This is to certify that the

dissertation entitled

Gene Expression in Sinorhizobium Meliloti

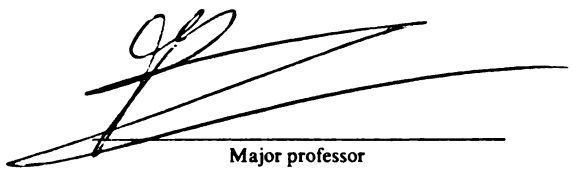
During Nutrient Deprivation

presented by

Mary Ellen Davey

has been accepted towards fulfillment
of the requirements for

Doctor of Philosophy degree in Microbiology



Major professor
Dr. Frans J. de Bruijn

Date August 20, 1999

GENE EXPRESSION IN *SINORHIZOBIUM MELILOTI* DURING NUTRIENT DEPRIVATION

By

Mary Ellen Davey

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1999

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ABSTRACT

GENE EXPRESSION IN *SINORHIZOBIUM MELILOTI* DURING NUTRIENT DEPRIVATION

By

Mary Ellen Davey

To persist, bacteria must adapt dynamically to their environment by switching on or off different suites of genes in response to their surroundings. Two major parameters that bacteria constantly monitor are nutrient status and oxygen tension. In soil, these resources are often scarce. Nutrients enter this ecosystem intermittently, however the diverse bacterial populations compete for these nutrients and they are quickly utilized. Consequently, the natural physiological state of indigenous soil bacteria is either dormancy or negligible growth. Understanding how bacteria are able to monitor and respond to their environment in this nutrient-deprived state is fundamental to our understanding of microbial biology. By mutagenizing the genome of *Sinorhizobium meliloti* with a Tn*5* derivative (Tn*5luxAB*), which generates transcriptional fusions resulting in bacterial bioluminescence when the gene fusion is expressed, a gene was identified in *S. meliloti* that is induced by environmental parameters which are representative of life in soil; that is, nitrogen or carbon deprivation, low oxygen tension, as well as during post-exponential stationary-phase growth, and by osmotic stress. The tagged gene was found to be part of an operon consisting of two open reading frames (ORF), which were designated

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ndiA and *ndiB* for nutrient deprivation induced genes. Comparison of the deduced amino acid sequences of both *ndiA* and *ndiB* to the protein databases did not reveal similarities with any known genes; therefore, they appear to be novel. In addition, a gene involved in the regulation of this operon was isolated, by carrying out a second round of mutagenesis on the primary mutant strain C22 with a Tn*3* derivative, Tn*1721*. A library of 3000 double mutant derivatives of C22 was screened for strains with altered luciferase expression patterns. One double mutant that failed to express the C22 luciferase fusion under any of the conditions tested was identified. This mutant contained a Tn*1721* insertion in a gene which encodes a protein with a high degree of similarity to the tryptophan-rich sensory protein, TspO, from *Rhodobacter sphaeroides*, as well as to the mitochondrial benzodiazepine receptor, pK18. Furthermore, proper environmental control of the *ndi-luxAB* reporter gene fusion was found to be restored after introduction of the *tspO* coding region *in trans*, under all inducing conditions tested. Thus, the experiments described here showed both the presence of a novel operon whose expression is induced by multiple environmental (stress) conditions, as well as a hitherto unidentified *S. meliloti* sensor/regulator locus involved in environmental control of gene expression.

To Tom,
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ACKNOWLEDGMENTS

I would like to thank my advisor Frans de Bruijn for supporting me in my studies as well as creating an environment in the lab which encouraged creative thinking. I would like to thank my committee members John Breznak, Tom Schmidt, and Peter Wolk for their many helpful criticisms and discussions. I would like to thank all the members of the "de Bruijn-lab" past and present, for their friendship. In particular, I thank Philipp Kapranov for the endless insightful discussions about life and science, and Jodi Trzebiatowski for her advice and support. I would also like to thank my classmates Debbie Hogan, Mark Johnson, and Dan Buckley for the amazing discussions during Euchre. Above all, I would like to thank my family for their love and support.

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Introduction

In nature, bacterial growth is restricted by a wide variety of environmental factors. One factor of particular interest is the lack of essential nutrients. In most natural settings, resources are scarce, therefore periods of negligible growth or dormancy are likely to be the typical physiological state of the microbial cell. The aim of this chapter is to present information pertaining to studies on the physiological adaptations of bacteria to nutrient deprivation, with special emphasis on the parallel resistance to other types of stresses that is acquired by cells when confronted with starvation. The regulatory pathways controlling gene expression during nutrient deprivation, as well as during other stresses will also be highlighted. In the first section, I will describe the general response of bacteria when deprived of nutrients, as well as the significance of understanding the starved state. Next, structural and physiological characteristics of non-sporulating bacteria during nutrient deprivation will be described. Third, information will be presented regarding protein expression profiles during nutrient deprivation, as well as the function of starvation/stress induced genes during stasis. Fourth, regulatory pathways known to control gene expression during nutrient-deprivation and under low oxygen tensions will be reviewed. Lastly, the environmental parameters that bacteria must contend with in soil, as well as the characteristics of my model soil organism, *Sinorhizobium meliloti*, will be discussed.

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The importance of starvation responses in bacteria

A remarkable feature of prokaryotes is their ability to adapt dynamically to their environment by switching on and off different suites of genes in response to their surroundings. In the natural setting, these surroundings are often comprised of a wide variety of physiologically stressful parameters, such as nutrient deprivation, low oxygen tensions, osmotic stress, and desiccation. Bacteria must respond to and contend with these highly variable conditions in order to survive. It is evident from the ubiquitous and diverse microbial communities that inhabit almost every niche on earth (86, 132), that these unicellular organisms have evolved distinct mechanisms which clearly allow them to withstand these perturbations. Studies have shown that bacteria respond to such environmental factors with a variety of changes in cellular morphology and physiology, allowing them to survive (15, 20, 70, 93).

Most natural habitats, such as soil and aquatic systems are nutrient poor. Resources enter these ecosystems only intermittently (e.g. from decomposing leaf litter or from surface run-off). Moreover, multiple metabolically complex microbial communities compete for the available substrates and they are rapidly and competitively utilized. In addition, in aquatic systems, oxygen tensions can quickly diminish below the surface layers, due, not only to utilization, but also to limited solubility of oxygen in water. In terrestrial systems, the availability of oxygen is also greatly influenced in this same manner. In essence, even though oxygen is one of the most plentiful

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gases in the atmosphere, most of the biosphere is limited for this element (58). This, in turn, severely affects the growth of strictly aerobic bacteria, which require oxygen for energy generation.

Hence, the natural setting of bacteria is characteristically nutrient and energy limited, and therefore, periods of non-growth during which cell biomass does not increase or periods of negligible growth are the rule rather than the exception (71, 100). Even though this starved state is fundamental to bacterial existence, we are only just beginning to understand how bacteria adapt to and function under such conditions. Some bacteria, such as those belonging to the genus *Bacillus*, respond to starvation by differentiating into morphologically distinct cell types (spores) that are extremely resistant to stresses, and an extensive amount of work has been carried out to investigate this differentiation process (57). While it is evident that the majority of bacteria do not form spores, studies have shown that "non-spore-forming" bacteria also enter into a stasis (absence of growth) survival state, in response to nutrient deprivation. During this response, major structural and physiological changes occur allowing the cells to persist without essential nutrients, and, in addition, the cells are resistant to a variety of other stresses.

A state of non-growth does not necessarily reflect an absence of metabolic activity. Bacteria persist in nature only if they can continually maintain a basic level of metabolic activity, even when nutrients are scarce. Accordingly, they have evolved mechanisms that allow them to capture substrates at concentrations that provide the essential nutrients for persistence, yet may not

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allow for an increase in biomass (44). A variety of terms, such as "pseudosenescence" (92) "somnicell" formation (100), or "dormancy" (39), have been used to describe this starved state. Bacterial cells in this state have features in common with spores, including the absence of "detectable" metabolic activity. Moreover, under "improved" environmental conditions, the cells can be induced to return to a physiologically active state. Thus, non-spore-forming bacteria in nature clearly exist in a variety of different starved states, although there has been controversy in the literature about how to determine and define these different states (40). Research investigating "starvation" in non-spore forming bacteria has been focused on two major areas. One field of investigation is that of the "spore-like" stage. These studies involve research on the viable but non-culturable (VBNC) state (83, 100) or viable, but not immediately culturable (NIC) state (40), and are concerned with studying the recovery of non-spore-forming bacteria from the starved state. The other main area is concerned with the response of bacteria as they enter the starved state. This area of research investigates the changes in the physiology of cells adapting from a eutrophic environment to nutrient-deprivation conditions. This area is the focus of this thesis.

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Starvation defined

Starvation most commonly refers to the final result of deprivation for an essential nutrient. This starved state can be brought about when a particular nutrient is completely utilized, or by resuspending cells in medium lacking a particular nutrient, such as phosphate, nitrogen, or carbon (104). Stationary phase is a term that refers to a state in which no net increase in numbers of cells occurs, and it is often used interchangeably with the starved state. However, a distinction should be made between stationary-phase cells and starved cells. The distinction made here is that stationary-phase describes cells in cultures that have stopped growing following balanced growth in a complex, or defined medium. In such cultures, the conditions that limit growth are not defined and, typically, growth is not limited by a single factor. On the other hand, in the case of starved cells the limiting nutrient is defined. Moreover, stationary-phase cells usually achieve high cell densities compared with starved cells and cell density can have a significant effect on the overall cellular responses (31). Furthermore, a distinct difference can be observed at the molecular level, where genes expressed by stationary-phase cells may or may not overlap with those genes that are expressed during starvation (64,106). Another term that is used through out this thesis is nutrient-deprivation. This term Is used to refer to the conditions that the cells are being exposed to, that is, resuspension in medium lacking either carbon or nitrogen.

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Response to nutrient limitation

Nutrient limitation is a condition in which all nutrients are available, however one or several nutrients are at concentrations that limit growth. When nutrients are limiting, bacteria can increase their chances of survival by a number of mechanisms, including the synthesis of higher-affinity enzymes or transport proteins specific for growth-limiting nutrients, utilization of alternative metabolic pathways to avoid possible blockages due to the lack of substrates, as well as decreased uptake of specific substrates that are available in excess (13, 63). In addition, depending on the relative concentrations of available nutrients, especially the ratio of carbon, nitrogen, and phosphorus, bacteria will synthesize storage polymers, such as polyphosphates and glycogen, as well as produce and release exopolysaccharides (73). These intracellular and extracellular storage compounds can be utilized later when more favorable growth conditions exist.

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Structural and physiological characteristics of starved cells

When an exogenous supply of an essential nutrient is completely exhausted, the starved state ensues. Some bacteria contend with starvation by differentiating into a morphologically distinct resistant form. Bacteria belonging to the genus *Bacillus* form endospores which are extremely resistant to a number of stresses including; desiccation, UV irradiation, and high temperature (24). *Myxococcus* spp. display another distinctive response to nutrient deprivation. These bacteria form elaborate multi-cellular aggregates and fruiting bodies when starved (38). Another stress resistant form is generated by *Azotobacter* species, which differentiate into a cyst-like form in response to nutrient deprivation by synthesizing a thick outer cell wall (58). Like spores, these cysts provide protection from environmental perturbations, especially desiccation; however, they are generally not as resistant to high temperatures as are spores.

While it is evident that many bacteria do not form classical spores, research has shown that under starvation conditions, such species, among them *Escherichia*, *Salmonella*, and *Vibrio* spp., do enter into a specific genetic program which results in major structural and physiological changes. The ultimate purpose of this new program is the persistence of the bacterial cells in the absence of growth (52, 64, 85).

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Various morphological changes are apparent in nutrient deprived cells (32). As *E. coli* cells enter into the stationary-phase, they become smaller and almost spherical in shape (73). This phenomenon has also been observed in *Vibrio* sp. S14 (78), as well as in a number of other marine bacteria, which have been shown to generate ultramicrocells, as small as 0.03 mm^3, during starvation (44). These markedly smaller cells are the result of continued cell division without an increase in biomass. In addition to an overall change in size, the cell cytoplasm is condensed and the volume of the periplasm increases during starvation (97). However, as indicated earlier, these cells are still potentially viable. For example, when the filtrates of water samples, that had been passed through a 0.2 μm filter, were enriched with dilute nutrient broth, "normally sized" cells of *Vibrio*, *Pseudomonas*, *Aeromonas*, and *Alcaligenes* were recovered (44).

The surface characteristics and adhesion properties of starved cells are different from those of cells growing under balanced growth conditions. Many marine bacteria become more hydrophobic, resulting in increased adherence properties when starved (44), and *E. coli* cells form aggregates in stationary phase (104). Changes in membrane fatty acid, peptidoglycan, and lipopolysaccharide composition have also been observed in a number of species (104).

As stated earlier, a bacterial cell must maintain a basal level of metabolic activity in order to persist. When the exogenous source of an

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essential nutrient has been exhausted, basic functions must be maintained through endogenous metabolism. The latter is defined as "the total metabolic reactions which occur within a living cell when it is held in the absence of compounds or elements which serve specifically as exogenous substrates" (79). Endogenous metabolism allows the cells to maintain a basal level of stored energy (ATP or other high energy phosphate compounds), as well as a sufficiently high proton motive force across the membrane. Another fundamental use of basal endogenous metabolism is to sustain the ability to transport substrates into the cell, should they become available again.

Endogenous storage polymers, as well as protein and RNA, mostly in the form of ribosomes, are used to sustain a basal level of endogenous metabolism. The rate of protein degradation in *E. coli* is approximately five-fold higher in starved cells compared to cells during balanced growth (59). An increase of sixteen-fold is observed in *Vibrio* sp. strain S14 during the first few hours of starvation (77). In addition, when cells are starved RNAse activity increases, which results in a decrease of 20 to 40% of the stable cellular RNA. Furthermore, the rate at which endogenous polymers, as well as protein and RNA are metabolized is carefully regulated (79). This control is evident in starved cells, which maintain the capacity to resume high rates of protein synthesis immediately after the addition of nutrients (1.).

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The protein expression profiles of starved cells

It has been demonstrated that when bacteria are nutrient deprived, energy derived from endogenous metabolism is used to synthesize macromolecules, including proteins (21, 78), RNA (78), lipids, and peptidoglycan (80). Specifically, Matin and his co-workers have demonstrated that *E. coli* cells continue to produce proteins for an extended period of time when deprived of exogenous carbon (21). To investigate starvation-induced gene expression at the whole cell level, "proteome" (the protein complement expressed by a genome) methods have been applied (37, 90). This approach uses two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE) of pulse labeled polypeptides (82) to separate proteins on the basis of their charge and their molecular mass, using isoelectric focusing in the first and SDS-PAGE in the second dimension. Radiolabeling of proteins permits a direct evaluation of the rates of synthesis of individual proteins (50), as well as their expression profiles under certain conditions or at specific times (123). Using the proteomic methods, a sequential synthesis of at least fifty proteins that are induced in response to carbon, phosphate, or nitrogen starvation has been documented in different non-sporulating bacteria (62, 78, 108).

Where many of these polypeptides are induced by the deprivation of a particular nutrient, some of them have been found to be induced by several or all of these conditions. For example, Spector *et al*, discovered that in

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Salmonella, twenty of the starvation-inducible polypeptides identified responded to two or more stress conditions and six of these proteins were shown to be produced in response to three or more starvation conditions (107). Similarly, studies in *E. coli* have shown the presence of fifteen polypeptides that were induced in response to carbon, phosphorus, or nitrogen deprivation conditions (62). Furthermore, analysis of *Vibrio* species has shown the presence of three polypeptides that are also induced in response to starvation for carbon, nitrogen, or phosphate (78). In addition, thirteen proteins were found to be induced by multiple starvation, that were not found in cells that were starved for the individual nutrients (78). These data indicate that alternative or additional signaling and regulatory pathways are involved when the cells are starved for several different nutrients at the same time.

Four distinct temporal patterns of protein expression have been observed in *E. coli* (21), as well as in several other non-spore forming bacteria (43). In general, one class of proteins is synthesized transiently during the very early stages of carbon starvation. The other three classes of proteins persist throughout starvation, with maximum synthesis at different time points, i.e., after 20-50 minutes of starvation, between 3 and 4 hours of starvation, or throughout starvation (21). In addition, the temporal pattern of protein synthesis was found to be the same when the cells were starved for either glucose or succinate. The expression profiles of cells grown under both succinate and glucose starvation conditions indicated that the bulk protein synthesis rate is reduced to approximately 40% at the onset of starvation. This reduced level of

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protein synthesis is maintained for 180 minutes during glucose starvation and for 90 minutes during succinate deprivation, at which time synthesis becomes non-detectable (21). Another common finding is that proteins that are expressed early in starvation are critical for long term stasis survival (78, 97).

The functions of starvation-induced proteins

Nyström and co-workers (81) have used 2-D-PAGE in combination with N-terminal amino acid sequencing analysis to investigate the changes in protein profiles and to identify protein products in cells that are starved for carbon. This global "proteomic" approach has shown that when *E. coli* cells are starved for carbon, an increased synthesis of glycolysis enzymes with a coinciding decrease in the synthesis of TCA cycle enzymes occurs. This expression profile is remarkably similar to that exhibited by anaerobically growing cells and two explanations for this similarity have been proposed. First, since superoxide radicals, which are detrimental to the cell, are a by-product of aerobic respiration, the down-regulation of proteins involved in aerobic respiration during starvation-stasis may be a defense mechanism which protects the cell from oxidative stress. In addition, the reduced respiratory activity would also prevent the over-utilization of valuable endogenous resources (81).

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In addition to the proteome research approach, genetic and molecular studies have been used to identify and characterize expression profiles of genes specifically induced during carbon starvation and stationary-phase. The expression of various carbon starvation (*cst*) genes have been studied by use of *cst*::*lacZ* (β-galactosidase) transcriptional fusions, both in *E. coli* (6) and in *S. typhimurium* (109). In addition, insertional mutagenesis with λplacMu (*lacZ*) has been employed to identify genes induced primarily during stationary-phase (130). However, β-galactosidase cannot be used to examine the expression of periplasmic or membrane localized proteins, because it cannot pass through the cytoplasmic cell wall. Therefore, studies with this reporter gene are restricted to proteins located in the cytoplasm. However, many of the starvation-induced genes that have been identified encode for periplasmic or membrane-localized proteins (2). In order to examine these proteins, the reporter gene *phoA* (alkaline phosphatase) has been used. Alkaline phosphatase is an excellent reporter gene for studying the expression of secreted or membrane associated proteins, because it is inactive in the cytoplasm, but can be readily assayed once it passes through the cytoplasmic membrane (60). Consequently, insertional mutagenesis with transposon *TnphoA* carrying the promoterless reporter gene *phoA* has been employed to identify such proteins (2, 3).

The discovery of specific functions for many starvation or stress-induced genes can be attributed to molecular studies of cells during stationary phase.

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Over the past 15 years, there has been intensive research by molecular microbiologists on stationary-phase gene expression (45). One of the key discoveries has been the identification of an alternative sigma factor, σ^S, which is encoded by the *rpoS* or *katF* gene (72, 114). Many of the genes expressed during stationary phase, or under carbon, nitrogen, or phosphate starvation, as well as in response to a diverse number of stresses (see below), are transcribed by RNA polymerase containing this alternative sigma factor (27). For a comprehensive review of σ^S - dependent genes and their function in *E. coli* and *Salmonella typhimurium*, the reader is referred to a review by Loewen *et al.*, (1998).

Even though the majority of σ^S - controlled genes are still unknown, several of the genes controlled by σ^S encode for proteins whose function is known (26). In the following section, a brief description of some of these stationary-phase-induced proteins will be presented. σ^S - controlled genes include those whose protein products are involved in providing energy to the cell; such as *cbdAB* which encode a cytochrome bd type oxidase (4),or *hya* encoding hydrogenase 1 (4), as well as *glpD*, the structural gene for aerobic glycerol-3-phosphate dehydrogenase (130). Another category of genes encode for proteins that are involved in transport of substrates, for example, *proP* is a transport system for glycine betaine and proline (66, 134). In addition, protein products involved in changes in cell morphology or surface properties have been identified. Among these are *bolA* which encodes a regulatory protein

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required for induced expression of PBP6, a penicillin-binding protein-carboxypeptidase (48), which, in conjunction with *ftsQAZ* functions in septum formation. Also, the expression of *osmB*, which encodes an outer membrane lipoprotein, is controlled by σ^s, and this protein may play a role in the formation of cell aggregates (26).

As indicated earlier, starved cells also become resistant to a variety of other physiological stresses. Several of the genes induced during stationary-phase growth encode protein products that are involved in general stress protection. Among these are genes which provide resistance to oxidative stress, such as *katE* and *katG* (34, 53) which encode catalases providing protection against peroxide. In addition, there are genes whose protein products provide protection during osmotic stress, such as *otsA*, *otsB* and *treA* which aid in the synthesis and transport of trehalose, an osmoprotectant which stabilizes both membranes and proteins (28, 112). Furthermore, there are proteins expressed during entry into starvation that function to repair DNA damage, such as *aidB* which is involved in DNA methylation damage repair (47, 127) or *xthA* an exonuclease III which is also involved in DNA repair (102).

Finally, some of the genes required for stasis survival are concerned with protein repair and maintenance. Since starved cells are unable to readily synthesize new proteins in response to environmental changes, they must rely on existing proteins to carry out essential cellular functions. Existing proteins are likely to become denatured or otherwise damaged due to environmental

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stresses (126). Therefore, mechanisms for repairing spontaneous damage, preventing denaturation, as well as assisting in renaturation of proteins after cessation of the stress conditions are critical for survival (63, 126). Two genes involved in damage repair have been identified. One is the *surA* gene (118) which encodes a periplasmic protein with peptidyl-prolyl isomerase activity (49). This activity repairs *cis*-conversions of prolines. Such conversions occur naturally in proteins and interfere with proper folding, therefore this damage must be repaired to recover function. Also, this isomerase is required for proper assembly of some outer membrane proteins. Therefore, it also appears to function as a chaperone (101). Another damage control gene is *pcm* (51), which encodes an L-isoaspartyl protein methyl transferase. Isoaspartate residues can also be formed spontaneously interfering with proper folding. Therefore, conversion of isoaspartate to L-aspartyl residues is essential for proper protein folding and function. In addition, genes encoding chaperones, such as GroEl and DnaK, which are under control of the heat-shock sigma factor (σ^{32}) also show increased expression during starvation. These proteins have been shown both to prevent denaturation of proteins, as well as aid in renaturing denatured proteins (116). Furthermore, even though these chaperones are not proteolytic *per se*, they appear to play a role in proteolysis. Strains with mutations in the *dnaK* gene have been found to impaired in protein degradation (41, 111). Two hypotheses as to how these chaperones function in proteolysis have been proposed (88). It may be that these proteins function as part of a protease complex or they may bind abnormal proteins in such a

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way as to make them more accessible to cleavage by proteases. However, the exact function of these proteins has not, as yet, been determined.

Regulation of starvation/stress induced genes

Transcribing an average 1kb *E. coli* gene just once and then translating it with five ribosomes consumes a minimum of 7000 "high energy" phosphates (7). Since energy efficiency is of critical importance to starved bacteria, an efficient mechanism for controlling gene expression must be employed. In bacteria, translation and transcription are concurrent. As mRNA is being synthesized translation commences immediately. Moreover, most mRNAs are not very stable. Therefore, the choice of templates that are available for translation by the pool of ribosomes, in essence, mirrors the transcriptional choices. It follows that extremely efficient control can occur at the level of transcript initiation and, in fact, most of the known global regulators do act at this level. It should be noted, however, that our present view may be biased due to limited evidence; therefore other mechanisms, such as controlling transcript elongation and termination, RNA processing, initiation of translation, and post-translational modifications may also play a significant role (7). Furthermore, the ability to select a variety of genes at different locations on the chromosome at the same time, without utilizing precious energy for transcribing and translating non-essential operons is extremely advantageous. This is known to be accomplished by two different mechanisms. First of all,

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histone-like proteins (IHF, H-NS, and HU) are involved in dynamically masking DNA, thereby controlling accessibility. For example, Integration Host Factor (IHF) has been found to be required for starvation survival, as well as the expression of fourteen glucose-deprivation-induced proteins (75,76).

Regulatory networks, such as the one depicted in Fig. 1 as a stimulus response pathway, are used by bacteria to orchestrate the coordinated expression of a number of different genes/operons (73). A stimulus, such as nutrient deprivation or osmotic shock, is detected by a sensor protein. The sensor protein then passes this signal on to a regulatory protein, either directly or indirectly via one or more transducers. The regulatory protein then acts on a number of target operons, which, in turn, may control the expression of other operons, thus creating a regulatory cascade. When the products of the response genes have reached the proper level, a feed-back response is generated. This feedback response can act at the level of signal production, transducer activity, or even regulator activity, or some combination there-of, thereby modulating the response. In this manner, a regulatory network allows for system equilibrium, since the expression of the cascade can be controlled at various levels resulting in stabilization at the appropriate amount of expression required for persistence under the new conditions (7).

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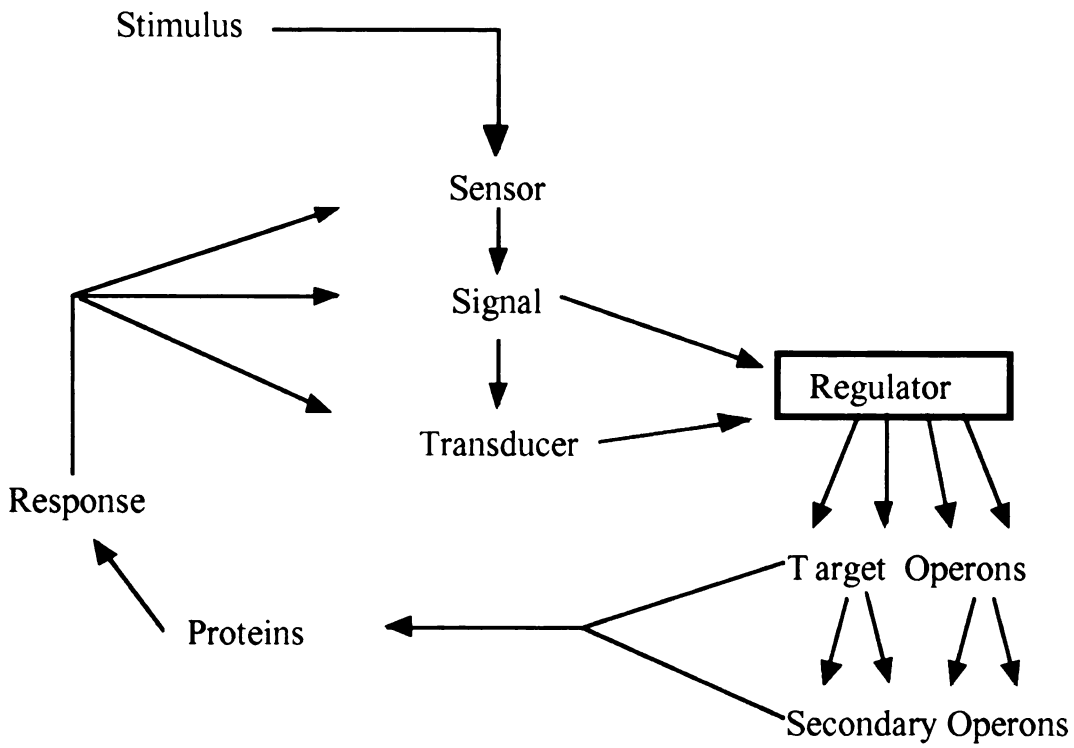

Figure 1. Regulatory network depicted as a stimulus-response pathway

Many operons, including those that contain genes required for the catabolism of certain sugars or amino acids, are controlled by such global regulatory networks, but are also independently regulated, creating an additional level of control (7).

It is evident that bacteria have evolved multiple mechanisms to orchestrate the expression of regulatory networks (7). In some cases, the mechanism used is the same as that described for a single operon, namely, via a repressor or activator protein that recognizes a particular promoter region; however, in the case of global regulatory networks, the promoter element is

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common to a set of operons. In other cases, coordinated regulation involves alternative sigma factors (see below), that direct RNA polymerase to specific promoter elements present in the target genes/operons. In addition, a combination of regulatory proteins and sigma factors can be utilized, as seen, for example, in the nitrogen utilization control pathway of a variety of bacteria (87). Finally, some regulatory networks involve other factors, including internal non-protein signaling molecules. For example, during the stringent response (amino acid starvation), as well as in response to other stresses, expression of various genes and operons appears to be regulated by the nucleotide guanosine tetraphosphate (ppGpp) in a manner that remains to be elucidated (9, 84). Selected examples of different control mechanisms and their significance during nutrient deprivation will be reviewed in the following sections.

Increased cAMP levels during general carbon deprivation

In *E. coli*, the only known regulatory system involved in monitoring carbon availability is the cyclic adenosine monophosphate/cAMP receptor protein (cAMP/CRP) system. cAMP is a key mediator of gene expression in Gram-negative bacteria (8). This molecule is synthesized from ATP by adenylate cyclase, which is encoded by the *cya* gene. It acts as an effector molecule by binding to CRP which subsequently activates transcription at target promoters.

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Studies using *cya* mutants of *E. coli* have shown that some of the genes induced in response to carbon starvation require cAMP for their induction. These proteins have been designated Cst (carbon starvation) proteins. However, another class of carbon starvation induced genes have been found to be cAMP independent; this group was named Pex (post-exponential) proteins (62). Moreover, it was discovered that the cAMP-independent (Pex) proteins are synthesized under various starvation and stress conditions, while the Cst proteins are specifically induced by the lack of carbon or in non-starving cultures supplemented with cAMP (6). Therefore, it has been proposed that the proteins regulated by cAMP levels (Cst) are specifically concerned with the response to starvation. For example, the *cstA* gene is specifically expressed by carbon-starved cells and functions in peptide utilization, and this increase in catabolic potential may be extremely advantageous when the more "typical" substrates become limited (103). On the other hand, the Pex proteins are expressed under various stresses and they appear to function in stress survival instead of the response to carbon starvation specifically; moreover, their gene expression appears to rely on alternative sigma factors, as well.

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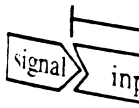

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Two-component systems

A major mechanism of signal transduction that bacteria use to sense and modulate gene expression in response to environmental stimuli is the so-called two-component system (see Blumenthal *et al.* [1996] for a comprehensive review). Nitrogen starvation, oxygen limitation, and changes in osmolarity are but a few of the environmental stresses that cells overcome by modifying their cellular physiology with the help of two-component systems (87). These bicomponent systems are composed of the following functional units: input sensor domains; output effector domains; and transmitter and receiver domains, as shown in Figure 2. Communication between transmitter and receiver domains is primarily accomplished by phosphorylation and dephosphorylation reactions. In essence the two-component systems consist of a sensory kinase and an associated response regulator.

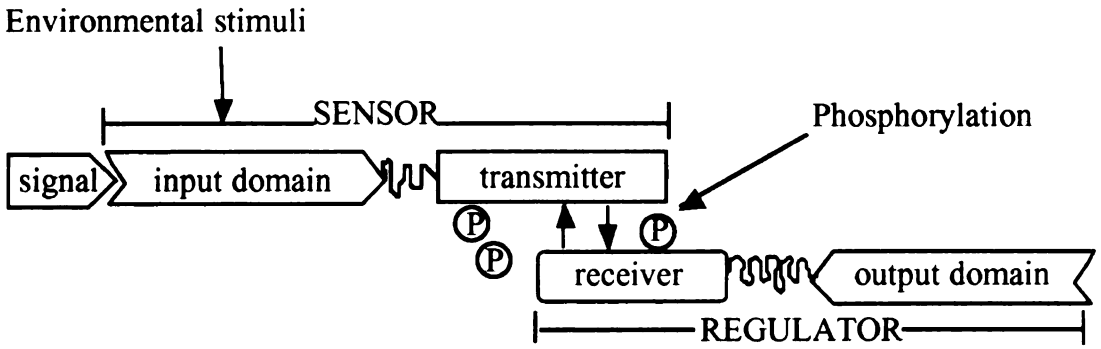

Figure 1. 2. Two-component signal transduction paradigm, consisting of an input sensor domain and an output effector domain with transmitter and receiver domains for protein-protein communication.

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The two-component regulatory systems most relevant here control gene expression in response to nitrogen availability, changes in osmolarity, the availability of different electron acceptors, as well as changes in oxygen tension, and include NtrB/NtrC for monitoring nitrogen availability, EnvZ/OmpR for responding to changes in the surrounding osmolarity, NarX/NarL which control the use of nitrate as a terminal electron acceptor during anaerobiosis, and ArcB/ArcA which controls aerobic respiration (87). As with most two-component systems, these regulatory circuits coordinate the expression of genes located in a variety of specific operons. The NtrB/NtrC system has been identified in many different bacteria, including a variety of enteric species, *Pseudomonas* spp., as well as *S. meliloti*, and is required for the regulation of the synthesis of glutamine synthetase and other enzymes important in nitrogen assimilation in response to changes in the availability of nitrogen (74, 99).

In *E. coli*, the EnvZ/OmpR system controls the ratio of the two outer membrane porins, OmpF and OmpC, in response to osmolarity. Under conditions of low osmolarity OmpF is favored, whereas in high osmolarity, OmpC is preferentially synthesized (120). The two porins are highly similar, but differ significantly in the diameters of the channels formed by each (94), thereby controlling passive diffusion.

An example of the interconnected nature of regulatory networks is found in the pathways controlling the adaptation of *E. coli* to different redox environments, in this regulatory scheme three global regulatory systems interact to control the use of energy sources in this bacterium (33). A key

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strategy of this facultative anaerobe is to channel electron transport from donors to terminal acceptors so that the drop in free energy is the maximal allowed with the available substrates. Thereby, the cells are able to exploit available energy sources to obtain the most energy.

The NarX/NarL two-component system controls anaerobic respiration by preferentially activating the operon encoding the major nitrate reductase when nitrate and molybdate are available. At the same time, this system represses the synthesis of other less beneficial anaerobic terminal reductases. In addition, NarX/NarL works in concert with another global regulator, Fnr (110). This regulator can function both as an activator and a repressor and its amino acid sequence shows a high degree of similarity to the catabolite repressor (CRP) described above. Fnr, however, has an added feature. This regulator contains a four-cysteine cluster that binds Fe^{2+}, and there is strong evidence that the transition between the +2 and the +3 states of the iron plays a role in redox sensing (33). In addition, *E. coli* utilizes a system (ArcB/ArcA) to control aerobic respiration (81). This control system is based on repression during anaerobiosis and relief of this repression when oxygen is available. The precise mechanism by which this regulation occurs has not been elucidated.

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Alternative sigma factors

Recent studies have firmly established that many mechanisms of transcriptional control of gene expression in response to different environmental stresses, including responses mediated by two-component systems, rely heavily on the utilization of distinct sigma factors. In *E. coli*, six distinct sigma factors have been found to be involved in the response to different environmental signals, including heat shock (σ^H or σ^{32}), nitrogen deprivation (σ^N or σ^{54}), extracytoplasmic stress (σ^E or σ^{24}), substrate gradients (σ^F or σ^{28}), iron deprivation (σ^{19}), and osmotic stress (σ^S or σ^{38}) specific factors (54). In addition, it is now evident that the σ^S subunit of RNA polymerase not only regulates gene expression during starvation and stationary-phase growth, but also serves as a master regulatory component of gene expression during general stress conditions, even when the cells are in exponential growth (25). In fact, σ^s controls such a large number of genes that it has even been described as the primary sigma factor. It has also been found to be essential for survival under the physiological stressful conditions that prevail in nature (25, 26). For example, in *E. coli*, *rpoS* mutants lacking σ^s fail to induce a significant subset of the carbon starvation induced polypeptides, including six of the Pex proteins. Moreover, these mutants are drastically impaired in the ability to survive starvation conditions, and are severely affected in their

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response to other stresses, such as heat shock, oxidation, and hyperosmosis (61). Therefore, it is becoming clear that σ^S is one of the key elements of the genetic response that enables *E. coli* to persist in its natural habitat under highly variable physiological conditions.

The soil environment

Although much is known about the changes in expression profiles under starvation conditions in a select group of enteric and marine bacteria, little information is available about the response of indigenous soil bacteria to such variables. It is likely that mechanisms for survival particular to this environment have evolved in bacteria whose normal habitat is soil. As shown in Fig. 3, soil constitutes an extremely complex heterogeneous environment with numerous fluctuating parameters that can influence microbial growth and survival (89). Moreover, like most natural habitats, soil is nutrient poor (133). Soil organic matter varies in concentration from 0.8-2.0%, with the bulk of the carbon in recalcitrant forms, such as humus, therefore bacteria indigenous to soil must constantly contend with nutrient deprivation (121).

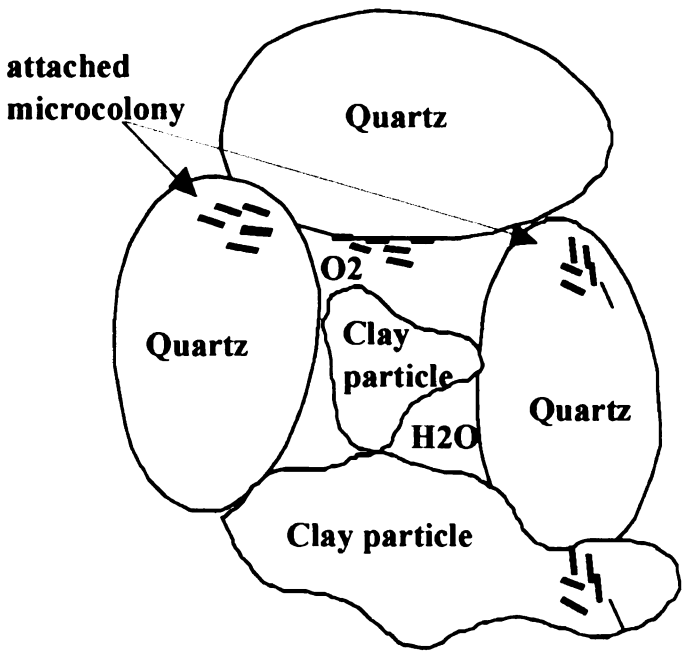

Figure 3. A soil aggregate showing bacteria localized on the surface as microcolonies, as well as the complexity and heterogeneity of bacterial niches found in a a single aggregate.

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Starvation studies with *Pseudomonas* spp.

In the past few years, the response of the ubiquitous soil bacterium, *Pseudomonas putida* KT2442, to carbon starvation conditions has been investigated. In these studies, *P. putida* KT2442 was tested along with two *E. coli* strains and one *S. typhimurium* strain for their ability to survive starvation. It was discovered that *P. putida* was fully viable after 20 days of carbon or multiple-nutrient starvation (17). On the other hand, *E. coli* and *S. typhimurium* cells showed a decrease in viability of one to two orders of magnitude after the first week (17). A *Vibrio* strain was also tested in a similar manner and it was found to be fully viable after two weeks in sea water (17). In addition, 2-D-PAGE analyses were performed and the results indicate that *P. putida* also synthesizes an array of new proteins in a temporal fashion, in response to deprivation of an exogenous carbon source. The function and regulation of these proteins have not, as yet, been elucidated (16).

However, more recently, the role of RpoS in regulating starvation gene expression, as well as in conferring stress tolerance has been examined in three different species of *Pseudomonas*. A *rpoS* mutant of *P. putida* strain KT2440, C1R1, was examined and showed reduced survival of carbon starvation, as well as reduced cross-protection to other types of stresses in cells that were carbon starved (96). In another study, it was found that *rpoS* mutations in *P. aeruginosa* resulted in a two - to three-fold decrease in resistance to a variety of stresses, although the effect was found to be less

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pronounced as that observed in *E. coli* cells (35). In addition, a two-component system (GacS/GacA) has been found to influence *rpoS* accumulation and the stress response of *P. fluorescens* Pf-5 (131). Strains with mutations in this two-component system are compromised in their ability to withstand oxidative stress. In addition, during entrance into stationary-phase, these mutants contained less than 20% of the wild-type levels of σ^s. Therefore, it is becoming evident that σ^s also plays an important role in stasis survival in *Pseudomonas* spp.

Sinorhizobium meliloti **as a model organism**

Bacteria belonging to the family *Rhizobiaceae* represent a unique group of organisms for research on environmental control of gene expression in soil. These bacteria form a mutualistic symbiosis with their leguminous host plant, in which a new specialized organ is formed, a nitrogen-fixing root or stem nodule (for a recent review see Spaink et al., 1998). The nodules that are formed provide the environs for the bacteria to survive and to reduce atmospheric dinitrogen to ammonia, which can then be assimilated by the plant, hence mutualism (55). The development of this symbiosis is a complex process and requires a constant exchange of signals between the symbionts. It is well established that plants influence the microbial community structure of the rhizosphere by both excreting growth promoting nutrients and releasing regulatory molecules that control bacterial gene expression (56, 91). In

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response, the rhizobia are known to produce signal molecules that alter the gene expression of their host plant (105, 122, 125).

Rhizobia persist in bulk soil with a scarcity of nutrients until they come in contact with their host plant. Hence, they persist in three distinct habitats: bulk soil, the rhizosphere; and the plant nodule. In order to do so, they must survive in the oligotrophic conditions of soil, competitively sense and utilize plant signaling molecules and growth promoting nutrients excreted from the rhizosphere, as well as adapt to the very different homeostatic environs within the host cell cytoplasm. Moreover, these three distinct modes of existence require that these bacteria consistently sense the environmental parameters and coordinately regulate gene expression to adapt to changes in their surroundings, even when they are starved for essential nutrients. Hence, these bacteria afford a unique model system in which to study environmental control of gene expression in an indigenous soil bacterium. Furthermore, *S. meliloti* is amenable to the use of molecular genetic techniques (18), both physical and genetic maps are available, and the determination of its genomic DNA sequence is well-advanced (29)

In addition, two aspects, in particular, of rhizobial biology add value to selecting these bacteria for studies on environmental control of gene expression. First, nodule occupancy competition studies between mutant and wild-type strains in order to assess the effect of mutations on symbiosis, provide a highly specific and unique assay (69). Second, during the intermediate stages of symbiotic development, the bacteria infect the plant

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nodule cells and differentiate into the "bacteroid state". This differentiation is accompanied by considerable morphological and physiological changes (124), which include adjustments in both nitrogen and carbon metabolism, as well as adaptation to the micro-aerobic milieu within the plant cell (12, 36). Moreover, bacteroids represent a non-growth or even terminally differentiated state, although controversy still continues as to whether bacteroids are, in fact, terminally differentiated (95). Nevertheless, this reversible or potentially even irreversible "nitrogen-fixing organelle"- state of bacteroids has features in common with the more universal bacterial "culturable" versus "non-culturable" state. Hence, another aspect of stasis survival can be investigated using rhizobia.

The responses of *S. meliloti* to environmental conditions

The response of rhizobia to nutrient deprivation or oxygen limitation is just beginning to be investigated. Recently, evidence for a general starvation response in *Rhizobium leguminosarum*, similar to that found in *E. coli* and *Vibrio* spp. has been reported (115). In addition, Uhde *et al*, (1997) have used transposon mutagenesis to identify *S. meliloti* mutants that are affected in stationary-phase survival. Some of the transposon tagged loci have been cloned and analyzed and found to be involved in amino acid metabolism and aerobic respiration. In addition, phosphate stress-induced genes in *S. meliloti*

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Studies on the regulatory mechanisms controlling gene expression during various stresses have, also, only just begun. A homologue of *rpoS* in *S. meliloti* still remains to be isolated, although the following data indicate that this sigma factor does exist. RpoS - dependent growth phase-regulated promoter elements from *E. coli* have been found to be recognized in *S. meliloti* as cells exit exponential growth and enter stationary-phase, indicating functional complementation (67). In addition, Southern hybridization experiments have identified DNA fragments that hybridize with the *rpoS* gene from *E. coli* (67). Accumulation of the nucleotide guanosine tetraphosphate (ppGpp) following amino acid, nitrogen, or carbon starvation has been reported in *S. meliloti* 1021 (Howorth, 1999). Interestingly, two other isolates (*S. meliloti* strain 41 and *R. tropici* CIAT899) were found not to produce ppGpp in response to these stresses (30). In addition, the ppGpp accumulating (stringent) strain 1021 showed much higher levels of intracellular ATP in response to nitrogen starvation, as compared to the ATP levels in the relaxed strains of rhizobia. Since nitrogen fixation requires a great deal of energy, it has been hypothesized that the ability to mount a stringent response and produce elevated levels of ATP may be directly related to the efficiency of nitrogen fixation (30). However, further studies need to be done to address the significance of these findings.

Two-component signal transduction pathways that sense and induce gene expression in response to the lack of nitrogen (Ntr system) (14); the

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presence of dicarboxylic acids (Dct BD system) (22, 128); low oxygen tension sensing (Fix LJ system) (5, 10, 19) as well as pH sensing (ActRS) (98, 117); have been identified in *S. meliloti*. The FixLJ is unique to rhizobia and is required for symbiotic nitrogen fixation (5). The DctBD and ActRS systems in rhizobia have strong sequence similarity to two corresponding systems (DctSR and RegAB, respectively) in *Rhodobacter* spp. The DctSR system in *Rhodobacter* has a similar function as its rhizobial counterpart, namely, synthesis of dicarboxylate transporters in response to certain environmental signals (22). However, ActRS and RegAB appear to represent distinct signal transduction pathways. The ActRS system is concerned with the response to low pH, whereas the RegAB system appears to be involved in the response to low oxygen tension (117). Although additional research is required to determine the exact mechanism and signal that is detected by these two systems, it may be that the sensor proteins in these systems respond to some environmental signal that is influenced by both low oxygen tensions and low pH.

Our laboratory has focused its studies on the nature and regulation of nutrient-deprivation-induced *S. meliloti* genes, and a collection of 70 genes expressed during carbon or nitrogen deprivation has been described (68, 69). As pointed out above, it is evident that bacteria frequently encounter an array of physiologically stressful parameters in their natural setting. Furthermore, certain stresses, such as nutrient deprivation or osmotic stress, trigger global regulatory circuits that control the expression of genes belonging to several

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pathways, which allow these bacteria to withstand various perturbations. Therefore, we hypothesized that by searching for *S. meliloti* regulatory genes involved in controlling gene expression under multiple stresses, global regulatory genes could be identified, and that the identification and characterization of such global regulatory genes might shed light on how this organism functions under the different physiological conditions it encounters, in nature. In addition, since many of the environmental parameters these bacteria encounter in soil; such as low oxygen tensions and osmotic changes, are also experienced *in planta*, we hypothesized that these global regulatory genes could control gene expression in both the free living state, as well as during symbiosis when the bacteria become endosymbiotic and differentiate into bacteroids.

Towards this goal, regulatory components for the induction of genes during nutrient deprivation were identified using successive rounds of transposon mutagenesis. A *S. meliloti* mutant (C22) which harbors a Tn*5luxAB* insertion in a gene that is induced by a variety of stresses; including carbon, nitrogen, or oxygen deprivation, osmotic stress, as well as during stationary-phase was identified after the first round of mutagenesis. This locus was designated *ndi* for nutrient deprivation induced, and it appears to be novel. The characterization of this locus will be discussed in Chapter 2. A second round of mutagenesis was subsequently used to identify genes involved in the regulation of the *ndi* locus. Strain C22 was mutagenized with transposon Tn*1721*, and a library of double mutants was screened for altered patterns of

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One of the double mutants (12,C-4) that lacks transcriptional activity of the *ndi* locus under any of the inducing conditions tested has proven to be of special interest. The Tn*1721* transposon was found to be inserted in a gene with a high degree of similarity to the mitochondrial benzodiazepine receptor (65), as well as to the outer membrane oxygen sensor protein (TspO) of *Rhodobacter sphaeroides* (136). Although the exact mechanism for TspO mediated regulation of gene expression is still not understood, there is evidence (135) that this outer membrane protein regulates the efflux of intermediates from the heme biosynthetic pathway (porphyrinogens) through the major porins, thereby controlling the expression of genes from certain operons by controlling the intracellular concentration of an effector molecule. Hence, TspO appears to represent a novel mechanism by which bacteria can regulate gene expression when stressed. The results of this study will be presented in Chapter 4.

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REFERENCES

1. **Albertson, N. H., T. Nystrom, and S. Kjelleberg.** 1990. Macro-molecular synthesis during recovery of the marine Vibrio sp. S14 from starvation. J Gen Microbiol. **136:**2201-2207.

2. **Alexander, D. M., K. Damerau, and A. C. St. John.** 1993. Carbohydrate uptake genes in *Escherichia coli* are induced by carbon starvation. Curr Microbiol. **27:**335-340.

3. **Alexander, D. M., and A. C. St John.** 1994. Characterization of the carbon starvation-inducible stationary phase-inducible gene *slp* encoding an outer membrane lipoprotein in *Escherichia coli*. Mol Microbiol. **11**(6):1059-1071.

4. **Atlung, T., K. Knudsen, L. Heerfordt, and L. Brondsted.** 1997. Effects of sigma S and the transcriptional activator AppY on the induction of the *Escherichia coli hya* and *cbdAB-appA* operons in response to carbon and phosphate starvation. J Bacteriol. **179**(7):2141-2146.

5. **Batut, J., and P. Boistard.** 1994. Oxygen control in *Rhizobium*. Antonie van Leeuwenhoek. **66:**129-150.

6. **Blum, P. H., F. D. Jovanovich, M. P. McCann, J. E. Schultz, S. E. Lesley, R. R. Burgess, and A. Matin.** 1990. Cloning and in vivo and in vitro regulation of cyclic AMP-dependent carbon starvation genes from *Escherichia coli*. J Bacteriol. **172:**3813-3820.

7. **Blumenthal, R. M., D. W. Borst, and R. G. Matthews.** 1996. Experimental analysis of global gene regulation in *Escherichia coli*, p. 1-79. *In* W. E. Cohen and K. Moldave (ed.), Progress in Nucleic Acid Research and Molecular Biology, vol. 55. Academic Press, New York, N.Y.

8. **Botsford, J. L.** 1981. Cyclic nucleotides in prokaryotes. Microbiol. Rev. **45**(4):620-642.

9. **Cashel, M., and K. E. Rudd.** 1996. The stringent response, p. 1410-1431. *In* F. C. Neidhart (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, DC.

10. **David, M., M.-L. Daveran, J. Batut, A. Dedieu, O. Domergue, J. Ghai, C. Hertig, P. Boistard, and D. Kahn.** 1988. Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. Cell. **54:**671-683.

11. **de Bruijn, F. J.** 1987. Transposon Tn5 mutagenesis to map genes. Meth Enzymol. **154:**175-196.

12. **Encarnacion, S., M. Dunn, K. Willms, and J. Mora.** 1995. Fermentative and aerobic metabolism in *Rhizobium etli*. J. Bacteriol. **177**(11):3058-3066.

13. **Ferenci, T.** 1996. Adaptation to life at micromolar nutrient levels: the regulation of *Escherichia coli* glucose transport by endoinduction and cAMP. FEMS Microbiol Rev. **18:**301-317.

14. **Fischer, H.-M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58**(3):352-386.

15. **Foster, J. W., and M. P. Spector.** 1995. How *Salmonella* survive against the odds. Ann Rev Microbiol. **49:**145-174.

16. **Givskov, M., L. Eberl, and S. Molin.** 1994. Reponse to nutrient starvation in *Pseudomonas putida* KT2442: two-dimensional electrophoretic analysis of starvation- and stress-induced proteins. J. of Bacteriol. **176:**4816-4824.

17. **Givskov**
to nutrien
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18. **Glazebr**
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19. **Gong, W.**
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20. **Graham,**
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21. **Groat, R.**
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22. **Gu, B., J.**
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23. **Harley, C**
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31. **Huisman**
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32. **Huisman**
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(ed.). *Esch*
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17. **Givskov, M., I. Eberl, S. Moller, L. K. Poulsen, and S. Molin.** 1994. Response to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol. **176**(1):7-14.

18. **Glazebrook, J., and G. C. Walker.** 1991. Genetic techniques in *Rhizobium meliloti*. Meth Enzymol. **204**:398-418.

19. **Gong, W., B. Hao, S. S. Mansy, G. Gonzalez, and M. A. Gilles-Gonzalez.** 1998. Structure of a biological oxygen sensor: A new mechanism for heme-driven signal transduction. Proc Natl Acad Sci USA **95**(15177-15182).

20. **Graham, P.** 1991. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Can J Microbiol. **38**:475-484.

21. **Groat, R. G., J. E. Schultz, E. Zychlinsky, A. Bockman, and A. Matin.** 1986. Staravtion proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. J Bacteriol. **168**(2):486-493.

22. **Gu, B., J. H. Lee, T. R. Hoover, D. Scholl, and B. T. Nixon.** 1994. *Rhizobium meliloti* DctD, a σ^{54} - dependent transcriptional activator, may be negatively controlled by a subdomain in the C-terminal end of its two-component receiver module. Mol Microbiol. **13**(1):51-66.

23. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter. Nucl Acids Res. **15**:2343-2361.

24. **Hecker, M., and W. s. a. U. Volker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol Microbiol. **19**(3):417-428.

25. **Hengge-Aronis, R.** 1996. Back to log phase: σ as a global regulator in the osmotic control of gene expression in *Escherichia coli*. Mol Microbiol. **21**(5):887-893.

26. **Hengge-Aronis, R.** 1996. Regulation of gene expression during entry into stationary phase, p. 1497-1512. *In* F. C. Neidhart (ed.), *Escherichia coli* and *Salmonella:* cellular and molecular biology, 2nd ed, vol. 1. ASM Press, Washington DC.

27. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpos* in early stationary phase gene regulation in E. coli. Cell. **72**:165-168.

28. **Hengge-Aronis, R., W. Klein, R. Lange, M. Rimmele, and W. Boos.** 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by rpoS and are involved instationary-phase thermotolerance in *Escherichia coli*. J Bacteriol. **173**:7918-7924.

29. **Honeycutt, R. J., M. McClelland, and B. W. Sobral.** 1993. Physical map of the genome of *Rhizobium meliloti* 1021. Journal of Bacteriology. **175**:6945-6952.

30. **Howorth, S. M., and R. R. England.** 1999. Accumulation of ppGpp in symbiotic and free-living nitrogen-fixing bacteria folowing amino acid starvation. Arch Microbiol. **171**:131-134.

31. **Huisman, G. W., and R. Kolter.** 1994. Sensing starvation: a homoserine lactone-dependent signaling pathway in *Escherichia coli*. Science. **265**(5171):537-539.

32. **Huisman, G. W., D. A. Siegele, M. N. Zambrano, and R. Kolter.** 1996. Morphological and physiological changes during stationary phase., p. 1672-1682. *In* F. C. Neidhart, I. R. Curtiss, J. L. Ingrham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology. ASM Press, Washington, DC.

33. **Iuchi, S**
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34. **Ivanova**
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35. **Jorgens**
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36. **Kahn, M**
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48. **Lange,**
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49. **Lazar, S**
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50. **Lemaux**
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33. **Iuchi, S., and E. C. C. Lin.** 1993. Adaptation of *Escherichia coli* to redox environments by gene expression. Mol Microbiol. **9**(1):9-15.

34. **Ivanova, A., C. Miller, G. Glinsky, and A. Eisenstark.** 1994. Role of *rpoS* (*katF*) in *oxyR*-independent regulation of hydroperoxidase I in *Escherichia coli*. Mol Microbiol. **12**:571-578.

35. **Jorgensen, F., M. Bally, V. Chapon-Herve, G. Michel, A. Lazdunski, P. Williams, and G. S. A. B. Stewart.** 1999. RpoS-dependent stress tolerance in *Pseudomonas aeruginosa*. Microbiology. **145**:835-844.

36. **Kahn, M. L., T. McDermott, and M. K. Udvardi.** 1998. Carbon and nitrogen metabolism in rhizobia, p. 461-485. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The Rhizobiaceae. Kluwer Academic Press.

37. **Kahn, P.** 1995. From genome to proteome: looking at the cell's protein. Science. **270**:369-370.

38. **Kaiser, D.** 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. Annu Rev Genet. **20**:539-566.

39. **Kaprelyants, A., J. C. Gottschal, and D. K. Kell.** 1993. Dormancy in non-sporulating bacteria. FEMS Microbiol Rev. **104**:271-286.

40. **Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer.** 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie van Leeuwenhoek. **73**:169-187.

41. **Keller, J. A., and L. D. Simon.** 1988. Divergent effects of a *dnaK* mutation on abnormal protein degradation in *Escherichia coli*. Mol Microbiol. **2**(1):31-41.

42. **Kim, Y., L. S. Watrud, and A. Matin.** 1995. a carbon starvation survival gene of *Pseudomonas putida* is regulated by σ^{54}. J Bacteriol. **177**:1850-1859.

43. **Kjelleberg, S.** 1993. Starvation in Bacteria. Plenum Press, New York and London.

44. **Kjelleberg, S., M. Hermansson, and P. Marden.** 1987. The transient phase between growth and nongrowth of heterotrophic bacteria, with emphasis on the marine environment. Ann Rev Microbiol. **41**:25-49.

45. **Kolter, R.** 1999. Growth in studying the cessation of growth. J Bacteriol. **181**(3):697-699.

46. **Kragelund, L., B. Christoffersen, O. Nybroe, and F. J. de Bruijn.** 1995. Isolation of *lux* reporter gene fusions in *Pseudomonas fluorescens* DF57 inducible by nitrogen or phosphorous starvation. FEMS Microbiol Ecol. **17**:95-106.

47. **Landini, P., L. I. Hajec, L. H. Nguyen, R. R. Burgess, and M. R. Volkert.** 1996. The leucine-responsive regulatory protein (LRP) acts as a specific repressor for sigma S-dependent transcription of the *Escherichia coli aidB* gene. Mol Microbiol. **20**(5):947-955.

48. **Lange, R., and R. Hengge-Aronis.** 1991. Growth phase-regulated expression of *bolA* and morphology of stationary-phase *Escherichia coli* cells are controlled by the novel sigma factor sigma S. J Bacteriol. **173**(14):4474-4481.

49. **Lazar, S. W., M. Almiron, A. Tormo, and R. Kolter.** 1998. Role of the *Escherichia coli* SurA protein in stationary-phase survival. J Bacteriol. **180**(21):5704-5711.

50. **Lemaux, P. G., S. I. Herendeen, P. L. Bloch, and F. C. Neidhardt.** 1978. Transient rates of synthesis of individual polypeptides in *E.coli* following temperature shifts. Cell. **13**:427-434.

51. **Li, C., J. K. Ichikawa, J. J. Ravetto, H. Kuo, J. C. Fu, and S. Clarke.** 1994. A new gene involved in stationary-phase survival located at 59 minutes on the *Escherichia coli* chromosome. J Bacteriol. **176**(19):6015-6022.

52. **Loewen, P. C., B. Hu, J. Strutinsky, and R. Sparling.** 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. **44**:707-717.

53. **Loewen, P. C., and B. L. Triggs.** 1984. Genetic mapping of *katF*, a locus that with *katE* affects the synthesis of a second catalase species in *Escherichia coli*. J Bacteriol. **160**:668-675.

54. **Lonetto, M. A., and C. A. Gross.** 1996. Nomenclature of sigma factors from *Escherichia coli* and *Salmonella typhimurium* and relationships to sigma factors from other organisms, p. 821. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC.

55. **Long, S. R.** 1996. Rhizobium symbiosis:nod factors in perspective. Plant Cell. **8**(10):1885-1896.

56. **Long, S. R.** 1989. Rhizobium-legume nodulation: Life together in the underground. Cell. **56**:203-214.

57. **Losick, R., P. Youngman, and P. J. Piggot.** 1986. Genetics of endospore formation in Bacillus subtilis. Annu Rev Genet. **20**:625-669.

58. **Madigan, M. T., J. M. Martinko, and J. Parker.** 1996. *Brock* Biology of Microorganisms, eigth ed. Prentice Hall, Upper Saddle River, NJ.

59. **Mandelstam, J.** 1963. Protein turnover and its function in the economy of the cell. Ann NY Acad Sci. **102**:621-636.

60. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc Natl Acad Sci USA **82**:8129-8133.

61. **Matin, A.** 1992. Genetics of bacterial stress response and its application, p. 1-15. *In* H. Pedersen, R. Mutharasan, and D. Dibiasio (ed.), Biochemical Engineering VII. Annals of the New York Academy of Sciences, vol. 665.

62. **Matin, A.** 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol Microbiol. **5**:3-10.

63. **Matin, A.** 1996. Role of alternative sigma factors in starvation protein synthesis - novel mechanisms of catabolite repression. Res Microbiol. **147**(6-7):494-505.

64. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Ann Rev Microbiol. **43**:293-316.

65. **McEnery, M. W., A. M. Snowman, R. R. Trifiletti, and S. H. Snyder.** 1992. Isolation of the mitochondrial benzodiazapine receptor: Association with the voltage-dependent anion channel and the adenine nucleotide carrier. Proc Natl Acad Sci USA**89**:3170-3174.

66. **Mellies, J., A. Wise, and M. Villarejo.** 1995. Two different *Escherichia coli proP* promoters respond to osmotic and growth phase signals. J Bacteriol. **177**:144-151.

67. **Miksch, G., and P. Dobrowolski.** 1995. Growth phase-dependent induction of stationary-phase promoters of *Escherichia coli* in different Gram-negative bacteria. J. Bacteriol. **177**(18):5374-5378.

68. **Milcamps, A., and F. J. deBruijn.** 1999. Identification of a novel, nutrient deprivation induced gene (*hmgA*) in *Sinorhizobium meliloti*, involved in Tyrosine degradation. Microbiology. **145**:935-947.

69. **Milcam**
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70. **Moriart**
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69. **Milcamps, A., D. M. Ragatz, P. Lim, K. A. Berger, and F. J. de Bruijn.** 1998. Isolation of carbon- and nitrogen-deprivation-induced loci of *Sinorhizobium meliloti* 1021 by Tn*5-luxAB* mutagenesis. Microbiology. **144:**3205-3218.

70. **Moriarty, D. J. W., and R. T. Bell.** 1993. Bacterial growth and starvation in aquatic environments., p. 25-48. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum, New York.

71. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1-23. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum press, New York, N.Y.

72. **Mulvey, M. R., and P. C. Loewen.** 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. Nucleic Acids Res. **17:**9979-9991.

73. **Neidhart, F. C., J. L. Ingraham, and M. Schaechter (ed.).** 1990. Physiology of the bacterial cell: A molecular approach. Sinauer Associates, Inc, Sunderland, MA.

74. **Ninfa, A. J., and B. Magasanik.** 1986. Covalent modification of the *glnG* product, NR_I, by the *glnL* product, NR_{II}, regulates the transcription of the *glnALG* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. **83:**5909-5913.

75. **Nyström, T.** 1995. Glucose starvation stimulon of *escherichia coli*: role of integration host factor in starvation survival and growth phase-dependent protein synthesis. J Bacteriol. **177**(19):5707-5710.

76. **Nyström, T.** 1994. The glucose-starvation stimulon of *Escherichia coli* : induced and repressed synthesis of enzymes of central metabolic pathways and role of acetyl phosphate in gene expression and starvation survival. Mol Microbiol. **12**(5):833-843.

77. **Nyström, T., N. Albertson, and S. Kjelleberg.** 1988. Synthesis of membrane and periplasmic proteins during starvation of a marine *Vibrio* sp. J Gen Microbiol. **134:**1645-1651.

78. **Nyström, T., K. Flardh, and S. Kjelleberg.** 1990. Responses to multiple-nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. J. Bacteriol. **172**(12):7085-7097.

79. **Nyström, T., and N. Gustavsson.** 1998. Maintenance energy requirement: what is required for stasis survival of *Escherichia coli*? Biochem Biophys Acta. **1365:**225-231.

80. **Nyström, T., and S. Kjelleberg.** 1989. Role of protein synthesis in the cell division and starvation induced resistance to autolysis of a marine vibrio during the initial stage of starvation. J Gen Microbiol. **135:**1599-1606.

81. **Nyström, T., C. Larsson, and L. Gustafsson.** 1996. Bacterial defense against aging: role of the *Escherichia coli* ArcA regulator in gene expression, readjusting energy flux and survival during stasis. EMBO. **15**(13):3219-3228.

82. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J Biol Chem. **250:**4007-4021.

83. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239-272. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum, Ney York.

84. **Ostling, J., K. Flardh, and S. Kjelleberg.** 1995. Isolation of a carbon stavation regulatory mutant in a marine *Vibrio* strain. J Bacteriol. **177:**6978-6982.

85. **Ostling, J., L. Holmquist, L. Flardh, B. Svenblad, A. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation and recovery of *Vibrio*, p. 103-123. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum, New York.

86. **Pace, N**
Science
87. **Parkins**
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88. **Parsell,**
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89. **Paul, E.**
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90. **Pedersen**
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94. **Pratt, L**
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99. **Rons**
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100. **Ros**
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101. **Ruc**
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102. **Sal**
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86. **Pace, N. R.** 1997. A molecular view of microbial diversity and the biosphere. Science. **276:**734-740.

87. **Parkinson, J. S.** 1995. Genetic approaches for signaling pathways and proteins. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-Component Signal Transduction. American Society for Microbiology, Washington, D.C.

88. **Parsell, D. A., and S. Lindquist.** 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet. **27:**437-496.

89. **Paul, E. A., and F. E. Clark.** 1989. Soil Microbiology and Biochemistry. Academic Press, San Diego, CA.

90. **Pedersen, S., P. L. Bloch, S. Reeh, and F. C. Neidhardt.** 1978. Patterns of protein synthesis in *E. coli*:a catalogue of the amount of 140 individual proteins at different growth rates. Cell. **14:**179-190.

91. **Phillips, D. A.** 1992. Flavonoids: Plant signals to soil microbes. Recent. Adv. Phytochem. **26:**201-231.

92. **Postgate, J.** 1967. Viability measurements and the survival of microbes under minimum stress., p. 1-21. *In* A. H. Rose and J. Wilkinson (ed.), Advances in Microbial Physiology. Academic Press, London.

93. **Potts, M.** 1994. Dessication tolerance of prokaryotes. Microbiol Rev. **58:**755-805.

94. **Pratt, L. A., and T. J. Silhavy.** 1995. Porin regulon of *Escherichia coli*, p. 105-127. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-Component Signal Transduction. American Society for Microbiology, Washington, DC.

95. **Quispel, A.** 1998. Evolutionary aspects of symbiotic adaptations: *Rhizobium*'s contribution to evolution by association., p. 495-496. *In* H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The Rhizobiaceae. Kluwer Academic Press.

96. **Ramos-Gonzalez, M. I., and S. Molin.** 1998. Cloning, sequencing, and phenotypic characterization of the *rpos* gene from *Pseudomonas putida* KT2440. J Bacteriol. **180**(13):3421-3431.

97. **Reeve, C. A., P. S. Amy, and A. Matin.** 1984. Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. J Bacteriol. **160**(3):1041-1046.

98. **Reeve, W. G., R. P. Tiwari, C. M. Wong, M. J. Dilworth, and A. R. Glenn.** 1998. The transcriptional regulator gene *phrR* in *Sinorhizobium meliloti* WSM419 is regulated by low pH and other stresses. Microbiology. **144**(3335-3342).

99. **Ronson, C. W., B. T. Nixon, L. M. Albright, and F. M. Ausubel.** 1987. *Rhizobium meliloti ntrA* (*rpoN*) Gene is required for diverse metabolic functions. J. Bacteriol. **169**(6):2424-2431.

100. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol Rev. **51**(3):365-379.

101. **Rudd, K. E., H. J. Sofia, E. V. Koonin, G. Plunkett, S. Lazar, and P. E. Rouviere.** 1995. A new family of peptidyl-prolyl isomerases. Trends Biochem Sci. **20:**12-14.

102. **Sak, B. D., A. Eisenstark, and D. Touati.** 1989. Exonuclease III and the catalase hydroperoxidase II in *Escherichia coli* are both regulated by the *katF* gene product. Proc Natl Acad Sci USA **86:**3271-3275.

103. **Schul**
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104. **Siegel**
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105. **Spain**
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103. **Schultz, J. E., and A. Matin.** 1991. Molecular and functional characterization of a carbon starvation gene of *Escherichia coli*. J Mol Biol. **218:**129-140.

104. **Siegele, D. A., and R. Kolter.** 1992. Life after log. J. Bacteriol. **174**(2):345-348.

105. **Spaink, H. P., A. Kondorosi, and P. J. J. Hooykaas.** 1998. The Rhizobiaceae. Kluwer Academic Press.

106. **Spector, M. P.** *1990.* Gene expression in response to multiple nutrient-starvation conditions in *Salmonella typhimurium*. FEMS Microbiol Ecol. **74:**175-184.

107. **Spector, M. P.** 1998. The starvation-stress response (SSR) of *Salmonella*, p. 233-279, Advances in Microbial Physiology, vol. 40. Academic Press.

108. **Spector, M. P., and C. L. Cubitt.** 1992. Starvation-inducible loci of *Salmonella typhimurium*: regulation and roles in starvation-survival. Mol Microbiol. **6**(11):1467-1476.

109. **Spector, M. P., Y. K. Park, S. Tirgari, T. Gonzalez, and J. W. Foster.** 1988. identification and characterization of starvation-regulated genetic loci in *Salmonella typhimurium* by using Mu d-directed *lacZ* operon fusions. J. Bacteriol. **170**(1):345-351.

110. **Spiro, S., and J. R. Guest.** 1990. Fnr and its role in oxygen-related gene expression in *Escherichia coli*. FEMS Microbiol. Rev. **75:**399-428.

111. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1988. *Escherichia coli* heat shock gene mutants are defective in proteolysis. Genes Dev. **2:**1851-1858.

112. **Strom, A. R., and I. Kaasen.** 1993. Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. Mol Microbiol. **8:**2202-2209.

113. **Summers, M. L., J. G. Elkins, B. A. Elliot, and T. R. McDermott.** 1998. Expression and regulation of phosphate stress inducible genes in *Sinorhizobium meliloti*. MPMI. **11**(11):1094-1101.

114. **Tanaka, K., Y. Takayanagi, N. Fujita, A. Ishihama, and H. Takahashi.** 1993. Heterogeneity of the principal sigma factor in *Escherichia coli*: the *rpoS* gene product σ^{38}, is a principal sigma factor of RNA polymerase in stationary phase *E. coli*. Proc Natl Acad Sci USA **90**(3511-3515).

115. **Thorne, S. H., and H. D. Williams.** 1997. Adaptation to nutrient starvation in *Rhizobium Leguminosarum* bv. phaseoli: analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. J. Bacteriol. **179**(22):6894-6901.

116. **Tian, G., I. E. Vainberg, W. D. Tap, S. A. Lewis, and N. J. Cowan.** 1995. Specificity in chaperonin-mediated protein folding. Science. **375:**250-253.

117. **Tiwari, R. P., W. G. Reeve, M. J. Dilworth, and A. R. Glenn.** 1996. Acid Tolerance in *Rhizobium meliloti* strain WSM419 involves a two-component sensor-regulator system. Microbiology. **142:**1693-1704.

118. **Tormo, A., M. Amiron, and R. Kolter.** 1990. *surA*, an Escherichia coli gene essential for survival in stationary phase. J Bacteriol. **172**(8):4339-4347.

119. **Uhde, C., R. Schmidt, D. Jording, W. Selbitschka, and A. Puhler.** 1997. Stationary-phase mutants of *Sinorhizobium meliloti* are impaired in Stationary-phase survival or in recovery to logarithmic growth. J. Bacteriol. **179**(20):6432-6440.

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120.**Van Alphen, W., and B. Lugtenberg.** 1977. Influence of osmolarity of the growth medium on th eouter membrane protein pattern of *Escherichia coli*. J Bacteriol. **131**:623-630.

121.**van Elsas, J. D., and L. S. van Overbeek.** 1993. Bacterial responses to soil stimuli, p. 55-79. *In* S. Kjelleberg (ed.), Starvation in bacteria. Plenum press, New York.

122.**van Rhijn, P., and J. Vanderleyden.** 1995. The *Rhizobium*-plant symbiosis. Microbiol Rev. **59**(1):124-142.

123.**VanBogelen, R. A., and F. C. Neidhardt.** 1990. Global systems approch to bacterial physiology: protein responders to stress and starvation. FEMS Microbiol Ecol. **74**:121-128.

124.**Vasse, J., F. deBilly, S. Camut, and G. Truchet.** 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bacteriol. **172**:4295-4306.

125.**Verma, D. P. S.** 1992. Signals in root nodule organogenesis and endocytosis of *Rhizobium*. Plant Cell. **4**:373-382.

126.**Visick, J. E., and S. Clarke.** 1995. Repair, refold,recycle: how bacteria can deal with spontaneous and environmental damage to proteins. Mol Microbiol. **16**(5):835-845.

127.**Volkert, M. R., L. I. Hajec, Z. Matijasevic, F. C. Fang, and R. Prince.** 1994. Induction of the *Escherichia coli aidB* gene under oxygen limiting conditions requires a functional *rpoS* (*katF*) gene. J Bacteriol. **176**:7638-7645.

128.**Wang, Y., L. Giblin, B. Boesten, and F. O'Gara.** 1993. The *Escherichia coli* cAMP receptor protein (CRP) represses the *Rhizobium meliloti dctA* promoter in a cAMP-dependent fashion. Mol Microbiol. **8**(2):253-259.

129.**Wei, X., and W. D. Bauer.** 1998. Starvation-induced changes in motility, chemotaxis and flagellation of *Rhizobium meliloti*. Appl Env Microbiol. **64**:1708-1714.

130.**Weichart, D., R. Lange, N. Henneberg, and R. Hengge-Aronis.** 1993. Identification and characterization of stationary phase-inducible genes in *Escherichia coli*. Mol. Microbiol. **10**(2):407-420.

131.**Whistler, C. W., N. A. Corbell, A. Sarniguet, W. Ream, and J. E. Loper.** 1998. The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor σ^S and the stress response in *Pseudomonas fluorescens* Pf-5. J Bacteriol. **180**(24):6635-6641.

132.**Whitman, W. B., D. C. Coleman, and W. J. Wiebe.** 1998. Prokaryotes: the unseen majority. Proc Natl Acad USA **95**(12):6578-6583.

133.**Williams, S. T.** 1985. Oligotrophy in soil: fact or fiction, p. 81-110. *In* M. Fletcher and G. D. Floodgate (ed.), Bacteria in Their Natural Environment. Academic Press Inc., Orlando, Fl.

134.**Xu, J., and R. C. Johnson.** 1997. Activation of the RpoS-dependent *proP* P2 transcription by the Fis protein in vitro. J Mol Biol. **270**:346-359.

135.**Yeliseev, A. A., and S. Kaplan.** 1999. A novel mechanism for the regulation of photosynthesis gene expression by the TspO outer membrane protein of *Rhodobacter sphaeroides* 2.4.1. in preparartion.

136.**Yeliseev, A. A., and S. Kaplan.** 1995. A sensory transducer homologous to the mammalian peripheral-type benzodiazepine receptor regulates photosynthetic membrane complex formation in *Rhodobacter sphaeroides* 2.4.1. J Biol Chem. **270**(36):21167-21175.

CHAPTER 2

Identification and Characterization of a Novel Locus (*ndi*) in *S. meliloti* induced by Nutrient-Deprivation

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Identification and Characterization of a Novel Locus (*ndi*) in *S. meliloti* induced by Nutrient-Deprivation

ABSTRACT

In order to identify nutrient deprivation genes in *Sinorhizobium meliloti*, 5000 Tn*5luxAB* -containing derivatives were screened for strains harboring luciferase reporter gene fusions induced by carbon and nitrogen deprivation, and by low oxygen tension. A collection of 22 *luxAB* gene fusions induced by nitrogen deprivation, 13 by carbon deprivation, and 34 by oxygen limitation was generated (26). One of theTn*5luxAB* containing *S. meliloti* strains was found to harbor a transcriptional fusion to a gene which is induced in response to carbon and nitrogen deprivation, oxygen limitation, osmotic stress, as well during entry into stationary–phase growth (designated ndi for nutrient deprivation induced). Comparison of the deduced amino acid sequences of the ORFs in the *ndi* locus to the protein databases at NCBI did not indicate similarity with any known genes, indicating that the *ndi* locus is novel.

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INTRODUCTION

Soil constitutes an extremely complex heterogeneous environment with a large number of fluctuating environmental parameters that affect microbial growth and survival (30). One factor of particular importance for bacterial growth and persistence in the soil is the lack of essential nutrients. Autochthonous soil bacteria are, more often than not, deprived of essential nutrients, consequently their typical physiological state in their natural habitat is likely to be either very slow growth or dormancy (27, 35). A relatively more nutrient-rich habitat in soil occurs around plant roots. The rhizosphere constitutes an ecological niche where nutrients are more readily available and certain bacteria have developed mechanisms to take advantage of this niche, such as members of the *Rhizobiaceae*, also generically referred to as rhizobia. Rhizobia establish symbioses with legume plants during which, a new specialized organ is formed, the nitrogen-fixing root nodule. These nodules provide the proper physiological conditions for the bacteria to survive in the absence of competing microflora, and to reduce (fix) atmospheric dinitrogen to ammonia, which is assimilated by the plant for growth (38, 43). Hence, rhizobia persist in three distinct habitats: bulk soil, the rhizosphere; and the plant nodule. In order to do so, they must survive the oligotrophic conditions in soil, competitively sense and utilize plant derived signaling molecules and growth promoting nutrients excreted from the rhizosphere, as well as adapt to the homeostatic environs of the infected plant host cell. Therefore, rhizobia

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provide a unique model system to investigate environmental control of gene expression in a bacterium indigenous to soil.

The role of rhizobia as symbionts and the signal transduction pathways involved in nodule ontogeny and symbiotic nitrogen fixation have been studied extensively (21, 38). However, little is known in regards to how these bacteria are able to persist and compete in the free-living state, in the soil and rhizosphere. In fact, general information on the molecular basis of starvation-survival of bacteria whose natural habitat is soil, such as pseudomonads or rhizobia, remains relatively scarce, in spite of the likelihood that bacteria, which have evolved in the soil habitat, have certain mechanisms for surviving in this environment that are distinct from those identified in enteric or marine isolates.

A few select bacteria, such as *Bacillus* and *Myxococcus* spp., contend with nutrient deprivation by differentiating into stress resistant spores, i.e., endospores and myxospores, respectively (14, 16). While it is evident that most bacteria do not form spores, research has shown that under starvation conditions, various species, among them *Escherichia*, *Salmonella*, and *Vibrio* spp., as well as a variety of other marine species, do enter into a specific genetic program which results in major structural and physiological changes, the ultimate purpose of which is the persistence of the species in the absence of growth (20, 22, 29, 39). Although the majority of other bacteria, including those commonly found in soil, do not readily appear to differentiate into a stress resistant state, it is likely that these bacteria also have a stasis survival state. For our studies on this topic, the common soil bacterium, *S. meliloti*,

was chosen (26). *S. meliloti* is a good model organism for studies on environmental control of gene expression in the soil and rhizosphere of plants, since it has a short doubling time, and is amenable to a variety of molecular genetic manipulations. Moreover, both genetic and physical maps are available, and the genomic DNA sequence of this organism is in the process of being determined in its entirety. *S. meliloti* induces nitrogen fixing root nodules on alfalfa (*Medicago sativa*), and numerous rhizobial genes involved in nodule otogeny and function have been identified (21, 38, 43)

Previously, the isolation of 33 *S. meliloi* strains with Tn5*luxAB* gene fusions induced by either carbon or nitrogen-deprivation, or both, was reported by Milcamps *et al.* (1998). Here we report the characterization of one of these strains (C22) that harbors a transcriptional fusion induced by a number of environmental conditions; including nitrogen or carbon deprivation, oxygen limitation, osmotic stress, as well as during entry into post-exponential stationary-phase growth. The locus containing this fusion has been designated *ndi* for nutrient deprivation induced, and DNA sequence analysis of the ORFs in the *ndi* locus has failed to reveal significant similarities with database entries, indicating that it represents a novel locus.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

Strain *S. meliloti* 1021 (Sm^R) has been described by Meade *et al.*, (1982), *E. coli* DH5α by Hanahan (1983), and *E. coli* HB101 by Boyer & Roulland-Dussoix (1969). Plasmid pRK2013 has been described by Ditta *et al.*, (1980). Plasmid pRL1063a (45) was kindly provided by Dr. Peter Wolk (Michigan State University). Plasmid pTB93 (Sp^R) was kindly provided by Sharon Long (8). pBluescript II SK (Ap^R) was obtained from Stratagene . pLAFR1 (IncP, Mob^+, Tra^-, Tc^r) has been described by Friedman *et al.*, (1982). pC22 is the plasmid recovered from strain C22 carrying the Tn*5-1063* transposon with flanking DNA on an *EcoR*I fragment (Km^r, this work). pC22lux is a plasmid carrying the entire *EcoR*I fragment of pC22 inserted into pTB93 (Sp^R, Km^r, this work).

S. meliloti strains were grown on or in rich TY medium (2) or minimal medium at 28°C, as indicated. The minimal medium used was GTS (26). *E. coli* strains were grown on or in rich medium (Luria-Bertani; LB) at 37°C, Antibiotics were added at the following concentrations: (i) *S. meliloti*: streptomycin (Sm) 250 μg ml^{-1}; kanamycin (Km), 200 μg ml^{-1}; tetracycline (Tc) 10 μg ml^{-1}; spectinomycin (Sp)50 μg ml^{-1}; (ii) *E. coli*: ampicillin (Ap), 100 μg ml^{-1};

(Km) 50 μg ml^{-1}; (Sp) 100 μg ml^{-1}; (Tc) 5 μg ml^{-1}. GTS-N is GTS medium lacking a combined nitrogen source. GTS-C is GTS medium lacking carbon. GTS+NaCl is GTS containing NaCl (400mM), GTS+sucrose is GTS containing sucrose (30%), which was used for testing the response to osmotic stress.

Survival during stationary phase

Strains 1021 and C22 were grown in GTS medium containing growth - limiting amounts of carbon (glucose 0.05%) and incubated on a rotary shaker for 10 days. Viable cells were determined by plate counts. In addition, the absorbance (O.D. 600 nm) of bacterial cultures was monitored over time, to evaluate bacterial growth, as well as lysis.

Testing of strains for substrate utilization

To test strains for the ability to utilize or oxidize a variety of different carbon sources, the GN Biolog Microplates from BiOLOG, (Hayward, CA) were used, as described by the manufacturer.

DNA isolation and manipulation

Plasmid DNA for restriction analysis and DNA sequencing was prepared using a Magic-mini kit from Promega Madison, WI. Chromosomal DNA was isolated from *S. meliloti* strains according to de Bruijn *et al.* (1994). All enzymes for DNA manipulations were purchased from Boehringer Mannheim or New England Biolabs and used as specified by manufacturers. Restriction enzyme digests and ligations were carried out as described by Sambrook *et al.* (1989). Probes were labeled with [α^{32} P]dATP using a random primer kit (Boehringer Mannheim) following the manufacturer's instructions. Plasmids were introduced into *E. coli* hosts by electroporation and into *S. meliloti* strains via triparental conjugation (5).

Transposon mutagenesis and reporter gene expression assays

Tn*5luxAB* reporter gene transposon mutants were generated, as described by Milcamps *et al.* (1998), and examined for luciferase activity under inducing conditions on agar plates using a Hamamatsu Photonic System model C1966-20 (Photonic Microscopy), as described by Milcamps *et al.* (1998).

Induction and quantitation of luciferase activity in cultures

To evaluate expression under nutrient deprivation conditions, strain C22 or strains harboring pC22Lux plasmids were grown in TY broth with the appropriate antibiotics for 36 hours, sub-cultured in GTS broth (1:100 dilution), and grown overnight to early exponential growth phase (O.D. between 0.1 and 0.3). The cells were centrifuged (rotor and tubes at room temperature) and the pellets resuspended in regular or modified GTS medium. The resulting cell suspensions were incubated in a shaker at 28°C and luciferase activity, as well as cell density were monitored, simultaneously, over time. To evaluate expression during the post-exponential growth phase, strain C22, or strains harboring pC22Lux plasmids, were grown in TY broth with the appropriate antibiotics for 24 hours and sub-cultured in TY or GTS broth (1:100 dilution). Subsequently luciferase activity, as well as cell density, were monitored, simultaneously, over time.

Luciferase activity was determined with a luminometer (model TD-20e, Turner Designs, Sunnyvale, CA) by placing a luminometer tube containing 10 μl of n-decyl aldehyde (Sigma, St. Louis, MO) into the cell of the luminometer, adding 140 μl of bacterial culture and immediately starting the analysis. The aldehyde solution was prepared in water (0.1% v/v) and vortexed for 10 minutes. Photons were counted for 20 seconds and data were recorded as light units (LU were converted to Relative Light Units (LU ml^{-1} $O.D._{600}{}^{-1}$).

Calibration of the luminometer by the method of Hastings & Weber (1963) was used to establish that one light unit equaled approximately $1.4x10^7$ photons.

Luciferase activity is dependent on the energy status of the cell, due to the requirement for reducing equivalents ($FMNH_2$) to catalyze the bioluminescent reaction (24), therefore a modified screening procedure was used for testing strains that had been grown under carbon deprivation conditions (26). To determine the luminescence of carbon-starved cells, a 500 μl aliquot of culture was mixed with an equal portion of complete GTS medium and vortexed briefly. This mixture was incubated at room temperature for 15 minutes and a 140 μl aliquot was used for luciferase activity determination, as described above.

A modified method was also used when the bacteria were grown under low oxygen tension (J. Trzebiatowski, unpublished). Oxygen is also required to catalyze the biolumenescence reaction. Therefore, to ensure that oxygen was not limiting when testing cultures that had been grown under low oxygen tension (1% oxygen in nitrogen), the cells were aerated, before testing, by pelleting a 750 μl aliquot of culture and then resuspending the pellet in an equal amount of fresh GTS medium. The resulting cell suspension was subsequently examined for luciferase activity, as described above.

DNA sequence analysis

Sequence analysis of DNA fragments carried in Bluescript vectors was performed using standard primers and primer walking strategies. The DNA sequencing was carried out at the DNA sequencing facility at Michigan State University. Initial DNA sequence analysis was carried out using the Sequencher software program (Gene Code Corporation, Ann Arbor, MI). ORFs were identified by analyzing the DNA sequence with a codon preference program based on codon usage for *S. meliloti* (C. Halling, University of Chicago, IL) and by examining the DNA sequence for start codons and Shine-Delgarno motifs at translational start sites indicated by the codon usage program. Putative promoter regions were identified by searching for characteristic motifs in the DNA sequence using the Predict Promoter for Prokaryotes program (32, 33, 34). Database searches were conducted through the NCBI Web page using the Gapped BLAST program (1).

RESULTS

S. meliloti strain C22 carries a Tn*5luxAB* insertion in a novel locus

The Tn*5luxAB* insertion mutant, C22, was isolated in a previous screen for *S. meliloti* strains carrying a *luxAB* reporter gene fusion induced under carbon deprivation conditions (26). The tagged locus was cloned by excision

from the genome as an *Eco*RI fragment (*Eco*RI does not cleave within Tn*5luxAB*), self-ligated and electroporated into *E. coli* (DH5α). The resulting plasmid (pC22) is self replicating due to the presence of an *oriV* within the Tn*5luxAB* transposon (45). DNA sequence analysis of the cloned Tn*5-luxAB* containing region (4.7 kb) revealed the presence of four open reading frames (see Fig. 2.1)

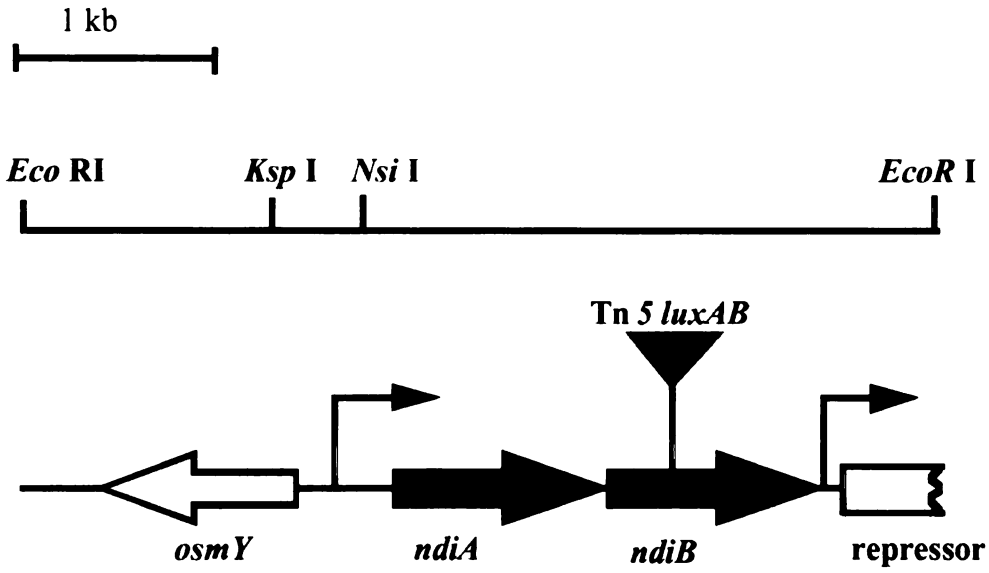


Figure 2.1 Map of the Tn*5luxAB* - tagged locus from strain C22. Insertion site of Tn*5luxAB* is indicated, along with selected restriction sites, ORFs, and putative promoter regions. The presumptive direction of transcription is indicated with arrows.

DNA motifs indicative of transcriptional terminators were not found between the Tn*5luxAB* tagged ORF and its neighboring ORF indicating that these two ORFs are likely to be part of one transcript. These two ORFs were designated *ndiA* and *ndiB* for nutrient deprivation induced genes A and B. The

Tn*5luxAB* insertion in strain C22 was found to be located near the middle of *ndiB*. Comparison of the deduced amino acid sequences of both *ndiA* and *ndiB* to the protein databases at NCBI failed to reveal significant similarity with any known genes. Therefore, they appear to be novel.

Two strategies were used to delimit the predicted promoter region of the *ndi* locus. First, plasmid pC22 was digested with *Eco*RI and the entire fragment was ligated to the broad host range vector pTB93 creating plasmid pC22lux. PC22lux was subsequently introduced into *S. meliloti* strain 1021. Induction of luciferase activity during oxygen, nitrogen, or carbon deprivation, osmotic stress, and stationary-phase was observed, indicating that the complete *ndi* promoter region is indeed contained within this fragment. In addition, a DNA sequence analysis program, Promoter Predictions for Prokaryotes (32, 33, 34) was used to examine the sequence for possible promoter regions. This program predicted a putative promoter region approximately 275 bp upstream of *ndiA* (see Fig. 2.1). In addition, the luciferase activity of plasmid pC22lux was examined in *E. coli*. In this host, the *ndi* locus displayed a constitutive expression pattern during growth in GTS medium, indicating that some promoter region is recognized in *E. coli* (see Fig. 2.2). However, it should be noted that it may not be the same promoter region that is recognized in *S. meliloti*. Furthermore, a decrease in expression was observed in *E. coli* under conditions that were inducing in *S. meliloti* (see Fig. 2.2). Therefore, the promoter region that is recognized in this heterologous host is not regulated by the same environmental signals.

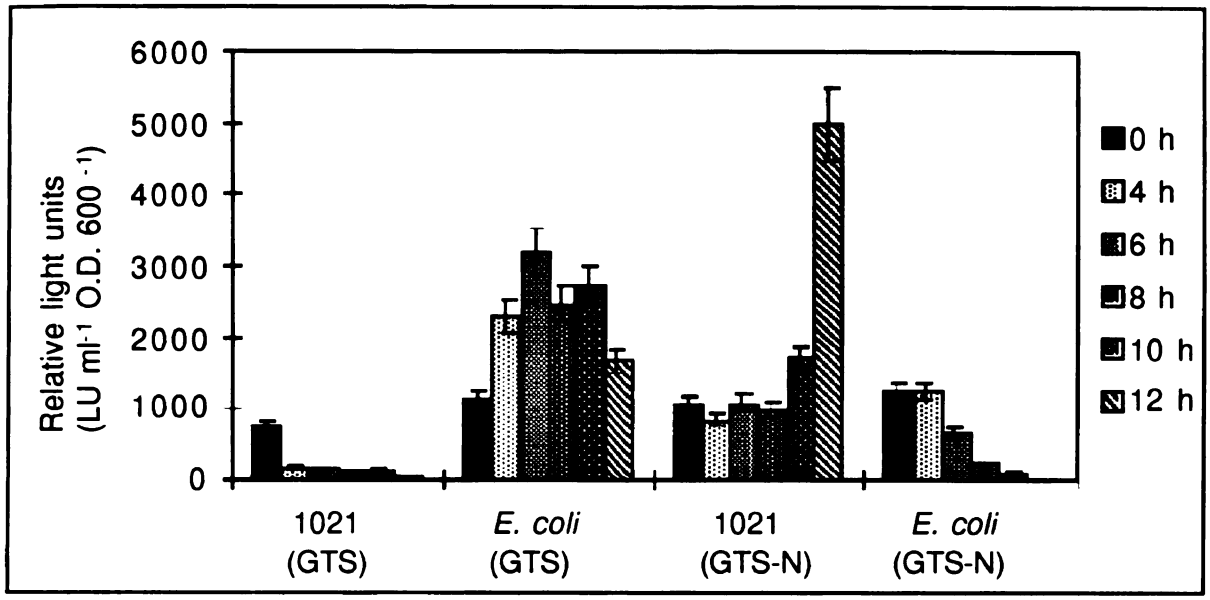


Figure 2.2. Plasmid pC22lux expression in wild-type and *E. coli* backgrounds after exposure to nitrogen deprivation for twelve hours

An ORF with a high degree of similarity (31% identity, 46% similarity) to various repressor proteins, such as PurR and LacI (37), was found adjacent and downstream of the *ndi* genes, along with a putative promoter region, as shown in Fig. 2.1. This putative promoter region was identified by the Predict Promoter Program (32, 33, 34). It is highly similar to the consensus (-35/ -10) promoter element of *E. coli* (12). In addition, there appears to be a divergent ORF at the 5' end of the *ndi* operon with significant similarity (41% identity, 60% similarity) to an osmotically induced periplasmic protein (*osmY*) from *E. coli* of unknown function (46). Interestingly, this gene has also been found to be induced by carbon starvation (15) and during stationary-phase (19), in addition to osmotic stress (46). It should be noted, however, that the similarity is restricted to the last 71 amino acids of the protein encoded by *osmY*. The first 130 amino acids of *osmY* do not show similarity to the ORF in the *ndi* region.

luxAB reporter gene expression in strain C22 is induced by O_2, N, and C deprivation, osmotic stress, as well as during stationary-phase

The expression of the chromosomal *ndiB*::Tn*5luxAB* fusion harbored by strain C22 was measured in Light Units (LU). The *ndi* promoter was found to be active at low levels throughout exponential growth and induced after one hour of carbon deprivation, two hours of nitrogen deprivation, two hours of osmotic stress, or four hours at reduced oxygen tension (see Figures 2.3, 2.4, 2.5, and 2.6). In addition, *luxAB* induction was observed in cells as they

entered stationary-phase in complex TY medium (see Fig. 2.7). This expression profile was also observed in cells grown in defined GTS medium (Data not shown). The observed expression pattern persisted for approximately 8 hours after entry into stationary-phase. Since luciferase activity is dependent on the energy status of the cell due to the requirement for reducing equivalents ($FMNH_2$) to catalyze the bioluminescent reaction, the persistence of expression during later stages of stationary phase was not evaluated.

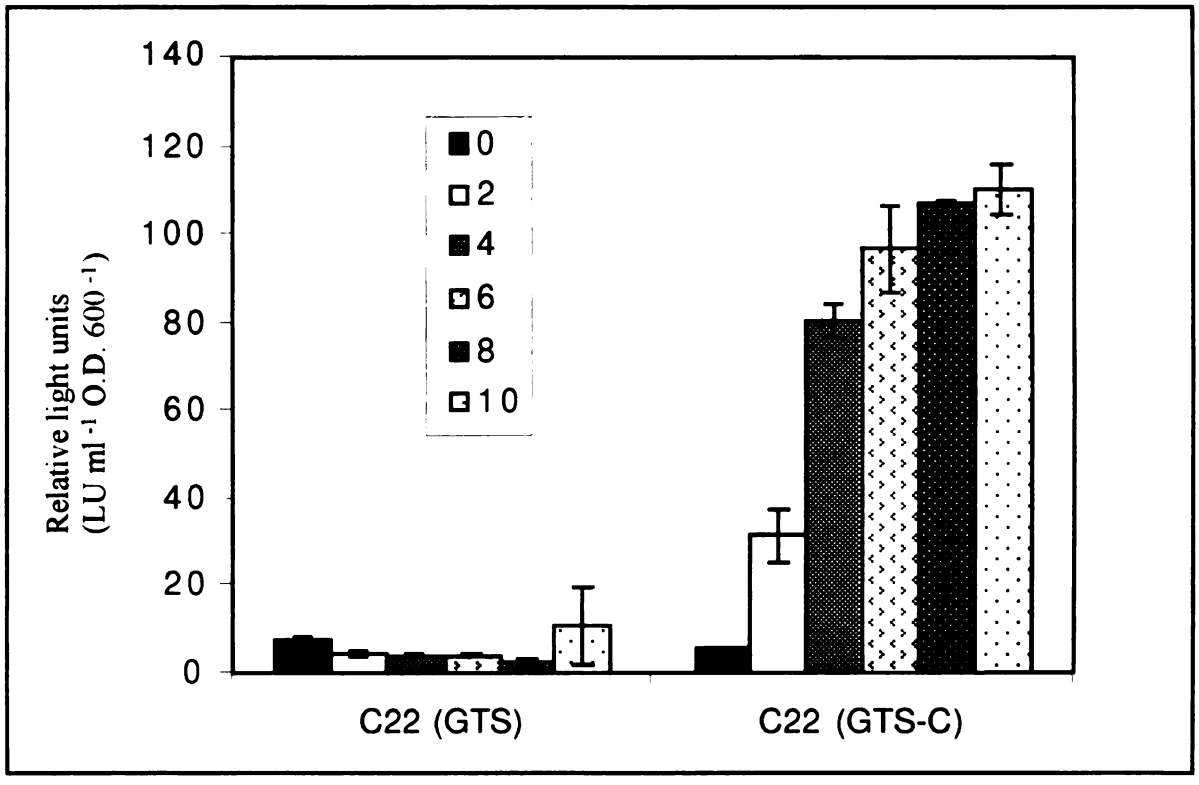


Figure 2. 3. *ndi*::Tn*5lux AB* expression during carbon deprivation. Luciferase activity was determined at two hour intervals for ten hours. Values of luminescence are defined as described in Materials and Methods.

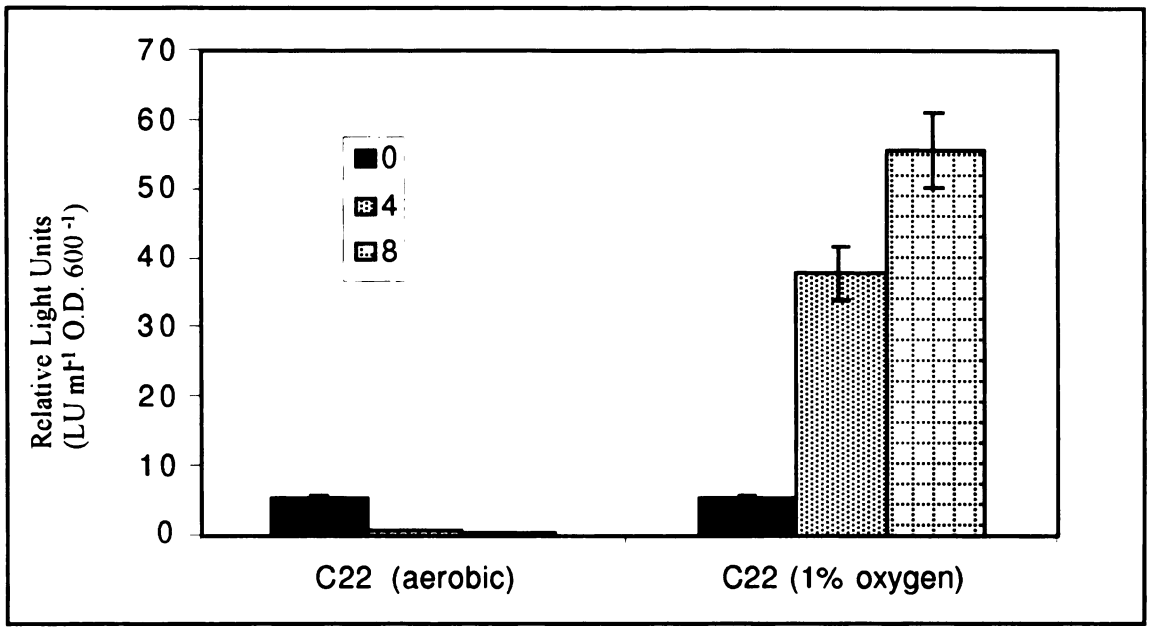


Figure 2.4. *ndi*::Tn*5luxAB* expression under low oxygen tension. Luciferase activity was determined after 4 and 8 hours of exposure to low oxygen tension.

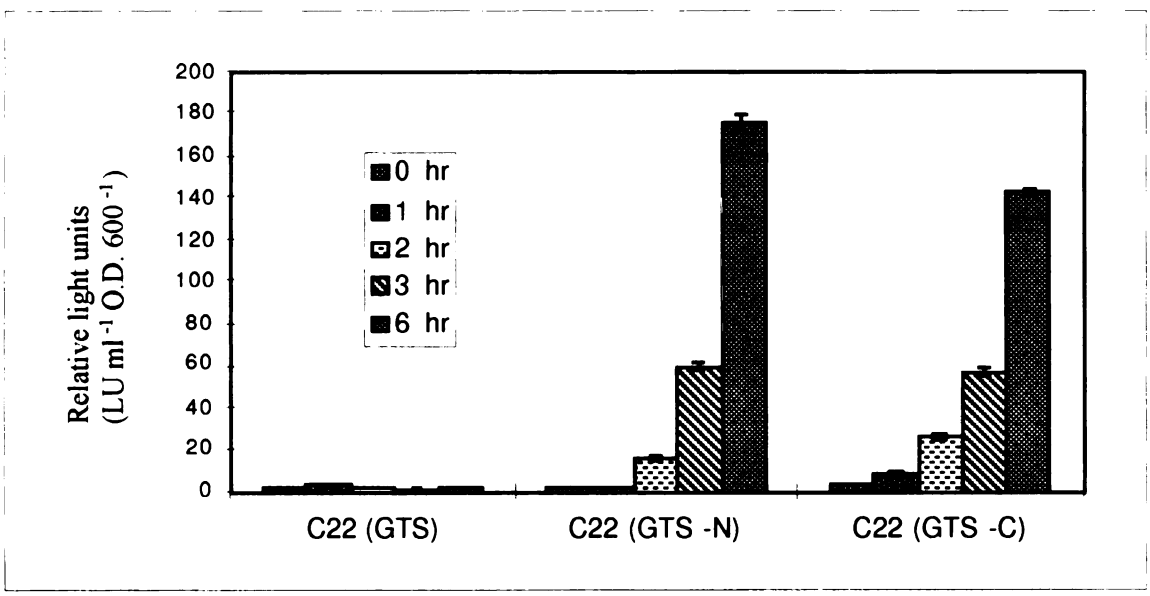


Figure 2.5. A comparison of *ndi* ::Tn*5luxAB* expression profiles during nitrogen and carbon deprivation . Luciferase activity was determined every hour for the first three hours, and then after six hours of exposure to nutrient deprivation.

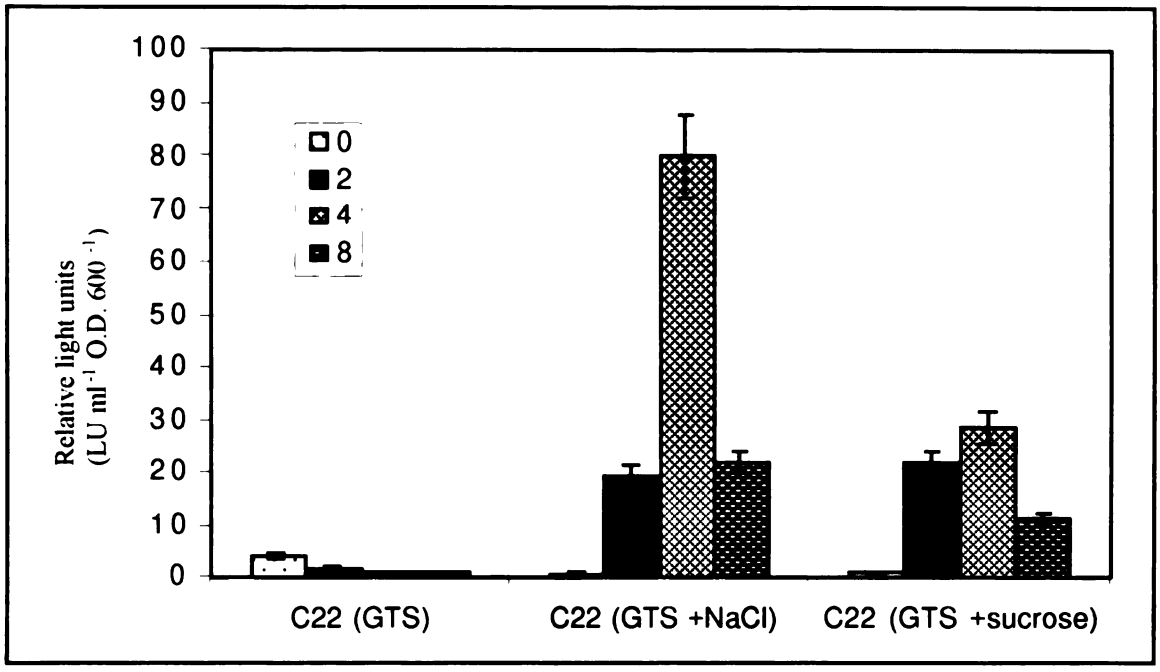


Figure 2.6. *ndi*::Tn*5luxAB* expression profile during osmotic stress. Luciferase activity was monitored over time for 8 hours. The osmolarity of GTS +sucrose (30% w/v) and GTS+NaCl (400mM) are comparable.

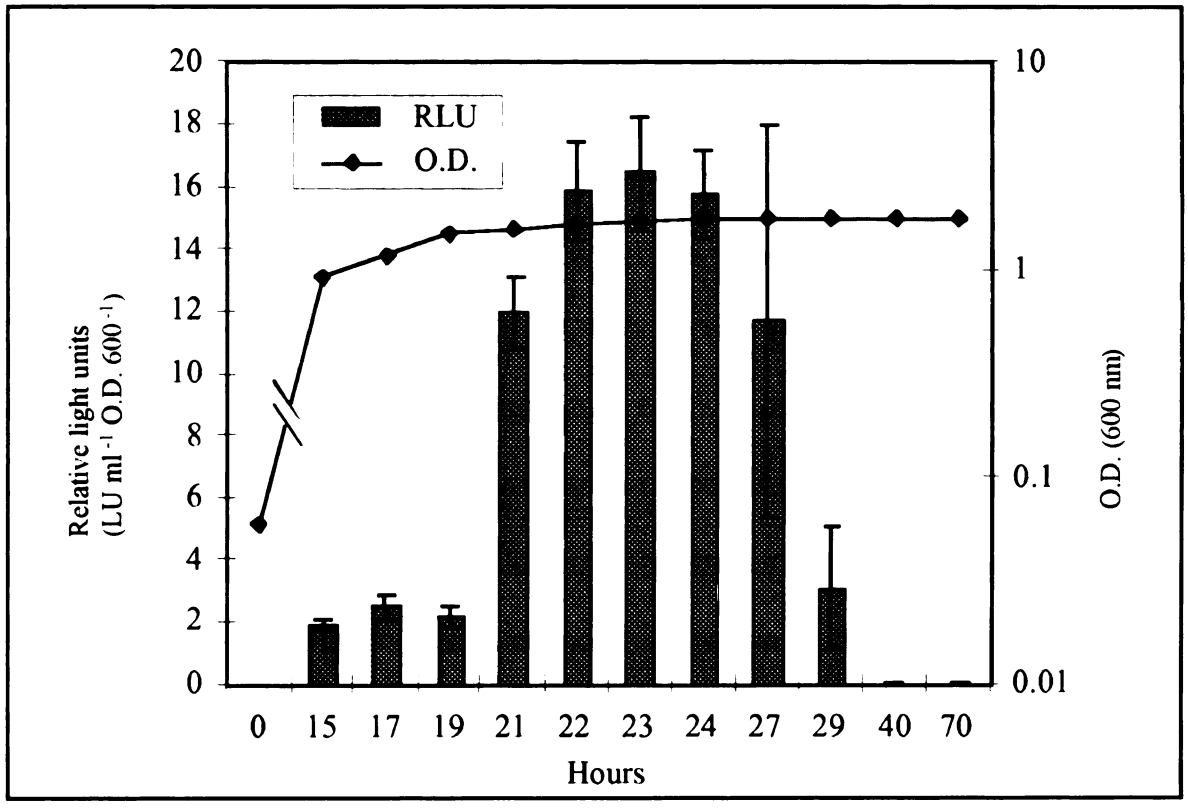


Figure 2.7. *ndi*::Tn*5lux AB* expression during stationary-phase in complex TY medium. 25ml cultures, in triplicate, were grown in a 125ml flasks with shaking at 28°C for 72 hours. Luciferase activity and absorbance were monitored at the specified intervals over time.

Phenotypic analysis of strain C22

Previously, it was found that mutant strain C22 was able to form nitrogen-fixation competent nodules (Nod+ Fix+) comparable to those formed by wild-type *S. meliloti* 1021 (31). In addition, it was found that strain C22 was significantly enhanced for nodule occupancy versus the wild-type strain when competed at a ratio of 1:10 (mutant to wild-type strain, respectively) (31). Growth experiments indicated that C22 has growth rates similar to that of the wild-type strain (see Fig. 2.8). In addition, survival experiments indicated that this mutant is not impaired in the ability to persist during carbon deprivation (see Fig. 2.9). Also, its carbon utilization pattern, as determined with Biolog plates, indicated that the mutant was not impaired in its ability to utilize the 95 substrates tested.

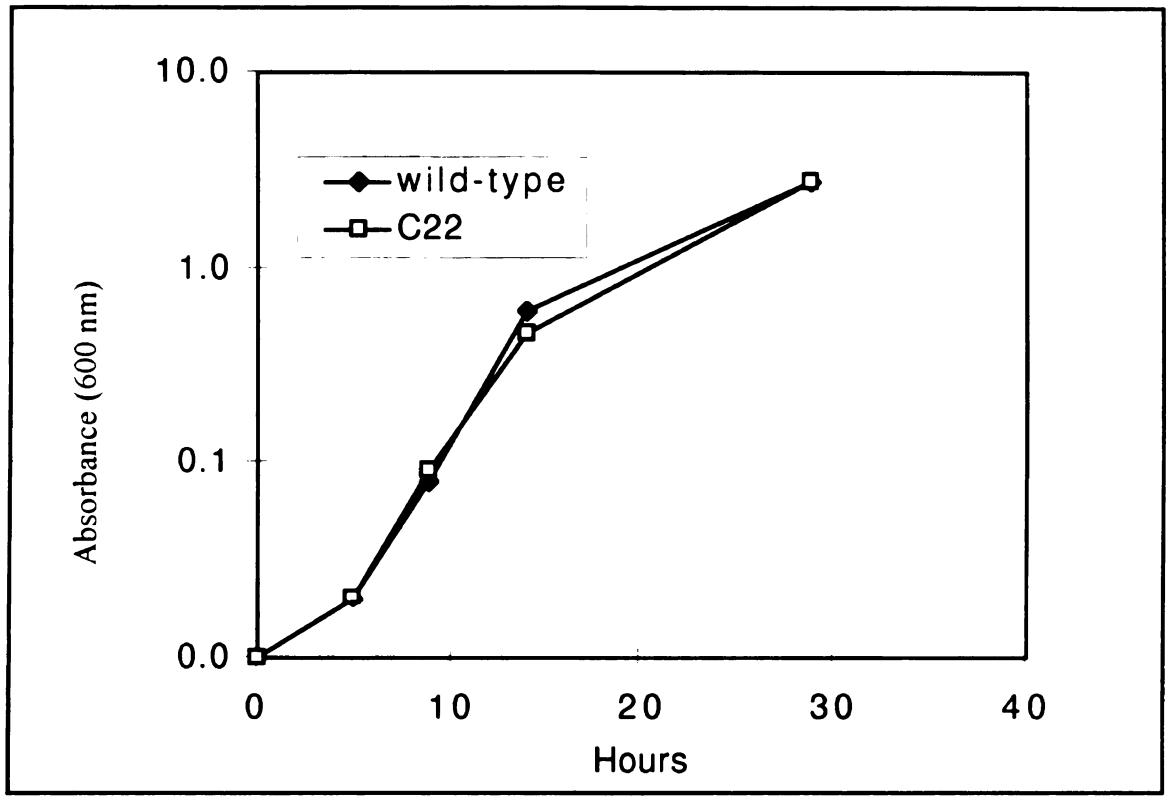


Figure 2.8. Growth of wild-type strain and mutant C22 in TY medium. 25 ml cultures, in triplicate, were grown in 125ml flasks with shaking at 28°C for 30 hours. Absorbance was monitored over time.

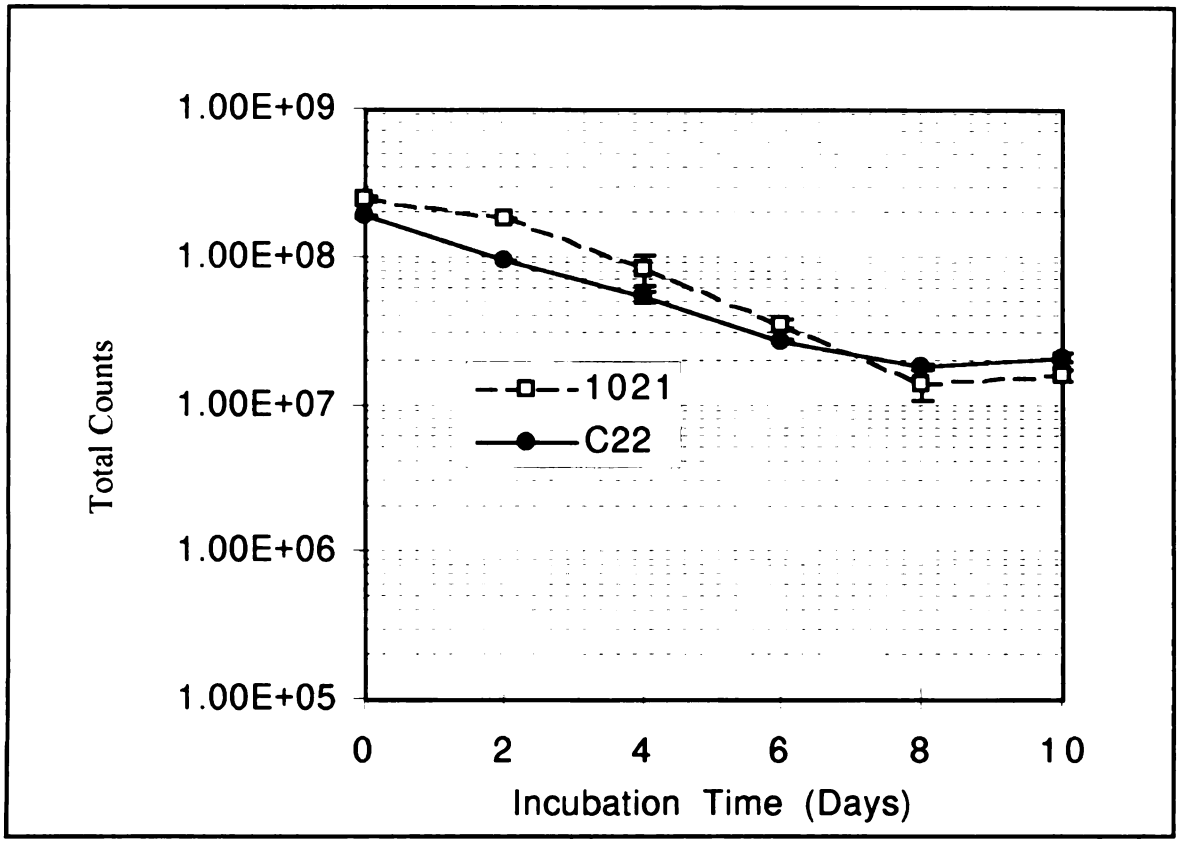


Figure 2.9. Survival during carbon deprivation. The strains were grown in GTS medium containing limiting amounts of carbon (0.05% glucose). 25ml culture were grown in 125ml flasks with shaking at 28°C for 10 days. Viable cells of each culture were determined every two days starting at day one after inoculation, by plating a dilution series on TY medium. The experiment was performed in triplicate.

DISCUSSION

We have been investigating gene expression during nutrient deprivation of S. *meliloti*, a bacterium indigenous to soil (25, 26). Here, we have described the characterization of a nutrient-deprivation-induced locus in *S. meliloti* strain 1021 that was identified by use of a Tn*5*-based transposon carrying *luxAB* as a reporter. This strain (C22) was previously reported as harboring a transcriptional fusion induced by both carbon and nitrogen deprivation (26). Further investigation revealed that this *luxAB* reporter gene fusion is also induced by oxygen limitation, osmotic stress, and during stationary-phase. In addition, DNA sequence analysis indicates that the tagged gene is part of an operon consisting of two ORFs, both of which appear to be novel. The two ORFs were designated *ndiA* and *ndiB* for nutrient deprivation induced genes A and B. The Tn*5luxAB* insertion is in *ndiB*, which is downstream of *ndiA*. Phenotypic analyses did not uncover a possible function of this gene. The mutant is not impaired in its ability to survive starvation. It is able to utilize similar carbon substrates for growth as the wild-type strain, and its growth rate is comparable to that of the wild-type strain.

The inability to assign a function to this nutrient-deprivation-induced *S. meliloti* locus is not unusual. For example, although the effect of nutrient-deprivation on *Pseudomonas putida,* a common soil isolate, has been investigated by other research groups (9, 10), and many proteins temporally

induced by carbon and nitrogen deprivation have been identified using 2-D-PAGE protein profiling, to date, few roles have been assigned to the array of proteins induced. In addition, transposon mutagenesis with Tn*5* derivatives carrying reporter genes has been used to generate collections of strains carrying gene fusions induced in stationary phase and under carbon or phosphate limiting conditions, in a variety of soil bacteria, such as *S. meliloti*, *P. putida* and *P. fluorescens* (17, 18, 42). Evidence for a general starvation response in *Rhizobium leguminosarum* similar to that found in *E. coli* and *Vibrio* sp. has been reported (41), and Uhde *et al*, (42) has identified *S. meliloti* mutants that are affected in stationary-phase survival. In addition, phosphate stress induced genes in *S. meliloti* (40), genes expressed during carbon or nitrogen deprivation (26), and starvation induced changes in chemotaxis, motility, and flagellation have also been reported (44). However, the exact function of most nutrient deprivation induced genes/proteins remains to be elucidated.

Since the *ndi* locus is controlled by a variety of environmental signals, experiments were performed to delimit the promoter region. The results of these studies indicate that the entire promoter region is located on the cloned fragment.

An ORF with a high degree of similarity to various repressor proteins was found adjacent and downstream to the *ndi* genes. This protein may be involved in regulating the *ndi* promoter, since expression analysis with plasmid pC22lux revealed extremely high background levels of luciferase activity

(approximately 200 fold higher than the chromosomal fusion) under non-inducing conditions (see Fig. 4.6 and 4.7) which may indicate a repressor protein being "titered-out" by the increased number of promoter templates available. However, further studies must be carried out to determine the possible involvement of this putative regulatory protein.

Many of the environmental stresses bacteria encounter in soil, such as low oxygen tensions, nitrogen and carbon limitation, as well as osmotic changes are also experienced by rhizobia during the development of a symbiotic association with their legume host plant. Indeed, many genes that encode for proteins that may potentially play a role in adaptation to the environment in the free-living state have been discovered through studies on their symbiotic phenotype (28). These proteins are involved in functions such as synthesis of respiratory complexes, synthesis of amino acids and other molecules, as well as carbon utilization. However, the precise role of these genes in adaptation to the environment in the free-living state needs to be further investigated, as well.

In summary, two primary findings are presented here. First, a promoter element has been identified that is regulated by a number of environmental signals; including carbon, nitrogen, and oxygen deprivation, as well as in response to osmotic shock, and during stationary-phase. Second, the two genes (*ndiA* and *ndiB*) that appear to be controlled by this promoter region do not show similarity to any previously characterized genes, therefore they appear to be novel.

The primary goal of our work has been the identification of genes in *S. meliloti* involved in regulating expression while it is persisting in the free-living state, in soil. Many of the environmental parameters that would be encountered in soil regulate the *ndi-luxAB* fusion described here. Therefore, strain C22, harboring the *ndi-luxAB* fusion, was selected for a second round of mutagenesis in order to identify such regulatory genes. The isolation and characterization of these loci will be discussed in the next two chapters.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zheng, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.

2. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188-198.

3. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol. **41:**459-472.

4. **Broughton, W. J., and M. J. Dilworth.** 1971. Control of leghemoglobin synthesis in snake beans. Biochem J. **125:**1075-1080.

5. **de Bruijn, F. J., and S. Rossbach.** 1994. Transposon mutagenesis, p. 387-405. *In* P. Gerhardt (ed.), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, D.C.

6. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. **77:**7347-7351.

7. **Friedman, A. M., S. R. Long, S. E. Brown, W. I. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene. **18:**289-296.

8. **Gage, D. J., T. Bobo, and S. R. Long.** 1996. Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J. Bacteriol. **178:**7159-7166.

9. **Givskov, M., L. Eberl, and S. Molin.** 1994. Reponse to nutrient starvation in *Pseudomonas putida* KT2442: two-dimensional electrophoretic analysis of starvation- and stress-induced proteins. J. of Bacteriol. **176:**4816-4824.

10. **Givskov, M., I. Eberl, S. Moller, L. K. Poulsen, and S. Molin.** 1994. Response to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol. **176**(1)**:**7-14.

11. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

12. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter. Nucl Acids Res. **15:**2343-2361.

13. **Hastings, J. W., and G. Weber.** 1963. Total quantum flux of isotropic sources. J Opt Soc. **53:**1410-1415.

14. **Hecker, M., and W. s. a. U. Volker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol Microbiol. **19**(3)**:**417-428.

15. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell. **72:**165-168.

16. **Kaiser, D.** 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. Annu Rev Genet. **20:**539-566.

17. **Kim, Y., L. S. Watrud, and A. Matin.** 1995. a carbon starvation survival gene of *Pseudomonas putida* is regulated by σ^{54}. J Bacteriol. **177:**1850-1859.

18. **Kragelund, L., B. Christoffersen, O. Nybroe, and F. J. de Bruijn.** 1995. Isolation of *lux* reporter gene fusions in *Pseudomonas fluorescens* DF57 inducible by nitrogen or phosphorous starvation. FEMS Microbiol Ecol. **17:**95-106.

19. **Lange, R., M. Barth, and R. Hengge-Aronis.** 1993. Complex transcriptional control of σ^S-dependent stationary-phase-induced and osmotically regulated *osmY* (*csi*-5) gene suggests novel roles for LRP, Cyclic AMP (cAMP) receptor protein-cAMP complex, and integration host factor in stationary-phase response of *Escherichia coli*. J. Bacteriol. **175**(24):7910-7917.

20. **Loewen, P. C., B. Hu, J. Strutinsky, and R. Sparling.** 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. **44**:707-717.

21. **Long, S. R.** 1996. Rhizobium symbiosis:nod factors in perspective. Plant Cell. **8**(10):1885-1896.

22. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Ann Rev Microbiol. **43**:293-316.

23. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutant of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**:114-122.

24. **Meighen, E. A., and P. V. Dunlap.** 1993. Physiological, biochemical and genetic control of bacterial bioluminescence. Adv Microb Phys. **34**:1-67.

25. **Milcamps, A., and F. J. deBruijn.** 1999. Identification of a novel, nutrient deprivation induced gene (*hmgA*) in *Sinorhizobium meliloti*, involved in Tyrosine degradation. Microbiology. **145**:935-947.

26. **Milcamps, A., D. M. Ragatz, P. Lim, K. A. Berger, and F. J. de Bruijn.** 1998. Isolation of carbon- and nitrogen-deprivation-induced loci of *Sinorhizobium meliloti* 1021 by Tn*5-luxAB* mutagenesis. Microbiology. **144**:3205-3218.

27. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1-23. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum press, New York, N.Y.

28. **Niner, B. M., and A. M. Hirsch.** 1998. How many *Rhizobium* genes, in addition to *nod*, *nif*/*fix*, and *exo*, are needed for nodule development and function? Symbiosis. **24**:51-102.

29. **Ostling, J., L. Holmquist, L. Flardh, B. Svenblad, A. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation and recovery of *Vibrio*, p. 103-123. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum, New York.

30. **Paul, E. A., and F. E. Clark.** 1989. Soil microbiology and biochemistry. Academic Press, San Diego, CA.

31. **Ragatz, D. M.** 1997. Carbon, nitrogen, and oxygen limitation-induced loci of *Rhizobium meliloti* isolated by Tn*5-luxAB* mutagenesis and their role in competition and survival. Diploma thesis. Michigan State University, East Lansing.

32. **Reese, M. G.** 1994. Diploma thesis. German Cancer Research Center, Heidelberg.

33. **Reese, M. G., and F. H. Eeckman.** 1995. "Novel neural network algorithms for improved eukaryotic promoter site recognition" Presented at The Seventh International Genome Sequencing and Analysis Conference, Hilton Head Island, South Carolina.

34. **Reese, M. G., N. L. Harris, and F. H. Eeckman.** 1996. "Large scale sequencing specific neural networks for promoter and splice site recognition" Presented at the Biocomputing: Proceedings of the 1996 Pacific Symposium.

35. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol Rev. **51**(3):365-379.

36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, New York.

37. **Schumacher, M. A., K. Y. Choi, H. Zalkin, and R. G. Brennan.** 1994. Crystal structure of the LacI member, PurR, bound to DNA: minor groove binding by α helices. Science. **266:**763-770.

38. **Spaink, H. P., A. Kondorosi, and P. J. J. Hooykaas.** 1998. The Rhizobiaceae. Kluwer Academic Press.

39. **Spector, M. P.** 1998. The starvation-stress response (SSR) of *Salmonella*, p. 233-279, Advances in Microbial Physiology, vol. 40. Academic Press.

40. **Summers, M. L., J. G. Elkins, B. A. Elliot, and T. R. McDermott.** 1998. Expression and regulation of phosphate stress inducible genes in *Sinorhizobium meliloti*. MPMI. **11**(11):1094-1101.

41. **Thorne, S. H., and H. D. Williams.** 1997. Adaptation to nutrient starvation in *Rhizobium Leguminosarum* bv. phaseoli: analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. J. Bacteriol. **179**(22):6894-6901.

42. **Uhde, C., R. Schmidt, D. Jording, W. Selbitschka, and A. Puhler.** 1997. Stationary-phase mutants of *Sinorhizobium meliloti* are impaired in stationary-phase survival or in recovery to logarithmic growth. J. Bacteriol. **179**(20):6432-6440.

43. **van Rhijn, P., and J. Vanderleyden.** 1995. The *Rhizobium*-plant symbiosis. Microbiol Rev. **59**(1):124-142.

44. **Wei, X., and W. D. Bauer.** 1998. Starvation-induced changes in motility, chemotaxis and flagellation of *Rhizobium meliloti*. Appl Env Microbiol. **64:**1708-1714.

45. **Wolk, C. P., Y. Cai, and J.-M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc Natl Acad Sci USA. **88:**5355-5359.

46. **Yim, H. H., and M. Villarejo.** 1992. *osmY*, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. J Bacteriol. **174**(11):3637-3644.

CHAPTER 3

Identification and Characterization of two *S. meliloti* Genes Tagged with Tn*1721*

Identification and Characterization of two *S. meliloti* Genes Tagged with Tn*1721*

ABSTRACT

A nutrient-deprivation-induced locus in *S. meliloti* strain 1021 was previously identified using a Tn*5*-based transposon carrying the *luxAB* genes as a reporter. The tagged gene in this strain (C22) was found to be part of an operon consisting of two open reading frames (ORFs). The two ORFs were designated *ndiA* and *ndiB* for nutrient deprivation induced genes A and B. Comparison of the deduced amino acid sequences of both *ndiA* and *ndiB* to the protein databases failed to reveal similarity with known genes. The expression of this locus was found to be induced by carbon and nitrogen deprivation, osmotic stress, oxygen limitation, and during stationary phase. To identify potential regulatory components involved in the induction of this locus, a second round of mutagenesis with a Tn*3* derivative, Tn*1721*, was performed on the primary mutant strain C22. Three double mutant strains were obtained displaying an altered *ndi*::Tn*5luxAB* expression pattern. Two strains showed a constitutive pattern of expression of the *ndi* locus, and one lacked transcriptional activity of the *ndi* locus under any of the inducing conditions examined. The latter will be described in Chapter 4. One of the double mutants displaying a constitutive luxAB reporter gene expression profile was found to

have an insertion in a gene with high similarity to an outer membrane protein from *E. coli* of unknown function. The other double mutant displaying a constitutive *luxAB* reporter gene expression profile, was shown to carry an insertion in a gene with significant similarity to a number of pyridine nucleotide-disulfide oxidoreductases from a variety of bacteria. Here, I will describe the isolation and partial characterization of these two loci.

At this time, there is no direct evidence that the constitutive *luxAB* expression pattern observed in the two double mutant strains is caused by Tn*1721* insertions in the two loci mentioned above. It is possible that a Tn*1721*-independent secondary mutation is responsible for the observed deregulation phenotype. Reconstruction of the Tn*1721* insertion mutations, creation of a non-polar insertion mutation in the two loci, and/or complementation experiments would have to be performed to provide evidence for the involvement of either locus in the regulation of *ndi*::Tn*5luxAB* expression. Therefore, the results presented in this chapter should be considered preliminary and incomplete.

INTRODUCTION

In nature, bacterial growth is restricted by a wide variety of environmental factors. One factor of particular importance is the lack of essential nutrients. In most natural settings, such as the soil, at least some essential nutrients are limiting, therefore periods of negligible growth or dormancy are the more typical physiological state of bacteria (21, 25). Understanding how bacteria are able to monitor, sense, and respond to their environment in this starved state is fundamental to our understanding of microbial biology and ecology. Some bacteria, such as *Bacillus* and *Myxococcus* spp., sporulate when starved (11, 12). However, the majority of bacteria do not appear to differentiate morphologically into these stress resistant forms. Research on *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio* spp., has shown that under starvation conditions, these non-sporulating bacteria do enter into a specific genetic program which results in a survival state. The ultimate purpose of this process is survival until conditions improve (13, 16, 23, 29).

I have been investigating environmental control of gene expression in the common soil bacterium, *S. meliloti*. This bacterium establishes a symbiosis with the legume, alfalfa, during which a new specialized organ is formed, the nitrogen-fixing root nodule. Nodules provide the proper physiological environs for the bacteria to survive in the absence of competing microbiota, and to reduce atmospheric dinitrogen to ammonia, that is

assimilated by the plant for growth (28). The role of rhizobia as symbionts has been studied extensively. However, little is known regarding the ability of these bacteria to persist in their free-living state, in the soil and rhizosphere. In fact, research on the starvation survival of bacteria whose natural habitat is soil, such as *Pseudomonas* or *Rhizobia* spp., has only just begun (see discussion). It is likely that these bacteria, which have evolved in this habitat, have distinct mechanisms for surviving in this environment.

Previously, the isolation of 33 *S. meliloti* strains with Tn*5luxAB* gene fusions induced by carbon or nitrogen deprivation, or both, was reported (20). In Chapter 2, the characterization of one of these strains (C22) that harbors a transcriptional fusion induced by oxygen, nitrogen, or carbon deprivation, osmotic stress, and stationary phase is discussed. This locus has been designated *ndi* for nutrient deprivation induced. In this chapter, I describe the findings of a second round of mutagenesis on mutant strain C22 designed to identify genes potentially involved in controlling the expression from the *ndi* locus. I will focus here on the description of two double mutant strains that display constitutive *ndi*::Tn*5luxAB* expression pattern. One of these double mutants was found to be carrying a Tn*1721* insertion in a gene with high similarity to an outer membrane protein of unknown function from *E. coli*. The other was shown to carry a Tn*1721* insertion in a gene with significant similarity to a number of pyridine nucleotide-disulfide oxidoreductases. The partial characterization of these loci will be described here. Since it has not yet been shown that the Tn*1721* insertions actually cause the observed phenotype (see

also Abstract), no conclusions can be made about the potential role of these two Tn*1721*-tagged loci in the regulation of *ndi*::Tn*5luxAB* expression.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

Strain *S. meliloti* 1021 (Sm^R) has been described by Meade *et al.,* (1982). *E. coli* DH5α by Hanahan (1983), and *E. coli* HB101 by Boyer & Roulland-Dussoix (1969). Double mutant strains 1,F-1 and 10,D-2 are C22 derivatives with a Tn*1721* insertion. Strain C22 is a derivative of strain 1021 carrying a Tn*5-1063a* insertion (*ndi*::Tn*51063*a; Km). Plasmid pRK2013 has been described by Ditta *et al.,* (1980), plasmid pACYC177 by Chang & Cohen, (1978) and plasmid pJOE105 by Schöffl *et al.*, (1981). pBluescript II SK (Ap^R) was obtained from Stratagene. pLAFR1 (IncP, Mob^+, Tra^-, Tc^r) has been described by Friedman *et al.*, (1982).

S. meliloti strains were grown on or in rich medium (TY; Beringer, 1974) or minimal medium at 28°C, as indicated. The minimal medium used was GTS (20). *E. coli* strains were grown on or in rich medium (Luria-Bertani; LB) at 37°C, Antibiotics were added at the following concentrations: (i) *S. meliloti*: streptomycin (Sm) 250 μg ml^{-1}; kanamycin (Km), 200 μg ml^{-1}; tetracycline (Tc) 10 μg ml^{-1}; spectinomycin (Sp) 50 μg ml^{-1}; (ii) *E. coli*: ampicillin (Ap), 100 μg ml^-

^{1}; (Km) 50 μg ml^{-1}; (Sp) 100 μg ml^{-1}; (Tc) 5 μg ml^{-1}. GTS-N is GTS medium lacking a combined nitrogen source. GTS-C is GTS medium lacking a carbon source.

Survival during stationary phase

Strains 1021 and 10,D-2 were grown in GTS medium (a 25ml culture in a 125 ml flask) with shaking at 28°C for 18 days. Viable cells of each culture were determined at regular intervals by plating a dilution series on TY medium. The data were recorded as colony forming units per ml (CFU/ml). The experiment was performed in triplicate.

Nodulation experiments

A preliminary screen of *S. meliloti* mutant strains 1,F-1 and 10,D-2 and was performed to examine their symbiotic phenotype by inoculation of rhizobial cultures on alfalfa (*Medicago sativa*) seedling roots. Alfalfa seeds were sterilized by soaking for 5 minutes in 70% ethanol, followed by 5 minutes in (bleach 5%), and rinsed thoroughly with sterile distilled water. The seeds were placed on sterile Whatman filter paper, in test tubes containing 20 ml of sterile nitrogen-free B+D liquid medium (4). Saturated cultures of rhizobial strains were diluted with sterile H_2O (1:5) and 1.0 ml aliquots were added to glass test tubes containing one week old alfalfa seedlings. Six plants were tested with

each strain. Inoculated plants were grown for 6 to 7 weeks in a growth chamber (16 hours light, 28°C) and examined for the presence or absence of nodules (Nod phenotype), as well as for a chlorotic plant growth phenotype, indicating defects in symbiotic nitrogen fixation (Fix phenotype).

DNA isolation and manipulation.

Plasmid DNA for restriction analysis and DNA sequencing was prepared using a Magic-mini kit from Promega Madison, WI. Chromosomal DNA was isolated from *S. meliloti* strains according to de Bruijn *et al.* (1994). All enzymes for DNA manipulations were purchased from Boehringer Mannheim or New England Biolabs and used as specified by manufacturers. Restriction enzyme digests and ligations were carried out as described by Sambrook *et al.* (1989). Probes were labeled with [α^{32} P]dATP using a random primer kit (Boehringer Mannheim) following the manufacturer's instructions. Plasmids were introduced into *E. coli* hosts by electroporation and into *S. meliloti* strains via triparental conjugation (7).

The Tn*1721* tagged loci from the double mutants 1,F-1 and 10,D-2 were isolated by ligating a mixed population of genomic DNA of fragment sizes between 12.0 and 15.0 kb, restricted with *Hin*dIII, to plasmid pACYC177. This fragment size population was chosen based on information obtained from Southern blot analysis using plasmid pJOE105 as a probe. Restriction mapping was used to select the clones containing the 5' end of Tn*1721*, plus

the flanking regions. The plasmids were designated pACYC.1F1 and pACYC.10D2, respectively. Finally, fragment sizes of 3.0 to 4.0 kb restricted with *Eco*RI and *Hin*dIII were inserted into pBluescript II KS for sequencing. A reverse primer was designed to the 5' end of Tn*1721* (5'TCAGGAAGTCAGGGCTATCA 3') and was used to obtain DNA sequence data from the site of insertion.

Genetic techniques

A protocol for Tn*1721* mutagenesis of *S. meliloti* was developed in our laboratory by Anne Milcamps. To perform the mutagenesis, *E. coli* strain DH5α, carrying plasmid pJOE105 (Tc) or pRK2013 (Km), and the *S. meliloti* insertion mutant, C22 (recipient), were grown in LB and TY, respectively. The cells were washed twice with TY to remove antibiotics. Both donor and helper strains (100 μl) were mixed with 400 μl of recipient cells. This suspension was sedimented in a microcentrifuge, the supernatant removed, and the cells were resuspended in 50μl of TY, and spotted on TY plates. After one day at 28°C, the mating mixtures were resuspended in sterile distilled water and plated on selective plates of GTS medium containing Sm (250 μg ml^{-1}), Km (200 μg ml^{-1}), and Tc (10 μg ml^{-1}). Three thousand transposon mutants were isolated, single colonies were purified, grown in liquid TY medium, and stored in microtiter plates at $^{-}$80°C. The resulting double mutant strains were screened for luciferase activity under inducing conditions using a photonic camera, as described by Milcamps *et al.* (1998).

DNA sequence analysis.

Sequence analysis of DNA fragments was carried out as described in Chapter 2.

RESULTS

Secondary mutations which render *ndi-luxAB* constitutive

The Tn*5luxAB* insertion mutant, C22, was identified in a previous screen for *S. meliloti* strains carrying a *luxAB* reporter gene fusion induced under carbon deprivation conditions (20). *S. meliloti* strain C22 carries a Tn*5luxAB* insertion in a novel locus that is induced by oxygen, nitrogen, and carbon deprivation, osmotic stress, and stationary-phase. The characterization of this locus is described in Chapter 2.

To identify genes that could be involved in the regulation of the *ndi* locus, strain C22 was mutagenized with a second transposon (Tn*1721*), as described in the Materials and Methods, and a library of 3000 double mutants was screened for altered patterns of *ndi*::Tn*5luxAB* expression (see Figure 3.1).

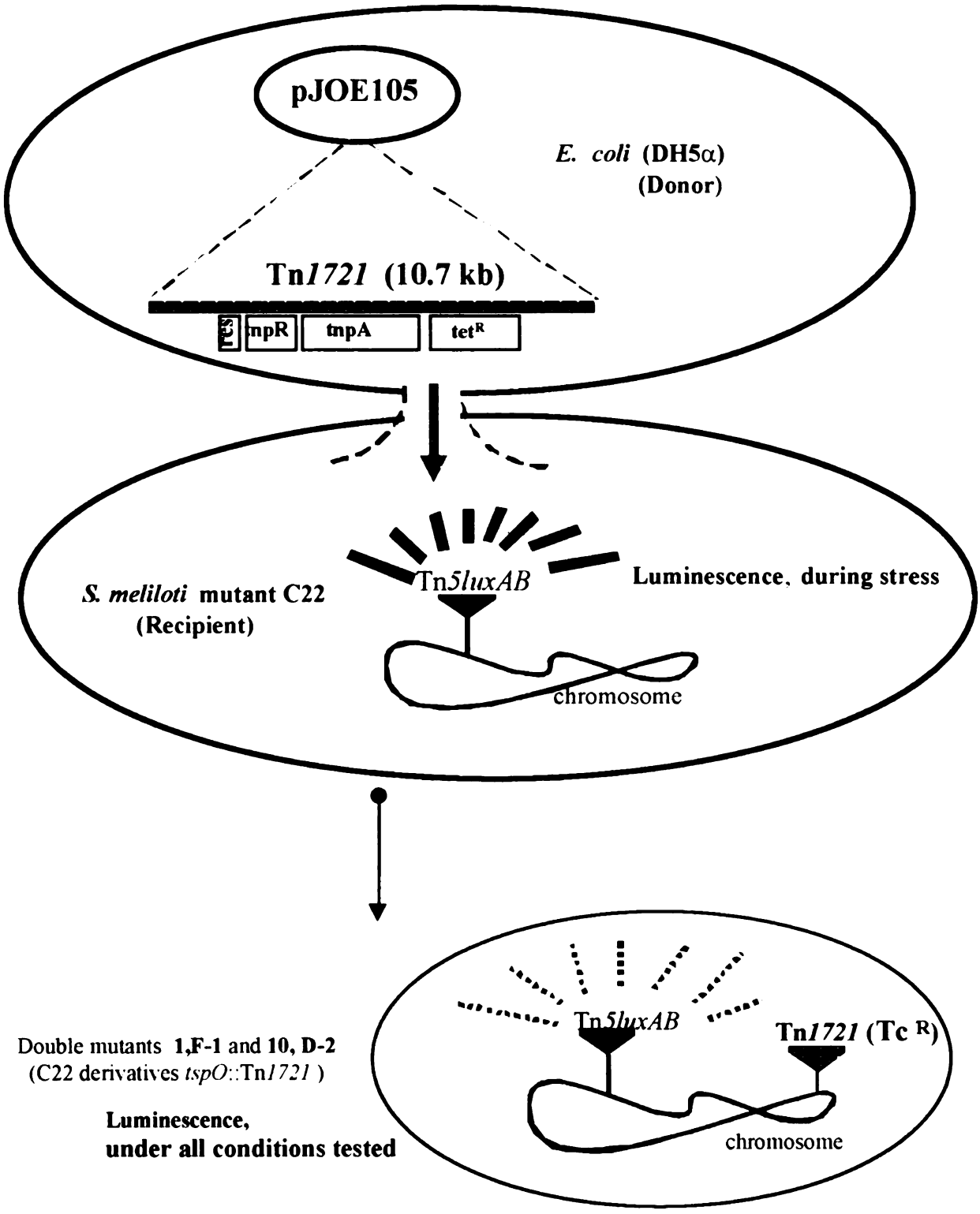


Figure 3.1. Secondary mutagenesis of mutant strain C22 (*ndi*::Tn*1721*) with Transposon Tn*1721*. 1, F-1 and 10,D-2 are double mutant strains that display a constitutive *luxAB* reporter gene expression pattern. See text for details.

Two mutants strains (1,F-1 and 10,D-2) displaying a constitutive *luxAB* reporter gene expression profile were identified, and Southern blot analyses of genomic DNA of strains 1,F-1 and 10,D-2 confirmed that these strains contained single Tn*1721* insertions in two distinct sites. The corresponding Tn*1721* tagged loci of these two strains were cloned as described in Materials and Methods. The site of insertion in strain 1,F-1 was found to be near the 3′end of a gene with a high degree of similarity (52% identity) to a hypothetical protein from *E. coli* of unknown function (see Figure 3.2). In addition, an ORF was identified 130 bp downstream of the tagged gene (data not shown) with a high degree of similarity (45% identity) to genes encoding penicillin–binding proteins from a variety of bacteria. These are high molecular weight proteins (approximately 850 amino acids in length), which appear to have two functions. They are involved in the biosynthesis of peptidoglycan of the cell wall, and they have the ability to bind penicillin (15).

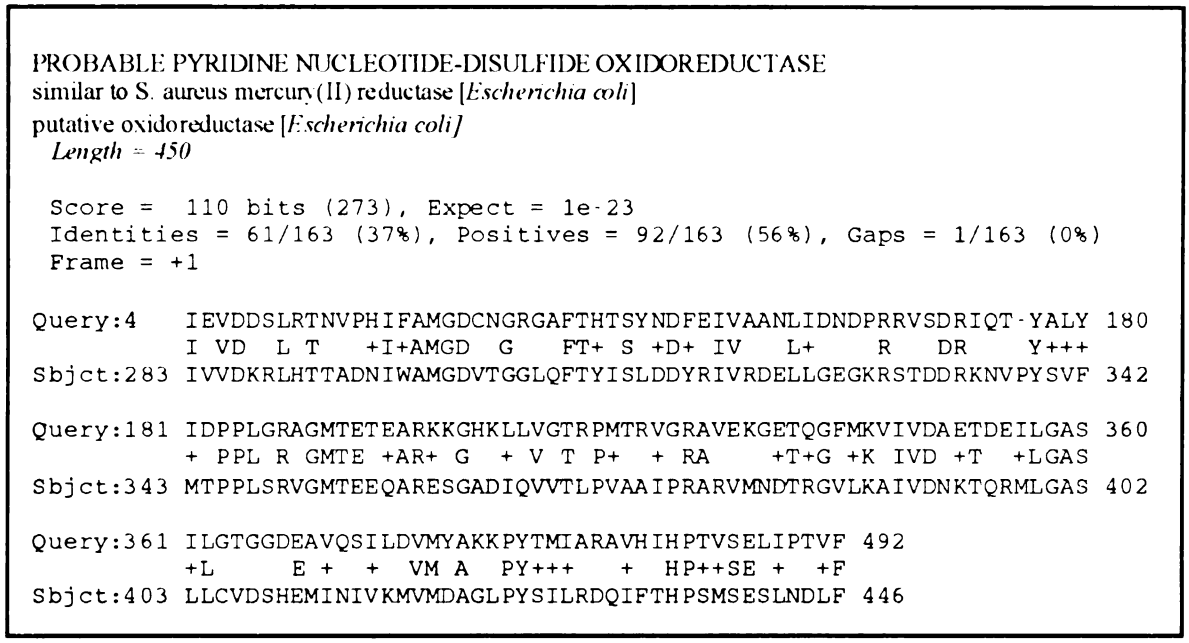
hypothetical protein [Escherichia coli]
Length = 1653

Score = 54.7 bits (129), Expect = 4e-07
Identities = 29/55 (52%), Positives = 35/55 (62%)
Frame = +1

```
Query:1      EFRSDRFVAAFDRSTGDNREITLAYVVRAVTPGTYDHPAANVEDMYRPQFSARTA 165
             EFR DRFVAA   +  + + +TL Y+ RAVTPGTY  P   VE MY PQ+ A  A
Sbjct:1592   EFRDDRFVAAV--AVDEYQPVTLVYLARAVTPGTYQVPQPMVESMYVPQWRATGA 1644
```

Figure 3. 2. Advanced BLAST results of the Tn*1721* tagged gene in mutant strain 1,F-1.

The site of insertion in strain 10,D-2 was found to be in a gene with a high degree of similarity (35-38% identity) to a variety of pyridine nucleotide-disulfide oxidoreductases. An example of one such alignment is shown in Figure 3.3. This class of reductases catalyzes disulfide bond formation and is primarily localized in the periplasmic space (3, 24).



PROBABLE PYRIDINE NUCLEOTIDE-DISULFIDE OXIDOREDUCTASE
similar to S. aureus mercury(II) reductase [*Escherichia coli*]
putative oxidoreductase [*Escherichia coli*]
Length = 450

```
 Score =  110 bits (273), Expect = 1e-23
 Identities = 61/163 (37%), Positives = 92/163 (56%), Gaps = 1/163 (0%)
 Frame = +1

Query:4   IEVDDSLRTNVPHIFAMGDCNGRGAFTHTSYNDFEIVAANLIDNDPRRVSDRIQT-YALY 180
          I VD  L T   +I+AMGD  G   FT+ S +D+ IV   L+    R   DR    Y+++
Sbjct:283 IVVDKRLHTTADNIWAMGDVTGGLQFTYISLDDYRIVRDELLGEGKRSTDDRKNVPYSVF 342

Query:181 IDPPLGRAGMTETEARKKGHKLLVGTRPMTRVGRAVEKGETQGFMKVIVDAETDEILGAS 360
          + PPL R GMTE +AR+ G  + V T P+  + RA    +T+G +K IVD +T  +LGAS
Sbjct:343 MTPPLSRVGMTEEQARESGADIQVVTLPVAAIPRARVMNDTRGVLKAIVDNKTQRMLGAS 402

Query:361 ILGTGGDEAVQSILDVMYAKKPYTMIARAVHIHPTVSELIPTVF 492
          +L     E +  +  VM A  PY+++   +  HP++SE +  +F
Sbjct:403 LLCVDSHEMINIVKMVMDAGLPYSILRDQIFTHPSMSESLNDLF 446
```

Figure 3.3. Advanced BLAST results of Tn*1721* tagged gene in mutant strain 10,D-2.

Phenotypic analysis of strains 10,D-2 and 1,F-1

Double-mutant strain 10, D-2 produced mucoid colonies on agar plates. Survival experiments indicated that this mutant is not impaired in the ability to persist during stationary phase (Figure 4). Both strains 10,D-2 and 1,F-1 were screened for their symbiotic phenotype by inoculation on alfalfa (*Medicago sativa*). Six alfalfa seedlings grown on sterile Whatman filter paper, in test tubes were inoculated with wild-type or mutant strains. Six additional plants were used as uninoculated controls. The experiment was performed twice. Both wild-type and mutant strains 10,D-2 or 1,F-1 infected plants were found to be nodulated and green, while the control (uninfected) plants were not nodulated and chlorotic after seven weeks. Therefore, the mutated locus in strain 10, D-2 does not appear to be required for survival during stationary phase, and neither gene was found to be absolutely required for the formation and function of the symbiotic association.

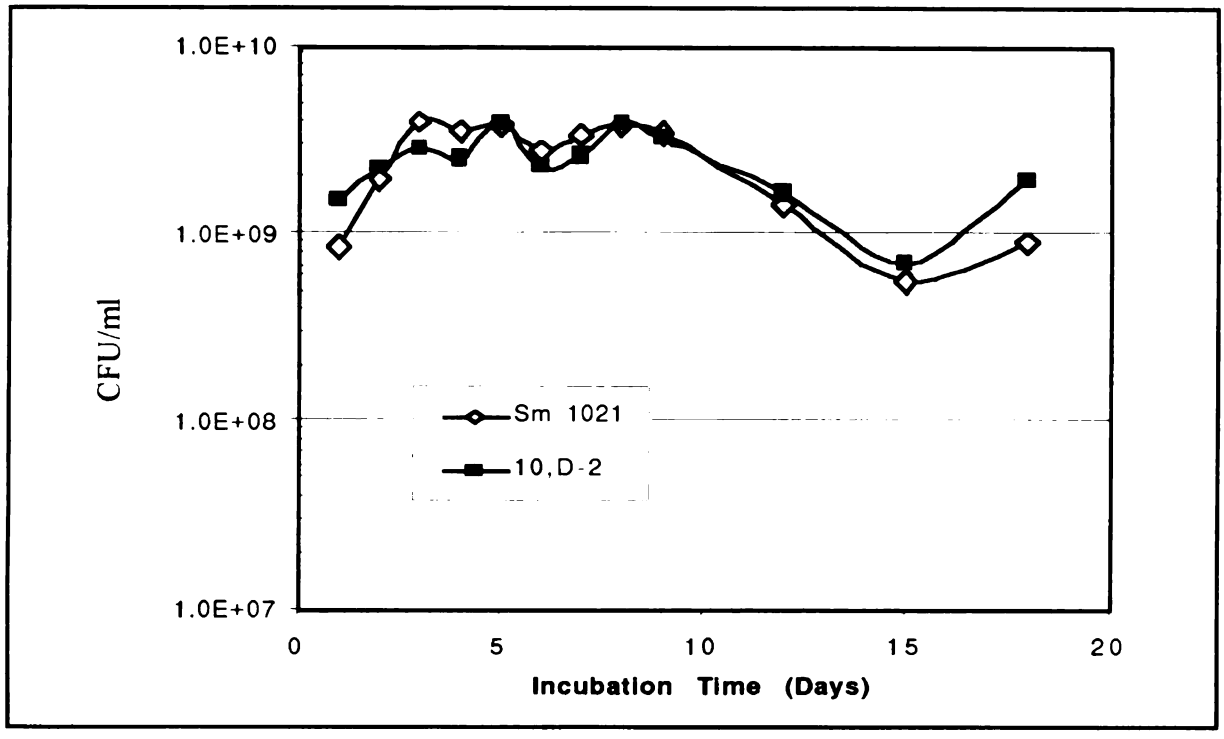


Figure 3.4. Survival of wild-type *S. meliloti* strain 1021 and double mutant strain 10,D-2 during stationary phase. See text for details.

DISCUSSION

In order to identify potential regulatory components for the induction of genes during nutrient deprivation, successive rounds of transposon mutagenesis were used. A *S. meliloti* mutant (C22) which harbors a Tn*5luxAB* insertion in a gene that is induced by a variety of stresses; including: carbon, nitrogen, or oxygen deprivation, osmotic stress, and stationary phase was identified after the first round of mutagenesis. This locus was cloned, sequenced, and was found to consist of two ORFs which we have designated *ndiA* and *ndiB* for nutrient deprivation induced genes A and B. The genes of this locus appear to be novel. A second round of mutagenesis was then used to identify genes potentially involved in the regulation of the *ndi* locus. Strain C22 was mutagenized with a second transposon (Tn*1721*), and a library of 3000 double mutants was screened for altered *luxAB* expression patterns.

Two strains were identified that showed a constitutive pattern of *ndi*::Tn*5luxAB* expression. One of the double mutants displaying a constitutive *luxAB* reporter gene expression pattern has an insertion in a gene with high similarity to an outer membrane protein of *E. coli* of unknown function. The other double mutant has an insertion in a gene with significant similarity to a number of pyridine nucleotide-disulfide oxidoreductases. Mutant reconstruction or complementation experiments were not performed on these double mutants to determine if the Tn*1721* insertions are responsible for the constituitive expression of the *ndi* locus. Therefore, I have no evidence that

these genes actually affect the expression of the *ndi* locus since it is possible that a Tn*1721*-independent secondary mutation is present in the genome which is responsible for the altered *ndi*::Tn*5luxAB* expression. However, I chose to characterize another locus that was also found to affect *ndi* expression. Both mutant reconstruction and complementation studies were carried out on this locus, which, once mutated, renders the *ndi* locus inactive. Its DNA sequence showed striking similarity to a gene encoding the tryptophan-rich sensory protein, TspO, from *Rhodobacter sphaeroides* (31). Because the TspO protein has been implicated in signal transduction, this locus became the primary focus of my investigation, and its characterization is described in the following chapter.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zheng, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.

2. **Bardwell, J. C. A.** 1994. Building bridges: disuphide bond formation in the cell. Mol Microbiol. **14**(2)**:**199-205.

3. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84:**188-198.

4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol. **41:**459-472.

5. **Broughton, W. J., and M. J. Dilworth.** 1971. Control of leghemoglobin synthesis in snake beans. Biochem J. **125:**1075-1080.

6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable muticopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol. **134**(3)**:**1141-1156.

7. **de Bruijn, F. J., and S. Rossbach.** 1994. Transposon mutagenesis, p. 387-405. *In* P. Gerhardt (ed.), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, D.C.

8. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. **77:**7347-7351.

9. **Friedman, A. M., S. R. Long, S. E. Brown, W. I. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene. **18:**289-296.

10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

11. **Hecker, M., and W. s. a. U. Volker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol Microbiol. **19**(3)**:**417-428.

12. **Kaiser, D.** 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. Annu Rev Genet. **20:**539-566.

13. **Loewen, P. C., B. Hu, J. Strutinsky, and R. Sparling.** 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. **44:**707-717.

14. **Long, S. R.** 1996. Rhizobium symbiosis:nod factors in perspective. Plant Cell. **8**(10)**:**1885-1896.

15. **Massova, I., and S. Mobashery.** 1998. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob Agets Chemother. **42**(1)**:**1-17.

16. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Ann Rev Microbiol. **43:**293-316.

17. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutant of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114-122.

18. **Meighen, E. A., and P. V. Dunlap.** 1993. Physiological, biochemical and genetic control of bacterial bioluminescence. Adv Microb Phys. **34:**1-67.

19. **Milcamps, A., and F. J. deBruijn.** 1999. Identification of a novel, nutrient deprivation induced gene (*hmgA*) in *Sinorhizobium meliloti*, involved in tyrosine degradation. Microbiology. **145:**935-947.

20. **Milcamps, A., D. M. Ragatz, P. Lim, K. A. Berger, and F. J. de Bruijn.** 1998. Isolation of carbon- and nitrogen-deprivation-induced loci of *Sinorhizobium meliloti* 1021 by Tn*5-luxAB* mutagenesis. Microbiology. **144:**3205-3218.

21. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1-23. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum press, New York, N.Y.

22. **Nakagawa, J., S. Tamaki, S. Tomioka, and M. Matsuhashi.** 1984. Functional biosynthesis of cell wall peptidoglycan by polymorphic bifunctional polypeptides. Penicillin-binding protein 1Bs of *Escherichia coli* with activities of transglycosylase and transpeptidase. J Biol Chem. **259**(22):13937-13946.

23. **Ostling, J., L. Holmquist, L. Flardh, B. Svenblad, A. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation and recovery of *Vibrio*, p. 103-123. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum, New York.

24. **Palmer, J. A., K. Hatter, and J. R. Sokatch.** 1991. Cloning and sequence analysis of the LPD-glc structural gene of *Pseudomonas putida*. J Bacteriol. **173**(10):3109-3116.

25. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol Rev. **51**(3):365-379.

26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Press, New York.

27. **Schoffl, R., W. Arnold, A. Puhler, J. Altenbuchner, and R. Schmitt.** 1981. The tetracycline resistance transposons Tn*1721* and Tn*1771* have three 38-base pair repeats and generate five-base pair direct repeats. Mol. Gen Genet. **181:**87-94.

28. **Spaink, H. P., A. Kondorosi, and P. J. J. Hooykaas.** 1998. The Rhizobiaceae. Kluwer Academic Press.

29. **Spector, M. P.** 1998. The starvation-stress response (SSR) of *Salmonella*, p. 233-279, Advances in Microbial Physiology, vol. 40. Academic Press.

30. **Wolk, C. P., Y. Cai, and J.-M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc Natl Acad Sci USA **88:**5355-5359.

31. **Yeliseev, A. A., and S. Kaplan.** 1995. A sensory transducer homologous to the mammalian peripheral-type benzodiazepine receptor regulates photosynthetic membrane complex formation in *Rhodobacter sphaeroides* 2.4.1. J Biol Chem **270**(36):21167-21175.

CHAPTER 4

A Homologue of the Tryptophan-Rich Sensory Protein, TspO, and FixL Regulate the Novel Nutrient-Deprivation-Induced *Sinorhizobium meliloti ndi* Locus.

ABSTRACT

A nutrient-deprivation induced locus in *Sinorhizobium meliloti* strain 1021 was identified by use of a Tn*5*-based transposon carrying *luxAB* as a reporter. The tagged gene is part of an operon consisting of two open reading frames (ORFs). The two ORFs were designated *ndiA* and *ndiB* for nutrient deprivation induced genes A and B. Comparison of the deduced amino acid sequences of both *ndiA* and *ndiB* to the protein databases failed to reveal similarity with any known genes. This locus is induced by carbon and nitrogen deprivation, osmotic stress, oxygen limitation, and stationary-phase. To identify regulatory components involved in the induction of this locus, a second round of mutagenesis with a Tn*3* derivative, Tn*1721* was performed on the primary mutant strain, C22. A double mutant strain was obtained that lacks transcriptional activity of the *ndi* locus under any of the inducing conditions tested. The Tn*1721* tagged gene was found to have a high degree of similarity with the tryptophan-rich sensory protein, TspO, from *Rhodobacter sphaeroides*, as well as to the mitochondrial benzodiazepine receptor, pK18. Proper environmental control of the *ndi-luxAB* reporter gene fusion was found to be restored after introduction of the *tspO* coding region *in trans*, under all inducing conditions tested.

INTRODUCTION

The enhancement of indigenous bacterial populations in soil or the introduction of genetically engineered microbes for bioremediation and agricultural applications requires a greater understanding of how bacteria function in their natural environment. Soil bacteria in their natural habitat are, more often than not, exposed to nutrient-deprivation conditions. In fact, the bulk of non-rhizosphere soil is so nutrient poor that it has been described as a nutritional desert (32). Consequently, the indigenous bacteria are frequently deprived of essential nutrients for extended periods of time, hence their typical physiological state in this habitat is likely to be either dormancy or very slow growth (26). In fact, the generation times of bacteria in the soil have been estimated to range from less than 1 to 80 per year (17, 26, 27, 32).

Even though this starved state is fundamental to bacterial existence, we are only just beginning to understand how the microbial cell functions under such conditions. Some bacteria, such as those belonging to the genus *Bacillus*, respond to starvation by differentiating into morphologically distinct spores that are extremely resistant to stress, and intensive research has been carried out to investigate this differentiation process (20). While it is evident that the majority of bacteria do not appear to form spores, studies have shown that these "non-spore-forming" bacteria also differentiate into a stasis survival state. During this differentiation they prepare themselves to persist without

essential nutrients, and, at the same time, they acquire parallel resistance to a variety of stresses (24, 28, 37).

The majority of soil bacteria fit into this "non-spore-forming" category (3). Therefore, understanding how these bacteria control gene expression during and after the initiation of deprivation conditions, and thereby persist in soil, is fundamental to our understanding of soil microbial ecology. To investigate such strategies, we have been studying environmental control of gene expression in the common soil bacterium, *Sinorhizobium meliloti*. This bacterium establishes a symbiosis with the legume, alfalfa, during which a new specialized organ is formed, the nitrogen-fixing root nodule, and the role of rhizobia as symbionts has been studied extensively (25, 47). However, little is known regarding the ability of these bacteria to persist in their free-living state, in the soil and rhizosphere. In fact, research on the starvation-survival of non-spore forming bacteria whose natural habitat is soil has only just begun. It is likely that these bacteria, which have evolved in this habitat, have distinct mechanisms for surviving in this particular environment.

Previously, the isolation of 33 *S. meliloti* strains with Tn5*luxAB* gene fusions induced by either carbon or nitrogen deprivation, or both was reported (34). In Chapter 2, the characterization of one of these strains (C22) that harbors a transcriptional fusion induced by oxygen, nitrogen, or carbon deprivation, osmotic stress, as well as during entry into post-exponential stationary-phase growth was described. This locus has been designated *ndi* for nutrient deprivation induced. In Chapter 3, we described selected findings

of a second round of mutagenesis on mutant strain C22 that identified genes involved in controlling the expression from the *ndi* locus. In this chapter, we will focus on the characterization of one of the double mutant strains, 12, C-4. The Tn*1721* tagged gene in this strain was found to have a high degree of similarity to the tryptophan-rich sensory protein, TspO, from *Rhodobacter sphaeroides*, as well as to the mitochondrial benzodiazepine receptor, pK18, of rat, mouse, human, and bovine. This double mutant (12,C-4) lacks transcriptional activity of the *ndi* locus under any of the inducing conditions tested. Moreover, induction of the *ndi-luxAB* reporter gene fusion is restored, under all inducing conditions, by introducing the *tspO* coding region, from either *S. meliloti* or *R. sphaeroides*, *in trans*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

Strain *S. meliloti* 1021 (Sm^R) has been described by Meade *et al.*, (1982), *E. coli* DH5α by Hanahan (1983), and *E. coli* HB101 by Boyer & Roulland-Dussoix (1969). Strain C22 is *S. meliloti* 1021 *ndi*::Tn*51063*a (Km). 12, C-4 is C22 tspO::Tn*1721* (Tc, Km). R-C22 is *S. meliloti* 1021 *tspO*::Tn*1721* (Tc), which was created by transducing *tspO*::Tn*1721* into 1021. Plasmid pRK2013 has been described by Ditta *et al.* (1980), and plasmid pJOE105 by Schöffl *et al.*, (1981). Plasmid pTB93 (Sp^R) was kindly provided by Sharon Long (13). pBluescript II SK (Ap^R) was obtained from Stratagene. pLAFR1 (IncP, Mob^+, Tra^-, Tc^r) has been described by Friedman *et al.*, (1982). pC22 is the plasmid recovered from strain C22 carrying the Tn*5-1063* transposon with flanking DNA on an *EcoR*I fragment (Km^r, this work). pC22lux is a plasmid carrying the entire *EcoR*I fragment of pC22 inserted into pTB93 (Sp^R, Km^r, this work). pTtspO is the plasmid used for complementation studies with the *S. meliloti* gene (Sp^R, this work). It carries the *ppu*MI - *Sal*I fragment of pBSR (see below) containing the *tspO*-like ORF from *S. meliloti* inserted into pTB93. Expression of the *tspO*-like ORF in this construct is controlled by the constitutive *trp* promoter from pTB93F (13). Plasmid pUI1126 was kindly provided by Sam Kaplan (54). pRstspO is the plasmid used for complementation experiments

with the *Rhodobacter sphaeroides tspO* gene. It carries the *BamH*I - *Kpn*I fragment containing the *tspO* gene from plasmid pUI1126 inserted into pTB93. Expression is under the control of the *trp* promoter from pTB93F. Plasmid pAY14A was kindly provided by Sam Kaplan (54). PTpK18 is the plasmid used for complementation studies with the rat mitochondrial benzodiazapine gene (pK18). It carries the *Hind*III - *Eco*RI fragment from pAY14A containing the PrrnB promoter and the pK18 coding region.

S. meliloti strains were grown on or in rich medium (TY; Beringer, 1974) or minimal medium at 28°C, as indicated. The minimal medium used was GTS (34). *E. coli* strains were grown on or in rich medium (Luria-Bertani; LB) at 37°C, Antibiotics were added at the following concentrations: (i) *S. meliloti*: streptomycin (Sm) 250 μg ml^{-1}; kanamycin (Km), 200 μg ml^{-1}; tetracycline (Tc) 10 μg ml^{-1}; spectinomycin (Sp) 50 μg ml^{-1}; (ii) *E. coli*: ampicillin (Ap), 100 μg ml^{-1}; (Km) 50 μg ml^{-1}; (Sp) 100 μg ml^{-1}; (Tc) 5 μg ml^{-1}. GTS-N is GTS medium lacking a combined nitrogen source. GTS-C is GTS medium lacking a carbon source. GTS+NaCl is GTS containing NaCl (400mM).

Survival during stationary phase

Strains 1021, 12,C-4, and R-C22 (A *S. meliloti* 1021 derivative created by transducing *tspO*::Tn*1721* into strain 1021) were were grown in GTS medium containing limiting amounts of carbon (0.025% glucose). A 25ml culture was grown in a 125ml flask with shaking at 28°C for 18 days. Viable

cells of each culture were determined at regular intervals by plating a dilution series on TY medium. The data were recorded as colony forming units per ml (CFU/ml). The experiment was performed in triplicate.

Nodulation experiments

S. meliloti mutant strain 12,C-4 was screened for its symbiotic phenotype by the protocol described in Chapter 3.

DNA isolation and manipulation.

See Chapter 2 for general protocols.

The Tn*1721* tagged locus from the double mutant, 12,C-4 was isolated by creating a partial genomic library in pBluescript II KS using genomic DNA of fragment sizes between 4.0 and 5.0 kb, following restriction with *Hin*dIII. This fragment size population was chosen based on information obtained from Southern blot analysis using a probe designed specifically for the first 350 bp of the 5' end of Tn*1721*. The ligation mixture was transformed into *E. coli* DH5α and colony hybridization experiments were carried out to identify the strain containing the Tn*1721* containing fragment. This clone was designated pBSR. A pLAFR1 library of *S. meliloti* 1021 (8) was probed with pBSR to isolate the genomic equivalent of the *S. meliloti tspO* locus.

Genetic techniques

A protocol for Tn*1721* mutagenesis of *S. meliloti* has been developed in our laboratory by Anne Milcamps (see Chapter 3).

Phage ϕM12 was used for general transduction as described by Finan *et al.*, (1984).

Induction and quantitation of luciferase activity in cultures

To evaluate expression under nutrient deprivation conditions, strains C22, 12,C-4, 1021 *ndi*::Tn*5luxAB*; *fixL*::Tn*5-233*, or strains harboring pC22Lux plasmids, were tested for induction and quantification of luciferase acitivity in culture using a luminometer, as described in Chapter 2.

DNA sequence analysis.

Sequence analysis of DNA fragments was carried out as described in Chapter 2. Alignments of deduced amino acid sequences were obtained using the pileup program (Genetics Computer Group, Madison, WI).

RESULTS

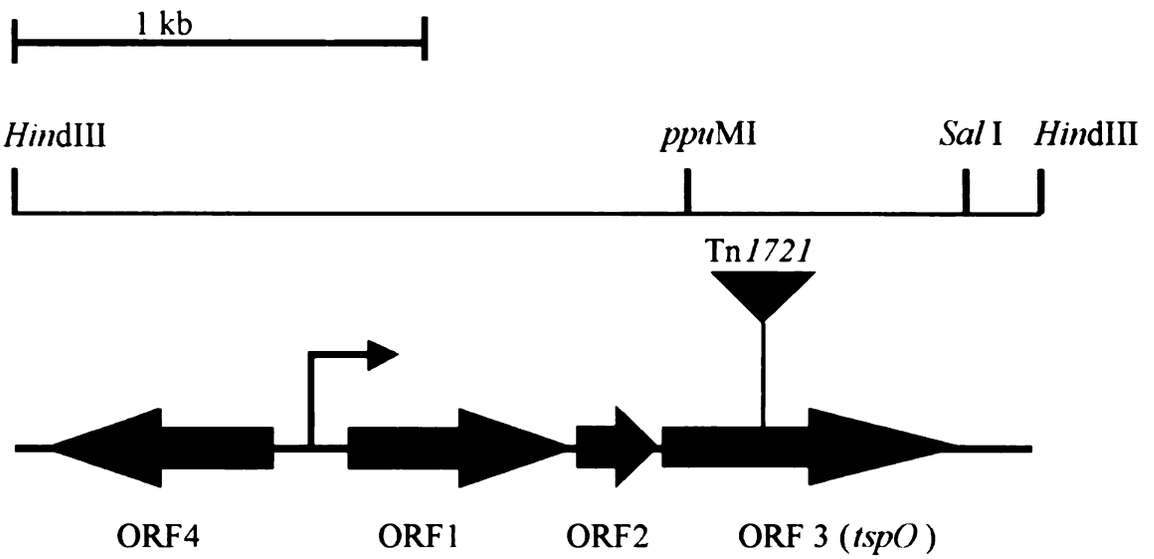
Isolation of a regulatory mutant of *S. meliloti* that abolishes *ndi*::Tn*5luxAB* expression

The Tn*5luxAB* insertion mutant, C22, was identified in a previous screen for *S. meliloti* strains carrying a *luxAB* reporter gene fusion induced under carbon deprivation conditions (34). *S. meliloti* strain C22 carries a Tn*5luxAB* insertion in the novel locus (*ndiAB*) that is induced by oxygen, nitrogen, and carbon deprivation, osmotic stress, and stationary-phase. The characterization of this locus is discussed in Chapter 2. In order to identify trans-acting regulatory loci affecting *ndi* expression, a second round of mutagenesis was performed on C22, as described in Chapter 3. This resulted in the identification of a gene (*tspO*) that abolishes expression of the ndi locus, once mutated.

A homologue of TspO regulates expression of the *ndi* locus

Strain 12,C-4 is a null double mutant in which the expression of the *ndi* locus was found to be silenced under all the previously determined inducing conditions. Southern blot analysis of genomic DNA of strain 12,C-4 confirmed that this strain contained a single Tn*1721* insertion and that the insertion of this transposon did not occur within the site of the initial Tn*5luxAB* insertion (data not shown). The corresponding Tn*1721* tagged locus was cloned as

described in Materials and Methods, and the structure and DNA sequence of the surrounding region was determined (see Fig. 4.1).



ORF	Function	Organism	Identity (%)
1	Transforming growth factor induced protein	*Synechocystis* sp.	46
	Major secreted immunogenic protein	*Mycobacterium bovis*	39
2	Unknown (*yuzA*)	*Bacillus subtilus*	55
3	Mitochondrial benzodiazepine receptor (pK18)	Rat, mouse, human, and bovine	48-50
	Tryptophan-rich sensory protein (TspO)	*Rhodobacter sphaeroides*	42
4	Epoxidase	*Methanobacterium thermoautotrophicum*	32

Figure 4.1. Map of the Tn*1721* tagged locus from "DARK" double mutant strain 12,C-4, with indication of the Tn*1721* insertion site, selected restriction sites, ORFs, protein sequence similarities, the organism with which the similarity was found, and percent identity. The presumptive direction of transcription is indicated with arrows.

The site of insertion was found to be in a gene with a high degree of similarity to genes encoding the mitochondrial benzodiazepine receptor protein pK18 of rat, human, mouse and bovine (50% identity, 65% similarity); as well as the outer membrane oxygen sensor protein (TspO) of *Rhodobacter sphaeroides* (42% identity, 67% similarity), (54, 55). The alignment of the corresponding amino acid sequences is shown in Figure 4.2.

Upstream (53 bp) of the *tspO*-like gene, a 225 bp ORF (ORF2) was found with a high degree of similarity (55% identity) to the *yuzA* gene from *Bacillus subtilus*, and encoding a protein of unknown function of approximately the same size (78 amino acids, see Fig. 4.1). Upstream of ORF2 (29 bp) another ORF (ORF1) was found in the same transcriptional direction, which had a high degree of similarity to genes encoding a transforming growth factor induced protein from *Synechocystis* sp. (46% identity) and a major secreted immunogenic protein from *Mycobacterium bovis* (39% identity). Both of these genes encode for secreted proteins that affect eukaryotic gene expression. A putative promoter region was identified just upstream of ORF1 by computer analysis, and a divergent ORF (ORF 4) upstream of the promoter region was identified with similarity to a gene encoding an epoxidase from *Methanobacterium thermoautotrophicum* (32% identity and 50% similarity). In addition, downstream of the *tspO*-like gene a divergent ORF encoding a protein with similarity (31% identity and 46% similarity) to a Tra protein from *Streptomyces lividans* plasmid pIJ101 was found (data not shown). Therefore,

it appears that three ORFs (ORF1; ORF2; and ORF3, which encodes TspO) constitute the Tn*1721* tagged operon.

Figure 4.2. Alignment of *S. meliloti* TspO deduced protein sequence (SmtspO) with the deduced amino acid sequences of loci encoding , pK18 from mouse (MpK18) (14), rat (RpK18) (50), human (HpK18) (43), bovine (BpK18) (38), *Rhodobacter capulatus* (Rccrtk) (2), *R. sphaeroides* TspO (RstspO) (54), a hypothetical protein (Bsytab) from *Bacillus subtilus* (23), and the hypothetical protein (SctspO) from *Synechocystis* sp. Strain PCC6803 (22). The alignment was created with the pile-up computer program (Genetics Computer Group, Madison, WI). Residues with similarity are framed and residues that are identical to SmtspO are shaded.

To determine if the "dark" phenotype of double-mutant strain 12,C-4 was, in fact, due to the mutation in the *tspO*-like gene, a plasmid (pTtspO) which constitutively expresses the coding region of only the *tspO*-like gene was introduced into strain 12,C-4 to test for complementation. This region was found to restore induction of the *ndi* locus under all conditions previously tested (see Figures 4.3 and 4.4). In addition, the tspO gene from *R. sphaeroides* was found to restore proper induction patterns when provided *in trans*, when expressed under the same constitutive (*trp*) promoter (see Fig. 4.5). However, interestingly, in these complementation experiments with the *Rhodobacter* gene the cells had to be always grown on GTS medium for testing complementation. The bacteria would not grow in the complex TY medium, in fact, this medium appeared to be lethal to the cells.

These complementation data not only indicates that the mutation in the *tspO* gene is responsible for the silencing of transcription from the *ndi* locus. In addition, it demonstrates that differential expression of TspO is not required for its regulatory control of the *ndi* locus, since it restores induction when it is being transcribed constitutively.

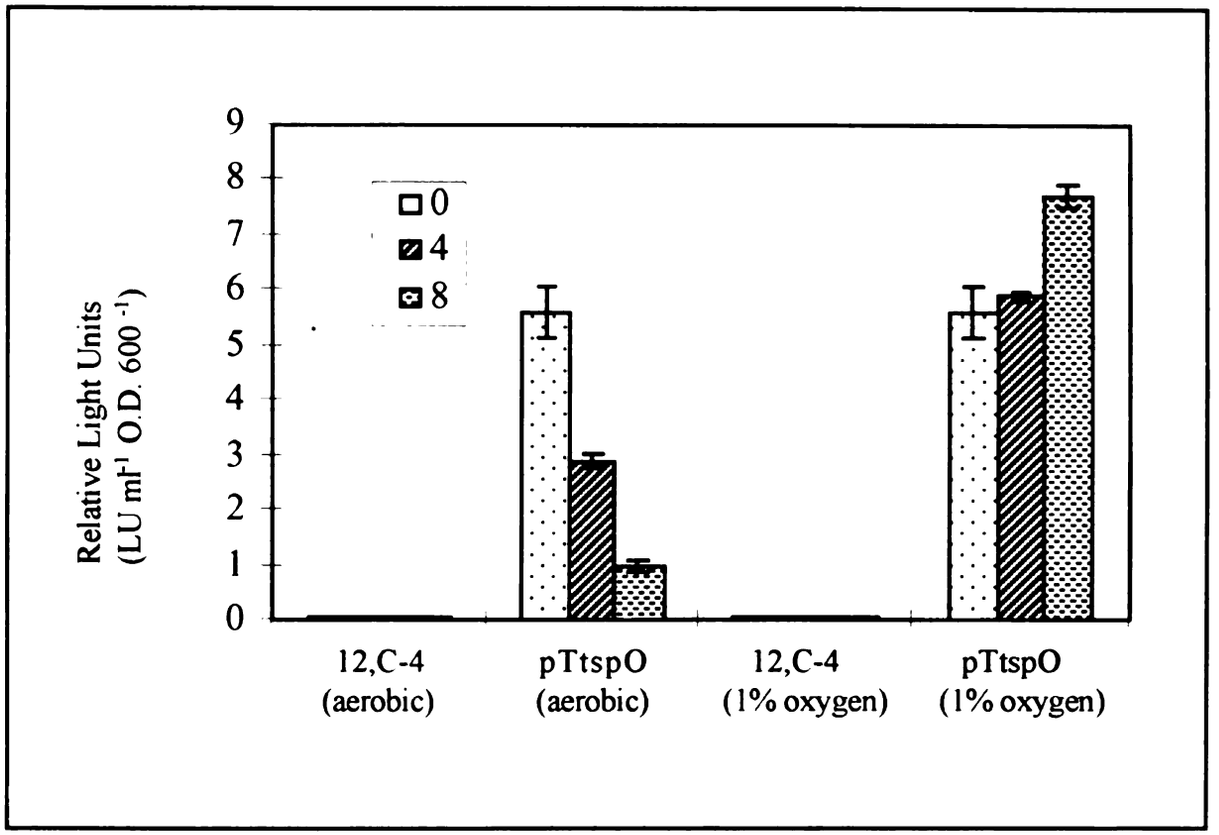


Figure 4.3 Complementation of 12,C-4 [a double mutant *S. meliloti* strain carrying a Tn*5*-1063 and a Tn*1721* insertion (*ndi*::Tn*51063*; *tspO*::Tn*1721*) that is not responsive under all conditions tested] under low oxygen tension by pTtspO, after 4 and 8 hours of exposure to low oxygen tensions. Values of luminescence are defined as described in Materials and Methods.

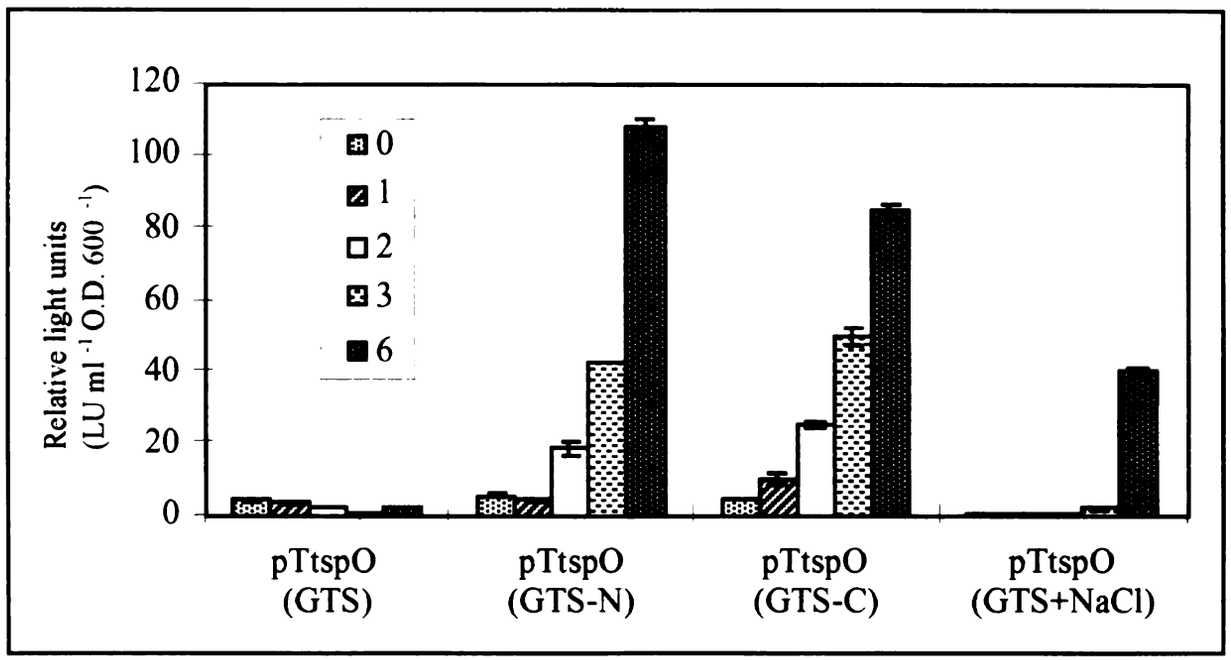


Figure. 4.4 Complementation of 12,C-4 during nitrogen and carbon deprivation, and osmotic stress, after 1, 2, 3, and 6 hours of exposure to different stresses.

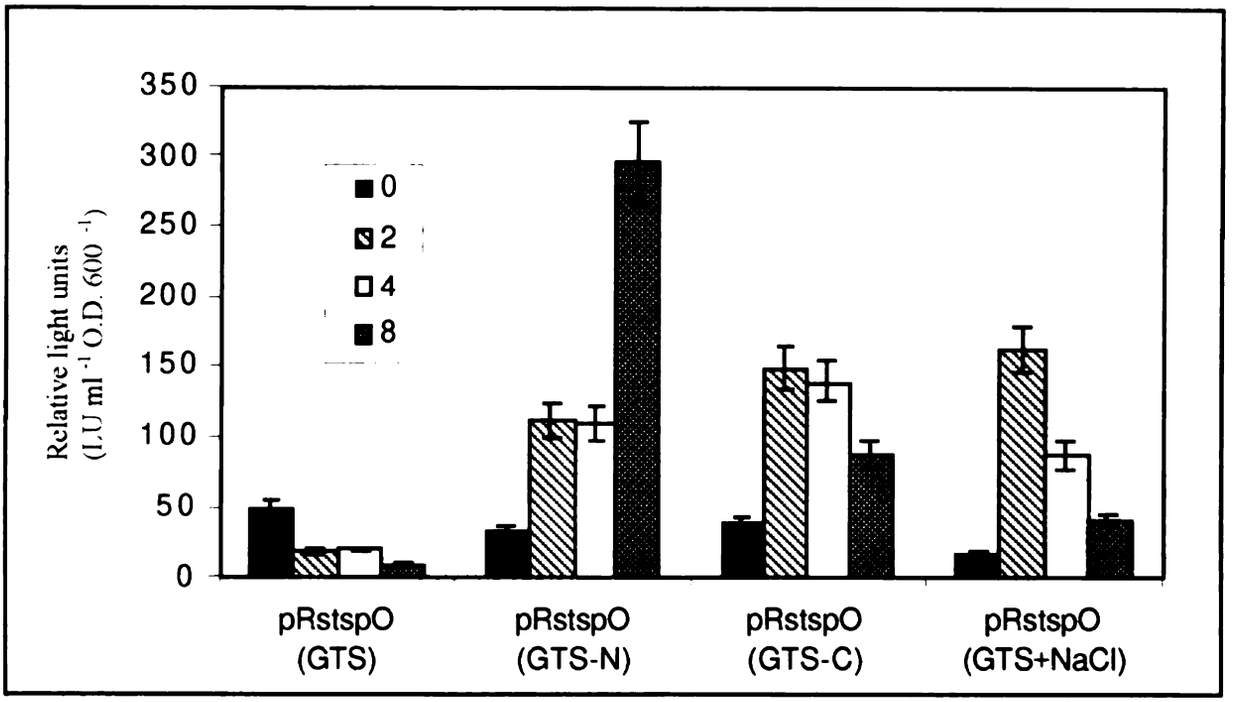


Figure. 4.5. Complementation of 12,C-4 with pRstspO during nitrogen and carbon deprivation, and osmotic stress, after 2, 4, and 8 hours of exposure to different stresses.

FixL is involved in regulating expression of the *ndi* locus

To evaluate the involvement of other signaling pathways in the control of *ndi* expression, plasmid pC22lux was introduced into *fixL*::Tn5 and *ntrC*::Tn5 mutant strains of *S. meliloti* 1021, and luciferase activity was monitored under inducing conditions over time. A similar expression profile was found in the wild-type and *ntrC* mutant backgrounds during nitrogen deprivation (see Fig. 4.6). However, a much lower level of luciferase expression was found in the FixL mutant strain versus the wild-type background, under oxygen limiting conditions (Fig. 4.7).

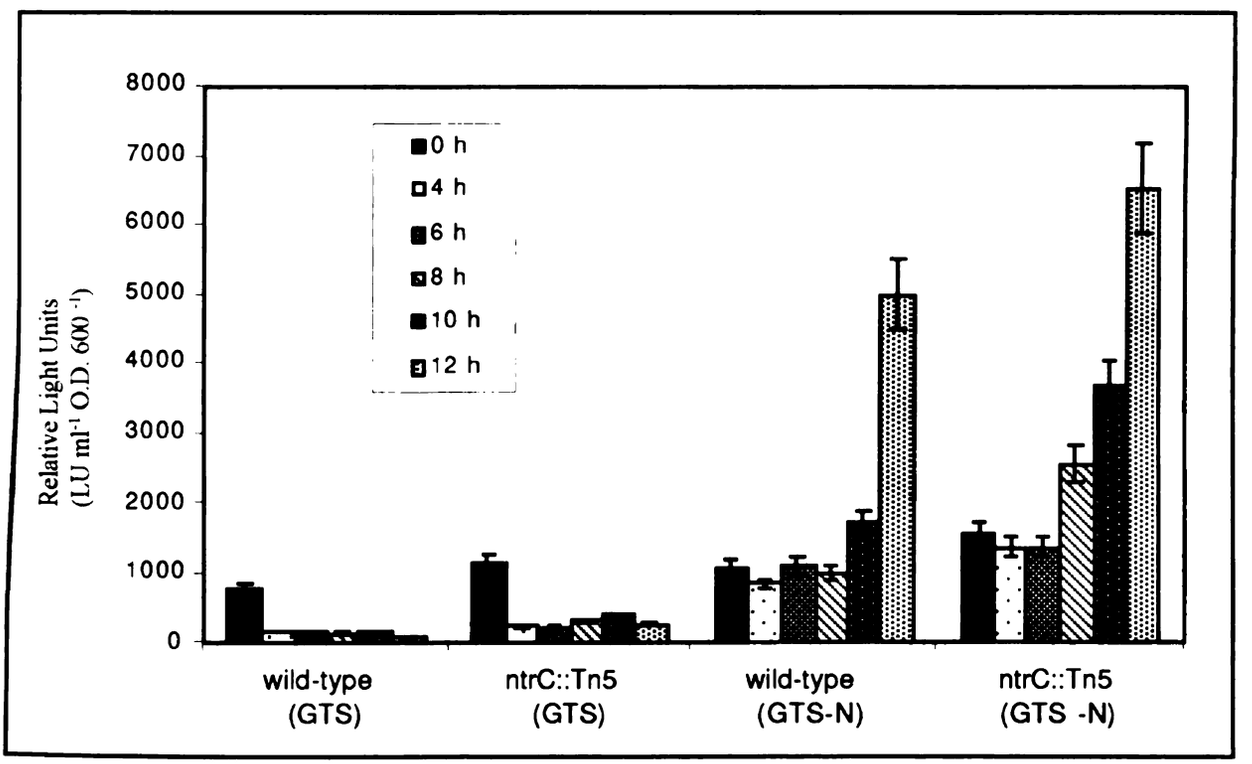


Figure 4.6. Plasmid pC22lux expression in wild-type and 1021 *ntrC*::Tn*5* mutant backgrounds after exposure to nitrogen deprivation for twelve hours.

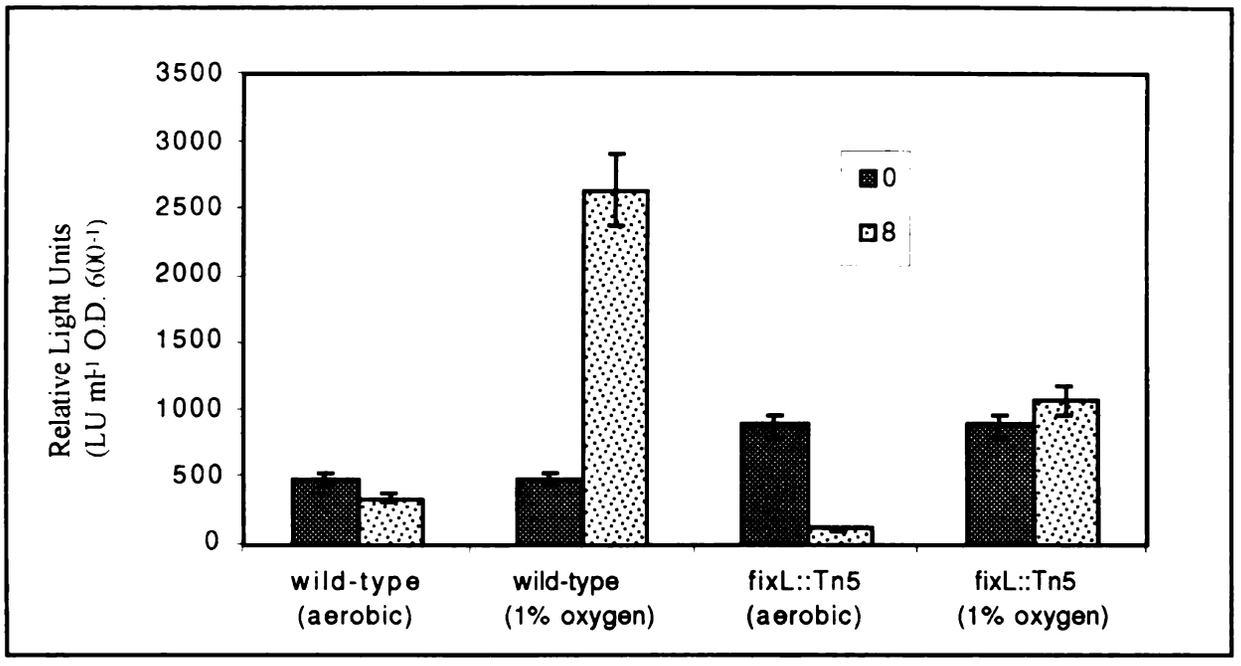


Figure 4.7 Plasmid pC22lux expression in wild-type and 1021 *fixL*::Tn*5* mutant backgrounds after exposure to low oxygen tensions for eight hours.

To further evaluate this observation, a double chromosomal (*S. meliloti* 1021 *fixL*::Tn*5-233*; *ndi*::Tn*5luxAB*) mutant was created by transducing the *ndi*::Tn*5luxAB* insertion into *S. meliloti* 1021 *fixL*::Tn*5-233*. This strain (*fixL*::Tn*5-233*; *ndi*::Tn*5luxAB*) was examined under oxygen limiting conditions. A decreased level of *ndi*::Tn*5luxAB* expression during oxygen deprivation was observed, as compared to that found in the wild-type background (see Fig. 4.8), reconfirming the data obtained with strains carrying the plasmid-borne fusion. Therefore, it appears that FixL is required for full induction of *ndi*::Tn*5luxAB* expression under low oxygen tensions. Furthermore, *ndi-lux* expression patterns were found to be unaltered under all other inducing conditions (carbon and nitrogen deprivation, and osmotic stress) in the *fixL* mutant (see Fig. 4.9).

Therefore, *fixL* involvement appears to be specific to sensing and responding to low oxygen tensions.

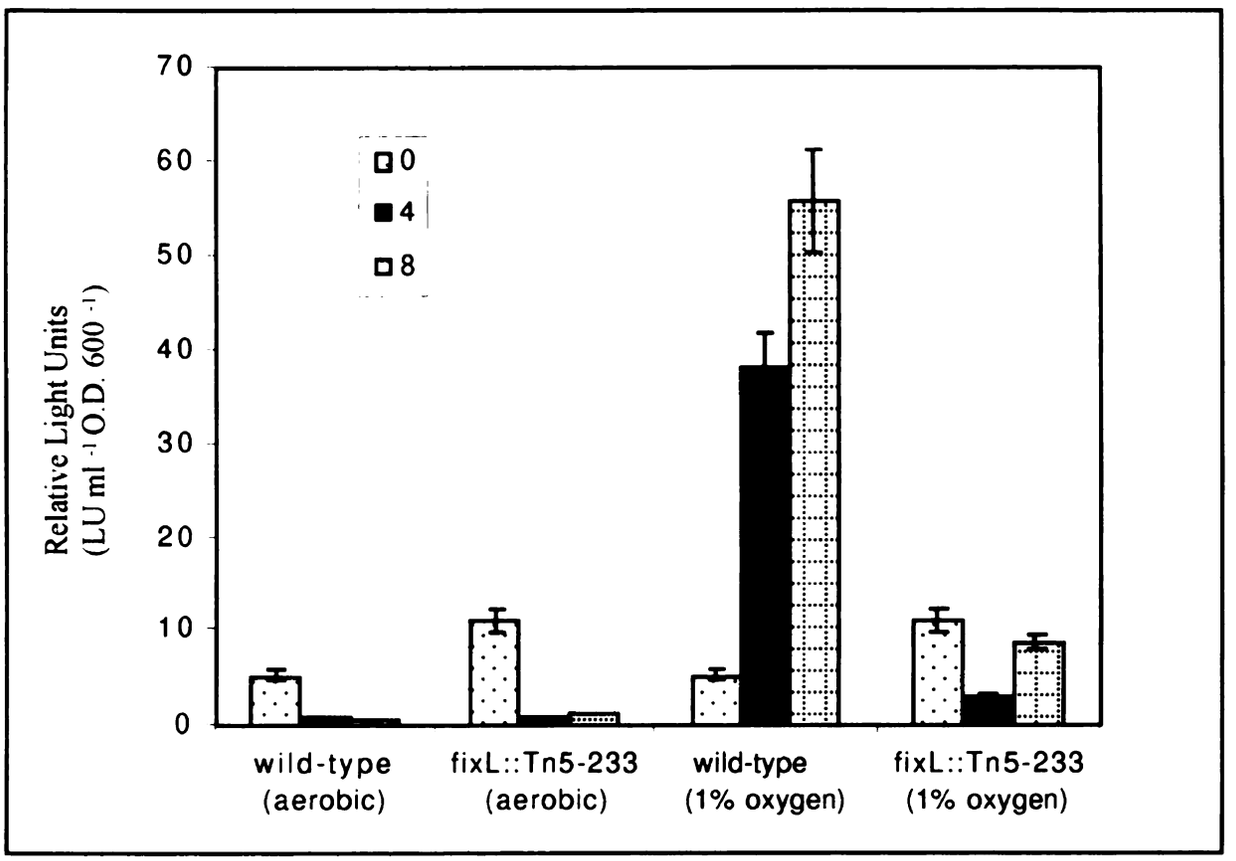


Figure 4.8. Chromosomal *ndi*::Tn*5luxAB* expression in wild-type and *fixL*::Tn*5-233* under low oxygen tension.

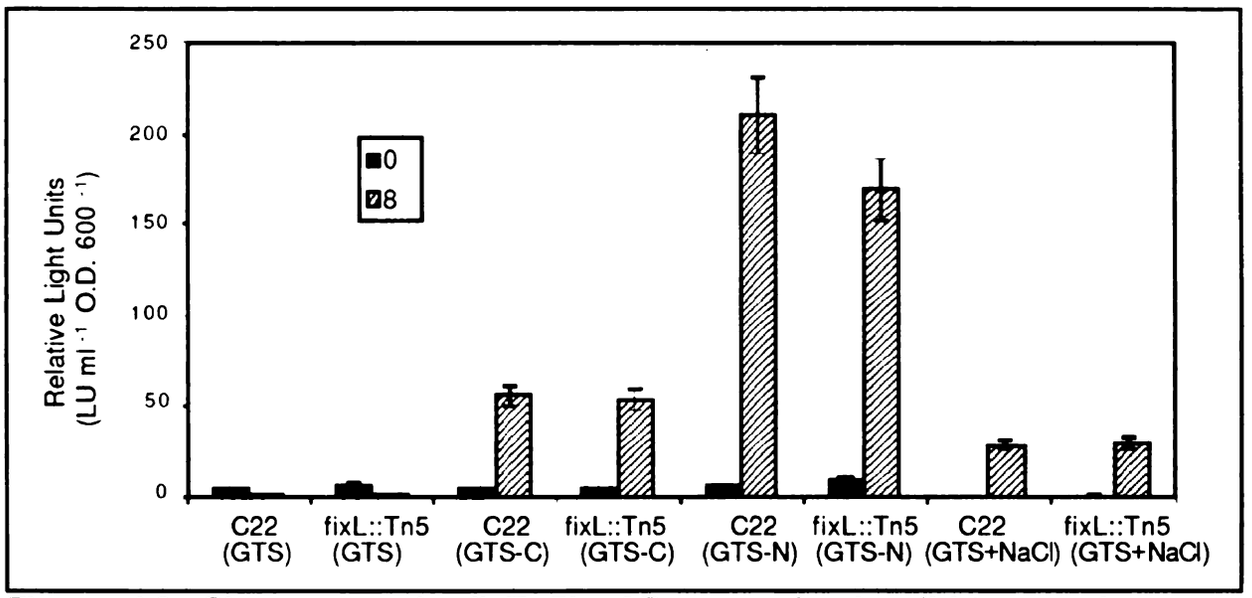


Figure 4.9. Chromosomal *ndi*::Tn*5luxAB* expression in wild-type and *fixL*::Tn*5-233* under carbon and nitrogen deprivation, and osmotic stress.

The specificity of TspO and FixL effects on *lux* expression in strain C22

Luciferase activity is very dependent on the energy status of the cell, therefore we hypothesized that the observed decrease in *ndi-lux* AB expression in the FixL and TspO mutants (see Fig. 4.10) could be the result of a general effect on the physiology of the cells. In order to evaluate whether the observed low levels of luciferase activity in the TspO and FixL mutants were due to a non-specific general physiological effect, double mutant strains CV2 *tspO*::Tn*1721* and CV2 *fixL*::Tn*5-233* (strain CV2 harbors a constitutive Tn*5-luxAB* fusion in a gene of unknown function) were created by transduction and examined under low oxygen tension (see Fig. 4.11). Relatively similar CV2-*luxAB* expression profiles under low oxygen tensions were observed in both mutants. Therefore, the decrease in expression of *ndi* locus in the FixL and TspO mutants is likely to be the direct result of these mutations. Furthermore, it should be noted that the high levels of luciferase activity from the CV2 fusion (100x greater than *ndi-luxAB*) indicates that these mutant strains (*tspO*::Tn*1721* or *fixL*::Tn*5-233*) have enough reducing power to catalyze a considerable amount of luciferase activity.

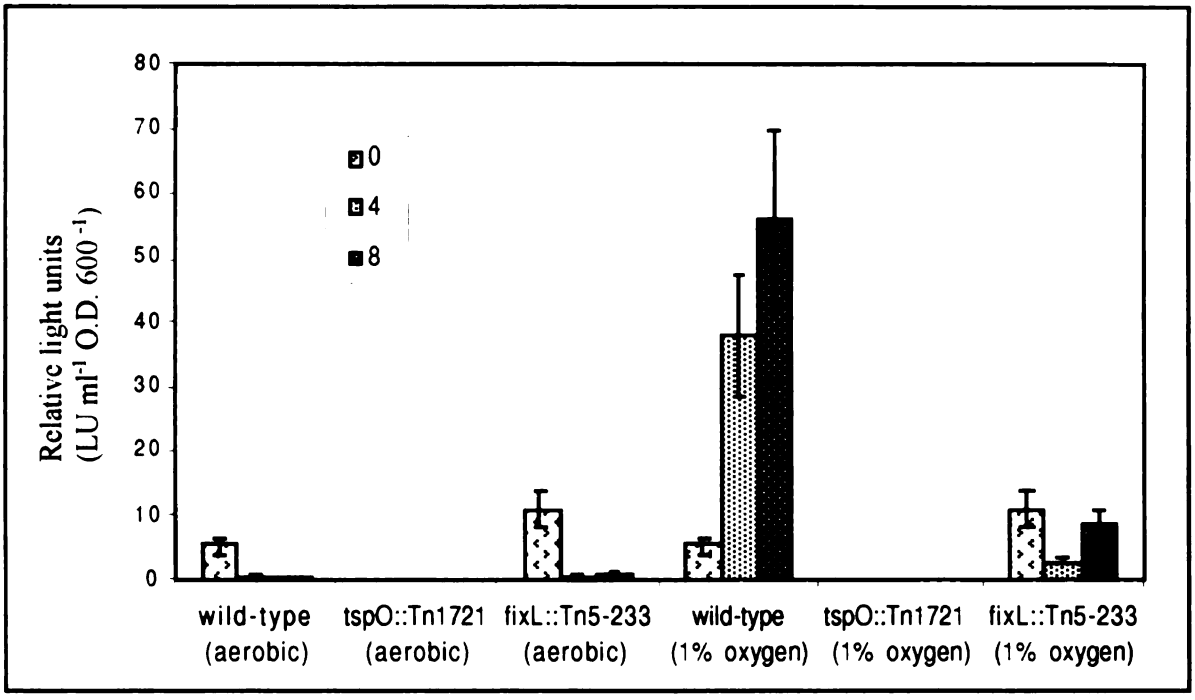


Figure 4.10 *ndi*::Tn*5luxAB* expression in wild-type strain, and *tspO* and *fixL* mutant strains.

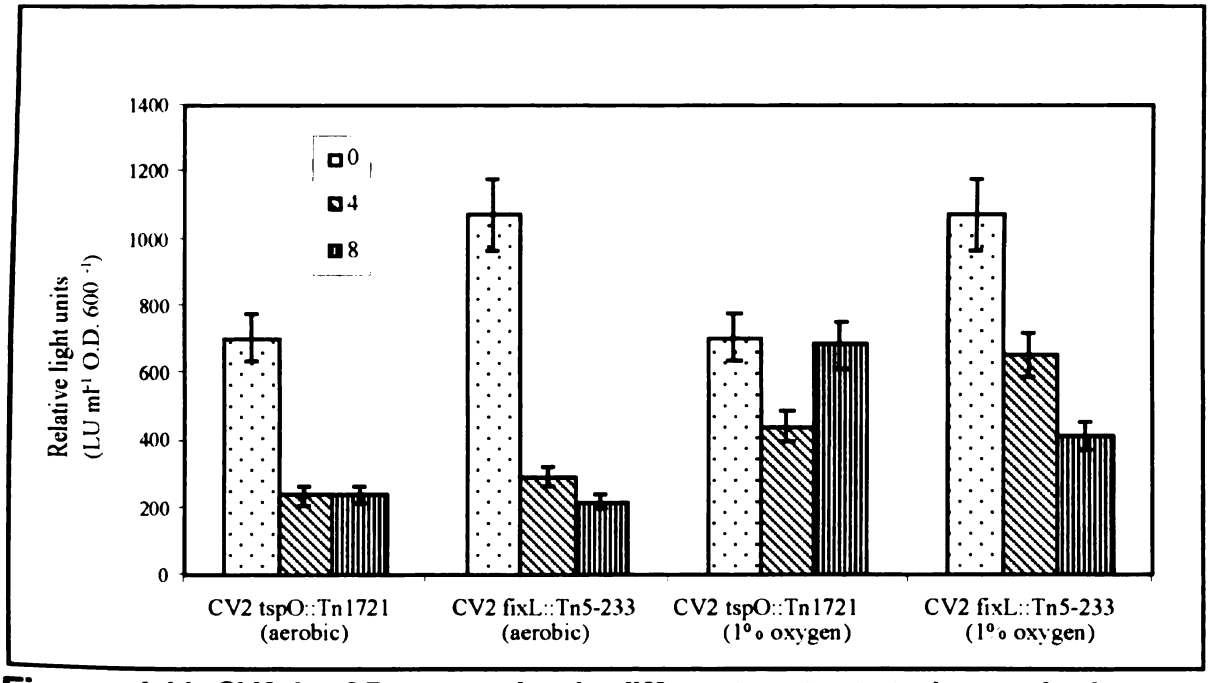


Figure 4.11 CV2-*luxAB* expression in different mutant strains under low oxygen tensions.

Phenotypic analysis of strain 12,C-4

Growth experiments indicated that strains C22 and 12,C-4, as well as R-C22 have growth rates that are similar to that of the wild-type strain. In addition, survival experiments indicate that these mutants are not impaired in the ability to persist during starvation (Fig. 4.12). Strain 12, C-4 was also screened for its symbiotic phenotype by inoculation on alfalfa (*Medicago sativa*). Six alfalfa seedlings grown on sterile Whatman filter paper, in test tubes were inoculated with wild-type or mutant strain. Six additional plants were used as uninoculated controls. The experiment was performed twice. Both wild-type and mutant strain 12,C-4 infected plants were found to be nodulated and green, while the control (uninfected) plants were not nodulated and chlorotic after seven weeks. Therefore, the mutated gene (*tspO*) in strain 12,C-4 does not appear to be absolutely required for the formation and function of the symbiotic association.

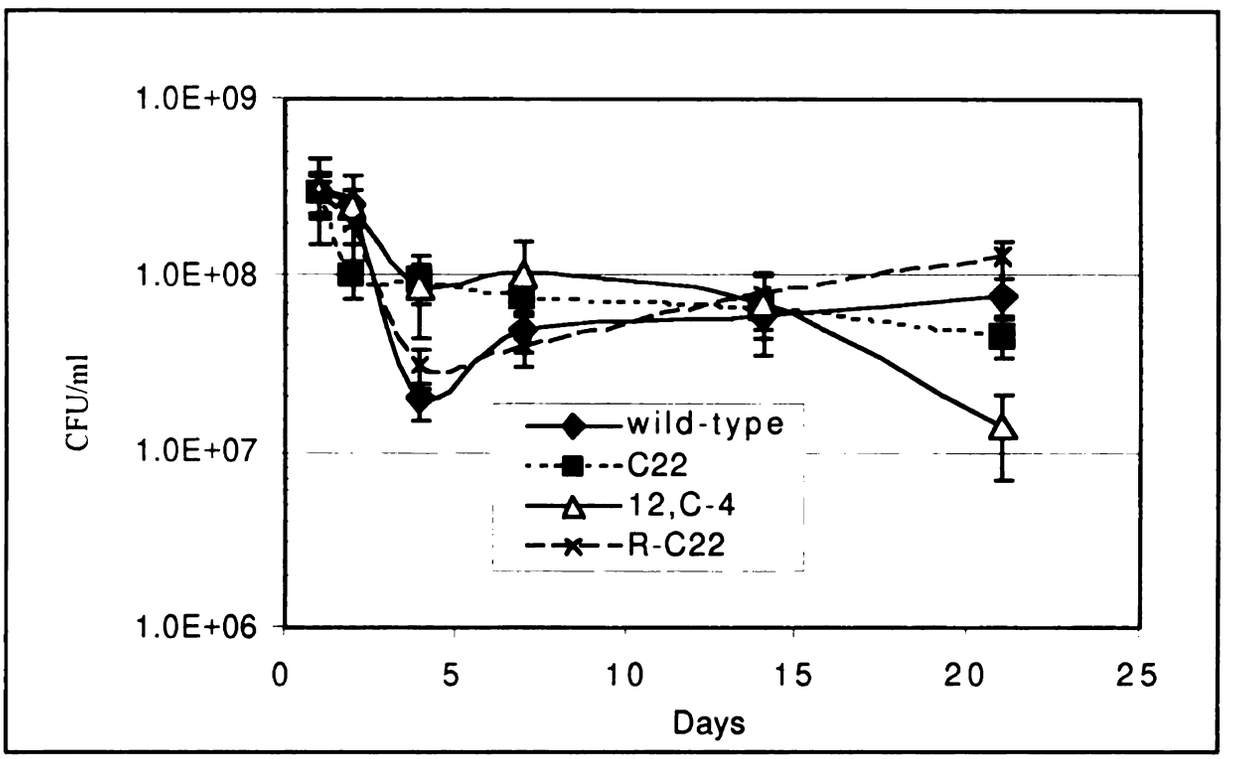


Figure 4.12. Survival of wild-type *S. meliloti* strain 1021, C22, 12,C-4, and R-C22 during stationary phase. The strains were grown in GTS medium containing limiting amounts of carbon (0.025% glucose). A 25ml culture was grown in a 125ml flask with shaking at 28°C for 18 days. Viable cells of each culture were determined at regular intervals by plating a dilution series on TY medium. The data were recorded as colony forming units per ml (CFU/ml). The experiment was performed in triplicate.

DISCUSSION

In order to identify regulatory components for the induction of genes during nutrient deprivation, successive rounds of transposon mutagenesis were used. A *S. meliloti* mutant (C22) was identified after the first round of mutagenesis, which harbors a Tn*5luxAB* insertion in a gene that is induced by a variety of stresses; including: carbon, nitrogen, or oxygen deprivation, osmotic stress, and stationary-phase. This locus was cloned, its DNA sequence determined, and the expression was examined in various regulatory mutant backgrounds. It was found to consist of two ORFs which we have designated *ndiA* and *ndiB* for nutrient deprivation induced genes A and B. The genes of this locus are novel and their expression is, in part, controlled by FixL, the sensor protein of a well described two-component system in *S. meliloti*, known to control gene expression during oxygen limitation (11, 15). A second round of mutagenesis was used to identify additional genes involved in the regulation of the *ndi* locus. Strain C22 was mutagenized with a second transposon (Tn*1721*), and a library of 3000 double mutants was screened for altered *lux* expression. A double mutant (12,C-4) was identified that does not express luciferase under any conditions tested. This 12,C-4 locus was then cloned, its DNA sequence determined, and the site of the Tn*1721* insertion was found to be in a gene with a high degree of similarity to the mitochondrial benzodiazepine receptor (29), as well as to the outer membrane oxygen sensor protein (*tspO*) of *Rhodobacter sphaeroides* (54).

To our knowledge, this is the first report of TspO involvement in signal transduction, in *S. meliloti*. Recently, however, this same locus was identified by Oke & Long (1999) in a screen for *S. meliloti* genes expressed predominantly in the nodule around the time of *bacA* expression (during the intermediate stages of nodule development). The function and role of this locus in the development of the symbiotic association is still under investigation.

The TspO outer membrane receptor does not appear to be ubiquitous, in nature. Homologues of the pK18 /TspO have been found in vertebrates and invertebrates; however, they have not been observed in *Saccharomyces cerevisiae* or in the majority of the genomes of prokaryotes whose DNA sequence has been completed. To date, orthologues of pK18 are only known to exist in purple photosynthetic bacteria and the cyanobacterium *Synechocystis*. Since a member of the alpha-subdivision of purple bacteria is the likely source of the endosymbiont that gave rise to the mammalian mitochondrion (51), the finding of the pK18 orthologue in *R. sphaeroides*, a member of this family, was of great interest. Moreover, Yeliseev *et al.*, (1997) demonstrated that the pK18 gene from rat could complement a TspO deficient strain of *R. sphaeroides*, indicating functional homology.

The finding of the pK18 orthologue in *S. meliloti* is also intriguing. On the one hand, given the fact that members of the genus *Sinorhizobium* are closely related to the alpha-subdivision of purple bacteria it is not surprising that similar proteins are retained in these organisms. On the other hand, since

only few prokaryotes contain this protein, it may be evolutionarily significant that mitochondria, members of the alpha-subdivision of purple bacteria that are the likely source of the endosymbiont that gave rise to the mammalian mitochondrion, and *S. meliloti*, an endosymbiont, all contain this protein.

In *Rhodobacter* spp., TspO is located in the outer membrane and is associated with the major outer membrane porins. In the mitochondrion the data indicate that pK18 is also localized to the outer membrane and is associated with the voltage-dependent anion channel (VDAC) (29). Moreover, both of these complexes bind and transport dicarboxylic tetrapyrrole intermediates of the heme biosynthetic pathway. This has been proposed as the likely mechanism by which TspO regulates gene expression in *Rhodobacter* (53). Based on this information, I propose that TspO may regulate gene expression in *S. meliloti* by the same mechanism, that is, porphyrin transport. A hypothetical model of this regulation, including the role of FixL, is depicted in Fig. 4.12. It has been reported that, under stress conditions, bacterial cells produce and secrete porphyrins (Pronk *et al.*, 1998 and references there in). We propose that heme itself or an intermediate of the heme biosynthetic pathway acts as an effector molecule or triggers the synthesis of another effector molecule that binds to the repressor, and then inhibits transcription of the *ndi* locus. On the other hand, efflux of porphyrin molecules, which appears to require TspO, is triggered and the molecules are transported out of the cell. When the concentration of porphyrins, or putative effector molecules is decreased, the repressor can no longer inhibit

transcription and expression of the *ndi* locus resumes. In the TspO mutant, however, the concentrations of porphyrins remains high under physiologically stressful conditions, therefore there is constant repression of the Ndi locus. In addition, FixL appears to be required for the full induction of the *ndi* locus under low oxygen tension. It is likely that FixL, either through the regulator FixJ or via another pathway, directly controls *ndi* expression when the oxygen tension is low. However, TspO appears to be epistatic to FixL, since no expression is observed in the TspO mutant, even though FixL is still functioning.

The mechanism by which TspO controls the transport of porphyrins and the nature of the signal that TspO senses still remains to be determined. In addition, further investigations are necessary to determine if these two related TspO homologues in *S. meliloti* and *Rhodobacter* spp. actually function in a similar manner, and respond to the same signals. Nonetheless, these two outer membrane receptors are involved in regulating gene expression and are likely to provide a new and important way to think about signal transduction in prokaryotes.

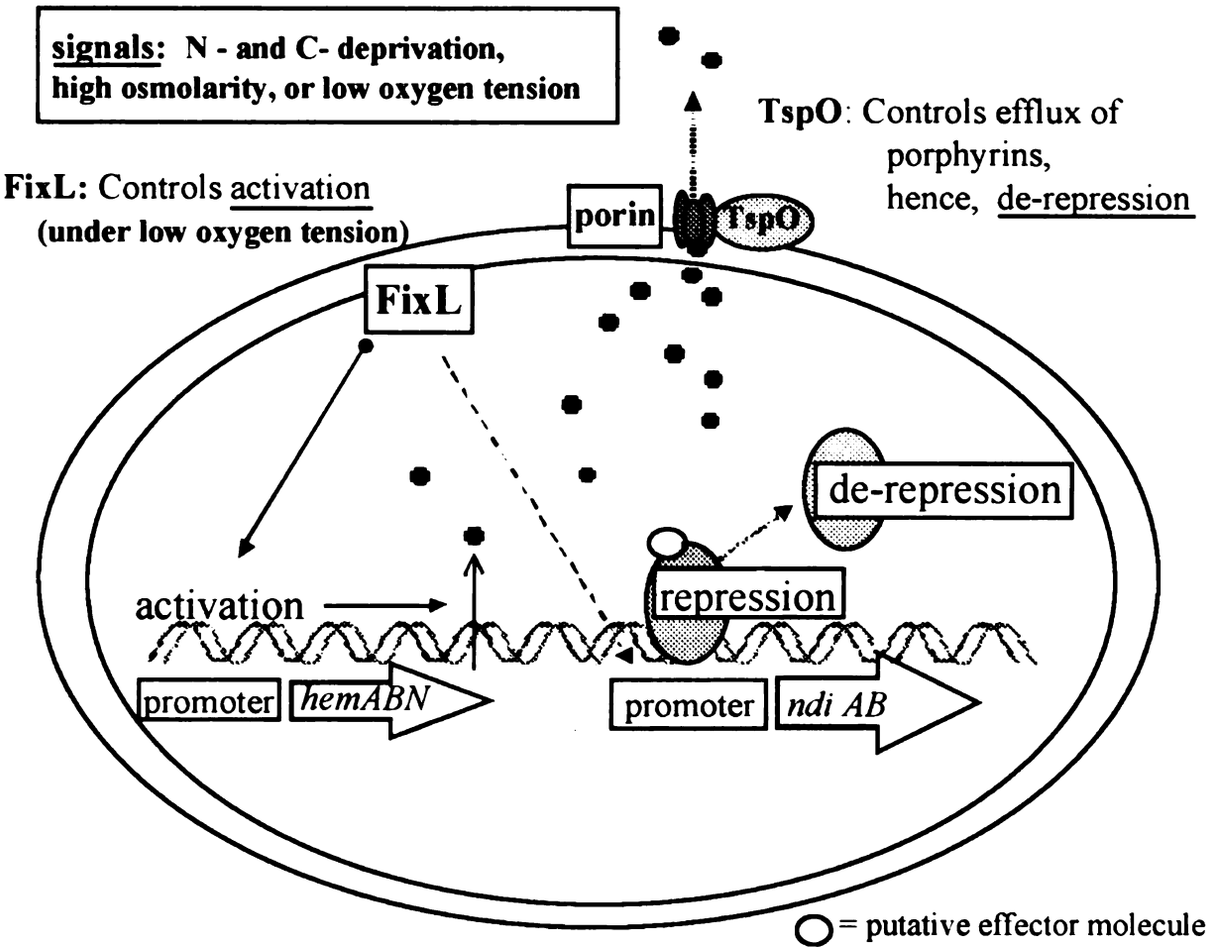


Figure 4.12. Hypothetical model of *ndiAB* regulation by TspO and FixL. See text for a detailed description of the above model. = porphyrin molecule.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zheng, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389-3402.

2. **Armstrong, G. A., M. Alberti, F. Leach, and J. E. Hearst.** 1989. Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. Mol Gen Genet. **216**(2-3):254-268.

3. **Atlas, R. M., and R. Bartha.** 1998. Microbial ecology: fundamentals and applications, 4th ed. Benjamin/Cummings, Menlo Park, CA.

4. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188-198.

5. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol. **41**:459-472.

6. **Broughton, W. J., and M. J. Dilworth.** 1971. Control of leghemoglobin synthesis in snake beans. Biochem J. **125**:1075-1080.

7. **de Bruijn, F. J., and S. Rossbach.** 1994. Transposon mutagenesis, p. 387-405. *In* P. Gerhardt (ed.), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, D.C.

8. **de Bruijn, F. J., S. Rossbach, M. Schneider, P. Ratet, S. Messmer, W. W. Szeto, F.M.Ausubel, and J. Schell.** 1989. *Rhizobium meliloti* 1021 has three differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. J Bacteriol. **171**(3):1673-1682.

9. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA. **77**:7347-7351.

10. **Finan, T. M., E. Hartwieg, L. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General Transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:120-124.

11. **Fischer, H.-M.** 1994. Genetic regulation of nitrogen fixation in Rhizobia. Microbiol Rev. **58**(3):352-386.

12. **Friedman, A. M., S. R. Long, S. E. Brown, W. I. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene. **18**:289-296.

13. **Gage, D. J., T. Bobo, and S. R. Long.** 1996. Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J. Bacteriol. **178**:7159-7166.

14. **Garnier, M., A. Dimchev, N. Boujard, M. J. Price, N. A. Musto, and V. Papadopoulos.** 1994. In vitro reconstitution of a functional peripheral-type benzodiazepine receptor from mouse Leydig tumor cells. Mol Parmacol. **45**:201-211.

15. **Gilles-Gonzalez, M. A., G. S. Ditta, and D. R. Helinski.** 1991. A haemprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. Nature. **350**:170-172.

16. **Gray, J. X., M. A. Djordjevic, and B. G. Rolfe.** 1990. Two gene that regulate exopolysaccharide production in *Rhizobium* sp. strain NGR234:DNA sequences and resultant phenotype. J Bacteriol. **172:**193-203.

17. **Gray, T. R. G.** 1976. Survival of vegetative microbes in soil, p. 327-364. *In* T. R. G. Gray and J. R. Postgate (ed.), The Survival of Vegetative Microbes. (Society for General Microbiology Symposium 26). Cambridge University Press, Cambridge.

18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.

19. **Hastings, J. W., and G. Weber.** 1963. Total quantum flux of isotropic sources. J Opt Soc Amer. **53:**1410-1415.

20. **Hecker, M., and W. s. a. U. Volker.** 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol Microbiol. **19**(3):417-428.

21. **Kaiser, D.** 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. Annu Rev Genet. **20:**539-566.

22. **Kaneko, T., S. Sato, H. Kotani, A. Tanaka, and E. Asamizu.** 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803.II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. **3:**109-136.

23. **Lapidus, A., N. Galleron, A. Sorokin, and S. D. Ehrlich.** 1997. Sequencing and functional annotation of the *Bacillus subtilus* genes in the 200 kb rrnB-dnaB region. Microbiology. **143:**3431-3441.

24. **Loewen, P. C., B. Hu, J. Strutinsky, and R. Sparling.** 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. **44:**707-717.

25. **Long, S. R.** 1996. Rhizobium symbiosis:nod factors in perspective. Plant Cell. **8**(10):1885-1896.

26. **Lynch, J. M.** 1988. The terrestrial environment, p. 224-248. *In* J. M. Lynch and J. E. Hobbes (ed.), Microorganisms in Action: Concepts and Applications. Blackwell Scientific, Oxford.

27. **Matin, A.** 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol Microbiol. **5:**3-10.

28. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Ann Rev Microbiol. **43:**293-316.

29. **McEnery, M. W., A. M. Snowman, R. R. Trifiletti, and S. H. Snyder.** 1992. Isolation of the mitochondrial benzodiazapine receptor: Association with the voltage-dependent anion channel and the adenine nucleotide carrier. Proc Natl Acad Sci USA **89:**3170-3174.

30. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutant of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114-122.

31. **Meighen, E. A., and P. V. Dunlap.** 1993. Physiological, biochemical and genetic control of bacterial bioluminescence. Adv Microb Phys. **34:**1-67.

32. **Metting, B.** 1985. Soil microbiology and biotechnology, p. 196-214. *In* R. P. Ovellette and P. A. Cheremisinoff (ed.), Biotechnology: Applications and Research. Technomic Publishers, Lancanster, PA.

33. **Milcamps, A., and F. J. deBruijn.** 1999. Identification of a novel, nutrient deprivation induced gene (*hmgA*) in *Sinorhizobium meliloti*, involved in Tyrosine degradation. Microbiology. **145:**935-947.

34. **Milcamps, A., D. M. Ragatz, P. Lim, K. A. Berger, and F. J. de Bruijn.** 1998. Isolation of carbon- and nitrogen-deprivation-induced loci of *Sinorhizobium meliloti* 1021 by Tn*5-luxAB* mutagenesis. Microbiology. **144:**3205-3218.

35. **Morita, R. Y.** 1993. Bioavailability of energy and the starvation state, p. 1-23. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum press, New York, N.Y.

36. **Oke, V., and S. R. Long.** 1999. Bacterial genes induced within the nodules during the *Rhizobium*-legume symbiosis. Mol Microbiol. **32**(4):837-849.

37. **Ostling, J., L. Holmquist, L. Flardh, B. Svenblad, A. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation and recovery of *Vibrio*, p. 103-123. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum, New York.

38. **Parola, A. L., D. G. Stump, D. J. Pepperl, K. E. Krueger, J. W. Regan, and H. E. Laird.** 1991. Cloning and sequencing of a pharmacologically unique bovine peripheral-benzodiazepine receptor isoquinoline binding protein. J Biol Chem. **266**(21):14082-14087.

39. **Pronk, A. F., J. Stigter, A. H. Stouthamer, F. J. de Bruin, and F. C. Boogerd.** 1998. Coproporphyrin excretion by *Azorhizobium caulinodans* under micro-aerobic conditions. Antonie van Leeuwenhoek. **74:**245-251.

40. **Reese, M. G.** 1994. Diploma thesis. German Cancer Research Center, Heidelberg.

41. **Reese, M. G., and F. H. Eeckman.** 1995. "Novel neural network algorithms for improved eukaryotic promoter site recognition" Presented at the Seventh International Genome Sequencing and Analysis Conference, Hilton Head Island, South Carolina.

42. **Reese, M. G., N. L. Harris, and F. H. Eeckman.** 1996. "Large scale sequencing specific neural networks for promoter and splice site recognition" Presented at the Biocomputing: Proceedings of the 1996 Pacific Symposium.

43. **Riond, J., M. G. Mattei, M. Kaghad, X. Dumont, J. C. Guillemot, G. L. Fur, D. Caput, and P. Ferrara.** 1991. Molecular cloning and chromosomal localization of a human peripheral-type benzodiazepine receptor. Eur J Biochem. **195:**307-312.

44. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol Rev. **51**(3):365-379.

45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, New York.

46. **Schoffl, R., W. Arnold, A. Puhler, J. Altenbuchner, and R. Schmitt.** 1981. The tetracycline resistance transposons Tn*1721* and Tn*1771* have three 38-base pair repeats and generate five-base pair direct repeats. Mol. Gen Genet. **181:**87-94.

47. **Shields, J. A., E. A. Paul, and W. E. Lowe.** 1973. Turnover of microbial tissue in soil under field conditions. Soil Biol Biochem. **5:**753-764.

48. **Spaink, H. P., A. Kondorosi, and P. J. J. Hooykaas.** 1998. The Rhizobiaceae. Kluwer Academic Press.

49. **Spector, M. P.** 1998. The starvation-stress response (SSR) of *Salmonella*, p. 233-279, Advances in Microbial Physiology, vol. 40. Academic Press.

50. **Sprengel, R., P. Werner, P. H. Seeburg, A. G. Mukhin, M. R. Santi, D. R. Grayson, A. Guidotti, and K. E. Krueger.** 1989. Molecular cloning and

expression of cDNA encoding a peripheral-type benzodiazepine receptor. J. Biol Chem. **264**(34):20415-20421.

51. **Woese, C. R.** 1987. Bacterial evolution. Microbiol Rev. **51:**221-271.

52. **Wolk, C. P., Y. Cai, and J.-M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc Natl Acad Sci USA **88:**5355-5359.

53. **Yeliseev, A. A., and S. Kaplan.** 1999. A novel mechanism for the regulation of photosynthesis gene expression by the TspO outer membrane protein of *Rhodobacter sphaeroides* 2.4.1. in preparation.

54. **Yeliseev, A. A., and S. Kaplan.** 1995. A sensory transducer homologous to the mammalian peripheral-type benzodiazepine receptor regulates photosynthetic membrane complex formation in *Rhodobacter sphaeroides* 2.4.1. J Biol Chem. **270**(36):21167-21175.

55. **Yeliseev, A. A., K. E. Krueger, and S. Kaplan.** 1997. A mammalian mitochondrial drug receptor functions as a bacterial "oxygen" sensor. Proc Natl Acad Sci USA. **94:**5101-5106.

CHAPTER 5

Conclusions and Future Directions

CONCLUSIONS AND FUTURE DIRECTIONS

Transposon mediated reporter gene fusion approach

We have used the reporter gene approach to identify and characterize the expression of nutrient deprivation - induced loci by mutagenizing *S. meliloti* 1021 with a Tn*5* derivative (Tn*5*-1063) containing the promoterless luciferase *luxAB* genes (12, 26). Tn*5* has been shown to be an extremely useful tool for random mutagenesis in many Gram-negative bacterial systems, including *S. meliloti* (2), and luciferase has proven to be a very useful reporter of bacterial gene expression (5), as well as a useful biomarker to tag and thereby track bacteria in the soil and rhizosphere (3, 13, 21). Previously, researchers in our laboratory have used Tn*5-1063* successfully in *Pseudomonas fluorescens* to isolate transcriptional fusions whose bioluminescence is induced under nitrogen and phosphate deprivation conditions (8), as well as in *S. meliloti* to isolate transcriptional fusions induced during carbon and/or nitrogen deprivation (15).

There are a few limitations to this transposon/reporter gene mediated approach: (i) The insertion of a transposon into a gene generally leads to inactivation, therefore genes that are essential can not be isolated with this method, since an insertion in such a gene would be lethal. However, this caveat did not pertain to these studies, since there was no selection for growth

during the screening procedure; (ii) the level of expression may be too low for detection of the reporter gene; and (iii) the transposon must be inserted in the correct orientation in respect to the promoter, therefore large libraries of mutants harboring transcriptional fusion need to be created and screened, which can be labor intensive. On the other hand, creating a mutation in a gene while at the same time being able to readily examine the expression profile of that gene with a reporter is a great advantage.

One potential disadvantage of using luciferase as a reporter gene for the study of genes induced by nutrient-deprivation is that luciferase activity is dependent on the energy status of the cell, due to the requirement for reducing equivalents ($FMNH_2$) to catalyze the bioluminescent reaction. Therefore, in theory, starved cells (especially carbon-deprived) are likely to be limited in stored energy and detection of enzyme activity could be misleadingly underestimated. However, by modifying our screening protocol when testing carbon-starved cells we appear to have overcome this limitation and we were clearly able to see an increase in expression during starvation. However, this too may underestimate the amount of expression, and the use of another reporter gene, such as the Green Fluorescent Protein (GFP) (5) that is not affected by the energy status of the cell may more accurately represent the level of expression. In addition, it was observed that the levels of luciferase activity in constitutive controls began to decrease significantly after 36 hrs of carbon starvation. Therefore, the duration of expression could not be assessed over a long peroid of time.

Search for genes involved in regulation during stress

The primary goal of this project was to identify regulatory circuits controlling stress-induced genes. Using successive rounds of transposon mutagenesis we have successfully identified such regulatory loci. The work described here in this thesis has been paralleled by an investigation by Anne Milcamps in our laboratory. For her studies, she chose to identify regulatory loci controlling the expression of a locus involved in the degradation of tyrosine whose expression is induced during both carbon and nitrogen deprivation, as well as in the presence of tyrosine (14). Using the same protocols as described here, she was able to identify a gene that encodes a protein with a high degree of similarity to a transcriptional regulator (Milcamps, in preparation), which appears to regulate gene expression in response to both carbon and nitrogen deprivation. These parallel projects clearly demonstrate the usefulness of this second site mutagenesis strategy to identify various components of regulatory pathways, including specific regulators, as well as more global "sensing" and regulatory proteins.

TspO as a regulator of stress-induced gene expression

Many of the environmental parameters bacteria encounter in soil, such as low oxygen tensions, nitrogen and carbon limitation, as well as osmotic changes, are also experienced *in planta*. Consequently, a number of genes have been identified during studies on symbiosis that are involved in both physiological adaptations in the free-living state, as well as for legume infection (16). These include functions such as synthesis of cytochrome complexes, amino acids, nucleotides, chaperonins (GroEL), as well as utilization of different carbon and energy sources (16). However, the potential role of these genes in the starvation/stress response of rhizobia has not, as yet, been explored. Interestingly, we have identified a locus (TspO) in *S. meliloti* involved in regulating gene expression during nutrient deprivation, osmotic stress, and under low oxygen tensions (see Chapter 4). At the same time, researchers in the laboratory of Sharon Long identified the same locus as being induced within the nodule during symbiosis when the rhizobia differentiate into bacteroids (18). Consequently, it appears that TspO represents a novel regulatory protein/mechanism that plays an important role in controlling gene expression during various physiologically stressful conditions, including the development of symbiosis.

As described in Chapter 4, TspO is a recently identified outer membrane receptor in *Rhodobacter* spp. that is associated with the major porins of the cell wall, and is involved in the cell's ability to sense oxygen levels (28, 29). A

homologue exists in mitochondria that is also localized to the outer membrane and is associated with a voltage-dependent anion channel (VDAC) (11). Moreover, both of these complex channels interact with these receptors to bind and transport dicarboxylic tetrapyrrole intermediates of the heme biosynthetic pathway, and this has been proposed to be the likely mechanism by which TspO regulates gene expression in *Rhodobacter* (27). However, the mechanism by which TspO controls the transport of porphyrins and the nature of the environmental signal to which TspO responds still remains to be determined.

Rhodobacter spp. synthesize the intracytoplasmic membrane system which contains bacteriochlorophyl and carotenoids in response to a decrease in oxygen tension (30). Therefore, it follows that the pathway of porphyrin biosynthesis would be induced in response to this environmental signal. However, one of the more curious aspects of these findings is that if the same mechanism in *Rhodobacter* is used in *S. meliloti*, then the synthesis of porphyrins would be induced in *S. meliloti* in response to carbon, nitrogen, or oxygen deprivation, as well as during osmotic shock. It has been documented that bacteria synthesize and excrete porphyrin molecules under certain conditions, such as iron deficiency and oxygen limitation (7). In addition, *Azorhizobium caulinodans*, a unique stem-nodulating symbiont of the tropical legume *Sesbania rostrata* (1) has been found to excrete coproporphyrin under micro-aerobic conditions. Moreover, the expression of the genes involved in the synthesis of this porphyrin molecule are controlled by FixL (17, 20). However,

the precise reason for this synthesis and excretion has not been determined. Under low oxygen tension, it is likely that the porphyrins are used to synthesize alternative cytochromes, in order to adapt to the change in redox. This may also be the case in response to stress, however, we are not aware of any reports of bacteria synthesizing and excreting porphyrins in response to carbon or nitrogen deprivation, or osmotic stress. On the other hand, TspO may regulate gene expression in response to stress via another unidentified mechanism, such as the one described below.

PAS domains: internal sensors

PAS domains are newly described sensor modules that monitor changes in light, redox potential, oxygen tensions, small ligands, as well as the overall energy status of the cell (25). Unlike the sensor domains of two-component systems, which are usually located within the periplasmic space, these modules are located in the cytosol, and therefore represent internal sensors. More than 300 PAS domains have been identified in diverse organisms (25). They have been found in all three kingdoms of life: *Archaea*, *Bacteria*, and *Eukarya*, and the designation PAS is simply an acronym formed from the names of the three proteins in which this domain was first recognized (25). Most PAS domains in prokaryotes are in histidine-kinase sensors, and these domains are known to detect the "signal" *via* a bound cofactor, such as heme or flavin. For example, FixL contains a PAS domain that overlaps with the

heme binding region of FixL. When oxygen levels are low, oxygen dissociates from the heme molecule located in the PAS domain changing the conformation of the PAS domain, which results in an altered structure and increased autophosphorylation activity of the transmitter, thereby activating signal transduction (4). Even though the finding of this internal sensing domain is a significant breakthrough, there are still many questions to address. The mechanism of signaling by PAS domains to downstream components of signal transduction pathways is not well understood, and the sensory role of the PAS domain for a variety of environmental signals remains to be determined.

Since TspO is involved in "sensing" many of the environmental parameters detected by the PAS domain, it is possible that TspO may also contain a similar "sensing" domain. Even though database searches have not identified a PAS domain in TspO, it may be that another unidentified domain is contained within this protein that is also involved in sensing changes in redox or the overall energy status of the cell.

Summary

Prokaryotes have a remarkable capacity to adjust themselves, both structurally and functionally, to changes in their surroundings. This holds true especially in their response to one of the most common environmental constraints that they encounter, namely, nutrient deprivation. Research over the

past two decades has provided strong evidence that not only spore-forming, but non-spore forming bacteria as well, differentiate into a stress-resistant, stasis-survival state (6, 10, 19). Moreover, because nutrients are scarce in most natural habitats, this starved state very likely represents the more typical physiological state of many bacteria in nature (22). Since the workings of the biosphere rely heavily on the activities of diverse microbes (9), understanding how different types of bacteria are able to monitor, sense, and respond to their environment in this more typical state is fundamentally important. We postulated that bacteria that are indigenous to soil are likely to have evolved particular mechanisms for persisting in this environment. Therefore, we chose to study nutrient-deprivation-induced gene expression in a ubiquitous soil bacterium, *S. meliloti*, and our investigations have, indeed, discovered a mechanism by which this bacterium modulates gene expression in response to a variety of stress conditions that is distinct, and is shared with only a few closely related bacteria. Finding this TspO/pK18 orthologue in *S. meliloti* that appears to work in concert with the two-component oxygen-sensing system FixLJ, and is apparently involved in multiple stress signaling pathways, in addition to oxygen sensing, is intriguing, and will likely form the basis for a variety of future studies pertaining to stress-induced gene expression in this bacterium.

Furthermore, although stasis-survival research in *S. meliloti* has only just begun, there is great potential for rapid progress in this well studied organism. This is possible because not only because numerous

environmentally controlled genes have been identified through studies on the development of the symbiotic association, but, in addition, the recent advances in determining the entire genomic sequence of this organism in combination with genomic arrays and DNA chip technology (23, 24) provides a significant advancement in technology that will make it quite feasible to address these issues of global regulation of gene expression during nutrient-deprivation.

REFERENCES

1. **de Bruijn, F. J.** 1989. the unusual symbiosis between the diazatrophic stem-nodulating bacterium Azorhizobium caulinodans ORS571 and its host, the tropical legume Sesbania rostrata, p. 457-493. *In* E. Nester and I. Kosuge (ed.), Plant-Microbe Interactions, vol. 3. McGraw Hill, New York.

2. **de Bruijn, F. J., and S. Rossbach.** 1994. Transposon mutagenesis, p. 387-405. *In* P. Gerhardt (ed.), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, D.C.

3. **De Weger, L. A., P. Dunbar, W. F. Mahaffee, B. J. J. Lugtenberg, and G. S. Sayler.** 1991. use of bioluminescence markers to detect *Pseudomonas* spp. in the rhizosphere. Appl Environ Microbiol. **57**:3641-3644.

4. **Gong, W., B. Hao, S. S. Mansy, G. Gonzalez, and M. A. Gilles-Gonzalez.** 1998. Structure of a biological oxygen sensor: A new mechanism for heme-driven signal transduction. Proc Natl Acad Sci USA **95**(15177-15182).

5. **Jansson, J. J., and F. J. de Bruijn.** 1999. Biomarkers and bioreporters to track microbes and monitor their gene expression, p. 651-665. *In* J. Davies (ed.), Manual of Industrial Microbiology and Biotechnology, 2nd ed. ASM Press, Washington DC.

6. **Kjelleberg, S.** 1993. Starvation in bacteria. Plenum Press, New York and London.

7. **Kragelund, L., B. Christoffersen, O. Nybroe, and F. J. de Bruijn.** 1995. Isolation of *lux* reporter gene fusions in *Pseudomonas fluorescens* DF57 inducible by nitrogen or phosphorous starvation. FEMS Microbiol Ecol. **17**:95-106.

8. **Madigan, M. T., J. M. Martinko, and J. Parker.** 1996. *Brock* : Biology of Microorganisms, eigth ed. Prentice Hall, Upper Saddle River, NJ.

9. **Matin, A., E. A. Auger, P. H. Blum, and J. E. Schultz.** 1989. Genetic basis of starvation survival in nondifferentiating bacteria. Ann Rev Microbiol. **43**:293-316.

10. **McEnery, M. W., A. M. Snowman, R. R. Trifiletti, and S. H. Snyder.** 1992. Isolation of the mitochondrial benzodiazapine receptor: Association with the voltage-dependent anion channel and the adenine nucleotide carrier. Proc Natl Acad Sci USA **89**:3170-3174.

11. **Meighen, E. A., and P. V. Dunlap.** 1993. Physiological, biochemical and genetic control of bacterial bioluminescence. Adv Microb Phys. **34**:1-67.

12. **Meikle, A., L. A. Glover, K. Killham, and J. I. Prosser.** 1994. Potential luminescence as an indicator of activation of genetically modified *Pseudomonas fluorescens* in liquid culture and in soil. Soil Biol Biochem. **24**:881-892.

13. **Milcamps, A., and F. J. deBruijn.** 1999. Identification of a novel, nutrient deprivation induced gene (*hmgA*) in *Sinorhizobium meliloti*, involved in Tyrosine degradation. Microbiology. **145**:935-947.

14. **Milcamps, A., D. M. Ragatz, P. Lim, K. A. Berger, and F. J. de Bruijn.** 1998. Isolation of carbon- and nitrogen-deprivation-induced loci of *Sinorhizobium meliloti* 1021 by Tn*5-luxAB* mutagenesis. Microbiology. **144**:3205-3218.

15. **Niner, B. M., and A. M. Hirsch.** 1998. How many *Rhizobium* Genes, in addition to *nod*, *nif*/*fix*, and *exo*, are needed for nodule development and function? Symbiosis. **24**:51-102.

16. **O'Brian, M. R.** 1996. Heme synthesis in the rhizobium-legume symbiosis: a palette for bacterial and eukaryotic pigments. J Bacteriol. **178**(9):2471-2478.

17. **Oke, V., and S. R. Long.** 1999. Bacterial genes induced within the nodules during the *Rhizobium*-legume symbiosis. Mol Microbiol. **32**(4):837-849.

18. **Ostling, J., L. Holmquist, L. Flardh, B. Svenblad, A. Jouper-Jaan, and S. Kjelleberg.** 1993. Starvation and recovery of *Vibrio*, p. 103-123. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum, New York.

19. **Pronk, A. F., J. Stigter, A. H. Stouthamer, F. J. de Bruijn, and F. C. Boogerd.** 1998. Coproporphyrin excretion by *Azorhizobium caulinodans* under micro-aerobic conditions. Antonie van Leeuwenhoek. **74**:245-251.

20. **Prosser, J. I.** 1994. Molecular marker systems for the detection of genetically modified microorganisms in the environment. Microbiology. **140**:5-17.

21. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol Rev. **51**(3):365-379.

22. **Schena, M., D. Shalon, R. W. Davis, and P. O. Brown.** 1995. Quantitative monitoring of gene expression patterns with complementary DNA microarray. Science. **270**:467-470.

23. **Shalon, D., S. J. Smith, and P. O. Brown.** 1996. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res. **7**:639-645.

24. **Taylor, B. L., and I. B. Zhulin.** 1999. PAS domains: Internal sensors of oxygen, redox potential, and light. Microbiol Mol Biol Rev. **63**(2):479-506.

25. **Wolk, C. P., Y. Cai, and J.-M. Panoff.** 1991. Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc Natl Acad Sci USA **88**:5355-5359.

26. **Yeliseev, A. A., and S. Kaplan.** 1999. A novel mechanism for the regulation of photosynthesis gene expression by the TspO outer membrane protein of *Rhodobacter sphaeroides* 2.4.1. in preparartion.

27. **Yeliseev, A. A., and S. Kaplan.** 1995. A sensory transducer homologous to the mammalian peripheral-type benzodiazepine receptor regulates photosynthetic membrane complex ormation in *Rhodobacter sphaeroides* 2.4.1. J Biol Chem. **270**(36):21167-21175.

28. **Yeliseev, A. A., K. E. Krueger, and S. Kaplan.** 1997. A mammalian mitochondrial drug receptor functions as a bacterial "oxygen" sensor. Proc Natl Acad Sci USA **94**:5101-5106.

29. **Zeilstra-Ryalls, J., M. Gomelsky, J. M. Eraso, A. Yeliseev, J. O'Gara, and S. Kaplan.** 1998. Control of photosystem formation in *Rhodobacter sphaeroides*. J Bacteriol. **180**(11):2801-2809.