced by growth on choline as sole carbon source, and has been predicted by Ras *et al.* (1991) to encode a quinoprotein dehydrogenase, based on sequence similarity with the product of the *moxF* genes of *P. denitrificans* (Harms *et al.*, 1987) and *Methylobacterium organophilum* (Machlin and Hanson, 1988). In addition, a high degree of similiarity with the product of the methanol dehydrogenase *medH* gene of *Methylophilis* W3a1 was observed (MEDLINE identifier: 93054513; Figure 3-1C).

Although significant ORFs were identified in each of the remaining carbon deprivation-induced loci, no significant similarity of these loci to sequences in the non-redundant protein databases at GenBank was found, suggesting that the tagged genes are novel.

Characterization of the chemotactic phenotype of strain C27 harboring a gene fusion with similarity to ribose transport genes

Strain C27, which carries a Tn*5*-1063 tagged locus with a high degree of similarity to ribose transport genes, was tested for its chemotactic response in a diffusion gradient chamber containing a 0.15% agarose gel across which a 4mM gradient of ribose was established (see Materials and Methods). Two diffusion gradient chambers were set up identically with ribose gradients and inoculated with 10 μL of late log cells (OD_{600}=1.0), from strains C27 or the wild-type, forming a column in the center of the gels (appears as a single point when viewed from

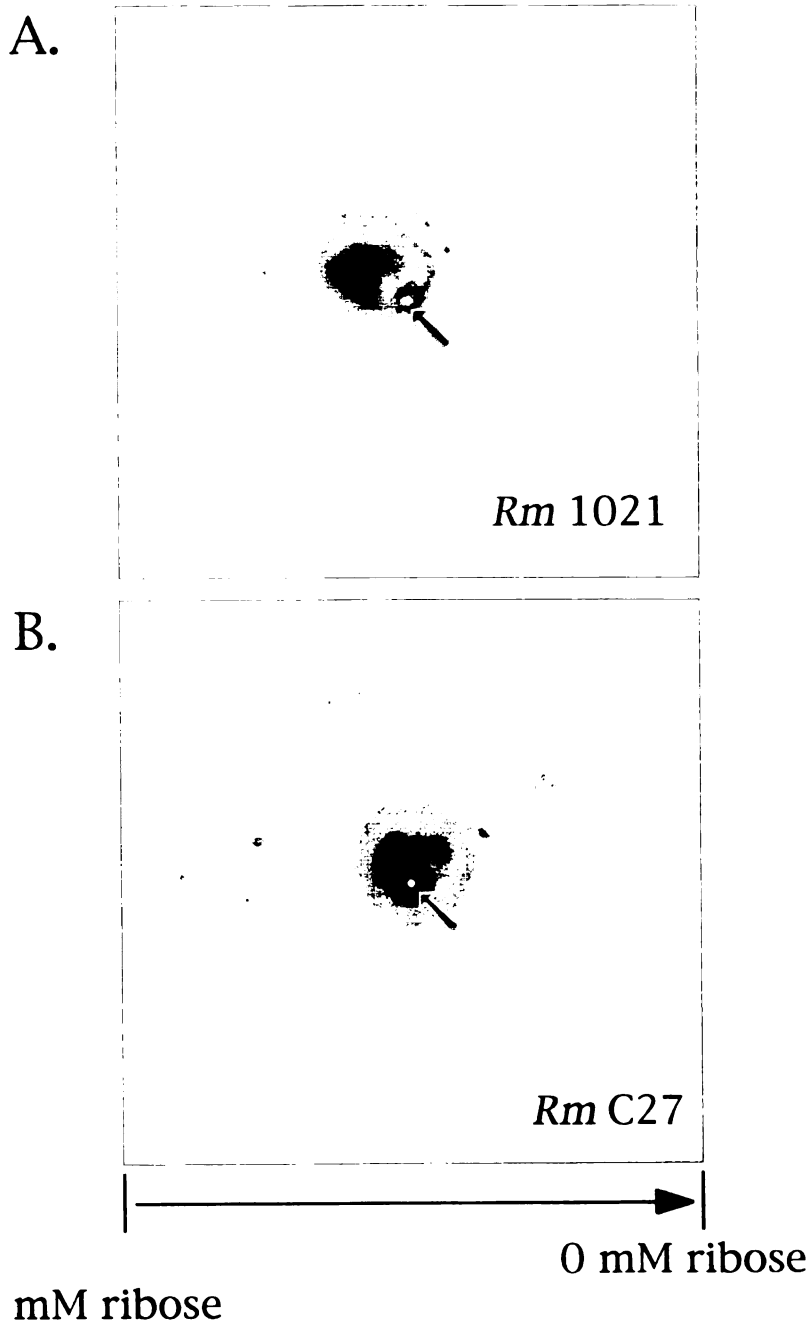

Figure 3-2. Chemotactic determination of strain C27 in a diffusion gradient chamber (DGC). In panel A, *R. meliloti* 1021 is inoculated in a 0.15% agarose gel to in which a 4mM gradient of ribose was established from left (high) to right (low), and grown for 3 days. In panel B, a separate DGC was inoculated with *R. meliloti* strain C27 carrying a Tn*5*-1063 tagged fusion with similarity to ribose transport genes, under the same conditions as panel A. Arrows indicate the point of inoculation.

above; Figure 3-2). The cells were allowed to grow and move for three days, at which point they were photographed. Strain C27 was able to grow with ribose as its sole carbon source, but did not move up the ribose gradient (Figure 3-2B), unlike the wild-type *R. meliloti* 1021 strain (Figure 3-2A). This provides proof that the Tn*5*-1063 insertion occurred in a gene necessary for ribose chemotaxis, probably the ribose transport gene *rbsA* (see sequence comparison results presented above and in Figure 3-1B).

DISCUSSION

Characterization of Tn*5*-1063 generated *luxAB* fusions induced by carbon deprivation.

Stress induced genes and gene products have been studied mainly through two dimensional gel electrophoresis in several bacteria (Spector and Cubitt, 1992; Matin, 1991; Givskov *et al.,* 1994). In this way, many proteins have been isolated, induced by one or multiple deprivation conditions. For some of these proteins, the correponding genes have been isolated. The use of reporter systems to isolate genes induced by deprivation conditions, however, has only been applied to a few bacterial systems including *Salmonella typhimurium*, resulting in the isolation of 7 *lacZ* gene fusions (Spector *et al.,* 1988), *Pseudomonas putida* (Kragelund *et al.,* 1995), and *Anabaena* Sp. (Cai and Wolk, 1997).

In this chapter, the use of *luxAB* as a reporter system in *R. meliloti* has resulted in the isolation of 12 loci that appear to be involved in the carbon deprivation response. The absence of a carbon source appears to cause the cell to induce the production of a number of carbon-transport proteins in its cellular membrane, making it more sensitive to carbon substrates in its environment, as well as more efficient at carbon uptake.

The total number of carbon deprivation-induced loci identified appears to be lower than was expected, given the large number of carbon deprivation-induced proteins in *E. coli* and other systems. This may be due to several factors. First, identifying carbon deprivation-induced *luxAB* fusions with this system was slightly awkward (due to $FNMH_2$ limitation) and may have reduced the ability of

deprivation promoters to be detected at the same level as those induced by nitrogen deprivation conditions. Second, it may be that the carbon deprivation-induced loci are required for normal growth and persistance, and therefore Tn*5* insertions would lead to lethal mutations. This possibility is increased due to the lack of Tn*5*-1063 fusions with homology to *E. coli* (and other bacterial) 'core' proteins induced by numerous stresses, such as the heat shock proteins described by Matin (1991). Third, the number of strains tested (5000) is likely be too small to adequately cover the genome (see chapter 7 for calculations). The occurrence of Tn*5*-1063 insertions within the same loci in two independent screenings—one induced by nitrogen deprivation (Tn*5*-1063 tagged loci in strains N5 and N9; see chapter 2) and one induced by carbon deprivation (Tn*5*-1063 tagged loci in strains C4 and C22)—suggests that this is not the case. However, chapter 7 presents data to support this third line of reasoning. Fourth, a large number of carbon-deprivation-induced loci may be post-transcriptionally regulated, rendering our approach unable to detect most of the carbon deprivation-induced loci. These possibilities will be examined in more detail in Chapter 7.

Of course, it may be that the number of carbon deprivation-induced loci in *R. meliloti* is simply smaller than the number of proteins induced in other bacteria, due to inherent differences in the kinds of proteins induced. This would support the hypothesis that soil bacteria such as *R. meliloti* have a different means of sensing and responding to environmental stresses than non-soil bactera. However, sequencing of the Tn5-1063 tagged loci, as well as physiological characterization of the strains carrying novel loci, needs to be completed before such conclusions can be drawn.

In spite of the possible limitations of the screening procedure used, the isolation of a locus from strain C47 with striking similarity to an ORF known to be induced by a poor carbon source (choline) is a good indication that we were successful in isolating carbon deprivation-induced loci. Also, the locus tagged in strain C27 is similar to *rbsA*, a gene involved in ribose transport. This gene, in addition to functioning as membrane-spanning carbon transport protein, is known to have a dual role as the primary receptor for chemotaxis toward ribose in *E. coli* (Bell *et al.*, 1986). Examination of chemotactic behavior in this Tn5-1063 tagged strain (C27) demonstrated it to be non-chemotactic for ribose. These data strongly suggest that the conditions applied for carbon deprivation were sufficient, if perhaps not completely adequate, to isolate carbon deprivation-induced loci.

MATERIALS AND METHODS

For bacterial strains and plasmids used, media and growth conditions, transposon mutagenesis, DNA isolation and manipulation, DNA sequence analysis, and nodulation and nitrogen-fixation assays see chapter 2 (Materials and Methods).

Screening of the Tn*5*-1063 insertion mutants for carbon deprivation-induced gene fusions

The *R. meliloti* strains carrying Tn*5*-1063 were spotted on a membrane filter (Nucleopore, Cambridge, MA) and incubated on solid GTS media for 48 hours. Luminescent colonies on the plate were visualized by spreading 50 µl of N-decanal inside the top of a glass petri-dish, placing the glass petri-dish top over the bottom of the plate, exposing the cells to N-decanal for 60 seconds, and measuring light emission using the Hamamatsu photonic system model C1966–20, as described by Wolk *et al.*, (1991).

Since the generation of $FMNH_2$, a cofactor required by luciferase, is closely correlated with the carbon status of the cells, and the levels of $FMNH_2$ appeared to be a limiting factor for luciferase activity in carbon starved cells, a screening procedure modified from that used in Chapter 2 was used. Colonies were transferred on filters to both GTS and GTS in which carbon sources had been omitted. The membrane filters on GTS without carbon were then transferred to regular GTS medium (containing succinate and glucose) and incubated for 30 minutes before visualization of luminescent colonies. This treatment was found to

provide sufficient energy for luciferase activity, while not interfering with the isolation of carbon deprivation-induced gene fusions (see the Results and Discussion sections above).

Testing of the Tn*5*-1063 tagged loci for induction by alternative carbon sources

The *R. meliloti* strains carrying Tn*5*-1063 were grown in 5 ml TY-Km^{200} media to saturation density (36 hrs), spotted on a membrane filter, and incubated on solid GTS media, containing 10 mM of each alternative carbon source tested, for 48 hours at 28°C. Colonies on the plates were then examined for light induction under the photonic camera as described above. It was not necessary to move the colonies to fresh plates, since all of the strains grew on all of the carbon sources indicating that energy was available for the luciferase reaction.

Use of a diffusion gradient chamber (DGC) to test chemotaxis

The DGC was kindly provided by David Emerson (Michigan State University), and set up according to the methods described in Emerson *et al.* (1994). The chamber was filled with 0.15% agarose, and a gradient of ribose was established ranging from 4mM to 0mM within the agarose chamber. A basal feed of GTS-Km^{100} media (without a carbon substrate) was flushed through the other two reservoirs to ensure that no other nutrients were limiting or formed a gradient. The gradient was allowed to form for two days, to ensure that it was complete, before inoculation with bacteria.

R. meliloti strains were grown to saturation (36 hrs) in 5 ml TY-Km^{200}, and a 10 ul portion of cells were inoculated by inserting the tip of a micropipette nearly to the bottom of the agarose and smoothly releasing the cells as the tip was removed, forming a single column of cells in the center of the chamber. *R. meliloti* strains in the agarose were allowed to grow for five days before the entire chamber was visualized photographically.

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CHAPTER 4

Isolation and Characterization of *Rhizobium meliloti* Genes Whose Promoters are Induced by Oxygen Limitation.

ABSTRACT

Oxygen levels in the soil environment vary drastically from microsite to microsite, as well as temporally due to changing water conditions within soil pores, and range from atmospheric levels to anaerobic conditions (van Elsas and van Overbeek, 1993). The ability to sense and respond to changing oxygen levels is one of the most important abilities of a soil microorganism, such as *Rhizobium meliloti*, which is also exposed to microaerobic (microoxic) conditions as an endosymbiont within the nodule of its host plant, alfalfa. Consequently, it has been of interest to understand how *R. meliloti* senses and responds to this microoxic condition. To investigate this question, the 5000 *R. meliloti* 1021 Tn*5-lux* insertion mutants that were screened for nitrogen and carbon deprivation (see chapters 2 and 3) were utilized to screen for gene fusions induced by microaerobiosis. The isolation of thirty-four gene fusions induced by microaerobiosis (1% O_2) is described. The strains harboring Tn*5-luxAB* fusions induced by microaerobiosis were tested for nodulation and nitrogen-fixation phenotypes. None were found to be Fix$^-$. Cloning and partial DNA sequence analysis of the transposon tagged loci revealed a variety of novel genes, as well as *R. meliloti* genes with sequence similarity to known bacterial loci. Genes identical to already described genes for exopolysaccharide synthesis (*exoO*), and for nitrogen fixation (*fixN*) were found, as well as genes with significant similarity to aldehyde dehydrogenases, amino acid transport proteins, and cytochrome oxidases.

INTRODUCTION

Rhizobia are non-fermenting aerobic bacteria which primarily use oxygen as their terminal electron acceptor (Long, 1989; Hirsch, 1992; Batut and Boistard, 1994). However, they are exposed to microoxic conditions during two distinct phases of their life-cycle, which comprise two distinct habitats: as free-living soil bacteria and as nitrogen-fixing plant endosymbionts. Both niches present a metabolic challenge to bacteria, in addition to other stresses they encounter in the environment such as the nutrient deprivation conditions described earlier (chapters 2 and 3). Thus, rhizobia have had to evolve mechanisms to sense and respond to changing oxygen conditions both in the soil and nodule environments.

Oxygen environment in the soil

As was stated in Chapter 1, the level of oxygen in the soil environment can change significantly over time with changing weather patterns, resulting in gradients of oxygen in the water films surrounding soil pores (Smiles, 1988; Metting, 1993). A microorganism in this environment can experience conditions ranging from standard atmospheric oxygen concentrations to virtually anaerobic conditions within a day (Foth, 1984). In addition, oxygen levels in the rhizosphere can be even lower than in the bulk soil, due to respiration by plant roots and microbial communities in the rhizosphere (Alexander, 1977). It is no surprise, then, that adaptation to changing oxygen conditions is one of the most important challenges of a soil microorganism, such as *R. meliloti*.

Oxygen environment in the nodule

The symbiotic association between rhizobia and their host plants results in a specialized ecological niche, the nodule (Figure 4-1). This specialized plant structure provides the physiological conditions required for the energy intensive nitrogen-fixation process (including high levels of bacterial respiration), while maintaining an intracellular free oxygen concentration less than 10nM, so that the oxygen sensitive nitrogenase enzyme is protected from irreversible oxygen damage (Batut and Boistard, 1994; Fischer, 1994; Kim and Rees, 1944).

The rhizobial nitrogen fixation genes are regulated in response to limited oxygen concentration via the two-component FixL/FixJ system (Fischer, 1994; Figure 4-2). FixL is a membrane-bound, oxygen-regulated, hemoprotein kinase and phosphatase that binds oxygen, resulting in an alteration of the Fe oxidation state, which in turn results in the autophosphorylation of FixL (Gilles-Gonzalez *et al.*, 1991), which is then able to phosphorylate FixJ (de Philip *et al.*, 1992; Fischer, 1994). Once phosphorylated, FixJ is able to act as a trascriptional activator in the induction of *nifA* and *fixK* (David *et al.*, 1988; de Philip *et al.*, 1990), which in turn regulate all of the *nif* and *fix* genes in *R. meliloti.* Kahn and Ditta (1991) have proposed that a conformational change occurs in FixJ when phosphorylated such that previously masked transcriptional activation sites in the C-terminus become unmasked. *In vitro* evidence by Da Re *et al.* (1994) supports this hypothesis.

Figure 4-2 illustrates the FixL/FixJ regulatory cascades (as well as the NtrB/NtrC regulatory cascade; see chapter 2) in species representative of the three rhizobial genera: *Rhizobium, Azorhizobium*, and *Bradyrhizobium.*

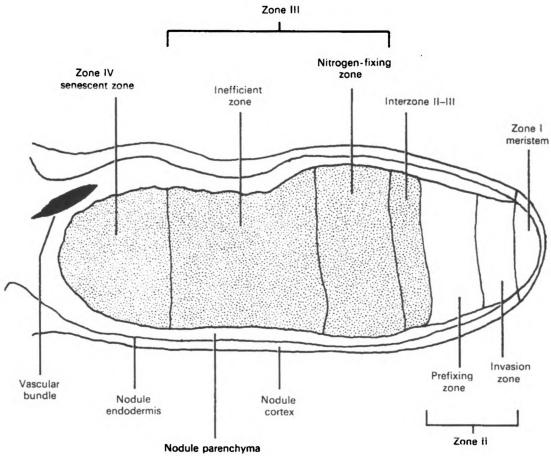
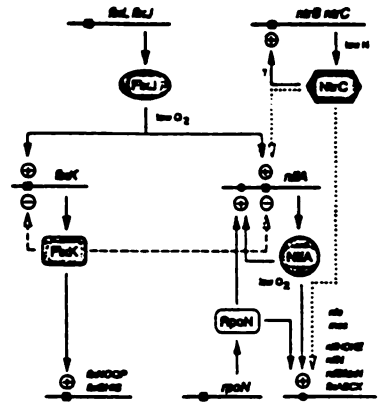
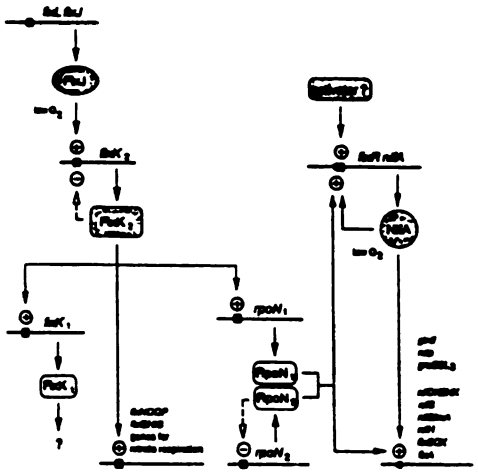


Figure 4-1. Diagram of an indeterminate nodule of alfalfa. The four zones of development are labeled, as well as various tissues. The characteristic meristem delineates the region where new cells are being added, causing the nodule to elongate. The central shaded region indicates the zones containing rhizobia. (Hirsch, 1992) For further details, see text.

A. *Rhizobium meliloti*



B. *Bradyrhizobium japonicum*



C. *Azorhizobium caulinodans*

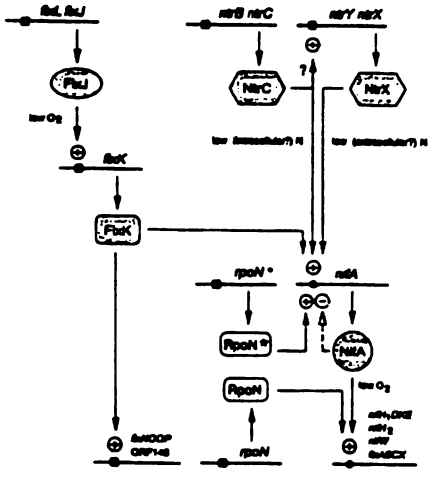


Figure 4-2. Comparative models of *nif* and *fix* gene regulation. Homologous regulatory proteins are symbolized identically in panels A, B, and C. Solid arrows and open arrowheads with dashed lines indicate positive and negative regulation, respectively. Dotted lines with open arrowheads denote NtrC-mediated activation of the *R. meliloti niHDKE, nifBfdxN,* and *fixABCX* promoters which is only observed in free-living cells, and is not relevant for symbiotic nitrogen fixation (from Fischer, 1994)., See text for further details.

Note that, although the *nif fix* genes are regulated differently in the three systems (primarily due to different NifA regulatory pathways), microaerobically induced gene expression occurs through the same FixL/FixJ two-component mechanism in all three genera.

FixK in rhizobia is part of a protein family of transcriptional regulators in bacteria, called the FNR family, which are responsible for many oxygen-regulated genes (Spiro, 1994; Fischer, 1994). The role of this transcriptional activator and repressor varies among bacterial genera. In most bacteria, regulation falls into one of two categories: regulation of the expression of genes involved in anaerobic metabolism (Spiro and Guest, 1991; Saffarini and Nealson, 1993; Zimmermann *et al.*, 1991; Cuypers and Zumft, 1993; Dispensa *et al.*, 1992), or regulation of genes necessary for nitrogen-fixation (Batut *et al.*, 1989; Colonna-Romano *et al.*, 1990; Anthamatten *et al.*, 1992; Kaminski *et al.*, 1991). However, FNR homologues have been described in other bacteria which are responsible for regulating other processes, such as bioluminescence (Meighen and Dunlap, 1993; Spiro, 1994) and cyanide synthesis (Sawers, 1991), and there are additional FNR homologues with unknown functions (Upadhyaya *et al.*, 1992; Irvine and Guest, 1993).

The symbiotic pattern of expression of N_2-fixation genes is tightly coupled to the physiology of the nodule, and expression has been shown to be induced at a sharply defined region of the nodule called interzone II-III (Soupène *et al.*, 1995; Figure 4-1). Figure 4-1 illustrates the different regions of an indeterminate nodule, such as those found on alfalfa. Note that bacteroids are present (indicated by shading) only inside of and within interzone II-III (i.e., in zones II-III, III, and IV). Evidence by Soupène *et al.* (1995) has verified the hypothesis that nitrogen fixation genes are regulated by oxygen according to a spatial program that is

related to the histological organization of the mature nodules. They found a steep decline in O_2 concentration approaching interzone II-III, attributed to the presence of an O_2 diffusion barrier. It is not known, however, whether interzone II-III itself restricts O2 diffusion, or what is the nature of the diffusion barrier.

To date, all microaerobically induced genes in rhizobia have been shown to be regulated via the FixL/FixJ system. However, as was stated in chapter 1, evidence from Zhulin *et al.* (1995) demonstrates that chemotactic behavior of *Rhizobium meliloti* in oxygen gradients is not regulated by the FixL/FixJ system.

Because of the success isolating nutrient deprivation-induced loci via the Tn*5-luxAB* method, I undertook the same approach to isolate gene fusions induced by microaerobic conditions. In this chapter, the generation and characterization of a collection of 34 *R. meliloti* mutants generated by random Tn*5*-1063 mutagenesis, carrying gene fusions induced by 1% oxygen, is described. Cloning and partial DNA sequence analysis of the transposon tagged loci revealed a variety of potentially novel genes, as well as *R. meliloti* genes with sequence similarity to known bacterial loci. Genes identical to already described genes for exopolysaccharide synthesis (*exoO*), and for nitrogen fixation (*fixN*) were found, as well as genes with significant similarity to dehydrogenases, carbon transport proteins, and cytochrome oxidases.

RESULTS

Isolation of strains carrying Tn*5*-1063 gene fusions induced by microaerobiosis

The screening procedure used for isolating microaerobically induced genes was modified, since it was unnecessary to move the filters bearing the colonies to new plates in order to "starve" them for oxygen. Preliminary experiments, however, made it clear that the results were more consistent and clear (stronger light emission) when the bacteria were plated on nucleopore filters, rather than directly on the media. A 1% oxygen mixture was chosen because it is as low as one can go before *R. meliloti* ceases to grow. Moreover, this concentration of oxygen has been shown to induce the FixL/FixJ regulated nitrogen fixation genes in rhizobia (Ditta *et al.,* 1987; Gilles-Gonzalez *et al.,* 1991; Reyrat *et al.,* 1993; Page and Geurinot, 1995). Using 1% oxygen induction conditions, 34 Tn*5*-1063 generated *luxAB* gene fusions were isolated whose bioluminescence was consistently increased at 1% O_2 (Table 4-1).

In order to test the nature of the transposition events involved in generating these strains, total DNA was isolated, digested with *Eco*RI or *Cla*I (which do not cut within the transposon; Wolk *et al.,* 1991), and hybridized with plasmid pRL1063a DNA as a probe. Only two of the thirty-four mutants displayed multiple bands (Table 4-1a), indicating that a single, simple transpositional event occurred in the majority of the strains, as observed with strains carrying fusions induced by nitrogen and carbon deprivation (chapters 2 and 3). The two strains with multiple hybridizing fragments were not further examined except as part of the nodulation and nitrogen fixation assays (Table 4-1b).

Table 4-1. Phenotypes of Tn5-1063 insertion mutants of R. meliloti 1021 induced by microaerobiolsis.

Strain	Symbiotic Phenotype [a]		Hybridizing Fragments [b]	Gene Similarity [c]
	Nod	Fix		
Ox1 /C101	+	+	one	NS
Ox2	+	+	one	NS
Ox3	+	+	one	*acoD*, aldehyde dehydrogenase
Ox4	+	+	one	*FixN*, nitrogen fixation
Ox6	+	+	one	*braF*, amino acid transport
Ox19	+	+	one	*FixN*, nitrogen fixation
Ox24	+	+	one	NS
Ox25	+	+	one	NS
Ox60	+	+	two	n.d.
Ox67	+	+	one	NS
Ox72	+	+	one	NS
Ox81	+	+	one	NS
Ox86	+	+	one	NS
Ox106	+	+	one	*exoO*, succinoglycan synthesis
Ox107	+	+	one	NS
Ox124	+	+	one	NS
Ox128	+	+	three	n.d.
Ox139	+	+	one	NS
Ox153	+	+	one	NS
Ox168	+	+	one	NS
Ox183	+	+	one	NS
Ox192	+	+	one	NS
Ox193	+	+	one	ORF6 in *cbbR* 3'end
Ox202	+	+	one	NS
Ox206	+	+	one	NS
Ox209	+	+	one	NS
Ox215	+	+	one	NS
Ox219	+	+	one	*CyoC*, cytochrome oxidase
Ox220	+	+	one	NS
Ox221/C22	+	+	one	NS
Ox222	+	+	one	NS
Ox223	+	+	one	NS
Ox226	+	+	one	NS
Ox249	+	+	one	NS

a) Symbiotic phenotype of alfalfa roots, inoculated with *R. meliloti* strains carrying Tn5-1063 tagged genes induced by microaerobiosis, for the presence of nodules on roots (Nod+) and the presence of nitrogenase as determined by acetylene reduction (Fix+).

b) Number of bands of genomic DNA cut with *Eco*RI or *Cla*I hybridizing to a probe of whole pRL1063a.

c) Genes sharing significant similarity with *R. meliloti* ORFs: *acoD* (Priefert *et al.*, 1992); *fixN* (Preisig *et al.*, 1993); *braF* (Hoshino & Kose, 1990); *exoO* (Becker *et al.*, 1993); *cyoC* (Chepuri *et al.*, 1990); NS=no significant similarity to known genes; n.d. = not determined.

The strains harboring gene fusions induced by 1% oxygen levels were examined for their symbiotic phenotype by testing their ability to form effective nodules on alfalfa seedlings. Nodule nitrogenase activity was measured by the acetylene reduction assay (see chapter 2, Materials and Methods). All of the strains were capable of forming nitrogen-fixation competent nodules (Nod^+ Fix^+) comparable to those induced by wild-type *R. meliloti* 1021 (summarized in Table 4-1b).

Sequence similarities of microaerobically induced Tn*5*-1063 tagged *R. meliloti* loci

The microaerobically induced *luxAB* fusions were cloned from the genome, and partial DNA sequences of the Tn5-1063 tagged loci were determined. The amino-acid sequences deduced from the primary DNA sequence were compared to sequences in the non-redundant protein databases at GenBank. By analyzing the codon usage in the primary sequence (see chapter 2, Figure 2-4) significant ORFs were identified in all the tagged loci (data not shown), and seven of the 34 loci analyzed were found to share significant similarity with known genes (Table 4-1b). However, the majority of the loci (27 out of 34) appear to be novel since they did not share significant similarity with GenEMBL sequences (as of 8/1/97).

The fusion in strain Ox1 was found to be the same as the previously identified carbon deprivation-induced fusion in strain C101 (chapter 3). Testing strain Ox1 for carbon deprivation induction revealed that it was indeed induced by both microaerobiosis and carbon deprivation. In addition, strain Ox221 was found to carry a fusion that had been identified both in the nitrogen deprivation-induced, and in the carbon deprivation-induced set of strains (strains N104 and C4/C22,

chapters 2 and 3). This interesting strain was thus the only one found to carry a *luxAB* fusion induced by all three stresses tested (see Discussion below). Sequence data from the Tn*5*-1063 tagged loci of strains Ox4 and Ox19 were identical, as were sequence data from the Tn*5*-1063 tagged loci of strains Ox81 and Ox86, thus indicating that these two sets of two strains each are clonal. Two of the Tn*5*-1063 tagged loci (in strains Ox4 and Ox106) correspond to already seqenced genes of *R. meliloti* (*fixN* and *exoO*; see Discussion below).

The Tn*5*-1063 tagged locus in strain Ox3 was found to be most similar (P(N) = 2.0 x 10^{-12}) to the aldehyde dehydrogenase gene *acoD* from *Alcaligenes eutrophus* (Priefert *et al.,* 1992), as well as to aldehyde dehydrogenases from *Rhodococcus* sp. (*thcA*; Nagy *et al.,* 1995), *E. coli* (*aldB*; Sofia, *et al.,* 1994; Xu and Johnson, 1995), and *Vibrio cholerae* (*aldA*; Parsot and Mekalanos, 1991)

The Tn*5*-1063 tagged locus in strain Ox4 (as well as in the identical strain Ox19) was found to match the *fixN* gene, which has been completely sequenced (Kahn *et al.,* 1993), and encodes a component of an alternative cytochrome-c containing heme/copper cytochrome oxidase required for nitrogen-fixation in rhizobia (Preisig *et al.,* 1993). There are two copies of this gene in *R. meliloti*, such that a single mutation does not result in a Fix^- phenotype. This observation was confirmed in this study (Table 4-1b).

The Tn*5*-1063 tagged locus in strain Ox6 was found to be most similar (P(N)=4.2 x 10^{-13}) to the *Pseudomonas aeruginosa braF* gene encoding a branched-chain amino-acid transport protein (Hoshino and Kose, 1990), as well as to amino-acid transport proteins in *E. coli* (*livG*; Adams *et al.,* 1990; Sofia *et al.,* 1994), and *Salmonella typhimurium* (*livG*; Matsubara *et al.,* 1992). The highest region of

similarity occurred in an ATP-binding motif common to active-transport proteins (Hoshino and Kose, 1990).

The Tn*5*-1063 tagged locus in strain Ox106 was found to match the *R. meliloti exoO* gene, which is part of an operon (*exoAMONP*) involved in succinoglycan (exopolysaccharide) biosynthesis (Glucksmann *et al.*, 1993; Becker *et al.*, 1993). Interestingly, the Tn*5*-1063 tagged insertion mutant in strain Ox106 displayed a Nod^+ Fix^+ phenotype (Table 4-1b), in contrast to other *exo* mutants of *R. meliloti*, which result in an empty nodule (Nod^+ Fix^-) phenotype (Finan *et al.*, 1985; Leigh *et al.*, 1987; Gray and Rolfe, 1990; Hirsch, 1992).

The Tn*5*-1063 tagged locus in strain Ox193 was found to be highly similar ($P(N)=8.5 \times 10^{-23}$) to an unknown ORF located 3' to CbbR, a LysR-type transcriptional activator in *Xanthobacter flavus* (van den Bergh *et al.*, 1993). This protein is required for expression of the autotrophic CO_2-fixation enzymes of *X. flavus*.

The Tn*5*-1063 tagged locus in strain Ox219 was found to have an extremely high similarities to cytochrome quinol oxidases from *Paracoccus denitrificans* (cytochrome ba(3) III, $P(N)=2.2 \times 10^{-44}$; Richter *et al.*, 1994), *E. coli* (*cyoC*, $P(N)=1.8 \times 10^{-34}$; Chepuri *et al.*, 1990), and *Acetobacter aceti* (cytochrome a1 chain III, $P(N)=3.9 \times 10^{-31}$; Fukaya *et al.*, 1993). Therefore, it seems likely that the Tn*5*-1063 tagged locus in strain Ox219 encodes a cytochrome quinol oxidase.

DISCUSSION

Characterization of Tn*5*-1063 generated *luxAB* fusions induced by microaerobiosis

Because of the success in isolating nitrogen deprivation-induced loci and carbon deprivation-induced loci in *R. meliloti* using Tn*5*-1063 tagged mutants, a similar approach was used to isolate microaerobically induced loci. In this chapter, the use of *luxAB* as a reporter system in *R. meliloti* resulted in the isolation of 34 loci involved in microaerobiosis, including genes involved in nitrogen fixation (*fixN*) and succinoglycan synthesis (*exoO*), as well genes with similarity to dehydrogenases, amino acid transport proteins, and cytochrome oxidases. The significance of the similarity of tagged loci to other genes is discussed below.

The number of genes isolated by microaerobiosis was higher than in the other two deprivation conditions tested (chapters 2 and 3). This may indicate that a larger number of genes is responsive to this testing condition, or it may be that the potential problems of using a transposon to tag genes of interest (discussed in chapters 2 and 3) do not apply to genes regulated by low oxygen concentration. In addition, the large number of novel genes tagged (27), similarly to the large number of novel genes described in chapters two and three, support the hypothesis that soil bacteria may have different genes than non-soil bacteria for dealing with environmental limitation conditions. As with the tagged loci described in chapters two and three, more work needs to be done characterizing the novel loci and their regulation, as well as complete sequencing of the tagged genes, before this hypothesis can be fully examined (see chapter 7 for further discussion of this important hypothesis).

The similarity of the locus in strain Ox3 to aldehyde dehydrogenases may indicate that a gene encoding an enzyme in a redox pathway, that is induced due to microaerobiolsis, has been tagged. The aldehyde dehydrogenases to which this locus has similarity are located on the chromosome near cytochrome oxidases and are believed to participate in the metabolism of various substrates (Nagy *et al.*, 1995). Therefore, this gene may function in cooperation with other redox enzymes and cofactors, in conjunction with alternative cytochrome oxidases, to provide the cell with the reducing equivalents it needs during microaerobiosis. This is particularly important while in the nodule, where the high ATP demands of nitrogen-fixation make it necessary to increase respiration rates (Fischer, 1994). Interestingly, expression of the *V. cholerae* gene (*aldA*) is regulated by ToxR, a transcriptional activator which regulates several virulence genes (Parsot and Mekalanos, 1991). This is reminiscent of the transcriptional activator FixK in *R. meliloti* which regulates several nitrogen fixation genes (Figure 4-2).

The microaerobic induction of the putative amino acid transport protein gene tagged in strain Ox4 is less simple to explain. Plant roots are known to exude many amino acids. Therefore, a microaerobically induced transport system may be involved in sensing the lower oxygen potential near the root and inducing transport genes which would help *R. meliloti* to compete for the available limited resources. Another possibility, suggested by the presence of an ATP-binding motif, is that the gene tagged in strain Ox4 is simply an active-transport protein of an unknown substrate. Best matches of the tagged loci to amino acid transporters may be coincidental, since these proteins belong to a family of proteins, called ABC transporters, which all bind ATP in order to actively transport a variety of

substrates. Complete sequencing of this locus and further comparison to GenBank sequences should resolve this question.

The interesting finding that the *R. meliloti exoO* gene is regulated by microaerobiosis constitutes the first report of any exopolysaccharide gene in *R. meliloti* being regulated by microaerobiosis. As mentioned in chapter 2, it is known that exopolysaccharide synthesis is decreased in the presence of ammonia (Ozga *et al.,* 1994), although it is not known how this regulation occurs. In addition, two of the *exo* genes characterized in this study (*exoYF*, see chapter 2) were isolated by nitrogen deprivation induction of their tagged genes, thus confirming the regulation of this operon in response to available nitrogen. Figure 4-3 illustrates the complete *exo* regulon, with the *exoFY* and *exoO* genes, tagged in this study, indicated. *R. meliloti* has apparently evolved two divergent operons responding to two environmental conditions it would find in the rhizosphere; microaerobiosis and nitrogen deprivation. This complicated regulatory cascade could help the bacterium compete in the soil by preventing the induction of unnecessary (and perhaps energy intensive) genes, while at the same time providing a mechanism to sense the presence of rhizosphere conditions leading to successful nodulation. Figure 4-3 also illustrates why an insertion in *exoO* (or any of the genes in this operon) is not Fix$^-$ due to a duplication of the *exoHKLAMONP* operon, unlike the Fix$^-$ phenotype of the *exoFY* mutants isolated in chapter 2.

The similarity of the locus in strain Ox193 to an ORF downstream from CbbR in *X. flavus* presents some interesting possibilities. CbbR is a LysR-type transcriptional activator that is required in *X. flavus* for the expression of autotrophic CO_2 fixation enzymes (van den Bergh *et al.,* 1993), suggesting that the Tn*5*-1063 tagged locus in strain Ox193 might be in an operon regulated by a

Figure 4-3. Map of the exo gene cluster on the second symbiotic megaplasmid of *R. meliloti* Rm1021. Boxes represent the open reading frames of the *exo* genes, and the arrows indicate the orientation of transcription. A duplicated region is indicated by a hatched box (adapted from Glucksmann *et al.*, 1993). For additional details, see the text.

transcriptional activator encoded nearby. Further sequence analysis of this tagged fusion, both upstream and downstream of the insertion site, would determine if this assertion is true.

Isolation of a known oxygen-regulated gene (*fixN*), as well as another putative cytochrome oxidase (in the Tn*5*-1063 tagged locus of strain Ox219), suggests that the conditions for oxygen limitation chosen were both adequate and sufficient for the identification of microaerobically induced loci. It was hypothesized that changes in oxygen levels would require the bacteria to respond by altering its ability to bind oxygen via induction of alternative cytochrome oxidases. Therefore, isolation and characterization of a tagged gene in *R. meliloti* with very high similarity to cytochrome oxidases was expected. What is, perhaps, unexpected is that more cytochrome oxidases were not found. This may be a true reflection of the induction of cytochrome oxidases by oxygen limitation, or may be related to

problems discussed in chapters 2 and 3 regarding the nature of the Tn*5*-1063 system. In addition, screening of 5000 Tn*5*-1063 insertion mutants is probably not sufficient to saturate the genome (see chapter 7 for calculations).

MATERIALS AND METHODS

For bacterial strains and plasmids used, media and growth conditions, transposon mutagenesis, DNA isolation and manipulation, DNA sequence analysis, and nodulation and nitrogen-fixation assays see chapter 2 (Materials and Methods).

Screening of the Tn*5*-1063 insertion mutants for microaerobically induced gene fusions

The *R. meliloti* strains carrying Tn*5*-1063 were spotted on a membrane filter (Nucleopore) and incubated on solid GTS minimal media for 36 hours at 28°C. The plates were then placed in a sealed oxygen chamber, that was continuously flushed with a 1% (± 0.1) oxygen-mixture for seven hours, in a growth chamber set to a temperature of 28°C. The concentration of oxygen was monitored using an oxygen electrode. At the end of the incubation period, the plates were removed from the oxygen chamber and briefly exposed to standard oxygen concentrations before the period of luminescence measurement in order to provide substrate (O_2) for the luciferase enzyme.

Luminescent colonies on the plate were visualized by spreading 50 µl of N-decanal inside the top of a glass petri-dish, placing the glass petri-dish top over the plate, exposing the cells to N-decanal for 60 seconds, and measuring light emission using the Hamamatsu photonic system model C1966–20, as described by Wolk *et al.* (1991).

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CHAPTER 5

Rhizobium meliloti Genes Induced by Nutrient Deprivation are Important for Competition in Nutrient Poor Soil.

Parts of this chapter have been presented at the 6th International Microbial Ecology Symposium (Lim *et al.*, 1993) and the 10th International Nitrogen Fixation Symposium (de Bruijn *et al.*, 1995), and have been submitted for publication in *Molecular Microbiology* (Ragatz *et al.*, 1997).

ABSTRACT

Competition between bacteria in the soil and rhizosphere for changing supplies of scarce nutrients is an important factor that distinguishes the soil microenvironment from other microhabitats. Little is known about how soil bacteria sense changing stress conditions, such as nutrient deprivation, and what role is played by the resulting genetic cascade in competition for survival in this harsh environment. Consequently, it has been of interest to address this question using *R. meliloti* strains with genes, tagged by Tn*5*-1063, induced under conditions of carbon or nitrogen deprivation. Three strains harboring Tn*5*-1063 insertions induced by carbon deprivation, and one strain harboring Tn5-1063 insertions induced by nitrogen deprivation, were selected for examination of persistence and competition with the wild-type strain 1021 in two soil types: a nutrient rich and a nutrient poor soil system. All four mutant strains exhibited a decline in bacterial numbers when inoculated in competition with the wild-type strain in nutrient poor soil, but exhibited no relative decline in numbers during persistence in this soil. No relative difference was found for either the mutant strains or the wild-type strain when inoculated in nutrient rich soil.

INTRODUCTION

The soil environment is highly oligotrophic, and bacterial growth rates are extremely slow under these conditions (see Chapter 1). Nutrients are either scarce or in a recalcitrant form making them inaccessible to the microflora (Alexander, 1977; Foth, 1984; Metting, 1993). When nutrients do become available, there is a burst of microbial cell growth by the indigenous populations which quickly scavenge any available nutrients and return the soil to its normal oligotrophic state (van Elsas and van Overbeek, 1993). Therefore, it would seem that the ability to sense and respond to environmental signals, such as nutrient deprivation, would be of benefit to the competition and survival of a species in the soil environment. This is the hypothesis to which this chapter is devoted.

Although much is known about bacterial gene expression patterns from laboratory studies involving growth of bacterial cultures under ideal conditions in terms of temperature, pH, and nutrients, comparatively little is understood about their behavior under unfavorable conditions, such as are found in the soil environment. Investigation of gene expression induced by nutrient deprivation conditions is rare, and has been largely studied in non-soil isolates. However, it is my contention that soil isolates may have different strategies for dealing with nutrient deprivation than non-soil isolates, such as enteric or marine bacteria. Recently, the effect of nutrient deprivation on *Pseudomonas putida,* a common gram-negative soil bacterium, has been reported (Givskov *et al.*, 1994), using 2D-PAGE protein analysis, the most common method for analyzing bacterial responses to nutrient deprivation, as has been pointed out in previous chapters. However, bacterial mutations in loci encoding these proteins carrying useful markers for tracking them in the soil have not been available thus far (Givskov, *et al.*, 1994;

Matin, 1991). Therefore, almost nothing is known about how mutations in the loci encoding stress induced proteins affect survival and competition in the soil.

One of the problems associated with studying the relative persistence or competition of (mutant) bacteria of interest has been how to track them in the soil environment. Traditionally, genetic markers such as antibiotic resistance genes have been used; however, the potential for lateral dissemination of genes encoding these antibiotic resistance proteins precludes extensive examination in studies of natural ecosystems (Tiedje *et al.,* 1989; Gustafsson and Jansson, 1993).

Consequently, many other marker systems have been developed to detect GEMs in the soil including genes encoding metabolic enzymes such as *lacZY* (encoding β-galactosidase and lactose permease; Kluepfel, 1993), *xylE* (encoding catechol 2,3 oxygenase; Saunders *et al.,* 1995), and *gusA* (encoding β-glucuronidase [GUS]; Wilson *et al.,* 1994, 1995) which are detected by their phenotypes when grown on appropriate media. Although these systems have some advantages in particular instances, two major problems of their use in soil systems are the potential of background marker enzyme activity in the indigenous microflora, and the requirement for growth and cultivation in the detection methods. New molecular techniques such as nucleic acid probes (Zeph and Stotzky, 1989), PCR amplification of DNA (Akkermans *et al.,* 1991; Smalla *et al.,* 1993) and rRNA (Hahn *et al.,* 1990), and a novel hybrid gene *phoE-caa* which can be detected using PCR, or immunological methods (Zaat *et al.,* 1994), have thus become invaluable tools for tracking bacteria of interest in the soil.

Other groups have focused on the use of visible markers such as the *Vibrio* sp. *lux* genes encoding luciferase (Prosser, 1994), and the beetle *luc* genes (Cebolla, *et al.,* 1993), which encode firefly and other beetle luciferases. The luciferase encoded by *luc* may be more sensitive (e.g., induced to a higher level) than the one encoded by *lux,* as well as having more versatility due to the

availability of *luc* derivatives that emit different wavelengths of light (Cebolla *et al.*, 1995; Jansson, 1995). New types of phenotypic markers continue to be discovered. Most recently, green fluorescent protein (GFP), cloned from jellyfish, has been employed as an environmental marker (Chalfie *et al.*, 1994) because it requires no exogenous substrate, unlike other light emitting proteins. See Jansson (1995) for an excellent review of these and many other marker systems being used to track bacteria in the soil and other environments.

The potential to use *luxAB* expression as a visible marker for soil and rhizosphere studies was one of the driving motivations for this study. After the isolation and characterization of strains carrying fusions induced by environmental stresses or other conditions, it was hoped that these strains could be used directly in the soil environment to assess the importance of their tagged genes for persistence and competition experiments. Studies with *R. meliloti* strains, carrying Tn*5*-1063 *luxAB* fusions expressed constitutively at a high level, in different soil environments were undertaken to test the sensitivity of a fiber optic-CCD camera system (D.M. Ragatz and F.J. de Bruijn, unpublished). Results of this study indicated that available equipment (including the fiber-optic system) for the detection of *luxAB* expression in *R. meliloti* within the soil matrix, was not sensitive enough to be used in detecting *luxAB* expression as an ecological marker. Therefore, it was necessary to use the antibiotic resistance markers present in the fusions for small, contained soil competition experiments, with the idea that they could be replaced later (via gene-replacement techniques) with a better marker (such as *luc* or GFP mentioned above), for larger, more extensive ecological studies in natural soil environments.

In general, when tagged bacteria have been studied in the soil environment, it has been to compare the persistence of these introduced strains versus the indigenous strains. For example, Tn*5*-tagged *Pseudomonas fluorescens* populations

were introduced into a sandy soil and showed a progressive decline in numbers (van Elsas *et al.,* 1991). The general assumption in experiments of this type is that the bacteria are not adversely affected by the introduction of the marker into the genome. In fact, one might imagine that the insertion of any type of foreign element into the bacterial genome might have a deleterious effect, however minor. However, more work has to be done with different markers in many different systems to establish whether or not this hypothesis holds true. Curiously, one report of *E. coli* growing in batch cultures revealed that the presence of IS50, the insertion sequence at the ends of the Tn*5* transposon, improved the growth of cells relative to their otherwise isogenic counterparts (Hartl *et al.,* 1983). This result did not depend on either the initial position, or the actual transposition of the IS50 element, or a transposon containing it. Thus, the presence of the IS50 element, itself, appears to mediate a physiological effect in the cells, rather than via inactivation of genes during transposition. Whether or not the IS50 insertion sequence and/or Tn*5* transposons would have a beneficial effect outside of the chemostat is not known, but is of obvious interest.

In this chapter, the use of *R. meliloti* with Tn*5*-tagged loci induced by carbon or nitrogen deprivation is reported as a model system to measure persistence and competition (survival) in soil systems. Sterile soil systems, containing either a nutrient rich soil or a nutrient poor soil, were examined first to minimize the influence of the indigenous microflora. The competition of four *R. meliloti* Tn*5*-1063 mutant strains (carrying tagged fusions induced by carbon or nitrogen deprivation) with an otherwise isogenic *R. meliloti* 1021 wild-type parental strain was examined over time. In addition, persistence of the *R. meliloti* mutant strains, as well as the wild-type strain, in the soil was examined. All four mutant strains exhibited a significant decline in bacterial numbers when inoculated in competition with the wild-type strain in nutrient poor soil, but exhibited no significant decline

in numbers during persistence in this soil. No significant decline in bacterial numbers over time was found for either the mutant strains or the wild-type strain when inoculated in nutrient rich soil.

RESULTS

Effect of mutations in deprivation-induced loci on persistence in the soil

Soil studies were initiated in order to examine the hypothesis that nutrient deprivation-induced genes are important for persistence and competition of *R. meliloti* in the environment. Mutant strain C101, carrying a Tn*5*-1063 fusion induced by carbon deprivation, and mutant strain N5 carrying a Tn*5*-1063 fusion induced by nitrogen deprivation, were inoculated in sterile, nutrient-rich (NR) and nutrient-poor (NP) soils, separately or in combination with the wild-type strain (see Materials and Methods). The soils were thoroughly mixed, and samples were taken weekly and plated on selective media to determine bacterial numbers. The results of these plate counts are presented in Figure 5-1, a grapth representative of two independent experiments for each mutant strain tested. No soil counts were taken at time zero (only culture concentration), therefore no zero points are plotted.

The Tn*5*-1063 tagged bacteria were tested for their ability to persist independently of wild-type bacteria by inoculating them in either NR or NP soil, and examining their plate counts over time. The mutant strains examined (C101 and N5), as well as the wild-type strain 1021, behaved similarly, showing little or no decline in plate counts (Figure 5-1A & C), although a difference (approximately ten-fold) could be observed between the relative numbers of bacteria growing in the NP soil versus NR soil.

In order to test the effect of the Tn5-1063 induced mutations on bacterial competition, C101 and N5 mutant strains were coinoculated with the wild-type strain at a 1:1 ratio in NR and NP soils. The mutant strains did not show any deviation nor decline in numbers vs. the wild-type in NR soil (Figure 5-1B & D; solid symbols and lines).

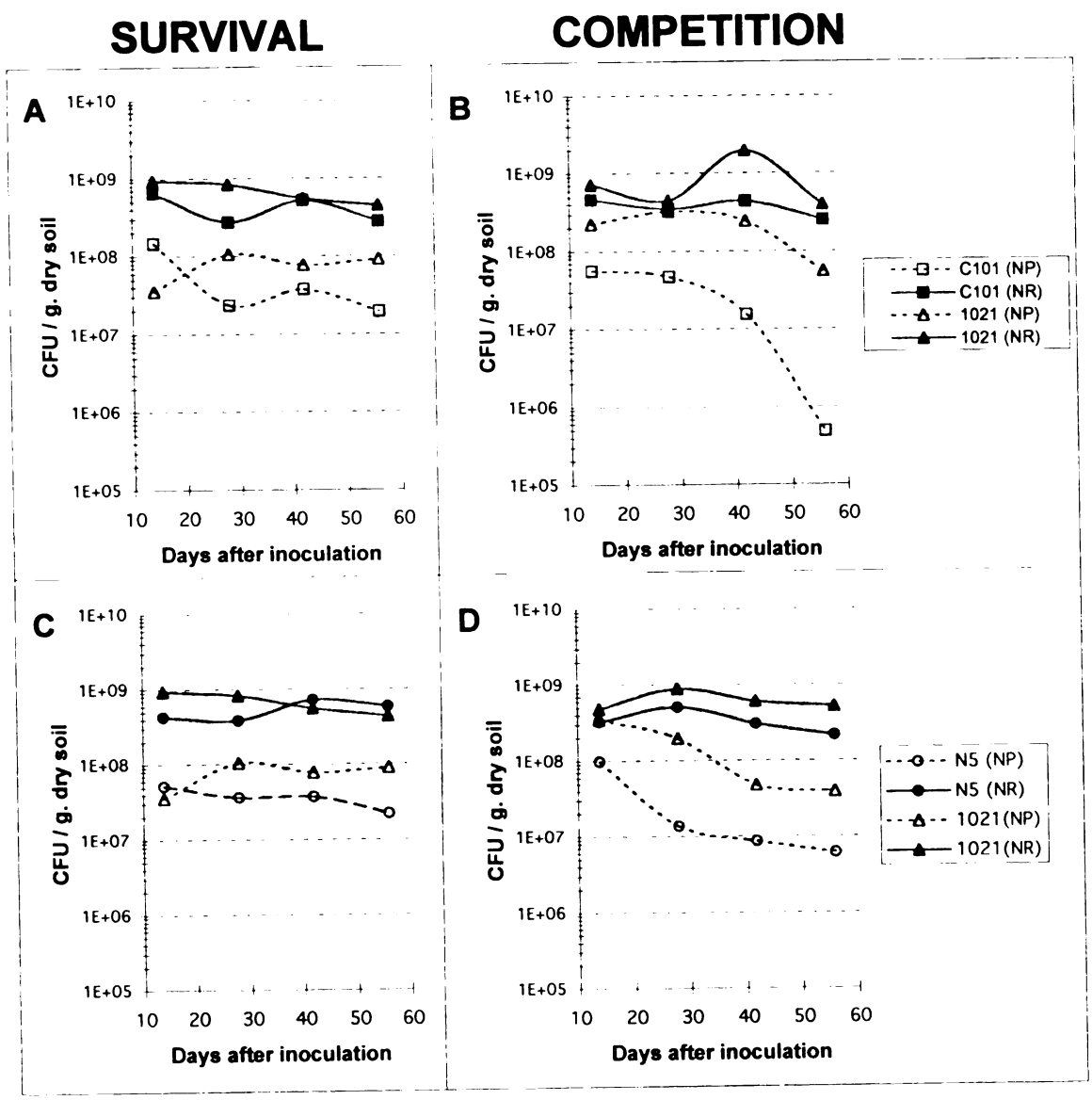

Figure 5-1. Persistence and competition of selected Tn5-1063 induced mutant strains in soil. In Panels A & C, the persistence of strains C101 (boxes), N5 (circles) and the wild-type strain Rm1021 (triangles) as single inocula in NR soil (closed symbols) and NP soil (open symbols; hatched lines) is shown. The X-axis indicates days after inoculation in soil, and the Y-axis indicates the colony forming units (CFUs) per gram of dried soil. In Panels B & D, the competitive ability of strain C101 (boxes) versus 1021 (diamonds), and strain N5 (circles) versus 1021 (diamonds) in dual 1:1 inoculation experiments is shown.

In contrast, an observed reduction in survival of the mutant strains versus the wild-type strain occurred in NP soil (Figure 5-1B & D; open symbols and dashed lines). This reduction was not as pronounced in strain N5 where the wild-type strain decreased as a similar rate. Weekly plate counts from serial dilutions of soil samples were carried out for a total of 56 days for mutant strains C101 and N5, as well as for the wild-type strain 1021.

Two other mutants, strains C18 and C27, carrying Tn*5*-1063 fusions induced by carbon deprivation, were examined for their persistence and competition in soil using the same experimental procedures. The functions of the loci harboring the Tn*5-luxAB* fusions in these two strains have been partially elucidated by sequence similarities (see Chapter 3), and are likely to encode an oxidoreductase (strain C18) and a ribose transport/chemotactic protein (strain C27). The results of the plate counts are presented in Figure 5-2, a graph representative of two independent experiments with each mutant strain tested. No soil counts were taken at time zero (only culture concentration), therefore no zero points are plotted. Weekly plate counts from serial dilutions of soil samples were carried out for a total of 42 days for mutant strains C18 and C27, as well as for the wild-type strain 1021.

Results similar to those presented in Figure 5-1A & C for strains C101 and N5 were observed with strains C18 and C27. In simple inoculation experiments in NR soil, no deviation or decline in plate counts over the time period tested were observed (Figure 5-2A & C). Again, an approximately ten-fold decrease of total counts could be observed comparing bacteria growing in NP versus NR soil.

As had been observed with strains C101 and N4, neither strain C18 nor C27 showed any decline in plate counts relative to the wild-type in coinoculation (competitive) experiments in NR soil (Figure 5-2B & D; solid symbols and lines).

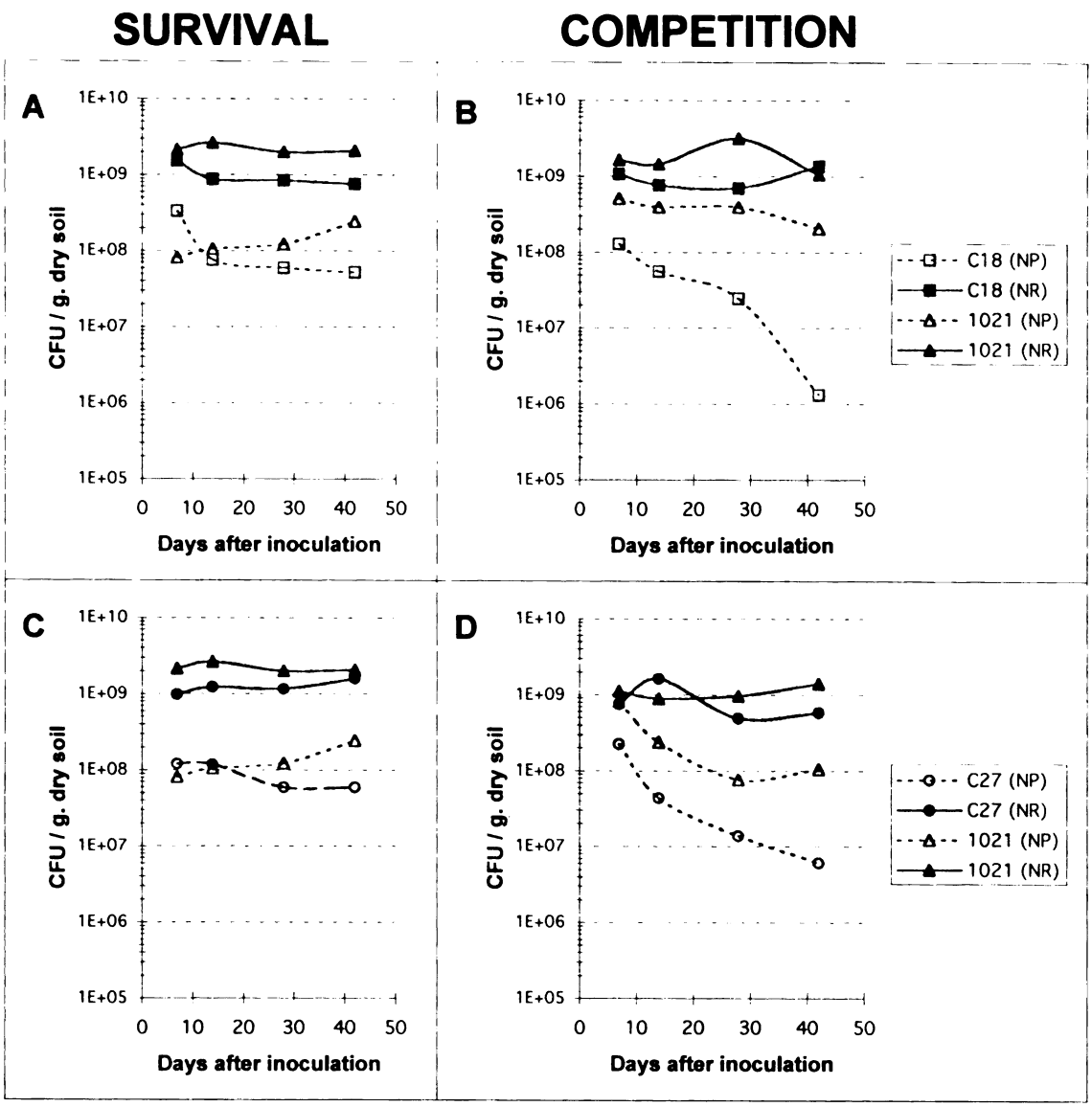

Figure 5-2. Persistence and competition of other selected Tn5-1063 induced mutant strains in soil. In Panels A & C, the persistence of strains C18 (boxes), C27 (circles) and the wild-type strain Rm1021 (diamonds) as single inocula in NR soil (closed symbols) and NP soil (open symbols; hatched lines) is shown. The X-axis indicates days after inoculation in soil, and the Y-axis indicates the colony forming units (CFUs) per gram of dried soil. In Panels B & D, the competitive ability of strain C18 (boxes) versus 1021 (diamonds), and strain C27 (circles) versus 1021 (diamonds) in dual 1:1 inoculation experiments is shown.

However, a large reduction in relative plate counts was observed in NP soil (Figure 5-2B & D; open symbols and dashed lines).

Overall, the relative reduction in bacterial plate counts was most pronounced for the strains harboring fusions induced by carbon deprivation, resulting in a decline to approximately 1.0% (strains C101 and C27) to less than 0.1% (strain C18) of their original numbers. The mutant strain N5 carrying a nitrogen deprivation-induced Tn5-1063 fusion showed a decline to approximately 10% of its original cell numbers in NP soil.

DISCUSSION

The large relative decline in bacterial numbers of mutant strain C101 only in the presence of wild-type bacteria in nutrient poor (NP) soil suggests a role for this induced locus; namely, as a mechanism to compete for, and utilize, unusual or 'poor' substrates as energy sources critical to the long-term survival of *R. meliloti* in competition with other microbes in the soil. Since the Tn*5*-1063 fusion in strain C101 is not induced by alternative carbon sources (see chapter 3, Table 3-1b), it may encode a protein that is near the top of a regulatory pathway, such as a sensor, making strain C101 especially susceptible to deprivation conditions in competition with the wild-type *Rhizobium.* In fact, the Tn*5*-1063 fusion in strain C101 is also induced by low oxygen levels (see Chapter 4), and thus makes sense as a regulatory protein. Alternatively, the gene fusion in mutant C101 may be involved in competition in some way that is not directly related to carbon assimilation, but to the deprivation condition itself. To date, no genes have been isolated in *R. meliloti* that are directly involved in competition in the soil. However, it is known that mutations in some of the *nod* genes, and in *nifA*, reduce both nodulation rate and competitiveness (Downie and Johnston, 1988; Sanjuan and Olivares, 1991). In addition, two genes have recently been isolated by Tn*5* mutagenesis which appear to be involved only in nodule competitiveness (Onishchuk *et al.,* 1994). The nature and regulation of these genes is unknown.

The mutant strains C18 and C27 were also remarkably affected by the presence of the wild-type bacteria in NP soil. The fusion induced in strain C18 is also responsive to alternative carbon sources (Table 3-1b) and appears to be an oxidoreductase, suggesting that it is being outcompeted by the wild-type strain 1021 for the scarce nutrients available in NP soil. It might also be sensitive to secretions from the wild-type owing to a mutation in a gene required for

degradation of potentially harmful bacterial waste products. The fusion induced in strain C27, in contrast, was shown to be similar to a ribose transport/chemotaxis operon. Unlike strain C18, strain C27 is only induced by one other carbon source tested, glucose. It would appear, then, that it was outcompeted for available carbon substrates in NP soil.

In comparison, strain N5 was less affected by the presence of the wild-type bacteria in competition for survival in NP soil, but the trend is still quite different than when strain N5 is inoculated in persistence experiments. Because the gene from this mutant has been identified as being involved in nitrate utilization, the most likely explanation of these results is that the mutant strain N5 is able to obtain some fixed nitrogen, perhaps as ammonia, even in the NP soil; however, not enough to completely alleviate the problem, which is most pronounced when the wild-type strain 1021 is also present. Presumably, the wild-type *Rhizobium* utilizes the soil nitrate, a nitrogen source inaccessible for the mutant strain N5. The observation (made after this set of experiments involving strain N5) made in chapter 2 that strain N5 is non-motile presents an alternative explanation for the reduction in plate counts of strain N5 versus the wild-type strain in NP soil. Lack of motility has been observed to be a competitive disadvantage to bacteria in the soil and rhizosphere (Lauffenburger *et al.,* 1982; Dowling and Broughton, 1986; Triplett and Sadowsky, 1992; Moens and Vanderleyden, 1996). Thus, the argument that the motility$^-$ phenotype (not coupled to the Tn5-1063 insertion, see chapter 2) is responsible for lower bacterial plate counts of strain N5 versus the wild-type 1021, is at least equally valid as the explanation presented initially. Only by repeating the experiment with the motility restored N5 strain (see chapter 2) could this question be answered.

The ability of these strains, as well as all other mutants tested, to survive in the soil and successfully inoculate alfalfa on their own is an indication that it is not

the presence of the Tn*5* transposon itself which is putting an insurmountable burden on the bacteria, but the lack of a functional protein necessary to compete with the wild-type strain during stressful (nutrient limiting) conditions. Examination of these and other deprivation-induced loci in more natural, i.e., non-sterile soils is the next step in understanding the roles of these loci, most of which do not have a phenotype or genetic similarity to known genes. This could lead to an enhanced understanding of soil microbial communities which would aid in the design and tracking of GEMs, as well as to a better understanding of how introduced chemicals and pollutants affect the microbial ecology of the soil.

MATERIALS AND METHODS

For bacterial strains and plasmids used, media and growth conditions, transposon mutagenesis, DNA isolation and manipulation, DNA sequence analysis, and nodulation and nitrogen-fixation assays see chapter 2 (Materials and Methods).

Soil Persistence and Competition Experiments

Two types of soil were used for seeding with bacteria; a nutritionally rich soil consisting of a 5:3:1 ratio of Metromix 510:sand:vermiculite, and a nutrient poor soil consisting only of sand. Metromix 510 is a professional plant growth medium consisting of sphagnum, peat moss, ash bark, and vermiculite, and is rich in carbon and nitrogen. Soils were sifted through a metal strainer to increase homogeneity, placed in small glass jars (100 grams per jar), and sterilized by autoclaving three times for 25 minutes, with 24 hours between each autoclaving.

Bacteria were grown to saturation (36 hours) in 5 ml of TY medium at 28°C. a 100 μl portion of culture was subcultured in 5 ml of TY medium and grown to a cell density of approximately OD_{600}=1.0. The cell density was adjusted by spectrometric measurement of optical density and subsequent centrifugation and resuspension (dilution or concentration) of the cultures in sterile distilled H_20 to a final cell density of OD_{600}=1.0, before 20 ml of cell culture was inoculated into the soil. For competition experiments, the bacterial cultures were centrifuged, and the pellets washed twice with sterile distilled H_2O, before resuspending them to a final cell density of OD_{600}=1.0. Ten ml of each culture to be examined was combined in a sterile tube and briefly vortexed before inoculation into the soil. The soil was thoroughly mixed with a sterile spatula. Experiments were carried out in duplicate.

Plate counts were determined by placing approximately one gram of soil into sterile 100 ml dilution blanks and determining the exact weight of the soil. Serial dilutions were carried out and two dilutions were plated three times on TY-plates containing Km^{200} and Sm^{250}, respectively. Initial counts were determined in triplicate with three soil samples from each jar, but this was found to be unnecessary, since the levels of error were found to be higher between dilution series than between soil replications (data not shown).

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CHAPTER 6

R. meliloti Genes Induced by Nutrient Deprivation and Affected in Soil Competition are Not Involved in Competition for Nodulation.

ABSTRACT

The rhizophere environment is characterized by plant root exudates, and higher bacterial numbers and diversity than in bulk soil (Bolton *et al.,* 1993), creating conditions where it is highly advantageous for a microorganism to respond quickly to the changing conditions in order to compete for available microsites. Rhizobia are particularly interesting in that they are competing not only for nutrients, but for sites that can lead to infection thread formation resulting in a microhabitat that is free from outside competition. Five mutant strains harboring Tn*5*-1063 insertions induced by carbon deprivation, as well as a constitutively expressed control, were selected and examined for competition for nodulation versus the wild-type 1021 at three different starting concentrations: 1:10, 1:1, and 10:1 mutant versus wild-type strains in two independent experiments. None of the mutant strains exhibited a loss of nodule competitiveness at any of the concentrations tested. Rather, two strains (C101 and C22) were significantly better than the wild-type strain at nodulation efficiency in both of the independent experiments.

INTRODUCTION

Rhizobia are unique soil bacteria that live in two distinct habitats: in the soil and rhizosphere, and in nodules induced on their host plant (Vincent, 1970). The unique nodule environment is separated from the 'outside', and thus presents an ideal biological system for examining bacterial competition in the absence of the complex microbial communities in the soil and rhizosphere. Because of this ideal system, and because of the importance of understanding and controlling the efficiency of biological nitrogen-fixation, more work has been carried out in the elucidation of the molecular mechanisms underlying competition for nodulation than in soil persistence and competition experiments (see e.g., McLoughlin *et al.*, 1987; Lagares *et al.*, 1992; Milner *et al*, 1992; Onishchuk *et al.*, 1994; Sharypova *et al*, 1994)

For example, Jiménez-Zurdo *et al.* (1995) obtained an *R. meliloti* Tn*5* insertion mutant in a proline dehydrogenase—which allows *R. meliloti* to utilize the amino acids ornithine and proline as sole carbon and nitrogen sources—which did not alter its ability to fix nitrogen, but drastically reduced its ability to compete for nodule occupancy (the ability of a particular rhizobial strain to occupy its host nodule; see further explanation below). In this case, competition could be understood in terms of the type of mutation involved. In other cases, however, nothing is known about the mutation in question except that it results in lowered nodulation competitiveness (Onishchuk *et al.*, 1994; McLoughlin *et al.*, 1987) or sometimes enhanced symbiotic effectiveness (Sharypova *et al.*, 1994). In *Rhizobium meliloti* and *Bradyrhizobium japonicum*, genes have been isolated on the megaplasmids, termed *nfe* genes (*n*odule *f*ormation *e*fficiency), which are involved in nodulation efficiency and competitiveness (Toro and Olivares, 1986; Sanjuan and Olivares, 1989; Chun and Stacey, 1994). In *R. meliloti*, expression of

the *nfe* genes has been found to be regulated by the NifA-NtrA regulatory system, which also controls the nitrogen fixation genes (Sanjuan and Olivares, 1991).

Examination of nodule competition is not without complications, however. Many factors have been shown to influence competitiveness including: genetic determinants of nitrogen fixation, production of polysaccharides, as well as environmental conditions such as temperature and soil pH (Noel and Brill, 1980; Boonker *et al.*, 1978; Beattie *et al.*, 1989). In addition, multiple-occupancy of a single nodule by more than one rhizobial strain has been observed in selected symbiosis, particularly in those giving rise to determinate nodules such as in *R. tropici*-bean (Wilson *et al.*, 1995; Sessitsch *et al.*, 1996). In other cases (e.g., *R. meliloti*-alfalfa) single-occupancy in a discrete nodule is the norm (Olivares *et al.*, 1980; Soto *et al.*, 1992; Goldman *et al.*, 1994). It may be that outside factors influence multiple-occupancy as well.

Because of the limited amount of nodules which are formed on the plant roots (Vincent, 1970; Amarger and Lobreau, 1982; Hirsch, 1992; regulated by unknown pathways), rhizobia are said to compete for nodule occupancy when two strains which can nodulate a particular host are both competing for the available sites which lead to nodulation. This competition occurs even when multiple-occupancy is the norm.

In this chapter the use of *R. meliloti* strains harboring Tn*5*-tagged loci induced by carbon deprivation as a model for measuring competition for nodulation is reported. Some of these strains (C18, C27, and C101) carry Tn*5*-1063 insertions in genes important for competition versus the wild-type strain 1021 in nutrient poor soil (see chapter 5). It is thought that because of the competitive nature of the rhizophere environment (see chapter 1), and because *R. meliloti* is an endosymbiont of alfalfa, genes responding to nutrient deprivation would be of benefit in competing for microsite occupancy on the roots of alfalfa

leading to subsequent root hair curling and nodule invasion. Therefore, mutations in such genes due to insertional inactivation by Tn*5*-1063 might lead to reduced nodulation effectiveness, or at the very least to no significant change in nodulation competitiveness phenotype. However, we found no competitive advantage for nodulation of wild-type strain 1021 versus any of the mutant strains tested, whereas two of the strains, C22 and C101, appear to be enhanced in their nodulation competitiveness phenotype.

RESULTS

Effect of mutations in deprivation-induced loci on competition for nodulation

Nodule competition studies were initiated in order to test the importance of the nutrient deprivation-induced genes for competition of *R. meliloti* for nodulation. Mutant strains C18, C27, and C101, carrying Tn*5*-1063 fusions induced by carbon deprivation (and oxygen limitation in the case of the locus tagged in strain C101), and shown to be outcompeted by the wild-type in nutrient poor soil (see Chapter 5) were chosen for co-inoculation experiments versus the wild-type strain 1021 on plant roots. Mutant strains C22 and C47, also carrying carrying Tn*5*-1063 fusions induced by carbon deprivation (and in the case of strain C22, carrying a fusion induced by nitrogen and oxygen limitation as well), were included in the nodule occupancy experiments., Strain CV1, bearing a constitutively expressed fusion, was also included in the analysis as an uninduced control. All six strains, as well as the wild-type strain, were grown in 5 ml TY liquid medium to saturation (36 hrs), reinoculated in larger volumes of TY, and grown to an approximate OD_{600} of 1.0 as determined by spectrophotometer. Cells were centrifuged, washed twice with ddH_2O, and resuspended in a volume of ddH_2O calculated to yield an OD_{600} = 1.0. Bacterial cultures were combined into test-tubes at three ratios of mutant to wild-type: 1:10, 1:1, and 10:1, and diluted 1:5 with ddH_2O before inoculating one week old alfalfa seedlings in large tubes with 1 ml of each condition (five tubes per condition tested). The results of two independent experiments, separated temporally, are presented in Figures 6-1A and 6-1B, respectively.

In the experiment shown in Figure 6-1A, none of the five mutant strains tested showed a relative decrease in nodule occupancy at any of the three

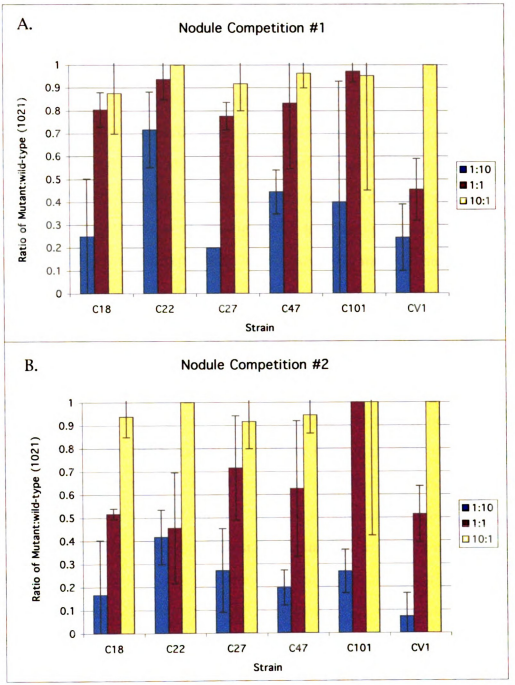

Figure 6-1. Competition for nodulation of selected Tn5-1063 induced mutant strains versus the wild-type 1021. In Panels A & B, the percentage number of nodules harboring mutant strains vs. the wild-type is shown in two independent experiments, respectively. Mutants were inoculated at 1:10 (blue bars), 1:1 (red bars), and 10:1 (yellow bars) ratios versus the wild-type. Nodules were individually harvested and plated to determine which strain was inhabiting the nodule.

inoculation ratios tested. In fact, the nodulation efficiency for all of the mutant strains was significantly higher than expected (<50% expected) at the 1:1 inoculation ratio, whereas the control strain CV1 was within normal limits at this ratio (Figure 6-1A). In addition, strains C22, C47, and possibly C101 (a large standard of error was observed) were significantly enhanced for nodule occupancy versus the wild-type strain at the 1:10 ratio of mutant strain to wild-type strain inoculation. Strain C22 was also enhanced for nodule occupancy versus the wild-type strain at the 10:1 ratio of mutant strain to wild-type strain inoculation (no nodules harboring the wild-type strain were recovered), making this strain the best competitor overall. No other strains deviated significantly from expected results at the 10:1 ratio, except for a slightly high value for the control strain, CV1 (no nodules harboring the wild-type strain were recovered).

The experiment was repeated using the same strains and conditions described above. The results, showing a similar pattern of nodule occupancy to the first experiment, are shown in Figure 6-1B. As before, none of the five mutant strains tested demonstrated a relative decrease in nodule occupancy at any of the three inoculation ratios tested. Unlike the first experiment, however, nodulation efficiency of only strain C101 (and possibly strains C27 and C47) was significantly higher than expected at the 1:1 innoculation ratio. In addition, nodulation efficiency of strain C22, but not strains C47 and C101, was again greater than expected at the 1:10 and 10:1 inoculation ratios. No other strains, including the control strain CV1, deviated significantly from their expected values at inoculation ratios of 1:10 and 10:1.

DISCUSSION

The data in these competition for nodule occupancy experiments clearly indicate that no competitive disadvantage is incurred by the insertion of Tn*5*-1063 in the loci of these selected mutants harboring fusions induced by carbon deprivation, under the conditions used in this experiment. In addition, the results presented in this chapter are very similar to those found by Anne Milcamps for competitive nodulation of three of the same *R. meliloti* Tn5-1063 tagged strains (C18, C27, and C101) versus the wild-type in a separate study (Ragatz *et al.*, 1997) using similar conditions. In this study, experiments were done in triplicate, and statistical analysis of the nodule occupancy data demonstrated that no significant difference between the mutant strains and the wild-type strain existed.

The data from both experiments is particularly surprising given that three of these mutants (C18, C27, and C101) were all shown to be at a competitive disadvantage in nutrient poor soils (see Chapter 5). However, unlike the soil system, bacteria inoculated on one week old alfalfa roots would not be starved due to the presence of nutrient exudates released by both the seeds and the plant roots into the B + D solution. This system then, may more resemble the nutrient rich soil system described in chapter 5, than the nutrient poor soil system in which the Tn*5*-1063 tagged loci were found to be important in competition.

Clearly, the hypothesis that the presence of Tn*5* in the genome would be detrimental in and of itself is not true in this system. One might even suspect it was advantageous, much like the IS50 element in *E. coli* batch cultures (Hartl *et al.*, 1983), due to the number of strains with increased nodulation competitiveness versus the wild-type strain. However, the contitutively expressing control, CV1, was nearly ideal in terms of its nodule occupancy at the various inoculation ratios.

Nevertheless, the increase in nodule competitiveness of most of the strains at one or more of the inoculation ratios is a surprising result.

Strains C22 and C101, in particular, were enhanced for nodulation competitivess in both of the experiments, and at more than one ratio. Neither of the cloned loci from these mutants show any similarity to known genes (see chapter 3, Table 3-1). In addition, the Tn*5*-1063 tagged genes they harbor are regulated by multiple stresses (see Chapter 4). This suggests that the Tn*5*-1063 tagged loci encode regulatory proteins of some sort, and that the presence of these genes (or regulons) in the genome presents a kind of energetic burden to the cell, such that it must be compensated for by some mechanism which improves competition for survival during deprivation conditions. It would be interesting to observe these strains in similar nodulation competitiveness experiments after they were exposed to deprivation conditions for a period of time. Nodule occupation competitiveness might be exprected to drop for these strains under such conditions, if the genes encoded by their tagged loci are indeed important for competition for survival prior to their exposure to plant roots.

As far as I know, this is the first report of Tn*5*-tagged loci with improved nodulation competitiveness in *R. meliloti*. Sharypova *et al.* (1994) isolated ten Tn*5*-induced mutants in *R. meliloti* with enhanced symbiotic effectiveness (Eff^{++}) as measured by an increase in the host plant's growth (dry weight), at the expense of nitrogen fixation in the root nodules. However, these strains were not tested for nodulation competitiveness versus the wild-type strain.

The observation that strains C22 and C101 are also induced by microaerobiosis suggests a regulatory pathway for their induction via the NifA-NtrA regulatory pathway. As was mentioned previously (chapter 4), part of this pathway is oxygen regulated (NifA) via the two-component FixL/FixJ system, and are the

same transcriptional regulators of the *nfe* locus involved in nodulation competitiveness of *R. meliloti* (Sanjuan and Olivares, 1991).

Overall, the results of these experiments do not appear to support the initial hypothesis, stated in chapter 1, that genes regulated by nutrient deprivation might be important for competition for nodulation. However, It may be that pre-starving the bacteria for nutrients, or inoculation of the bacteria into the B + D media (which contains no carbon or nitrogen sources) at a time prior to plant germination, would yield completely different results from those obtained in this experiment.

MATERIALS AND METHODS

For bacterial strains and plasmids used, media and growth conditions, transposon mutagenesis, DNA isolation and manipulation, DNA sequence analysis, and nodulation and nitrogen-fixation assays see chapter 2 (Materials and Methods).

Nodule Competition Experiments

Alfalfa seeds (*Medicago sativa* bv. *cardinal*) were sterilized by soaking for three minutes in 95% ethanol, followed by three minutes in 0.1% $HgCl_2$ and rinsed thoroughly with sterile distilled water. Three seeds were placed on a piece of sterile Whatman 3MM filter paper in test tubes containing 20 ml of sterile, nitrogen-free B+D liquid medium (Broughton and Dilworth, 1971). Five tubes were used for each strain at each of the three concentrations tested.

Bacteria were grown to saturation (48 hours) in 5 ml of TY medium at 28°C. 100 μl of culture was subcultured in 5 ml of TY medium and grown to a cell density of approximately OD_{600}=1.0. The cell density was adjusted by spectrophotometric measurement of optical density and subsequent centrifugation and resuspension (dilution or concentration) of the cultures in sterile distilled H_20 to a final cell density of OD_{600}=1.0. Mutant cultures were combined with the wild-type 1021 at ratios of 1:10, 1:1, and 10:1, diluted to a 1:5 concentration, and applied to one week old alfalfa roots at a final volume of 1ml. Each culture was briefly vortexed before inoculation into the tubes.

Nodule occupancy was determined by collecting the nodules from two to three tubes (yielding approximately 20–40 nodules per condition) containing 8-10 week old plants, surface sterilizing them in 10% bleach for 3 minutes in individual

1 ml microtubes, and rinsing them three times with sterile ddH_2O. Nodules were crushed with sterile micro-pestles and plated on TY medium containing Sm^{250} or Km^{200} and grown for three days at 28°C. Cultures which grew on both antibiotics were streaked in TY plates with Sm^{250}, and individual colonies were picked for plating on TY-Km^{200} plates.

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CHAPTER 7

Conclusions and Future Directions

CONCLUSIONS AND FUTURE DIRECTIONS

Throughout this thesis it has been stated, and reiterated, just how harsh and oligotrophic the soil environment can be, and how bacteria need to sense and respond to environmental signals in order to persist and compete in their environment. Microbial ecologists have traditionally taken two approaches to studying microorganisms in the soil: (i) an ecological approach marked by the examination of bacteria directly and how they compete with other microorganisms for resources and survive in the midst of harsh environmental conditions, and (ii) a molecular approach marked by the examination of individual enzymes necessary for the cell to survive and compete successfully in its environment and how the individual genes for these enzymes are regulated. I have tried to combine the two approaches in the hope that they would complement one another in the elucidation of not only genes and their regulators, but in the understanding of the role of such genes for competition and survival in the natural environment.

At the outset of the research described in this thesis, very little was known about environmentally regulated gene expression in soil bacteria, and only a little more was known in non-soil bacteria. Since that time, nearly seventy loci have been cloned and sequenced in *R. meliloti* which respond to nutrient or oxygen limitation conditions (chapters 2–4), and some of the Tn*5*-1063 tagged loci have been examined for their importance in the persistence and competition of the strains harboring them in the soil and in nodulation competitiveness (chapters 5 and 6).

The large number of genes isolated in response to nutrient and oxygen limitation is comparable to the number of proteins found to be induced by stresses in other systems (Matin, 1991; Givskov *et al.*, 1994). However, the lack of overlap of genes induced by multiple-stresses is decidedly different than what had been seen in non-soil isolates, and supports the hypothesis, presented in chapter 1, that

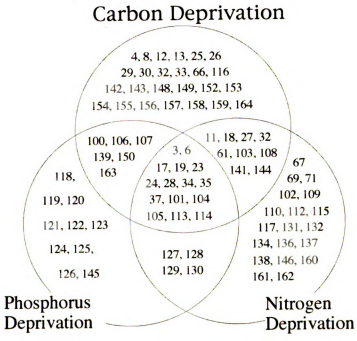
soil bacteria have a different means of regulating gene expression in response to environmental stresses than non-soil bacteria. This interesting hypothesis is discussed in greater detail below.

Overlap of loci induced by multiple stresses

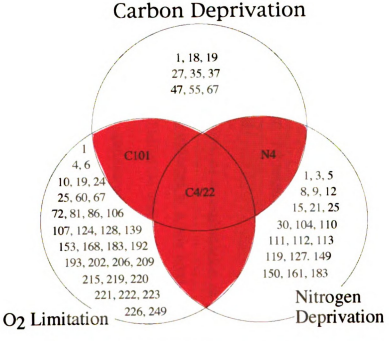
In this collection of 69 mutants with gene fusions induced by nutrient deprivation and microaerobiosis, only one locus (tagged in strains C4 and C22, see chapters 3 and 4) was found to be induced by all three stresses, and only two other loci (tagged in strains N4 and C101, see chapters 2 and 3) were found to be induced by two stresses, in contrast to what has been seen in other systems, such as *E. coli* and *S. typhimurium*, in which the presence of deprivation-induced proteins was screened via the use of 2D-PAGE (Matin, 1991).

Figure 7-1 shows a ven diagram adapted from Matin (1991) that compares the overlap of proteins induced by three stresses in *E. coli* with the overlap of Tn*5*-tagged loci regulated by three stresses in *R. meliloti*. It is immediately apparent that there is a large difference in the number of overlapping proteins in *E. coli* compared to the very few overlapping genes in *R. meliloti*. This difference could be due to an inherent difference in deprivation-induced gene regulation between *R. meliloti* and other non-soil type bacteria, or due to the difference in selecting for Tn*5*-tagged promoter induction versus protein induction. Two lines of reasoning suggest the latter. First, the use of reporter systems *lacZ* and *luxAB* in *S. typhimurium* and *P. fluorescens*, respectively, yielded similar results to ours with the isolation of few mutants induced by multiple stresses (Spector *et al.*, 1988; Kragelund *et al.*, 1995). Secondly, the similarity between the number of proteins induced by carbon deprivation in *P. putida*, a common soil isolate, to other systems

Figure 7-1. Ven diagrams illustrating the overlap of loci induced by multiple stresses. The overlap of proteins in *E. coli* induced by three stresses, and the Tn5-1063 tagged loci in *R. meliloti* induced by three stresses, is shown. Each number represents either an induced protein, in the case of *E. coli*, or a Tn*5*-tagged locus in the case of *R. meliloti* . *E. coli* data are from Matin (1991).



Escherichia coli *



Rhizobium meliloti

* adapted from Matin, 1991

such as *E. coli* (Givskov *et al.*, 1994), suggests that the use of Tn*5*-*luxAB* versus 2D-PAGE protein analysis is the main reason for these differences. This may be explained in several ways: (i) The loci may be induced at such a low level that they could not be visualized with luciferase as a reporter; (ii) insertion of Tn*5* in these loci may be lethal; (iii) the number of mutants generated may not cover the full extent of the genome; or, (iv) regulation of these proteins may be primarily at the post-transcriptional level.

Any of these reasons, or a combination of them, is plausible. In this study, many of the fusions were induced at a low level, but were not retained for further study because low-level induction was hard to reproduce accurately. It is also likely that the 5000 mutants generated was not enough to completely saturate the genome. Based on the size of the *R. meliloti* genome (6.5 Mb; Honeycutt *et al.*, 1993), and the average number of genes found in bacterial genomes thus far (1743 predicted coding regions in *Haemophilus influenzae* [1.8 Mb; Fleischmann *et al.*, 1995]; 1738 in *Methanococcus jannashii* [1.6 Mb; Bult *et al.*, 1996]), approximately 1 Kb per coding region in *R. meliloti* is estimated, yielding an estimate of 6500 total loci. Given a random distribution of Tn*5*-1063 insertions in the genome, and the fact that the Tn*5*-1063 must insert in the proper orientation to generate *luxAB* fusions, It is estimated that 43,290 individual Tn*5*-1063 insertions would be necessary to achieve a 99% confidence level (z=2.33) of one hit per gene in the proper orientation, thus approaching saturation of the genome. However, given the highly polar nature of Tn*5* mutations, and the high occurance of multiple loci regulated by a single promoter (regulons), many fewer insertions would be necessary to ensure that all regulons were mutagenized

In addition, post-transcriptional regulation of gene expression might occur during many deprivation-induced conditions. The observed instability of bacterial mRNA has led to the suggestion that this would provide the bacterial cell with a

very sensitive method of dealing with sudden environmental changes (Gros *et al.*, 1961; Jacob and Monod, 1961), and during non-deprivation conditions the mRNA would be quickly recycled. Although much less studied in bacteria, more and more proteins, including a lipoprotein encoded by *lpp*, the outer membrane protein OmpR, and thioredoxin TrxA, are being shown to be regulated via mRNA degradation (O'Hara *et al.*, 1995). In addition, mRNA stability has been shown to be regulated via polyadenylation in ribosomal protein S15 encoded by *rpsO* (Hajnsdorf *et al.*, 1995) and RNA I, which encodes the antisense repressor of replication of ColE1-type plasmids (Xu *et al.*, 1993; Xu and Cohen, 1995). Thus, mRNA stability is probably highly underestimated as a mechanism of protein regulation in bacteria (Hajnsdorf *et al.*, 1994).

Importance of Tn5-1063 tagged genes regulated by nutrient deprivation for persistence and competition in the soil and in nodule occupancy

Perhaps the overall guiding theme of this thesis has been the recurring contrast of soil microorganisms versus non-soil microorganisms. Because of the harsh, oligotrophic conditions of the soil environment, it seems clear that bacterial genes regulated in response to environmental stresses might be very important to the microorganism when competing for available resources and niches. In addition, the hypothesis that such genes might be different, and/or differently regulated in soil bacteria compared with non-soil enterics or aquatic microorganisms, is compelling. In chapter 5 of this thesis, the competition of selected strains—harboring Tn*5*-1063 insertions in nutrient regulated loci—versus the wild-type strain 1021 supports the hypothesis that the tagged genes are important for competition and survival when the soil is lacking in nutrients. The nutrient poor soil used in this study was a sandy soil that is typical of many native soils such as in

Eastern Michigan. Thus, conditions even within this restricted environment reflect real soil environments, and lend even more weight to the observed results.

In contrast to the soil, competition for nodulation experiments yielded unexpected and surprising results which go counter to the hypothesis presented in chapter 1. However, the conditions of the experiment may not have presented a sufficient environmental challenge to the tested strains, and more work needs to be done with pre-deprivation conditions before any final conclusions can be drawn. Nevertheless, the apparent increase in nodulation competitiveness of strains C22 and C101 (both harboring gene fusions regulated by multiple stress conditions) versus the wild-type strain suggests that genes involved in global regulation of stress induced pathways may carry a competitive burden under certain circumstances that is apparently outweighed by its potential to protect the cell while in harsh, oligotrophic, naturally occurring soil and rhizosphere environments.

Search for Regulators Responsive to Stress Conditions

The next obvious step in this research is to isolate and characterize the regulators of the tagged loci described in this thesis. Carbon, nitrogen and oxgyen limitation responsive regulators have already been described in many bacteria including *R. meloliti* (see chapters 2–4; Szeto *et al.*, 1987; Matin, 1991; Fischer, 1994; Arcondeguy *et al.*, 1997). It would be of interest to see if the Tn*5*-1063 tagged loci described in this thesis are also regulated by any of the the known two-component (e.g., FixL/FixJ or NtrB/NtrC) regulators or, in the case of carbon-deprivation-induced genes, the cAMP/CRP regulators. Tn*5*-1063 tagged loci *not* regulated by any of these described systems would then be the most interesting candidates for a secondary mutagenesis approach in the isolation of the unknown regulatory proteins.

Researchers in the Frans de Bruijn lab (Anne Milcamps and Mary Ellen Davies) have already begun to look for regulators of selected carbon and nitrogen deprivation-induced loci, using the mini-Tn*3* transposon. Random insertion of this transposon within the genome of the strain of interest, should lead to loss of *luxAB* induction (no light production) if a positive regulator controlling this promoter fusion is inactivated via the insertion of mini-Tn*3*. Of course, insertion of the mini-Tn*3* within the *luxAB* genes would also lead to this phenotype, so Southern analysis using *luxAB* and mini-Tn*3*-labeled probes would be necessary to distinguish between these possibilities. Another regulator one might expect to see using this method is a repressor of the tagged gene of interest. Insertional inactivation of a repressor would lead to a constitutively expressing phenotype (always producing light). A last possiblility is that such an insertion would occur in a regulator at the top of a cascade of regulators leading to a pleiotropic phenotype that would be hard to characterize, but extremely interesting.

The elucidation, via sequence comparison, of many of the Tn5-1063 tagged genes has provided clues into the physiological response of *R. meliloti* while subjected to stress conditions, and may in turn help lead to a better understanding of how soil microoganisms survive in such harsh environments. In addition, the tagged genes and their promoters may be useful markers to test the nutrient conditions of a particular environment of interest, or to regulate genes of interest (such as for biodegradation or plant growth) in the soil and rhizosphere.

Taken together, the data presented in this thesis answer many of the questions posed in chapter 1, and provide a definite direction for the continued examination of the overall hypothesis that soil bacteria have a different set of genes and regulators required for sensing and responding to environmental stresses.

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