IDENTIFICATION AND CHARACTERIZATION OF COLD SHOCK LOCI IN *SINORHIZOBIUM MELILOTI* 1021

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1999

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF COLD SHOCK LOCI IN *SINORHIZOBIUM MELILOTI* 1021

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In order to survive and persist in the soil, *Sinorhizobium meliloti* must be able to adapt to a wide range of temperatures. To investigate *S. meliloti*'s response to low temperature, transposon mutagenesis was performed on *S. meliloti* strain 1021 using the reporter transposon, Tn*5*-1062. Tn*5*-1062 carries the promoterless *luxAB* genes of *Vibrio harveyi* which encode bacterial luciferase. Luciferase activity can be easily assayed by exposing the cell culture to aldehyde and measuring the luminescence of the sample. Luminescence of the transposon recipients was assayed at 30°C and following a temperature downshift to 15°C. Seven transposon insertions were isolated that exhibited increased luminescence at 15°C. Five of these insertions were in either a 16S or 23S *rrn* gene. Further investigation revealed that *luxAB* insertions in all three of the *rrn* operons in *S. meliloti* 1021 showed increased luminescence following a temperature downshift. To characterize their apparent temperature regulation, the *rrn* promoter regions were sequenced and the transcription start sites mapped. Each 16S rRNA gene is preceded by a 354 bp leader region that is conserved between the three operons. A single promoter having significant similarity to the *rrn* P1 promoters of

Escherichia coli was located upstream of each *rrn* operon. The *rrn*A promoter was fused to the *luxAB* genes at +1 and +172 and recombined into the inositol locus of *S. meliloti* 1021. Study of the two promoter-*luxAB* fusions revealed that the 5' end of the rRNA transcript was required for the maximum temperature-dependent increase in luminescence. Increased luminescence in the +172 fusion resulted from the combined effects of increased translation, increased message stability, and increased promoter activity at 15°C. A rrn^P-*luxAB* fusion to +187 also showed significantly increased luminescence following a temperature downshift from 30°C to 15°C. To investigate the factors influencing this increase, transposon mutagenesis of *S. meliloti* 1021 was performed using the transposon, Tn*5*-233. Several transposon insertions were isolated that increased the luminescence of the rrn^P-*luxAB* fusion and the original 16S *rrn*::Tn*5*-1062 insertion mutants at 30°C. Two of these Tn*5*-233 insertions were located in an ORF whose deduced product showed significant similarity to Ribonuclease E of *E. coli*. Ribonuclease E is an essential endoribonuclease involved in the processing and degradation of mRNA and rRNA. In *S. meliloti* 1021, the expression of this gene increased transiently following a temperature downshift from 30°C to 15°C.

ACKNOWLEDGMENTS

First, I want to thank my advisor, Mike Thomashow, for his encouragement and guidance during my graduate study and in the completion of this dissertation. I would also like to thank the members of my guidance committee, Frans de Bruijn, Lee Kroos, Rich Lenski and Tom Schmidt, for taking the time to read this dissertation and for their helpful comments over the years. Several people worked with me on different aspects of this project. Deane Lehmann was the first to isolate cold-induced transposon insertion mutants and helped me get started, and keep going, as a graduate student. Kevin O'Connell contributed greatly to this project through his own research efforts and sage advice. Patty Spagnuolo and Beth Seymour provided technical assistance in screening for "down" mutants and in mutant analysis, respectively. Peter Wolk and his laboratory kindly provided use of their photonic system and the reporter transposon, Tn*5*-1062. I would also like to thank Sylvia Rossbach and Anne Milcamps for their helpful advice. A special thanks goes to all the members (past and present) of Mike Thomashow's and Rebecca Grumet's labs. It has really been a privilege. Finally, I would like to thank the Center for Microbial Ecology and the Department of Microbiology for funding this work and providing this opportunity for graduate study.

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CHAPTER 1

Literature Review

Introduction

Temperature is an important physical factor that has a great impact on bacterial growth and survival. Although bacteria have been isolated from extremely hot and extremely cold environments, individual bacterial species are limited to a narrower range (30-40°C) of growth temperatures (Russell, 1990). Organisms adapted to relatively high temperatures are classed as thermophiles, while those capable of growth at or near 0°C are defined as psychrotrophs or psychrophiles. Psychrotrophs have optimum growth temperatures above 15°C while psychrophiles have maximum growth temperatures of less than 20°C. Organisms like *Escherichia coli*, *Bacillus subtilis*, and *Sinorhizobium meliloti* are described as mesophiles showing optimum growth at 30 to 37°C.

At low temperature, several cellular structures and processes can be negatively affected. Enzymatic reactions slow down and may become limiting as temperature decreases. Protein conformation can also be affected by low temperature resulting in the inactivation or improper regulation of enzymes. Membrane fluidity decreases with temperature, potentially affecting the activity of

any membrane-bound enzyme. Specifically, a negative effect of low temperature on substrate uptake has been demonstrated (Nedwell and Rutter, 1994). The conformation of nucleic acids can also be negatively affected by temperature through the potential stabilization of unfavorable secondary structures at low temperatures. Changes in nucleic acid conformation could impact many cellular functions including: transcription, translation, DNA replication, ribosome synthesis and regulation of gene expression.

In considering adaptation to low temperature or the response to a "cold shock", it is important to note that "cold" is a relative term. To a psychrophilic bacterium, 8°C may be an optimum growth temperature. Yet, to the mesophilic bacterium *E. coli*, 8°C constitutes an extreme low temperature stress. Low temperature stress may then be considered to be exposure to temperatures below those to which the organism is adapted. It is interesting to note that a cold shock response has been observed in both psychrophilic and mesophilic bacteria albeit at different temperatures. While comparative studies of psychrophilic and mesophilic bacteria can be informative in examining different adaptations to low temperature, they are necessarily limited as any observed differences may result from species variation as opposed to being adaptative changes. Studying the response of a single organism to different temperatures allows a more straightforward comparison. This type of study is particularly relevant to soil microorganisms like *Sinorhizobium meliloti* that must adapt to seasonal, diurnal

and spatial temperature variation in order to persist in the environment.

This review will focus on the cold shock response in *Escherichia coli* as most of the proteins that are induced in response to low temperature have been identified in this bacterium. Characterizing the functions of these proteins and their importance to low temperature growth will aid in the understanding of low temperature stress and how it affects cellular physiology. Preliminary studies of the cold shock response have also been performed in very diverse bacteria. The cold shock proteins identified in these studies will be compared to those characterized in *E. coli*. Comparison of the different responses will be helpful in determining the degree to which the cold shock response is conserved among different bacteria. Finally, the regulation of the cold shock response will be discussed. The cellular mechanisms for sensing and responding to low temperature stress have been examined most thoroughly in *E. coli*. The ribosome has been proposed to be the sensor of low temperature stress and its adaptation to low temperature to be the main objective of the cold shock response. This model will be discussed along with the regulation of CspA, the major *E. coli* cold shock protein, and its potential role as a regulator in the cold shock response.

Cold shock proteins in *Escherichia coli*

The cold shock response in *E. coli* is characterized by the induction of at least 16 cold shock proteins, the repression of heat shock protein synthesis and the

continued expression of ribosomal proteins despite a transient inhibition of overall protein synthesis. Three cold shock proteins, ribosome binding factor A (RbfA), cold-shock DEAD-box protein A (CsdA) and translation initiation factor 2 (IF 2), are associated directly with the ribosome. RbfA is a 15 kDa protein with a pI of 6.05 that is associated with the free 30S ribosomal subunit and is thought to be involved late in ribosome maturation or translation initiation (Jones and Inouye, 1996). Following a cold shock from 37°C to 15°C, wild type cells show a transient change in their ribosome sedimentation profile indicating a temporary block in the initiation of translation. Immediately after the cold shock, there is an increase in 70S ribosomes and the 50S and 30S ribosomal subunits and a corresponding decrease in polysomes. A similar change in the ribosome sedimentation profile occured in a *rbfA* knockout mutant indicating that RbfA was required for efficient translation initiation. Significantly, the cold shock response was found to be constitutively induced in the *rbfA* knockout mutant following a cold shock from 42°C to 15°C. Several cold shock proteins, including NusA, H-NS, CsdA, CspA and CspB, were expressed at levels 2-5 times higher than in the wild type strain. Conversely, overexpression of RbfA on a multicopy plasmid shortened the growth lag normally observed following a cold shock by 50%.

A second cold shock protein associated with the ribosome is CsdA, a 70kDa protein with a pI of ~9 (Jones *et al.*, 1996). CsdA was predicted to be an RNA helicase because of its similarity to other DEAD-box proteins. Jones *et al.* (1996)

have confirmed that CsdA does have helix-destabilizing activity and propose that this activity may be important in removing secondary structures in mRNAs at low temperature, since CsdA is almost exclusively associated with the 70S ribosomes at 15°C. While a *csdA* knockout mutant shows no difference in growth from its parent strain at 37°C, it has an increased generation time of 16 hours at 15°C compared to the 8 hour generation time of the wild type strain. When the cold shock response was examined in the *csdA* mutant, no difference in protein expression was observed until 3 hours following the temperature shift from 37°C to 15°C. The expression of the heat shock proteins, DnaK and GroEL, remained low in the *csdA* mutant although they are normally derepressed within three hours. This effect was specific to low temperature as DnaK and GroEL were expressed normally following a typical heat shock from 28°C to 42°C.

CspA, CspB, CspG and CspI are the most highly expressed cold shock proteins (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Nakashima *et al.*, 1996; Wang *et al.*, 1999). These four proteins are members of the *E. coli* CspA family that shares significant similarity with the cold shock domain of the eukaryotic Y-box protein family (Wistow, 1990). The nine proteins in this family range in size from 69 to 74 amino acids, have pI's of 5.5 to 10.7, and are from 46 to 91% identical at the amino acid level (Yamanaka *et al.*, 1998). The structure of CspA has been determined and found to sonsist of five β-strands forming a β-barrel (Newkirk *et al.*, 1994; Schindelin, *et al.*, 1994). Each member of the CspA family contains

two RNA binding sites, RNP1 and RNP2, that are perfectly conserved among the cold-induced members (Yamanaka *et al.*, 1998). Because CspA was the first cold-induced member identified, most functional studies have focused on this protein. CspA is strongly induced following a cold shock and constitutes more than 10% of protein synthesis within an hour of the temperature shift (Goldstein *et al.*, 1990). Since CspA is so quickly induced, it was initially investigated as a transcriptional activator. In fact, when CspA was overexpressed on a multicopy plasmid, the expression of several cold shock proteins was increased following a temperature downshift (Jones *et al.*, 1992b). *In vitro* experiments with the *hns* promoter region showed that while CspA enhanced activity of this promoter, the purified protein bound only weakly to a DNA fragment containing the promoter in gel shift and DNA footprinting experiments (La Teana *et al.*, 1991; Brandi *et al.*, 1994). CspA, however, appeared to enhance the binding of RNA polymerase to the promoter region. The authors suggested that CspA might be stabilizing the RNA polymerase open complex by binding the DNA strands separated by the polymerase (Brandi *et al.*, 1994). CspA has also been ascribed a role in translation as a possible RNA chaperone (Jiang *et al.*, 1997). In contrast with earlier results, Jiang *et al.* (1997) showed that CspA was capable of binding only ssDNA and ssRNA and not dsDNA. Additionally, CspA bound cooperatively to RNA and destabilized intermolecular and intramolecular structures. Jones *et al.* (1996) have proposed that secondary structures formed in mRNAs at low temperature could

inhibit their translation. Additionally, it was suggested that CsdA and CspA could aid in translation through the unwinding of secondary structures by CsdA followed by the stabilization of the single-stranded conformation by CspA. Further, the moderate affinity of CspA to RNA would allow the ribosome to easily displace bound CspA proteins as it translated the message.

Two additional cold shock proteins, trigger factor and Hsc66, are thought to function as molecular chaperones. The identification of molecular chaperones as cold shock proteins is quite interesting since molecular chaperones are usually associated with heat stress. Trigger factor is thought to promote folding of nascent polypeptides as it is associated with the 50S ribosomal subunit and has been crosslinked to nascent peptides (Kandror and Goldberg, 1997). Trigger factor has also been shown to have peptidyl prolyl isomerase activity. Interestingly, this step in the folding of proteins is often rate-limiting and may be particularly sensitive to low temperature. Although trigger factor has been shown to associate with the heat shock protein GroEL and to enhance its activity, these two proteins have opposite responses to temperature. In fact, overexpression of trigger factor at high temperature actually decreases cellular viability. Following incubation for one week at 4°C, a non-permissive growth temperature for *E. coli*, overexpression of trigger factor increased survival from 15% to 40% while the reduction of trigger factor expression led to a decrease in survival to only 1%.

The second molecular chaperone, Hsc66, has 42% similarity to the heat

shock protein DnaK and can prevent aggregation of proteins *in vitro* (Silberg *et al.*, 1998). Hsc66 is encoded by the gene *hscA* which is cotranscribed with *hscB*, a gene encoding a co-chaperone, Hsc20. Although the transcript containing both genes is induced at low temperature, only the Hsc66 protein levels have been examined (Lelivelt and Kawula, 1995). An insertion mutant of *hscA* was not greatly altered in growth following a cold shock although the expression of 5 proteins was altered at low temperature. The characterization of trigger factor and Hsc66 as cold shock proteins suggests that multiple steps in protein synthesis are negatively affected by low temperature in *E. coli*.

The remaining cold shock proteins in *E. coli* are involved in cellular functions not directly related to protein synthesis (Jones *et al.*, 1987). Two enzymes of the pyruvate dehydrogenase complex are cold-induced, dihydrolipoamide acetyltransferase and pyruvate dehydrogenase (lipoamide). The cold shock protein, NusA, associates with RNA core polymerase and is involved in transcriptional termination, antitermination and pausing. Polynucleotide phosphorylase is a 3' to 5' exonuclease that interacts with Ribonulease E to degrade mRNA. RecA regulates the SOS response for DNA repair and is a key enzyme in homologous recombination. DNA gyrase introduces negative supercoils into the chromosome. Finally, H-NS shows increased expression during the cold shock response (La Teana *et al.*, 1991). H-NS is an abundant DNA-binding protein that negatively affects the expression of several unrelated

genes including the *rrn* operons (reviewed in Atlung and Ingmer, 1997). Binding of H-NS to the *rrn* promoter region specifically counteracts activation by FIS protein (Afflerbach *et al.*, 1998). In an *hns* insertional mutant expressing no detectable H-NS protein, growth was significantly inhibited at 12°C and 25°C but only slightly affected at 37°C (Dersch *et al.*, 1994).

Most of the cold shock proteins in *E. coli* are not expressed exclusively at low temperature but are also important at optimum growth temperatures. Presumably, the function of these proteins increases in importance at lower temperatures. One example is the cold-sensitive growth of an H-NS null mutant. As described above, the cold shock proteins are involved in a variety of celluluar functions indicating that low temperature probably affects many aspects of cell function. Protein synthesis appears to be particularly affected as 9 of the 16 cold shock proteins are involved in either translation, RNA conformation, or protein folding. The remaining cold shock proteins are involved in transcription, gene regulation, metabolism, RNA degradation and DNA conformation and repair.

Cold shock proteins in other bacteria

The cold shock response has been investigated in many different bacteria. Most bacteria examined induced the synthesis of several proteins following a temperature downshift. CspA, in particular, appears to be highly conserved as it has been detected in hyperthermophiles, thermophiles, mesophiles and

psychrotrophs. In several species, multigene families have been identified that contain from 3 to 9 CspA homologs. In each case, at least one of these proteins is increased in expression at low temperature (Graumann *et al.*, 1996; Mayr *et al.*, 1996; Michel *et al.*, 1997; Wouters *et al.*, 1998). In the psychrotrophic bacteria, *Pseudomonas fragi* and *Bacillus cereus*, four and five Csp homologs have been identified, respectively (Michel *et al.*, 1997; Mayr *et al.*, 1996). While all four proteins are induced by low temperature in *P. fragi*, the expression of two proteins termed cold acclimation proteins (Caps), remained high while the other two proteins were transiently induced. Surprisingly, the transiently induced proteins were also induced by heat shock and are,therefore, referred to as temperature adaptation proteins (Taps). In *B. cereus*, two of the five CspA-like proteins were constitutively expressed at a higher level at 7°C as compared to 30°C. Having multiple copies of CspA-like proteins is not necessarily a characteristic of psychrotrophic bacteria as only one CspA homolog has been identified in the psychrotroph *Arthrobacter globiformis* (Berger *et al.*, 1997). This protein, CapA, is expressed constitutively at a higher level at 4°C as compared to 25°C. Multigene families of CspA homologs were also found in mesophilic strains. *Lactococcus lactis* MG1363 contains five CspA homologs that are differentially regulated in response to low temperature (Wouters *et al.*, 1998). The *L. lactis* CspE protein is constitutively expressed while the remaining CspA homologs are induced following a shift from 30°C to 10°C. All four proteins responded

transiently to the cold shock although CspB and CspD were induced at least 30-fold while CspA and CspC were induced less than 10-fold. Interestingly, CspA and CspC have a pI of ~9 which is unusually high for the cold-induced CspA homologs. In *B. subtilis*, three CspA homologs have been characterized (Graumann, *et al.*, 1996). While all three are induced following a cold shock, CspB and CspC are induced during stationary phase as well (Graumann and Marahiel, 1999). Unlike most bacterial strains, no CspA homologs were found in the cyanobacteria *Anabaena variabilis* M3 (Sato, 1995). Interestingly, four of the five RNA-binding proteins identified in *A. variabilis* were induced by a temperature downshift from 38°C to 22°C. While these RNA-binding proteins are not homologous to CspA, they may serve a similar function at low temperature.

Although the proteins induced by the cold shock response have been enumerated using 2D-PAGE, most of the individual proteins have not been identified. Sixteen of the 37 proteins observed to increase in *Bacillus subtilis* following a temperature shift from 37°C to 15°C have been identified (Graumann, *et al.*, 1996). Several of these proteins were similar to cold shock proteins in *E. coli*. As described above, three homologs of the *E. coli* cold shock protein, CspA, were identified in addition to the ribosomal proteins S6 and L7/L12. While individual ribosomal proteins have not been identified as being cold-induced in *E. coli*, the continued synthesis or induction of groups of ribosomal proteins has been noted (Jones *et al.*, 1987; Jones *et al.*, 1996). The ribosomal protein S21 has been

characterized as being cold-inducible in both *Anabaena variabilis* (Sato, 1994) and in *Sinorhizobium meliloti* 1021 (O'Connell and Thomashow, 1999). Cyclophilin, a peptidylprolyl isomerase encoded by *ppiB*, has been identified as a cold shock protein in *B. subtilis*. The *E. coli* cold shock protein, trigger factor, also has peptidylprolyl isomerase activity. *B. subtilis* also induces several enzymes involved in amino acid synthesis and glycolysis: CysK, IlvC, Gap and TIM.

An additional *E. coli* cold shock protein, polynucleotide phosphorylase, while not identified as a cold shock protein in *B. subtilis*, was characterized as such in *Yersinia enterocolitica*. In fact, synthesis of polynucleotide phosphorylase (PNP) was required for growth at 5°C in this psychrotroph (Goverde *et al.*, 1998). Strains carrying transposon insertions in this gene were unable to form colonies at 5°C yet were indistinguishable from the wild type strain at 30°C. While cold shock experiments were not performed on this strain, amounts of PNP were compared in cultures grown constantly at 30°C or 5°C. Levels of PNP protein and *pnp* mRNA were 1.6-fold higher at the lower temperature.

Two RNA helicases were identified in *Anabaena* sp. strain PCC 7120 that showed increased expression following a temperature downshift of 10°C (Chamot *et al.*, 1999). Both CrhC and CrhB belong to the DEAD-box family of RNA helicases as does the *E. coli* cold shock gene, *csdA*. While *crhB* expression was induced under several conditions beside cold stress, *crhC* was induced only at low temperature. Although CrhC is a DEAD-box RNA helicase like CsdA, it shows

more similarity to another *E. coli* RNA helicase, RhlE.

Although no fatty acid desaturase has been identified as a cold shock protein in *E. coli*, cold induced desaturases have been identified in *B. subtilis* and at least two cyanobacteria. In *B. subtilis*, a *des-lacZ* fusion was induced 10 to 15-fold following a temperature shift from 37°C to 20°C (Aguilar *et al.*, 1998). Levels of unsaturated fatty acids increased at 20°C but were not required for viability as a *des* null mutant showed growth similar to that of the parent strain at both 37°C and 20°C. Of the four desaturases identified in the cyanobacterium *Synechocystsis* sp. PCC 6803, three are clearly induced following a temperature downshift albeit at different rates (Los *et al.*, 1997). In *Synechococcus* sp. strain PCC 7002, three desaturases, DesA, DesB, and DesC, have been identified (Sakamoto and Bryant, 1997). The expression of all three genes was induced within five minutes of a shift from 38°C to 22°C. The three genes were also expressed more highly in cells grown continuously at 22°C. Insertion mutants of *desA* and *desB* were unable to grow at 15°C although the strains showed different effects at higher temperatures (Sakamoto *et al.*, 1998). While both mutant strains were able to grow normally at 38°C, the *desA* mutant showed a decreased growth rate at 22°C. Interestingly, the mutants were not affected in photosynthesis but were limited in their ability to take up nutrients from the growth medium.

Unlike *E. coli*, *B. subtilis* induced several proteins in response to both heat and cold shocks. Of the six TIPs or temperature-inducible proteins, only CheY

was identified. CheY is involved in regulating rotation of the flagella. As mentioned earlier, a set of four temperature-inducible proteins were identified in the psychrotroph *Pseudomonas fragi* as well (Michel *et al.*, 1997). Since these proteins were induced following both cold shock and heat shock they were designated temperature adaptation proteins (Taps). Surprisingly, two of these proteins, TapA and TapB were identified as homologs of the *E. coli* major cold shock protein, CspA. While not all CspA homologs are induced by cold shock, none had been identified as heat shock proteins previously.

Relatively few cold shock proteins besides the CspA homologs have been identified in bacteria other than *E. coli*. The widespread occurrence of proteins similar to CspA does suggest, however, that the cold shock response is a well conserved response. Comparison of the best characterized species, *E. coli* and *B. subtilis*, demonstrates that parallels do exist in the types of proteins induced. Further similarities were found in diverse strains such as the cyanobacteria *Anabaena* and the psychrotroph, *Y. enterocolitica*. Characterizing the specific functions of the CspA proteins of *E. coli* and the RNA-binding proteins of cyanobacteria may reveal interesting parallels as well. Differences between the cold shock responses were also found. Unlike *E. coli*, both *B. subtilis* and *P. fragi* synthesized a set of proteins that responded to both heat shock and cold shock. Considering the opposing nature of the two stresses, characterizing the function of these general temperature proteins should prove very interesting.

Regulation of the cold shock response in *Escherichia coli*

As stated earlier, the cold shock response in *E. coli* is characterized by the overall inhibition of protein synthesis with the exception of the increased synthesis of the cold shock proteins. The cold shock proteins in *E. coli* can be divided into two classes (Thieringer *et al.*, 1998). Class I includes those proteins induced greater than 10-fold following a temperature downshift while class II contains those proteins that are induced from 2 to 5-fold. The cold-induced members of the CspA family and CsdA are class I proteins and are induced within an hour following a temperature downshift (Wang *et al.*, 1999). This response is transient and the levels of synthesis decline within three hours. The remaining cold shock proteins are class II proteins which are also induced quickly, reaching maximum synthesis by three hours (Jones *et al.*, 1987).

In *E. coli*, depending on the extent of the temperature downshift, overall cellular protein synthesis is inhibited for 2-4 hours following the temperature shift. In fact, this inhibition of ribosome activity by low temperature has been proposed to act as the signal stimulating the cold shock response. The evidence for this connection arose in part from a study that linked ribosome activity to the regulation of both heat shock and cold shock (VanBogelen and Neidhart, 1990). When cells were exposed to non-lethal concentrations of antibiotics affecting different aspects of ribosome function, the pattern of protein synthesis resembled either a cold shock response or a heat shock response depending on the antibiotic

used. The antibiotics that induced a response similar to a cold shock appeared to slow or block activity of the ribosome. The effects of amino acid starvation and ppGpp concentration on the cold shock response were investigated as well (Jones *et al.*, 1992a). Amino acid starvation or overproduction of the regulatory nucleotide, ppGpp, prior to a temperature downshift resulted in the delay or prevention of the cold shock response. Conversely, a *relA spoT* mutant that produced no ppGpp showed no growth lag when shifted to 10°C as compared to the 2 hour growth lag displayed by the wild type strain. Thus, the alteration of ribosome function either through the action of certain antibiotics or through amino acid starvation influenced the regulation of the cold shock response.

The "Cold-Shock Ribosome Adaptation" model proposed by Jones and Inouye (1996) takes into account the negative effect of low temperature on ribosome function and the potential regulatory interaction between ribosome activity and the cold shock response. According to this model, translation of most cellular transcripts is inhibited by a shift to low temperature signalling a cold shock response. In contrast to most cellular transcripts, the transcripts encoding cold shock proteins and ribosomal proteins can be translated by "non-adapted" ribosomes due to some unique property of these mRNAs. The newly synthesized cold shock proteins interact with the ribosome to adapt it to function at low temperature thus allowing overall protein synthesis to resume. If this model is correct, inhibiting the ribosome by some other mechanism besides low temperature

should induce a full cold shock. Ten of the fourteen cold shock proteins assayed increased following the addition of the "cold shock" antibiotic, tetracycline (VanBogelen and Neidhart, 1990). This partial cold shock response indicates that additional signals may be required.

In studying the regulatory mechanisms controlling the expression of the cold shock genes, attention has focused on CspA as a potential regulator. Overexpression of CspA, such that CspA levels were increased five-fold above normal levels following a cold shock, resulted in the increased induction of at least five cold shock proteins (Jones *et al.*, 1992b). One of the cold shock proteins affected by overexpression of CspA was DNA gyrase. CspA was further implicated in the regulation of DNA gyrase as it was shown to bind the *gyrA* promoter (Jones *et al.*, 1992b). CspA enhances the expression of the H-NS protein as well (La Teana *et al.*, 1991). While CspA did not bind strongly to the *hns* promoter, it did appear to enhance the binding and activity of RNA polymerase (La Teana *et al.*, 1991; Brandi *et al.*, 1994). A *cspA* null mutant was not greatly affected in growth or protein expression at either 37°C or 15°C with the exception of the increased expression of CspB, CspG, and CspE (Bae *et al.*, 1997). Presumably, the increased expression of these related proteins was able to compensate for the lack of CspA. Strains containing multiple deletions of CspA family genes will be necessary to assess the possible regulatory functions of these proteins.

The regulation of CspA, itself, has also been studied extensively. In most of these studies, cultures of *E. coli* were grown to mid-log phase at 37°C prior to a shift to 10 or 15°C. In these studies, the level of *cspA* mRNA was extremely low at 37°C and CspA protein synthesis was not detectable. These observations led to the conclusion that CspA was specifically synthesized for low temperature conditions. A recent study contradicts this conclusion as CspA was observed to be highly expressed at 37°C when stationary phase cells were first introduced into a fresh medium (Brandi *et al.*, 1999). Although expression of CspA declined as cell density increased, it remained detectable by Western blot. Previous studies had quantified the synthesis of CspA without regard to the pool of CspA protein already present in the cell. Measuring CspA levels by Western blot, Brandi *et al.* (1999) did find that CspA levels increased following a cold shock although the degree of induction ranged from 3 to 30-fold depending on the age of the initial culture. Regardless of the amount of CspA present in the cells at 37°C, the same level of CspA protein was attained following the cold shock. The finding that CspA is highly expressed at 37°C following the transfer of stationary phase cells to fresh medium may relate to the suggestion by Jones *et al.* (1992a) that the effect of a cold shock is similar to that of a nutritional upshift. In both cases, the capacity of the cell for protein synthesis is insufficient relative to the concentration of charged tRNAs present.

Following a cold shock, *cspA* mRNA levels were induced within 30

minutes of the temperature downshift. This change in message level did not result from a burst in promoter activity but rather from increased stability of the *cspA* message. Three nucleotide substitutions introduced just upstream of the *cspA* Shine-Dalgarno sequence significantly stabilized the *cspA* mRNA at 37°C, increasing the half-life from 12 seconds to 30 minutes CspA protein was highly expressed in this strain at 37°C indicating that the *cspA* promoter was active at both temperatures (Fang *et al.*, 1997). Cloning the *lpp* promoter in front of the *cspA* gene further illustrated the role of message stability in the induction of CspA synthesis (Fang *et al.*, 1997). The *cspA* message transcribed from the lpp^{P}-*cspA* fusion was identical to the wild type *cspA* mRNA. When the lpp^{P}-*cspA* fusion was integrated into the chromosome of a Δ*cspA* strain, no CspA protein was detected at 37°C despite the high constitutive activity of the *lpp* promoter. CspA protein was detected only after a cold shock to 15°C. Further characterization of *cspA* mRNA stability following a cold shock from 37°C to 15°C demonstrated that *cspA* mRNA was not permanently stabilized at low temperature. The *cspA* message reached a maximum half-life of over 60 minutes within the first half hour at 15°C. Message stability then decreased to a 10 minute half-life after an additional hour at 15°C (Goldenberg *et al.*, 1996).

The *cspA* gene and the other class I cold shock genes, *cspB*, *cspG*, *cspI*, and *csdA*, have a long untranslated region (UTR) at the 5' end of their message that is 145 to 226 bp in length (Figure 1.1) (Wang *et al.*, 1999; Jiang *et al.*, 1996).

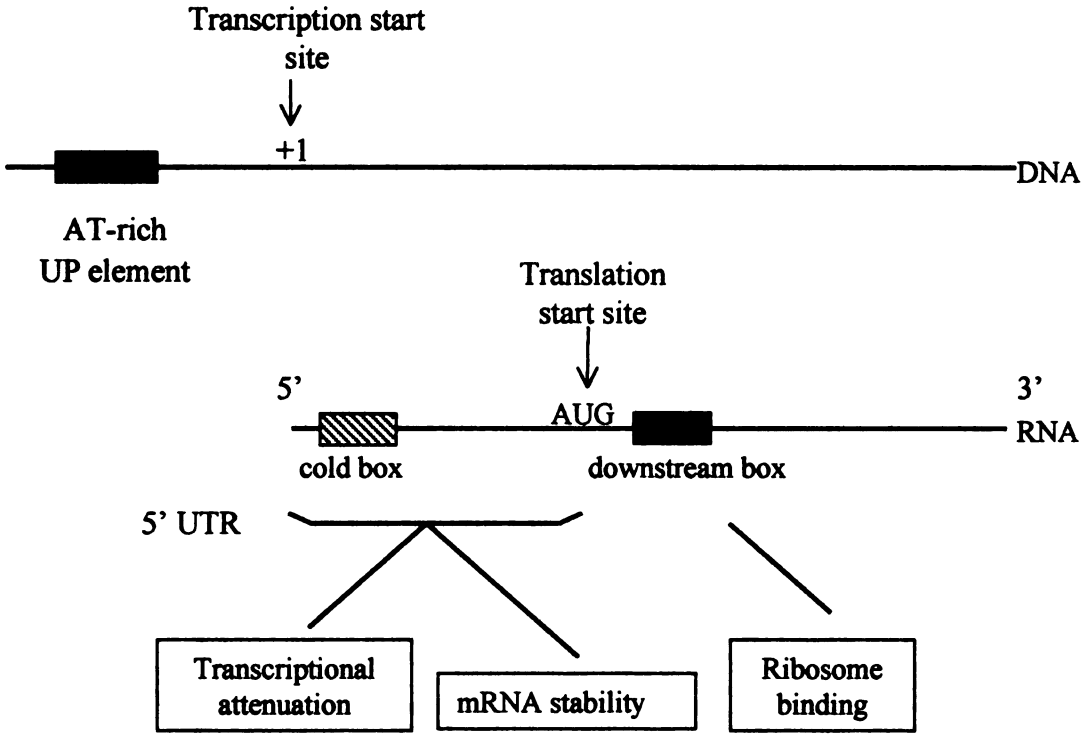

Figure 1.1. Regulatory elements controlling the expression of the cold shock protein, CspA. This figure is adapted from Thieringer *et al.* (1998).

Transcriptional fusions including the full 5' UTR of *cspA* showed increased reporter gene expression following a cold shock (Goldenberg *et al.*, 1997; Mitta *et al.*, 1997; Jiang *et al.*, 1996). While a fusion to +26 showed comparable message stability at 37°C, the full UTR sequence was required for transient stabilization at low temperature (Mitta *et al.*, 1997). Within the first 26 bp of the *cspA* message, there is an 11 bp conserved sequence known as the cold box (Figure 1.1). Overexpression of this sequence leads to the prolonged inhibition of cellular protein synthesis and the prolonged synthesis of the class I cold shock proteins following a temperature downshift (Jiang *et al.*, 1996; Fang *et al.*, 1998). The effect on protein synthesis achieved by the overexpression of this sequence indicates that a repressor protein may be binding this site after the cold shock. In addition, each of the class I cold shock proteins has a cold box sequence in its 5' UTR suggesting that these genes are coordinately regulated.

The 5' UTR alone does not account for the full induction of the CspA protein at low temperature. Mitta *et al.* (1997) constructed both transcriptional and translational fusions in their study of *cspA* gene expression. Although a transcriptional fusion to the *lacZ* gene showed induction of β-galactosidase activity following a cold shock, the 5' end of the *cspA* coding sequence was required for full induction. This region contains a "downstream box" that is complementary to the decoding region of the 16S rRNA thereby increasing the affinity of this message for the ribosome (Figure 1.1) (Sprengart and Porter, 1997).

The location and sequence of the downstream box is conserved among all of the cold-inducible *cspA* homologs in *E. coli* (Wang *et al.*, 1999; Mitta *et al.*, 1997). The downstream box in the cold shock gene transcripts may explain why these mRNAs can be translated immediately following a temperature downshift prior to adaptation of the ribosome to low temperature.

Bae *et al.* (1997) have proposed that CspA negatively regulates its own expression. In the construction of a Δ*cspA* mutant, only the *cspA* coding region was removed leaving an intact *cspA* promoter and 5' UTR. While the deletion of the coding region did not result in a growth defect, the expression of the *cspA* 5' UTR was altered. Both transcription and stability of the 5' UTR were affected following a cold shock. Immediately after a temperature downshift, the half-lives of both the wild type *cspA* transcript and the mutant *cspA* 5' UTR were over 60 minutes. The level of *cspA* 5' UTR in the Δ*cspA* mutant was approximately 3-fold higher than the level of the wild type gene. These results indicate that immediately after a cold shock the transcription of the *cspA* gene in the mutant strain has increased. Three hours after the cold shock both the *cspA* and *cspB* transcripts in the mutant strain had almost doubled in stability relative to the wild type strain. The authors suggest that CspA may affect its own expression in two ways. First, as a RNA chaperone, CspA may bind its own message and enhance its degradation. A second mechanism may be through transcriptional attenuation, potentially mediated through the binding of CspA to its own message (Bae *et al.*,

1997).

The "Cold-Shock Ribosome Adaptation" model proposed by Jones and Inouye (1996) describes one mechanism involved in sensing temperature and stimulating the cold shock response, but it is likely that other mechanisms are involved as well. The exact regulatory mechanisms leading to the induction of cold shock protein synthesis are still not clear. While overexpression of CspA led to increased levels of several cold shock proteins, other data suggests that CspA acts as a RNA chaperone. The regulation of CspA, itself, involves the interaction of several mechanisms. The extended 5' UTR is responsible for *cspA* message instability at 37°C and its transient stabilization after a cold shock. In addition, the cold box at the 5' end of the *cspA* transcript appears to be involved in negative regulation at low temperature, possibly through the binding of CspA to its own transcript. The downstream box may account for the extremely efficient translation of the *cspA* message at low temperature. Whether the *cspA* promoter plays a significant role during a cold shock is still not known as no +1 fusion has been examined at low temperature. Finally, the growth phase dependent regulation of CspA synthesis described by Brandi *et al.* (1999) may give new insights into the regulation of the CspA family of proteins and the cold shock response.

The cold shock response in *Sinorhizobium meliloti*

Sinorhizobium meliloti 1021, as a soil bacterium, must survive a wide range of temperatures, including those well below its optimum growth temperature of 30°C. Considering the apparent conservation of the cold shock response among different bacterial species, it seems likely that *S. meliloti* 1021 possesses a mechanism for adapting to sub-optimal temperatures. Preliminary studies of a related strain, *Rhizobium meliloti* A2, performed by Cloutier *et al.* (1992) indicated that this bacterium was capable of protein synthesis at temperatures below its minimum growth temperature of 7°C. However, the identity of the proteins synthesized at low temperature, their relative synthesis at optimal temperature, and their importance for the survival of *R. meliloti* A2 at low temperature was not determined. In our study of *S. meliloti* 1021, we hoped to identify those genes whose expression increased at low temperature, to assess their importance to growth at low temperature, and to begin to characterize their regulation in response to temperature.

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CHAPTER 2

Identification of low temperature induced genes in *Sinorhizobium meliloti* 1021

Summary

Transposon mutagenesis of *Sinorhizobium meliloti* 1021 was conducted in an effort to identify genes whose products might be involved in adaptation to sub-optimal growth temperatures. The transposon Tn*5*-1062 containing the promoterless *luxAB* genes of *Vibrio harveyi* was introduced into the wild type strain *S. meliloti* 1021. Approximately 23,000 transposon recipients were screened for those exhibiting increased luminescence after a temperature shift from 30°C to 15°C. Seven strains were isolated. Sequencing of the insertion sites revealed that in five of the strains the transposon had inserted in either a 16S or a 23S rRNA gene. Southern blot analysis indicated that the transposons had inserted into two of the three ribosomal RNA operons present in *S. meliloti* 1021. Direct mutagenesis of the three ribosomal RNA operons indicated that the *luxAB* genes were affected by the temperature shift to a similar extent in each of the three operons. The upstream region of the ribosomal RNA operon containing the transposon in strain RM3166 was isolated and cloned in front of the *luxAB* genes

on the broad host range plasmid, pRK290. This 605 bp fragment was shown to contain the sequences required to promote the temperature-dependent expression of the *luxAB* genes.

Introduction

As a soil bacterium, *Sinorhizobium meliloti*, must adapt to a wide range of temperatures. While the response of microorganisms to high temperature is relatively well understood, the adaptive response of bacteria to low and freezing temperatures is just beginning to be described. The cold shock response has been examined in several bacterial species including several Rhizobia (Cloutier *et al.*, 1992), *Bacillus subtilis* (Graumann *et al.*, 1996), *Escherichia coli* (Jones *et al.*, 1987), *Lactococcus lactis* (Panoff *et al.*, 1994), and the psychrotrophs *Arthrobacter globiformis* SI55 (Berger *et al.*, 1996), *Bacillus psychrophilus* (Whyte and Inniss, 1992), and *Pseudomonas fragi* (Michel *et al.*, 1997). The cold shock response occurs following a sudden drop in growth temperature and involves the induction of a set of proteins, termed the cold shock proteins. Overall protein synthesis is often inhibited immediately following a temperature downshift resulting in a temporary growth lag.

While the cold shock response has been observed in several bacterial species and yeast, it has been studied most thoroughly in *E. coli*. Most of the induced proteins, or cold shock proteins, are also synthesized at optimum temperatures and are only induced between 2 and 10-fold (Jones *et al.*, 1987). These proteins include polynucleotide phosphorylase, NusA, IF2α/β, RecA, dihydrolipoamide acetyltransferase, pyruvate dehydrogenase, DNA gyrase A, H-

NS (La Teana *et al.*, 1991), RbfA (Jones and Inouye, 1996), Hsc66 (Lelivelt and Kawula, 1995), and trigger factor (Kandror and Goldberg, 1997). These proteins are known to have roles in important cellular processes including mRNA degradation, transcriptional termination and antitermination, translation, chromosome structure, energy metabolism and protein structure. In addition, several ribosomal proteins continue to be synthesized and in some cases are reported to be moderately induced despite the overall inhibition of protein synthesis (Jones and Inouye, 1994; Jones *et al.*, 1996).

Another class of cold shock proteins includes those which are expressed primarily at low temperatures. Four of these proteins, CspA, CspB, CspG and CspI, are highly induced following a cold shock from 37°C to 15°C (Goldstein *et al.*, 1990; Lee *et al.*, 1994; Nakashima *et al.*, 1996; Wang *et al.*, 1999). The synthesis of CspA alone accounts for 13% of total protein synthesis within an hour of a temperature downshift to 15°C. These proteins are members of the CspA family of proteins that share significant similarity with the cold shock domain of the eukaryotic Y-box protein family (Wistow, 1990). Proteins homologous to CspA have been identified in many bacteria. In addition to being implicated in the transcriptional regulation of two cold shock genes, *hns* and *gyrA*, CspA has also been shown to bind cooperatively to single-stranded RNA and to destabilize secondary structure (La Teana *et al.*, 1991; Jones *et al.*, 1992b; Jiang *et al.*, 1997). Because of its affinity for RNA, CspA has been proposed to function as a

RNA chaperone in preventing formation of secondary structures that could interfere with translation of mRNA. Another protein, CsdA, is also expressed predominantly at low temperature although it is not induced as highly as CspA and CspB (Jones *et al.*, 1996). CsdA contains the DEAD motif which is highly conserved among RNA and DNA helicases and has been shown to unwind double-stranded RNA. As CsdA is associated with both ribosomal subunits at low temperature in approximately stoichiometric amounts, it has been speculated that CsdA may aid in translation at low temperature by removing deleterious secondary structures in mRNA.

Protein synthesis has been shown to be the most cold-sensitive process in *Escherichia coli* and, specifically, the initiation of translation has been shown to be inhibited at low temperatures (Friedman *et al.*, 1971). Interestingly, a set of antibiotics that inhibit ribosome function can stimulate a cold shock response at 37°C when added to cultures in non-lethal amounts (VanBogelen and Neidhart, 1990). Based on these observations and the induction of several proteins involved in translation, a model of the cold shock response as a mechanism for adapting the ribosome to low temperature has been developed (Jones and Inouye, 1996). The actions of the cold shock proteins RbfA, CsdA, IF2 and CspA are thought to enable the ribosome to function at low temperature. These proteins are either associated with the ribosome itself or involved in destabilizing secondary structures in RNA. CsdA and CspA are thought to interact with mRNA, removing

any secondary structures that may interfere with their translation.

Whether this model is accurate for the cold shock response in *Sinorhizobium meliloti* 1021 is not clear. While Cloutier *et al.* (1992) showed that several proteins continued to be synthesized in *Rhizobium meliloti* A2 following a cold shock to temperatures as low as 0°C, the identity and function of these proteins is unknown. Clearly, there is much to be learned about the response of *Sinorhizobium meliloti* 1021 to low temperature.

In performing transposon mutagenesis of *S. meliloti* 1021, we hoped to identify those genes whose expression was induced following a temperature downshift. This method was thought to give an advantage over two-dimensional gel electrophoresis in allowing the isolation and tagging of the induced genes. A Tn*5* derivative, Tn*5*-1062, was used in this study as Tn*5* transposons have been shown to work well in the mutagenesis of *S. meliloti* (Milcamps *et al.*, 1998). This reporter transposon carries the promoterless *Vibrio harveyi luxAB* genes encoding bacterial luciferase (Meighen, 1991). Luciferase releases photons when catalyzing the following reaction: $FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O$. The *luxAB* genes are expressed when the transposon inserts in the correct orientation downstream of an active promoter. In this way, Tn*5*-1062 can be used as an indicator of promoter activity. Since the enzyme substrate, dodecyl aldehyde, can penetrate the cellular membrane, luciferase activity can be assayed without disrupting the bacterial cells and the same colonies can be assayed multiple times.

We present here the isolation of seven cold-inducible Tn*5*-1062 insertion mutants. Five of these insertions are located within ribosomal RNA genes. These insertions are mapped to two of the three *rrn* operons known to exist in *S. meliloti* 1021 (Honeycutt *et al.*, 1993). Sequences required for the cold-inducible luminescence of the *luxAB* genes were isolated on a 605 bp *Bgl*II-*Sac*I fragment of one of the *rrn* promoter regions.

Results

Isolation of transposon mutants induced by low temperature.

Sinorhizobium meliloti 1021 was mutagenized with the transposon Tn*5*-1062 carrying the promoterless *luxAB* gene cassette (Figure 2.1B). When inserted in the correct orientation downstream of an active promoter, the transposon's *luxAB* genes are transcribed as part of the endogenous transcript. The transposon was moved into the cells on the plasmid pRL1062 via triparental matings (Figure 2.1A). This plasmid is not replicated in *S. meliloti* 1021, so any cell gaining kanamycin resistance must have the transposon inserted in the chromosome. After selecting for kanamycin resistance, the resulting colonies were screened for those that exhibited increased luminescence after a temperature downshift from 30°C to 15°C or from 30°C to 10°C.

Most transposon recipients showed a slight increase in luminescence after 4 to 6 hours at the lower temperature (Figure 2.2). This 2 to 5-fold increase was considered to be a background increase in activity resulting from a greater stability of either the *luxAB* mRNA or the luciferase protein at the lower temperature. Three rounds of mutagenesis and screenings were performed resulting in the isolation of seven mutant strains showing markedly increased luminescence after a temperature downshift. The first round of screening involved a temperature shift from 30°C to 10°C. The mutant, RM3166 (Figure 2.3A), was isolated after

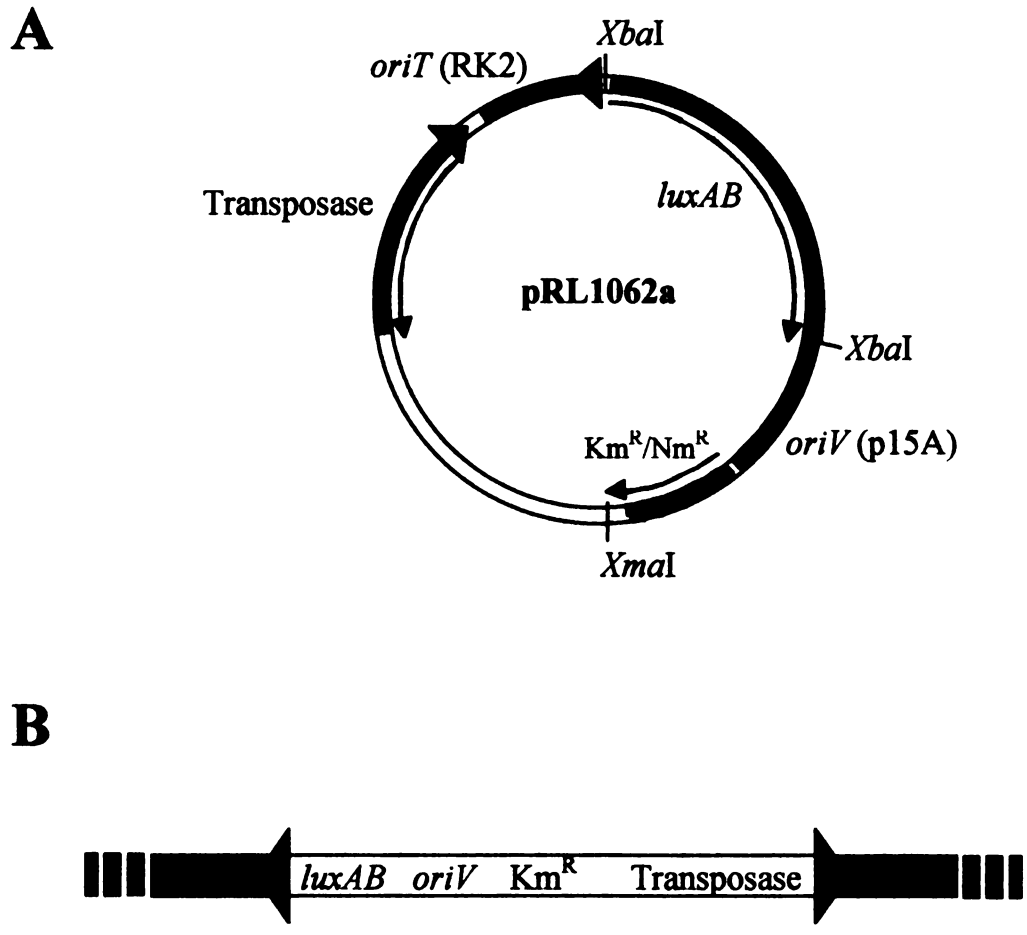


Figure 2.1. Structure of pRL1062a and Tn*5*-1062 inserted in genome. The plasmid pRL1062a was used to move Tn*5*-1062 into *S. meliloti* via conjugation. (A) The plasmid carries the origin of transfer, *oriT*(RK2), and the transposon delimited by 52 bp of the IS*50*L, ◀ , and its inverted repeat, ▶ , in IS*50*R. The *oriV*(p15A) located within the transposon supports replication of this plasmid in *E. coli* but not in *S. meliloti*. Arrows indicate the direction of transcription of the various genes. The *luxAB* genes are located within the *Xba*I fragment. (B) Tn*5*-1062 is shown after transposition into the bacterial genome. The inverted repeats of IS*50*L, ◀ , and IS*50*R, ▶ , delimit the transposon sequence. If the transposon inserts in the genomic DNA downstream of an active promoter in the correct orientation, the *luxAB* genes will be transcribed.

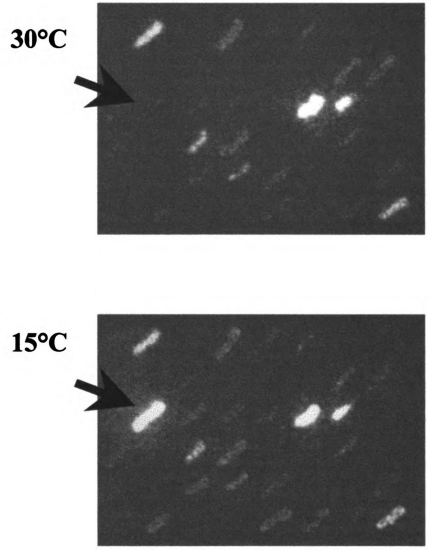


Figure 2.2. Screen for Tn*5*-1062 inserts induced by a temperature downshift. 48 Tn*5*-1062 recipients were streaked on TY agar containing kanamycin (200 μg/ml) and streptomycin (250 μg/ml). The plates were incubated for 2 days at 30°C, exposed to aldehyde and the luminescence recorded with a photonic camera (top photo). The plates were transferred to 15°C for 5 1/2 hours, re-exposed to aldehyde and observed again (bottom photo). The arrow indicates the mutant strain RM603.

Figure 2.3. Set of seven Tn*5*-1062 inserts induced by a temperature downshift. (A) Strains were cultured on TY agar plates at 30°C for two days and then assayed for luminescence. The plates were then shifted to 15°C for 5 hours before being assayed again. (B) Strains were grown in TY broth to an A_{600} of approximately 0.4 and assayed for luminescence before (30°C) and after (15°C) a temperature downshift. Each column represents the mean $\pm$ SD of three measurements per strain. This graph is adapted from O'Connell *et al.* (1999).

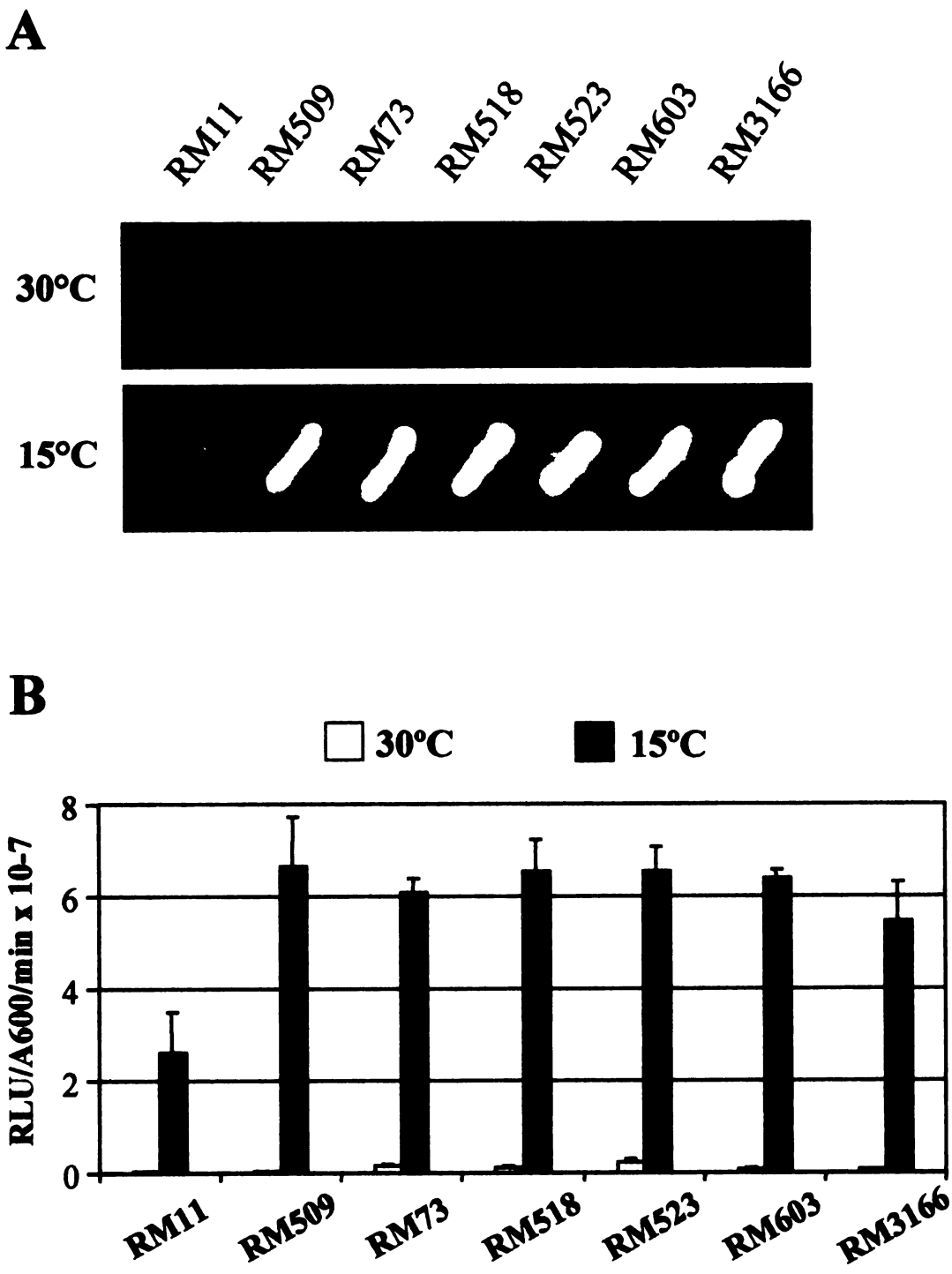

A
RM11
RM509
RM73
RM518
RM523
RM603
RM3166
30°C
15°C
B
30°C
15°C
RLU/A600/min x 10-7
8
6
4
2
0
RM11
RM509
RM73
RM518
RM523
RM603
RM3166

Figure 2.3

examining approximately 17,000 kanamycin resistant colonies. Five additional strains were isolated, including RM518, RM523, and RM603, in a second round of mutagenesis and screening using a temperature shift from 30°C to 15°C (Figure 2.3A). Approximately 5,000 transposon recipients were screened in this round. One thousand more mutants were analyzed in a third round of screening utilizing the same temperature shift but a minimal growth medium, GTS, instead of the rich medium, TY, used previously. This last screen resulted in the isolation of strain RM73 (Figure 2.3A).

The seven Tn*5*-1062 recipient strains consistently showed a marked increase in light production after a temperature downshift when colonies were assayed for luciferase activity with a photonic camera. In order to more accurately quantitate the change in luminescence, the mutant strains were grown in broth and light production was quantified using a luminometer (Figure 2.3B). When analyzed in this way, luminescence could be adjusted to cell density allowing more accurate comparisons between samples and time points. The cultures were grown up at 30°C and assayed immediately prior to being shifted to 15°C and five hours after the temperature shift. The mutant strains showed large increases in luminescence after a cold shock. Luminescence in mutants RM11 and RM509 increased over 100-fold while luminescence in the remaining mutants increased between 26 and 76-fold.

Identification of the cold induced loci. In order to characterize the transposon insertion sites, the Tn*5*-1062 transposons along with the neighboring genomic DNA were cloned out of each strain. The Tn*5*-1062 transposon contains an origin of replication, p15a, recognized in *Escherichia coli* allowing the transposon to be isolated and maintained as a plasmid outside of *S. meliloti* (Figure 2.1A). Genomic DNA isolated from the strains was typically digested with an enzyme that did not cut within the transposon. In some cases an enzyme that did cut within the transposon was used but was selected such that the region encoding kanamycin resistance and containing the origin of replication remained intact. The resulting restriction fragments were circularized and transformed into *E. coli* DH5α.

The genomic DNA flanking the transposons was sequenced and compared to known sequences. The DNA contiguous with the transposon in RM3166 was nearly identical to a region of a 16S rRNA gene in *R. meliloti* LMG 613 (Figure 2.4) while the transposon insertion site in RM73 was very similar to a 23S rRNA gene in *Agrobacterium vitis* (Figure 2.5). Analysis of the remaining mutants revealed that the transposons in 5 of the 7 mutants were inserted in rRNA genes (Table 2.1). The transposons in mutants RM518, RM603 and RM3166 were in 16S rRNA genes and the transposons in RM73 and RM523 were inserted in 23S rRNA genes. The two remaining insertion mutants, RM11 and RM509, contained insertions in an operon encoding homologs of the *E. coli* proteins, CspA and S21

Figure 2.4. Sequence alignment of the RM3166 transposon insertion site and the 16S *rrn* gene of *Rhizobium meliloti* LMG 613. DNA contiguous with the transposon in RM3166 was sequenced. A BLASTN search (Altschul *et al*, 1997) revealed that the sequence was similar to the 16S *rrn* of *Rhizobium meliloti* LMG 613 (accession number, X67222). **A** = RM3166, **B**= *R. meliloti* LMG 613, "|"= identity, ":" = mismatch. Gaps were included in sequence (-) to facilitate alignment.

```
A    77 GGGCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGA
        ||||||||||||||||||||||||||||||||||||||||||||||||||
B   840 GGGCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGA

A   127 ACCTTACCAGCCCTTGACATCCCGATCGCGGATACGAGAGATCGTATCCT
        ||||||||||||||||||||||||||||||||||||||||||||||||||
B   890 ACCTTACCAGCCCTTGACATCCCGATCGCGGATACGAGAGATCGTATCCT

A   177 TCAGTTCGGCTGGATCGGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGT
        ||||||||||||||||||||||||||||||||||||||||||||||||||
B   940 TCAGTTCGGCTGGATCGGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGT

A   227 GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTA
        ||||||||||||||||||||||||||||||||||||||||||||||||||
B   990 GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTA

A   277 GTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCG
        ||||||||||||||||||||||||||||||||||||||||||||||||||
B  1040 GTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCG

A   327 AGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGC
        ||||||||||||||||||||||||||||||||||||||||||||||||||
B  1090 AGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGC

A   377 TACACACGTGCTAC-ATGGTGGTGACAGTGGGCAGCGAGACCGCGAGGTC
        ||||||||||||||:||||||||||||||||||||||||||||||||||
B  1140 TACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGACCGCGAGGTC

A   426 GACTGAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGA
        ||:::||||||||||||||||||||||||||||||||||||||||||||
B  1190 GAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGA

A   476 GTGCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCATGCTGC-GTGA
        ||||||||||||||||||||||||||||||||||||||||||||:||||
B  1240 GTGCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCATGCTGCGGTGA

A   525 ATACGTTCCGG 535
        |||||||||:|
B  1290 ATACGTTCCCG 1300
```

Figure 2.4

Figure 2.5. Sequence alignment of the RM73 transposon insertion site and the 23S *rrn* gene of *Agrobacterium vitis*. The DNA bordering the transposon in RM73 was sequenced. A BLASTN search (Altschul *et al*, 1997) revealed significant identity to the 23S *rrn* gene in *A. vitis* (accession number U45329). **A** = RM73, **B** = *A. vitis*, "|"= identity, ":" = mismatch. Gaps were included in sequence (-) to facilitate alignment.

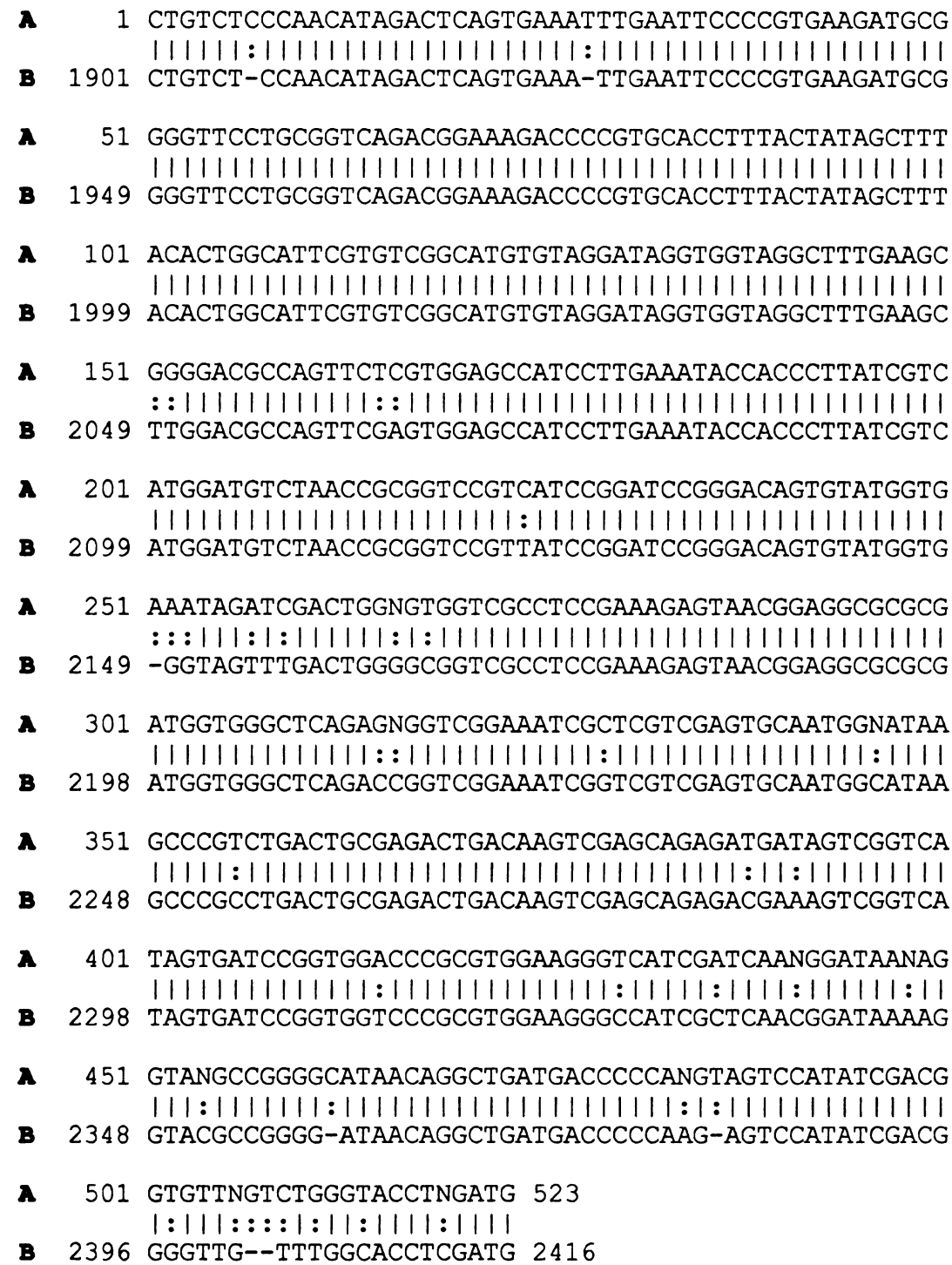

A 1 CTGTCTCCCAACATAGACTCAGTGAAATTTGAATTCCCCGTGAAGATGCG
B 1901 CTGTCT-CCAACATAGACTCAGTGAAA-TTGAATTCCCCGTGAAGATGCG
A 51 GGGTTCCTGCGGTCAGACGGAAAGACCCCGTGCACCTTTACTATAGCTTT
B 1949 GGGTTCCTGCGGTCAGACGGAAAGACCCCGTGCACCTTTACTATAGCTTT
A 101 ACACTGGCATTCGTGTCGGCATGTGTAGGATAGGTGGTAGGCTTTGAAGC
B 1999 ACACTGGCATTCGTGTCGGCATGTGTAGGATAGGTGGTAGGCTTTGAAGC
A 151 GGGGACGCCAGTTCTCGTGGAGCCATCCTTGAAATACCACCCTTATCGTC
B 2049 TTGGACGCCAGTTCGAGTGGAGCCATCCTTGAAATACCACCCTTATCGTC
A 201 ATGGATGTCTAACCGCGGTCCGTCATCCGGATCCGGGACAGTGTATGGTG
B 2099 ATGGATGTCTAACCGCGGTCCGTTATCCGGATCCGGGACAGTGTATGGTG
A 251 AAATAGATCGACTGGNGTGGTCGCCTCCGAAAGAGTAACGGAGGCGCGCG
B 2149 -GGTAGTTTGACTGGGGCGGTCGCCTCCGAAAGAGTAACGGAGGCGCGCG
A 301 ATGGTGGGCTCAGAGNGGTCGGAAATCGCTCGTCGAGTGCAATGGNATAA
B 2198 ATGGTGGGCTCAGACCGGTCGGAAATCGGTCGTCGAGTGCAATGGCATAA
A 351 GCCCGTCTGACTGCGAGACTGACAAGTCGAGCAGAGATGATAGTCGGTCA
B 2248 GCCCGCCTGACTGCGAGACTGACAAGTCGAGCAGAGACGAAAGTCGGTCA
A 401 TAGTGATCCGGTGGACCCGCGTGGAAGGGTCATCGATCAANGGATAANAG
B 2298 TAGTGATCCGGTGGTCCCGCGTGGAAGGGCCATCGCTCAACGGATAAAAG
A 451 GTANGCCGGGGCATAACAGGCTGATGACCCCCANGTAGTCCATATCGACG
B 2348 GTACGCCGGGG-ATAACAGGCTGATGACCCCCAAG-AGTCCATATCGACG
A 501 GTGTTNGTCTGGGTACCTNGATG 523
B 2396 GGGTTG--TTTGGCACCTCGATG 2416

Figure 2.5

Table 2.1. Identification of transposon insertion sites in Tn*5*-1062 mutants.

Strain	Sequence analyzed	Best gene match	Organism[a]	BLASTN score[b]	E value[b]
RM518	150 bp	16S *rrn*	*Azospirillum* sp. Z29621	248	2e-64
RM603[c]	252 bp	16S *rrn*	*R. meliloti* LMG 613 X67222	500	e-140
RM3166	538 bp	16S *rrn*	*R. meliloti* LMG 613 X67222	854	0.0
RM73	523 bp	23S *rrn*	*Agrobacterium vitis* U45329	731	0.0
RM523	323 bp	23S *rrn*	*Agrobacterium vitis* U45329	490	e-137

[a]The organism name and sequence accession number is given for the best sequence match for each insertion site.
[b]The BLASTN score and E value are given as reported in each BLASTN search (Altschul *et al*, 1997). The E value indicates how many sequences from the database would be expected to receive the indicated BLASTN score by chance.
[c]This sequence was identical to portions of seven 16S rRNA sequences including the one listed.

(O'Connell and Thomashow, 1999).

Mapping of transposon insertions to specific *rrn* operons. Honeycutt *et al.* (1993) identified three *rrn* operons in *S. meliloti* 1021. To determine whether the apparent temperature-dependent regulation was specific to a particular set of ribosomal RNA genes, the transposon inserts were mapped to the individual *rrn* operons. The enzyme *Sac*I does not cut within or between the 16S or 23S rRNA genes, allowing three fragments representative of the three *rrn* operons to be resolved on a Southern blot (Figure 2.6A, RM1021). Southern blot analysis of RM3166 and RM603 showed that the transposon was in the *rrn* operon contained in fragment 1 as this band was missing in these strains and another, larger band was present (Figure 2.6). In RM518, RM73, and RM523 the transposons were inserted in the *rrn* operon represented by fragment 2 as this band had been shifted due to the presence of the transposon (Figure 2.6). The question then arose as to whether an insert in the third operon would also be induced by low temperature. When a vector containing a fragment of the 16S *rrn* gene fused to the *luxAB* genes was recombined into all three of the *rrn* operons, the inserts appeared to be regulated by temperature in a similar fashion (data not shown).

Fusing the 16S *rrn* upstream region to the *luxAB* genes. Because all of the transposons were inserted well within the rRNA genes, the *luxAB* transcript was joined to a significant amount of either the 16S or 23S rRNA in each mutant. The ribosomal RNA transcript contains a large amount of secondary structure,

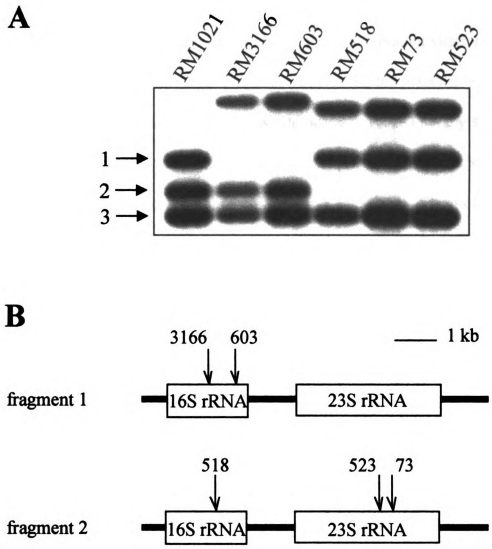


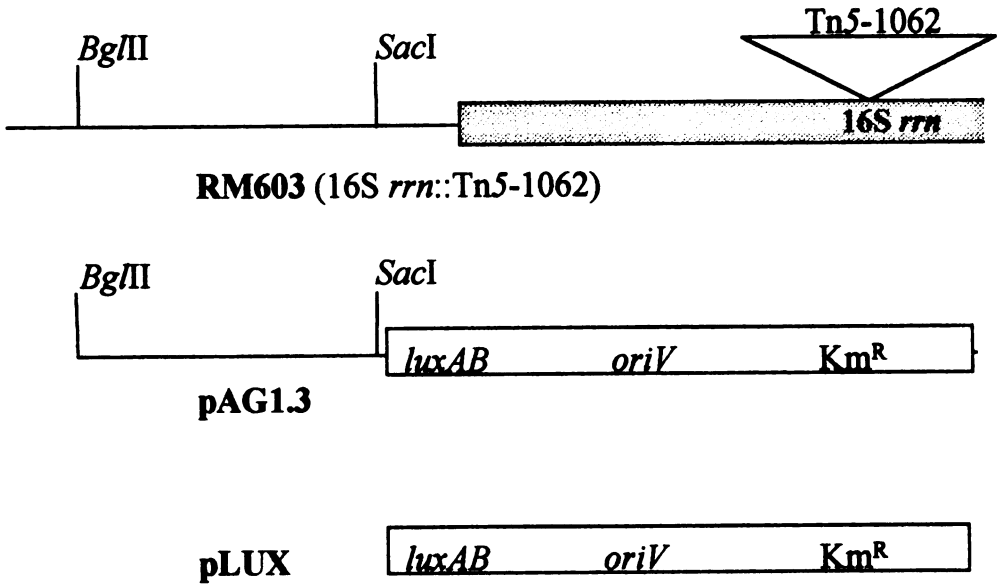
Figure 2.6. Mapping the transposon inserts to the *rrn* operons. (A) Genomic DNA isolated from the mutant strains and *S. meliloti* 1021 was digested with *Sac*I and electrophoresed on an agarose gel. The DNA was tranferred to a nylon membrane and probed with ^{32}P-labeled 23S rDNA. The three *rrn* operons are separated onto three fragments, see RM1021. The presence of Tn*5*-1062 within an operon is shown by the increase in size of a band relative to its position in the wild type sample, RM1021. (B) Maps of two of the three *rrn* operons are shown. Based on sequence comparison and the Southern blot analysis (A), the relative positions of the different transposon insertion sites are depicted.

undergoes significant processing and interacts with the ribosomal proteins causing concern that even the partial rRNA transcript in each mutant strain may adversely affect the expression of the *luxAB* genes. Therefore, the plasmid, pAG1.3, was constructed so that the *luxAB* genes would not be preceded by any structural rRNA. Based on the location of the transposon within the 16S rRNA gene in RM3166 as compared to the *E. coli* 16S rRNA gene, the location of the mature end of the 16S rRNA gene was predicted to be approximately 1 kb upstream of the transposon insert. In *E. coli*, two tandem *rrn* promoters are located 190 bp from the 5' end of the 16S rRNA gene (Srivastava and Schlessinger, 1990). After allowing for this leader region, the *Bgl*II-*Sac*I fragment was selected for cloning as the *rrn* promoter was predicted to be within that region (Figure 2.7A). The plasmid, pAG1.3, was constructed by cloning the *Bgl*II-*Sac*I fragment from the 16S *rrn* upstream region in front of the *luxAB* genes on the broad host range plasmid pRK290 (Figure 2.8). The plasmid, pLUX, was constructed to serve as a negative control and is identical to pAG1.3 except for not containing the *Bgl*II-*Sac*I fragment (Figure 2.7A).

The two plasmids were moved into the wild type strain, RM1021, and their luminescence compared to an original insertion mutant, RM603 (Figure 2.7B). The transposon in RM603 is inserted in the same 16S rRNA gene as is tagged in RM3166. Both RM1021/pAG1.3 and RM1021/pLUX emitted measurable light although pLUX contained no exogenous promoter. The luminescence from

Figure 2.7. Effect of a temperature downshift on the luminescence of the *luxAB* transcriptional fusion, pAG1.3. (A) The structures of pAG1.3, pLUX, and RM603 are shown. A 605 bp *Bgl*II-*Sac*I fragment containing a 16S *rrn* upstream region was subcloned from RM3166 and used to make a transcriptional fusion to *luxAB* (see Figure 2.8). The activity of this fusion was compared to that of RM603 (16S *rrn*::Tn*5*-1062) and pLUX, a plasmid identical to pAG1.3 but lacking the *Bgl*II-*Sac*I fragment. (B) GTS medium containing Km (50 μg/ml) was inoculated 1:1000 and allowed to grow to an A_{600} of 0.1-0.2 at 30°C. The cultures were sampled immediately prior to shifting the culture flasks to a 15°C water bath and 1, 2, 4, and 6 hours afterward. The aliquots were assayed for luciferase activity as described in Materials and Methods. Three cultures were grown for each construct and three aliquots taken from each flask at every time point. The relative light unit (RLU) measurements were averaged for each flask and divided by the A_{600} to correct for culture density. Each bar represents the average of values from three flasks ± the standard deviation.

A


BglII
SacI
Tn5-1062
16S rrn
RM603 (16S rrn::Tn5-1062)
BglII
SacI
luxAB
oriV
KmR
pAG1.3
pLUX
luxAB
oriV
KmR

B

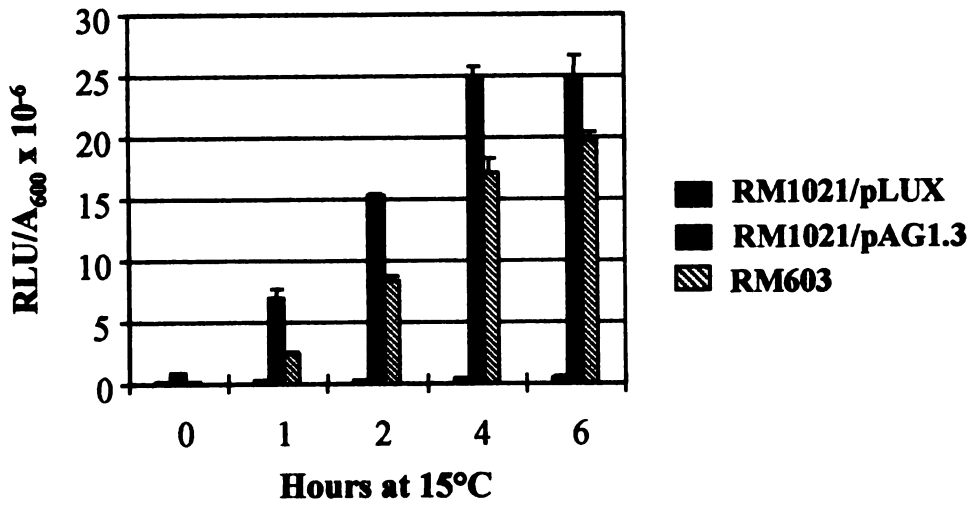

RLU/A600 x 10-6
30
25
20
15
10
5
0
0
1
2
4
6
Hours at 15°C
RM1021/pLUX
RM1021/pAG1.3
RM603

Figure 2.7

Figure 2.8. Construction of pAG1.3 and pLUX. The *rrn* promoter region directing the transcription of the *luxAB* genes in strain RM3166 was cloned using *Xma*I to form the plasmid, pAG1X. The *BamH*I fragment containing the promoter region and a portion of the 16S rRNA gene was then isolated from this plasmid and cloned into the *BamH*I site of pBluescript SK forming pAG1X7. The *BamH*I-*Sac*I fragment containing the *rrn* promoter region was isolated and treated with T4 DNA polymerase to form blunt ends. This fragment was then ligated to the larger *Bgl*II fragment of pRL1062 that had been treated with the Klenow fragment of DNA polymerase. The resulting intermediate plasmid was digested with *Bgl*II and *Xho*I. This fragment contained a 605 bp fragment of genomic DNA in front of the left portion of Tn*5*-1062. The plasmid pRK290 was digested with *Bgl*II and *Sal*I and the largest band isolated. The *Bgl*II-*Xho*I fragment was ligated to the *Bgl*II-*Sal*I fragment of pRK290 to form pAG1.3 which was then moved into *S. meliloti* 1021 via triparental mating. The plasmid, pLUX, was constructed to serve as a negative control in the luciferase assays. This plasmid was made by digesting pRL1062 with *Bgl*II and *Xho*I and isolating the fragment containing the *luxAB* genes. This fragment was then ligated to the *Bgl*II-*Sal*I fragment of pRK290 described above. This plasmid is identical to pAG1.3 but lacks the 605 bp *rrn* promoter fragment sub-cloned from RM3166. Restriction enzyme sites used in a cloning step are indicated by an asterisk. The 52 bp inverted repeats at each end of the transposon are represented by arrowheads.

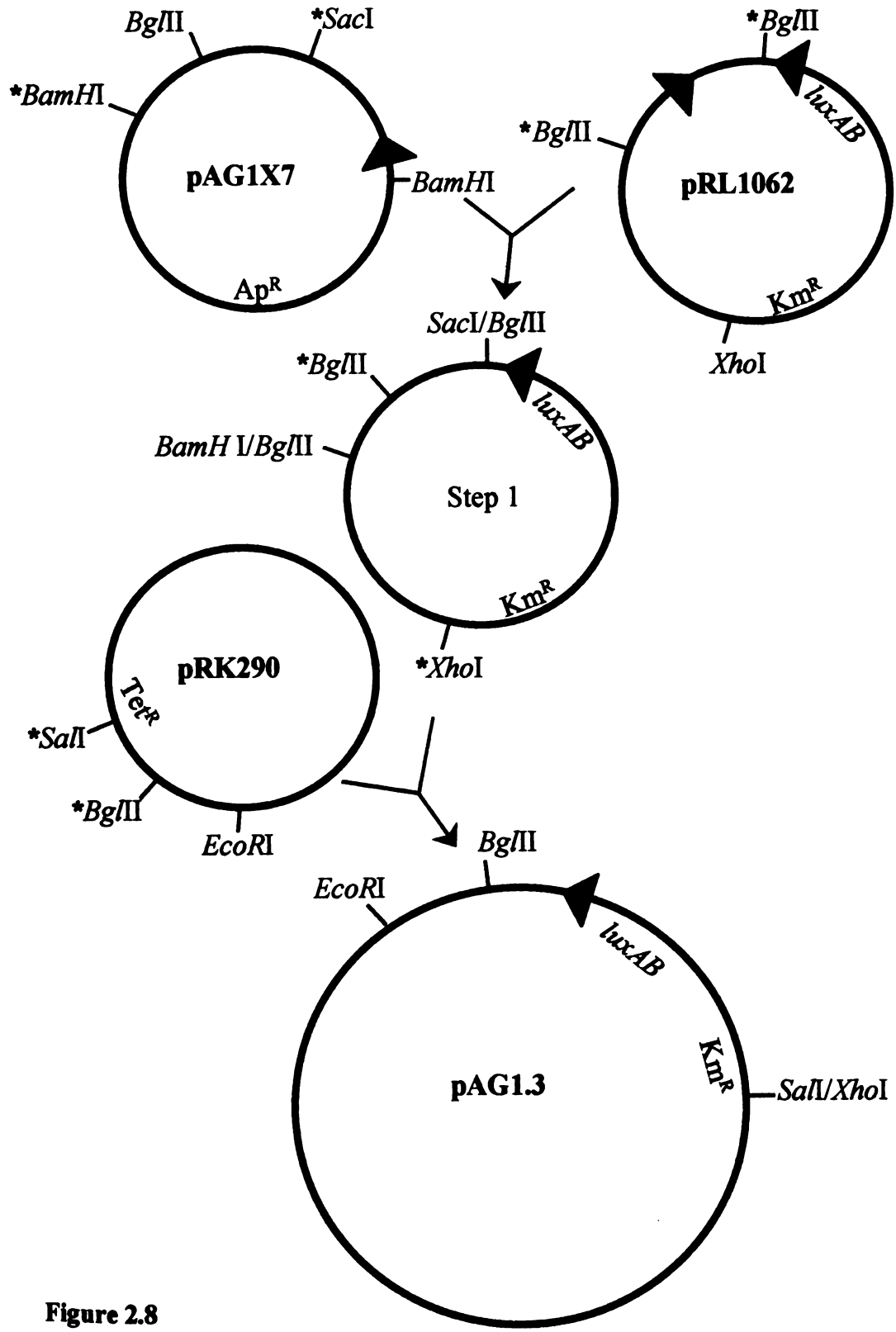

BglII
*SacI
*BamHI
pAG1X7
BamHI
Ap^R
*BglII
luxAB
*BglII
pRL1062
Km^R
XhoI
SacI/BglII
*BglII
luxAB
BamH I/BglII
Step 1
Km^R
*XhoI
pRK290
Tet^R
*SalI
*BglII
EcoRI
BglII
EcoRI
luxAB
Km^R
pAG1.3
SalI/XhoI

Figure 2.8

RM1021/pLUX is most likely the result of transcription originating from pRK290 sequences serving as a cryptic promoter. The amount of luminescence produced by RM1021/pAG1.3 at 30°C was approximately 10-fold higher than that produced by RM603. This may be due to the multiple copies of the plasmid-borne promoter-*luxAB* fusion as compared to the single transposon insert in RM603. While both RM1021/pAG1.3 and RM603 increased in luminescence to comparable levels after the temperature downshift, the calculated inductions, 28-fold and 219-fold respectively, were quite different. The difference in induction is primarily due to the relatively elevated luminescence of RM1021/pAG1.3 at 30°C. While the induction of RM1021/pAG1.3 luminescence is lower than that of RM603, the 28-fold increase was still higher than the 4.6-fold increase in luminescence from RM1021/pLUX. The increase in luminescence observed with RM1021/pLUX is comparable to what was observed when screening the random Tn*5*-1062 inserts for induced loci (Figure 2.2). Thus, the *Bgl*II-*Sac*I fragment contained sequences required for the cold-induced expression of the *luxAB* genes in RM1021.

Analysis of *luxAB* transcript levels following a temperature downshift. The induction of luminescence in the *rrn* insertion mutants is quite high and varies significantly between different growth media. For instance, the increase in luminescence in RM603 when grown in TY broth is approximately 74-fold (Figure 2.3) as compared to the 219-fold induction measured during growth in GTS

medium (Figure 2.7). To determine whether the increase in luminescence was paralleled by an increase in *luxAB* message, RNA was isolated from two *rrn* insertion mutants and RM1021/pAG1.3, before and after a temperature downshift. Strains RM518 and RM73 contain Tn*5*-1062 insertions in a 16S or a 23S rRNA gene respectively (Figure 2.6). Figure 2.9 shows the result of a Northern blot probed with ^{32}P-labeled *luxAB* DNA. In all three strains, levels of the *luxAB* transcript increased within an hour at 15°C and were still elevated after 6 hours. The pattern of message accumulation is different than the pattern of luminescence at 15°C (compare to Figure 2.7B). While maximum levels of the *luxAB* transcript are reached within 1-2 hours, luminescence is still increasing up to 6 hours following the temperature shift. The Northern blot did confirm, however, that levels of *luxAB* transcript increased at 15°C in the insertion mutants and in RM1021/pAG1.3.

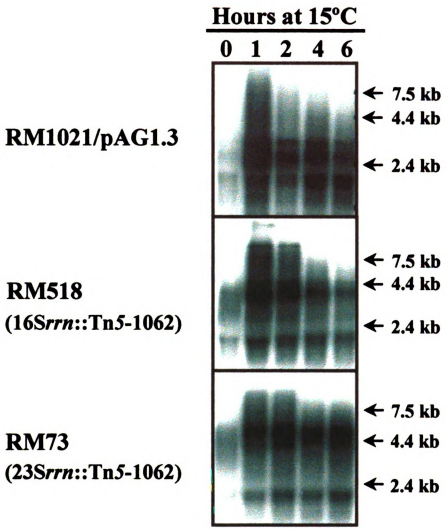


Figure 2.9. Levels of *luxAB* transcript following a temperature downshift from 30°C to 15°C. Strains RM1021/pAG1.3, RM518 and RM73 were grown in TY broth at 30°C until reaching an A_{600} of 0.2. Aliquots were taken before shifting the flasks to a 15°C shaking water bath and 1, 2, 4, and 6 hours following the temperature downshift. RNA was isolated from 5 ml aliquots as described in Materials and Methods. 2 μg of each RNA sample were separated on an agarose gel and transferred to a nylon membrane. The membranes were probed with a ^{32}P-labeled *luxAB* DNA fragment.

Table 2.2. Bacterial strains

Strains	Description	Reference or source
Sinorhizobium meliloti		
RM1021	Str^R; derivative of SU47	Meade *et al*, 1982
RM11	Km^R; RM1021 containing Tn*5*-1062 insert in ORF2	O'Connell and Thomashow, 1999
RM509	Km^R; RM1021 containing Tn*5*-1062 insert between *cspA* and ORF2	O'Connell and Thomashow, 1999
RM73	Km^R; RM1021 containing Tn*5*-1062 insert in the 23S *rrn*B gene	This study
RM518	Km^R; RM1021 containing Tn*5*-1062 insert in the 16S *rrn*B gene	This study
RM523	Km^R; RM1021 containing Tn*5*-1062 insert in the 23S *rrn*B gene	This study
RM603	Km^R; RM1021 containing Tn*5*-1062 insert in the 16S *rrn*A gene	This study
RM3166	Km^R; RM1021 containing Tn*5*-1062 insert in the 16S *rrn*A gene	This study
Escherichia coli		
DH5α	*supE44 hsdR17 recA1 thi-1* Δ*lacU169*(ϕ 80*lacZ*Δ*M15*) *endA1 gyrA96 relA1*	Hanahan, 1983

Table 2.3. Plasmids

Plasmids	Description	Reference or source
pRL1062a	Nm^R/Km^R; vector used in Tn*5*-1062 mutagenesis and construction of pAG1.3 and pLUX	Cohen *et al*, 1998
pRK2013	Km^R; helper plasmid used to mobilize plasmids into RM1021 strains	Figurski *et al*, 1979
pAG1X	Km^R; *Xma*I fragment subcloned from RM3166 containing left half of Tn5-1062 and upstream sequences	This study
pAG1X7	Ap^R; *BamH*I fragment containing the *rrn* promoter subcloned from pAG1X and cloned into *BamH*I site of pBluescript SK	This study
pRK290	Tet^R; broad host range (RK2) plasmid used in constructing pAG1.3 and pLUX.	Ditta *et al.*, 1980
pAG1.3	Km^R; 605 bp *Bgl*II/*Sac*I fragment containing the *rrn* promoter, subcloned from pAG1X7, and cloned in front of the *luxAB* genes in pRK290	This study
pLUX	Km^R; *Bgl*II/*Xho*I fragment of pRL1062 cloned between the *Sal*I and *Bgl*II sites of pRK290	This study

Discussion

Seven cold-inducible Tn*5*-1062 insertion mutants were isolated in this study. Five insertions are within the 16S and 23S rRNA genes of two ribosomal RNA, *rrn*, operons, while the other two insertions are within an operon containing genes similar to *cspA* and *rpsU* of *E. coli*. All three of the *rrn* operons found in *S. meliloti* 1021 appear to be coordinately regulated, as the *luxAB* genes recombined into the third *rrn* operon also showed cold-inducible luminescence (data not shown). While these results suggest that more ribosomal RNA is required by the cells following a cold shock, a previous study of the relationship of ribosome synthesis and growth rate did not come to this conclusion (Ryals *et al.*, 1982). The identification of the *rrn* operons as potential cold-inducible loci in *S. meliloti* was, therefore, a novel and unexpected finding.

Following a temperature downshift from 30°C to 15°C, *S. meliloti* 1021 slows from a generation time of 2.4 hours to 22 hours (Lehmann, 1994). An increased demand for ribosome synthesis and, therefore, increased transcription of the *rrn* operons is unexpected in view of this decline in growth rate. Ryals *et al.* (1982) examined the amount of stable RNA present in *E. coli* cells when grown at different temperatures and found that the amount of stable RNA and, therefore, the number of ribosomes per cell, remained the same in the temperature range from 40°C to 20°C. This observation suggests that at these temperatures the different

cellular enzymes are affected similarly such that the translational capacity of the cell remains proportional to the demand for protein synthesis. A study of a psychrophilic *Pseudomonas* demonstrated that if growth rate was held constant, the RNA content of the cell increased at the lower temperature (Harder and Veldkamp, 1967). In this case, it was proposed that the cells synthesized more ribosomes in order to compensate for the decline in translation rate. Synthesizing more ribosomes would presumably provide the translational capacity required to maintain the same growth rate. The crucial difference between these two experiments is most likely the manipulation of the growth rate and not the use of different bacteria. A possible conclusion from these experiments would be that *rrn* expression can be regulated by temperature but a net increase in the number of ribosomes is not necessary if growth rate is allowed to vary as temperature declines.

Protein synthesis has been shown to be particularly sensitive to low temperatures in many mesophilic bacteria (Broeze *et al.*, 1978). In *E. coli*, it is the initiation of translation that is inhibited below 8°C, resulting in the accumulation of the ribosomal subunits and cessation of growth (Friedman *et al.*, 1971). In fact, Jones and Inouye (1996) have proposed the "Cold-Shock Ribosome Adaptation" model. In this model, the cold shock response is viewed primarily as an adaptive response to a decline in translational capacity. The cold shock proteins are thought to adapt the ribosome for optimum function at low temperature. Three of

the cold shock proteins in *E. coli*, translation initiation factor 2, RbfA and CsdA, are associated directly with the ribosome and its function. RbfA and CsdA have been shown to be particularly important at low temperature. In this model of the cold shock response, synthesis of the ribosome is not being induced but a direct connection between the cold shock response and ribosome function is made.

Another aspect of the cold shock response in *E. coli* is the drop in level of guanosine 3', 5'-bis(pyrophosphate), or ppGpp, immediately following a cold shock (Mackow and Chang, 1983). This nucleotide is involved in regulating the stringent response (Cashel *et al.*, 1996). Levels of ppGpp increase following a nutrient downshift, repressing the transcription of rRNA and tRNA and inducing the expression of biosynthetic enzymes. In this way, higher levels of ppGpp are associated with a sudden decline in growth rate and lower levels with a nutrient shift up or sudden increase in growth rate. Paradoxically, this relationship is reversed following a temperature downshift when the levels of ppGpp fall even as growth rate declines. It has been proposed that the levels of ppGpp decrease following a cold shock in *E. coli* in response to the sudden inhibition of translation initiation (Jones *et al.*, 1992a). This inhibition of translation is thought to lead to a surplus of charged tRNAs. A similar condition would occur following a nutrient upshift. While the response of ppGpp levels to a temperature downshift has not been measured in *S. meliloti* 1021, the response to amino acid starvation is similar to that observed in *E. coli* (Howorth and England, 1999). If ppGpp levels decrease

following a temperature downshift in *S. meliloti* as in *E. coli*, transcription from the *rrn* operons could potentially increase.

Most studies of cold shock have focused on analyzing changes in protein synthesis using two-dimensional gel electrophoresis. These studies would not be able to detect increased synthesis of rRNA. If, however, the increased synthesis of rRNA were leading to the synthesis of more ribosomes, one would expect to see increased synthesis of the ribosomal proteins. In *E. coli*, several ribosomal proteins continue to be synthesized following a cold shock and in some cases appear to be induced (Jones *et al.*, 1987; Jones *et al.*, 1996). Although selected ribosomal proteins have been shown to be cold-inducible in other organisms, including *S. meliloti* -- S6 and L7/L12 in *Bacillus subtilis* (Graumann *et al.*, 1996) and S21 in *Anabaena variabilis* (Sato, 1994) and *Sinorhizobium meliloti* 1021 (O'Connell and Thomashow, 1999) -- the coordinate induction of all the ribosomal proteins has not been observed. It is, however, possible that a novel mechanism exists in *Sinorhizobium meliloti* and that the ribosomal proteins are cold-induced. While the Tn*5*-1062 mutagenesis of *S. meliloti* 1021 identified only one cold-inducible ribosomal protein, the possible existence of other induced ribosomal proteins cannot be eliminated (O'Connell and Thomashow, 1999). Because the regulation of ribosomal protein synthesis in *E. coli* occurs post-transcriptionally (Keener and Nomura, 1996), the luminescence of the *luxAB* genes inserted in a ribosomal protein gene may not accurately reflect the regulation of that ribosomal

protein's synthesis.

Another aspect of cold shock involves its predicted effect on RNA secondary structure. It has been proposed that secondary structures in RNA affect translation at low temperature but altered secondary structure may also affect rRNA processing and consequently ribosome synthesis. Two of the cold shock proteins in *E. coli*, CspA and CsdA, are thought to interact with RNA at low temperature. A role for CspA as a RNA chaperone has been proposed because of its ability to bind single-stranded RNA and destabilize secondary structures in RNA (Jiang *et al.*, 1997). CsdA has been shown to unwind double-stranded RNA and shows sequence similarity to the DEAD box family of RNA helicases (Jones *et al.*, 1996). That CspA and CsdA are induced after a cold shock suggests that mRNA and perhaps rRNA are negatively affected at low temperatures. At low temperature, RNA molecules may form aberrant secondary structures that could interfere with their function. In addition, rRNA processing has been shown to be inhibited at low temperature in the yeast, *Saccharomyces cerevisiae* (Kondo *et al.*, 1992). One of the cold shock proteins identified in this organism is NSR1, a nucleolin-like protein that is involved in ribosomal RNA maturation (Kondo *et al.*, 1992). Inhibition of rRNA processing would necessarily inhibit ribosome synthesis. This inhibition may in turn activate a feedback mechanism that could stimulate transcription from the *rrn* promoters. So there may be two factors leading to a decline in translational capacity: inhibition of ribosome function and

the inability to synthesize more functional ribosomes.

A final consideration involves the isolation of only highly induced loci in our mutagenesis and screen. This result was unexpected as several moderately induced loci have been identified in other organisms including *Escherichia coli* (Jones *et al.*, 1987), *Arthrobacter globiformis* (Berger *et al.*, 1996), *Lactococcus lactis* (Panoff *et al.*, 1994), and *Bacillus subtilis* (Graumann *et al.*, 1996). After extensive screening, only two classes of induced loci were isolated: the *rrn* operons and the operon containing homologs of the major cold shock protein CspA and the ribosomal protein S21. Yet, Cloutier *et al.* (1992) showed that a strain of *Sinorhizobium meliloti* continued to synthesize several proteins following a temperature downshift to 0°C or 5°C. Although the mutagenesis appeared to be thorough, the sensitivity of the screen for altered luminescence may have been limited. The luminescence of the average insertional mutant appeared to be affected by the temperature shift. This led to a background level of variable luminescence that made it difficult to identify moderately induced loci that increased by only 3 to 5-fold.

Further experiments are required to assess the effect of a cold shock on ribosomal RNA regulation in *S. meliloti*. The testing of direct promoter fusions is needed to accurately measure changes in *rrn* promoter activity in response to temperature shifts. While the *rrn* promoter was brought closer to the *luxAB* genes in constructing pAG1.3, the exact location of the promoter is still unknown.

Intervening sequences between the start of transcription and the *luxAB* genes may interfere with the measurement of *rrn* promoter activity. The construction and testing of direct promoter-*luxAB* fusions is described in the following chapter.

Materials and Methods

Bacteria and growth conditions. Cultures of *Escherichia coli* DH5α were grown aerobically at 37°C in LB broth (Sambrook *et al.*, 1989) or on solid LB medium containing 1.4% agar. Cultures of *Sinorhizobium meliloti* were grown aerobically at 30°C in either tryptone-yeast extract medium, TY, (Beringer, 1974), or GTS medium (Kiss *et al.*, 1979). Cultures were maintained on solid TY or GTS medium containing 1.4% agar, or frozen to -80°C in TY broth containing 15% glycerol. *S. meliloti* was cultured on solid media containing the following antibiotics when appropriate: kanamycin, Km (200 μg/ml) and streptomycin, Sm (250 μg/ml). *E. coli* was grown on plates containing ampicillin, Ap (100 μg/ml), Km (100 μg/ml), or tetracycline, Tet (10 μg/ml) when appropriate. A lower concentration, 50 μg/ml, of Km and Sm was used in broth cultures for both bacteria.

Transposon mutagenesis of *Sinorhizobium meliloti* 1021. Wild type *S. meliloti* 1021 was subjected to mutagenesis by Tn*5*-1062 carried on the plasmid pRL1062 (Figure 2.1) (Cohen *et al.*, 1998). The plasmid pRL1062 was introduced into *S. meliloti* 1021 via triparental matings (de Bruijn and Rossbach *et al.*, 1994). Cells were isolated from overnight cultures of *E. coli* DH5α carrying the plasmids pRL1062 and pRK2013 and also from two-day old cultures of *S. meliloti* 1021.

These cells were rinsed once and then resuspended in TY broth before being mixed and spotted on TY agar plates. The plates were incubated at 30°C for 12-16 hours. Individual mating spots were resuspended in a growth medium and dilutions were spread on selective plates containing Km and Sm and incubated at 30°C. When single colonies had formed (3-5 days), they were transferred to new plates, grown up at 30°C (2 days), and assayed for luciferase activity under different conditions. In some cases the colonies were not transferred to fresh plates before being assayed. Three sets of mutagenesis and screening were performed. In the first set which resulted in the isolation of RM3166, over 17,000 colonies were screened for increased luminescence after a temperature shift from 30°C to 10°C. A second screening of approximately 5,000 colonies resulted in the isolation of RM518, RM523 and RM603 by looking at increased luminescence after a temperature shift from 30°C to 15°C. In the third mutagenesis and screening, transposon recipients were selected and screened on GTS medium. In this case approximately 1,000 colonies from eleven matings were examined and one mutant, RM73, showed significantly increased luminescence at 15°C.

Detection and quantification of bacterial luciferase activity. Luciferase activity was measured differently depending on the culture conditions used to grow the bacteria. If the bacteria were grown on a solid agar medium, luminescence was measured with the Hamamatsu Photonic System model C1966-20 (Photonic Microscopy, Oak Brook, IL). The Berthold LUMAT luminometer

LB 9501 (Wallac Inc., Gaithersburg, MD) was used to measure aliquots of liquid bacterial cultures. Bacteria grown on agar plates were exposed to the enzyme substrate, N-decyl aldehyde, for two minutes prior to being assayed for light production for 59 seconds. The aldehyde was spread on a glass petri dish which was then placed over the agar plate. The Hamamatsu system converts photon counts to an image displayed on a monitor. This system can also quantitate the amount of light produced by each colony.

Bacteria grown in broth culture were assayed quantitatively for light production. In this assay, the N-decyl aldehyde was dispersed in an aqueous solution of bovine serum albumin (20 mg/ml) at a concentration of 1 μl/ml. A 5-10 μl aliquot of the cell culture was added to 50-100 μl of the aldehyde solution, vortexed for 30 seconds, then assayed for 60 seconds. The measured luminescence was reported in arbitrary units, RLU, and adjusted for cell number by dividing this number by the A_{600} of the culture. All assays were performed at room temperature.

DNA isolation, cloning and sequencing. Genomic DNA was isolated from 3-5 ml cultures of *S. meliloti* according to a standard CTAB extraction protocol (Ausubel *et al.*, 1987) with one modification. Prior to extraction with CTAB, the bacterial lysate was passed several times through an 18-gauge needle using a sterile syringe in order to partially shear the DNA. This step reduced sample viscosity and facilitated extraction of the samples with phenol and chloroform.

The Tn*5*-1062 transposons and their contiguous genomic DNA segments were cloned out of the transposon recipients and transformed into *E. coli* DH5α. Genomic DNA isolated from the mutants was digested with the appropriate restriction enzyme and circularized. The transposons contain an origin of replication, p15A, that is recognized by *E. coli* DH5α allowing the ligation mixture to be electroporated directly into *E. coli* DH5α. The circularized transposons were selected by spreading on plates containing Km.

Plasmid DNA was isolated using a Qiagen maxi-prep column (Qiagen, Inc., Valencia, CA) or a standard alkaline lysis method (Sambrook *et al.*, 1989). DNA sub-cloned from strain RM3166 was sequenced manually using the T7 Sequenase v.2 kit according to the recommended protocol (Amersham Life Science, Inc., Cleveland, OH) and examined using standard gel electrophoresis and autoradiography techniques. DNA subcloned from all other mutants was sequenced using an automatic fluorescent sequencing method. The clones were sequenced at the MSU-DOE-PRL DNA Sequencing facility using the ABI Catalyst 800 for Taq cycle sequencing and the 373A Sequencer for the analysis of the products. Sequences to the left of the transposons were obtained using the primer MT47 5'-CTAAGCTGCCTCCATCC-3' which binds within the *luxAB* gene cassette. Sequences to the right of the transposon were obtained using the primer MT84 5'-CACATGGAATATCAG-3' which is specific for sequences within the IS*50*R of the transposon. Both primers were synthesized by the Macromolecular

Structure Facility located in the Department of Biochemistry at Michigan State University.

Southern blotting. DNA fragments were separated on an agarose gel and transferred to a nylon membrane in 20X SSC via capillary action according to the standard protocol (Sambrook *et al.*, 1989) with two modifications. Instead of the acid depurination step, the gel was first exposed to UV light for 10 minutes in the UV Stratalinker 2400 (Stratagene, La Jolla, CA). After transfer, the DNA was crosslinked to the nylon membrane by exposure to 20 mjoules of light energy in the UV Stratalinker. The blot was hybridized to a ^{32}P-labeled probe following a protocol based on the one described in Sambrook *et al.*, 1989. The pre-hybridization and hybridization solutions contained 6X SSC, 0.5% SDS, and 0.25% nonfat dry milk. Hybridization was performed at 60-65°C in a Robbins Hybridization Incubator Model 400 (Robbins Scientific Corp., Sunnyvale, CA). The probe was prepared from a band isolated fragment by a random priming method (Feinberg and Vogelstein, 1983). The blots were washed four times in a solution of 0.1X SSC and 0.1% SDS at 60-65°C. The blot was then exposed to film.

DNA sequence analysis. The DNA contiguous with the transposons was sequenced by priming out of the ends of the transposons. The resulting sequences were joined at the nine base pair direct repeat created during transposition and compared to the non-redundant database including GenBank, EMBL, DDBJ, and

PDB sequences using the BLASTN algorithm (Altschul *et al.*, 1997).

Isolation of RNA. RNA was isolated from *S. meliloti* strains according to a modified protocol derived from that found in Ausubel *et al.* (1987) for the isolation of RNA from gram negative bacteria. Bacteria were harvested from a 3-20 ml aliquot of cell culture to which rifampicin had been added to a final concentration of 0.2 mg/ml. The cell pellets were resuspended in 1.4 ml of protoplasting buffer and 80 µl of 50 mg/ml of lysozyme added. The tubes were incubated on ice 5-10 minutes. The protoplasts were collected by centrifugation for 2 minutes at 7,000 rpm. The pellets were resuspended in 0.5 ml of gram negative lysing buffer, mixed with 15 µl of diethylpyrocarbonate, DEPC, and incubated for 5 minutes at 37°C. Tubes were chilled briefly on ice, mixed with 250 µl of 5M NaCl, incubated 10 minutes on ice and centrifuged for 10 minutes. The supernatant was removed, added to a tube containing 1 ml ethanol and stored overnight at -20°C. The RNA was collected by centrifuging for 15 minutes at 4°C, dried under vacuum and resuspended in DEPC-treated water. Samples were quantified using a spectrophotometer and measuring the A_{260} and A_{280}.

Northern blots. RNA samples were treated with formaldehyde and electrophoresed on formaldehyde/agarose gels according to standard protocols (Sambrook *et al.*, 1989). RNA was transferred from the gel to a nylon membrane in 10X or 20X SSC via capillary action according to the standard protocol (Sambrook *et al.*, 1989) with one modification. After transfer, the RNA was

crosslinked to the membrane by exposure to 20 mjoules of light energy in the UV Stratalinker 2400 (Stratagene, La Jolla, CA). The membranes were pre-hybridized at 42°C in the following solution: 50% formamide, 5X SSC, 50mM potassium phosphate, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate, and 100μg/ml denatured DNA. After a minimum of 2 hours of pre-hybridization treatment, ^{32}P-labeled probe was added to the solution and incubated overnight at 42°C in a Robbins Hybridization Incubator Model 400 (Robbins Scientific Corp., Sunnyvale, CA). The probe was prepared from a band isolated fragment by a random priming method (Feinberg and Vogelstein, 1983). The membranes were treated first at room temperature with three washes of 2X SSC and 0.5% SDS. After one wash in 0.1X SSC and 0.5% SDS, the temperature was raised to 50°C for two more washes in the same solution. Washes were continued until the spent wash solution was no longer detectably radioactive.

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CHAPTER 3

Characterization of the *Sinorhizobium meliloti* 1021 *rrn* promoters

Summary

The promoter regions of the three *rrn* operons of *S. meliloti* 1021 were isolated from a genomic clone bank and sequenced. The three promoter regions share a large section of identity beginning with the 16S structural gene and continuing 408 bp 5' of the predicted mature end of the 16S rRNA. The start of transcription was determined by primer extension and ribonuclease protection assays. A single promoter was located 354 bp upstream of the predicted fully processed ends of the three 16S rRNA transcripts. These promoters are very similar in structure to the *rrn* P1 promoters in *Escherichia coli*. Two promoter fragments were fused to the *luxAB* genes and recombined into the *S. meliloti* genome in order to study the regulation of the *rrn* promoters. Fragment A began with the +1 nucleotide and contained only the promoter and upstream regions. Fragment B contained 172 bp of the 5' end of the *rrn* transcript in addition to the *rrn* promoter. The 172 bp region included in fragment B was primarily responsible for the increased luminescence after a cold shock. The +1 fusion

showed only a 6-fold increase in luminescence compared to the 42-fold increase observed with the +172 fusion. Examination of the *luxAB* mRNA levels at 30°C and 15°C revealed that the greater increase of luminescence by fragment B resulted from the apparent inhibition of translation at 30°C and the increased stability of the *luxAB* transcript in this *rrn-luxAB* fusion. The *rrn* promoter fragments were also cloned in front of the *uidA* reporter gene. The *rrn-uidA* fusions showed a transient accumulation of reporter gene transcript following a temperature downshift. In addition, the 5' end of the *rrn* transcript included in fragment B was demonstrated to have antitermination activity.

Introduction

The rate of protein synthesis in bacteria is primarily determined by the number of ribosomes per cell. Since ribosome synthesis requires the coordinate synthesis and assembly of over fifty proteins and three ribosomal RNA molecules, it represents a significant investment of cellular resources. Consequently, many overlapping mechanisms appear to be in place such that ribosome number is tightly correlated with cellular growth rate under most conditions. The cellular growth rate is in turn primarily influenced by the nutrient content and temperature of the growth medium.

The regulation of ribosome synthesis has been extensively studied in *Escherichia coli* (for reviews Keener and Nomura, 1996; Gourse *et al.*, 1996). The expression of the *rrn* operons has been identified as the primary target for regulation. Each operon normally produces a single transcript containing the 16S, 23S and 5S rRNAs which is then processed into the individual rRNAs. The competition for binding of the ribosomal proteins by the newly transcribed rRNAs and the mRNAs encoding the ribosomal proteins coordinates the synthesis of rRNA with the synthesis of the ribosomal proteins. If no rRNA is present to bind to the ribosomal proteins, then the ribosomal proteins bind to their own mRNAs and prevent their translation. While the expression of the *rrn* operons involves the initiation of transcription, transcriptional antitermination, and the processing of the

rRNA transcript, the initiation of transcription is thought to be the key regulatory target.

As stated above, the synthesis of ribosomes and consequently the activity of the *rrn* promoters is positively correlated with the growth rate of the cell. Studies of the relationship of growth rate to ribosomal synthesis have focused on variations in the nutritional content of the growth medium. Three different regulatory mechanisms have been proposed to interact with the *rrn* promoters under different situations including FIS-dependent activation, growth rate-dependent regulation and stringent control. Upstream of the *rrn* promoters in *E. coli*, there are three binding sites for the FIS protein (Ross *et al.*, 1990). The binding of FIS to the *rrn* promoters contributes significantly to the activation of these promoters. The significance of FIS binding has been noted particularly in relation to the different growth phases of a bacterial culture. The most rapid growth occurs during exponential phase and then declines as the medium is exhausted and the culture enters stationary phase. This cycle continues if the stationary phase cells are introduced into fresh medium and resume rapid growth. FIS protein is implicated in the positive regulation of the *rrn* promoters because its concentration is highest during the stages of rapid growth and then declines during slow growth. Appleman *et al.* (1998) have shown that the concentration of FIS in the cell is directly related to its binding of the *rrn* promoters so that only at high cellular concentrations are the FIS binding sites fully occupied. The H-NS protein

has been shown to counteract the FIS-dependent activation of the *rrn* P1 promoters in *in vitro* and *in vivo* experiments (Tippner *et al.*, 1994; Afflerbach *et al.*, 1998). The concentration of the H-NS protein was shown to increase by approximately 5-fold as the culture slowed from exponential growth and entered stationary phase supporting the idea that H-NS is important in reducing expression of the *rrn* P1 promoter during the transition into stationary phase (Afflerbach *et al.*, 1998). While an earlier study found no effect of H-NS on *rrn* expression during this stage of growth (Aviv *et al.*, 1996), Afflerbach *et al.* (1998) suggested that the different strains were not maintained at the same growth rate thus preventing an accurate assessment of the effect of H-NS.

Growth rate-dependent control involves the regulation of the *rrn* promoters in response to different growth rates achieved in media of different nutrient contents. This type of control requires only the core *rrn* promoter (-41 to +1) and is thought to involve the cellular ATP and GTP concentrations (Gaal *et al.*, 1997). The levels of ATP and GTP are positively correlated with the cellular growth rate and either ATP or GTP is the first nucleotide incorporated into all of the *rrn* transcripts. The concentration of the initiating nucleotide greatly affects the stability of the RNA polymerase-*rrn* promoter complex so that stability is enhanced at higher concentrations resulting in a higher rate of transcription. Levels of the nucleotide guanosine 3', 5'-bis(pyrophosphate), or ppGpp, also vary with growth rate and were initially thought to mediate growth rate control.

However, an *E. coli* strain completely lacking ppGpp still exhibited growth rate-dependent control (Gaal and Gourse, 1990).

The stringent response refers to the cellular response to starvation for amino acids (see review Cashel *et al.*, 1996). This response is characterized by a dramatic increase in ppGpp levels and the simultaneous activation or repression of various genes. The levels of ppGpp also respond to variations in other nutrients but ppGpp has not always been shown to be the key regulator in these responses. In response to starvation for amino acids, ppGpp acts a negative regulator of rRNA and tRNA transcription and a positive regulator of certain biosynthetic genes.

The regulation of *rrn* operon expression in response to temperature is not fully understood. Ryals *et al.* (1982) found that stable RNA content and synthesis remained constant when cultures of *E. coli* were grown at temperatures between 20°C and 40°C. The levels of ppGpp, however, were found to vary inversely with temperature. Mackow and Chang (1983) also found that ppGpp levels decreased in *E. coli* following temperature downshifts from 40° to 23°C; however, they observed that the RNA synthesis rates changed as well. RNA synthesis fell initially after the temperature downshift only to increase again as the ppGpp levels continued to decrease. The inverse relationship between ppGpp and RNA synthesis was supported in this study while the absolute amounts of ppGpp required to affect the rRNA regulation changed with temperature. Another interesting finding concerns the relationship between the stringent response and

cold shock response in *E. coli*. Jones *et al.* (1992) found that the two responses were antagonistic and that mutants lacking ppGpp were able to more quickly adapt to low temperature. While this study did not examine *rrn* synthesis directly, many of the proteins up-regulated at low temperature are involved in transcription and translation and may be regulated similarly to the *rrn* operons. Thus, the nucleotide ppGpp may be important to the temperature regulation of the *rrn* operons although more precise studies must be conducted to determine the contributions of other regulatory systems.

As described in Chapter 2, we performed transposon mutagenesis of *S. meliloti* 1021 using the reporter transposon Tn*5*-1062 which carries the promoterless *luxAB* genes encoding *Vibrio harveyi* luciferase. Seven cold-induced insertion mutants were isolated in the study, including five mutants with insertions in ribosomal RNA genes. While these insertions were distributed between only two of the three *rrn* operons present in *S. meliloti* 1021, recombination of the *luxAB* genes into the third 16S rRNA gene revealed that the third *rrn* operon showed temperature-dependent expression of the *luxAB* genes as well. Preliminary characterization of the *rrn*A promoter indicated that a 605 bp *Bgl*II-*Sac*I fragment cloned in front of the *luxAB* genes led to temperature-dependent luminescence and accumulation of the *luxAB* transcript.

We report here the further characterization and sequencing of the *rrn* promoters and the role of temperature in their regulation. The three *rrn* promoters

present in *S. meliloti* 1021 were isolated from a cosmid clone bank and sequenced. The transcriptional start sites were mapped indicating the presence of a single promoter controlling the expression of each *rrn* operon. Two *rrn* promoter fragments were fused to the *luxAB* and *uidA* reporter genes, recombined into the inositol locus of *S. meliloti* 1021, and their regulation in response to a temperature downshift examined. The first 172 bp of the rRNA transcript appeared to be primarily responsible for the increase in luminescence observed in the original *rrn*::Tn*5*-1062 insertions. Luminescence in the *rrn* promoter +1 fusion to *luxAB* increased only 4 to 6-fold following a temperature downshift from 30°C to 15°C. The greater increase in luminescence by the *rrn*::Tn*5*-1062 insertions and the *rrn* promoter +172 fusion to *luxAB* resulted from the apparent inhibition of translation in these strains at 30°C. The 5' end of the *rrn* transcript also increased stability of the *luxAB* transcript following the temperature downshift. The *rrn* promoter fusions to the *uidA* gene showed a transient increase in *uidA* transcript following a temperature downshift. Further, the *rrn* promoter +172 fusion to the *uidA* gene indicated that the 5' end of the *rrn* transcript has antitermination activity.

Results

DNA sequence analysis of the three *rrn* promoter regions in *S. meliloti* 1021. The promoter regions of the three *rrn* operons of *S. meliloti* 1021 were isolated in order to study their regulation. A *S. meliloti* 1021 cosmid clone bank was probed with MT119, an oligomer complimentary to a central region of the 16S rRNA gene. The three *rrn* promoter regions were isolated on three separate cosmids. The sequence 5' to that encoding the mature 16S rRNA was determined for each of the three operons (Figure 3.1). Two operons, *rrn*B and *rrn*C, were identical over the first 441 bp of this "leader" region while the sequence of *rrn*A diverged after 392 bp. The designations *rrn*A, *rrn*B, and *rrn*C, refer to those operons isolated on fragments 1, 2, and 3, respectively (Figure 2.6). Because the *luxAB* genes behave similarly in response to a temperature downshift when recombined into each of the three operons, it seems likely that the regulatory elements required for this response are present within the region shared by all three *rrn* operons.

Transcriptional mapping of the *rrn* promoters. In order to precisely locate the *rrn* promoters, the 5' ends of the *rrn* transcripts were identified using primer extension analysis. The primer MT179 (Figure 3.1) was chosen for this assay because it is complementary to sequences downstream of the *Bgl*II-*Sac*I fragment cloned into pAG1.3 (Figure 2.7). This fragment was able to support

Figure 3.1

Figure 3.1. Sequence comparison of the three *Sinorhizobium meliloti* 1021 *rrn* promoter regions. The three *rrn* promoters were isolated from a clone bank and sequenced. Dashes indicate sequence identity to *rrn*B. The promoter regions of *rrn* A, B, and C, refer to the *rrn* operons isolated on fragments 1, 2, and 3, respectively (Figure 2.6). The putative -35 and -10 hexamers are indicated and the lower case letters represent bases that differ from the *E. coli rrn* P1 consensus sequences (Keener and Nomura, 1996): (-35) TTGTc/gA and (-10) TATAAT. The +1 identifies the 5' end of the pre-16S rRNA as determined by primer extension. Sequences complementary to the primers used in the primer extension assays, MT179 and MT261, are underlined. The 5' end of the mature 16S rRNA was predicted based on sequence identity to *R. sphaeroides* (Dryden and Kaplan, 1993).

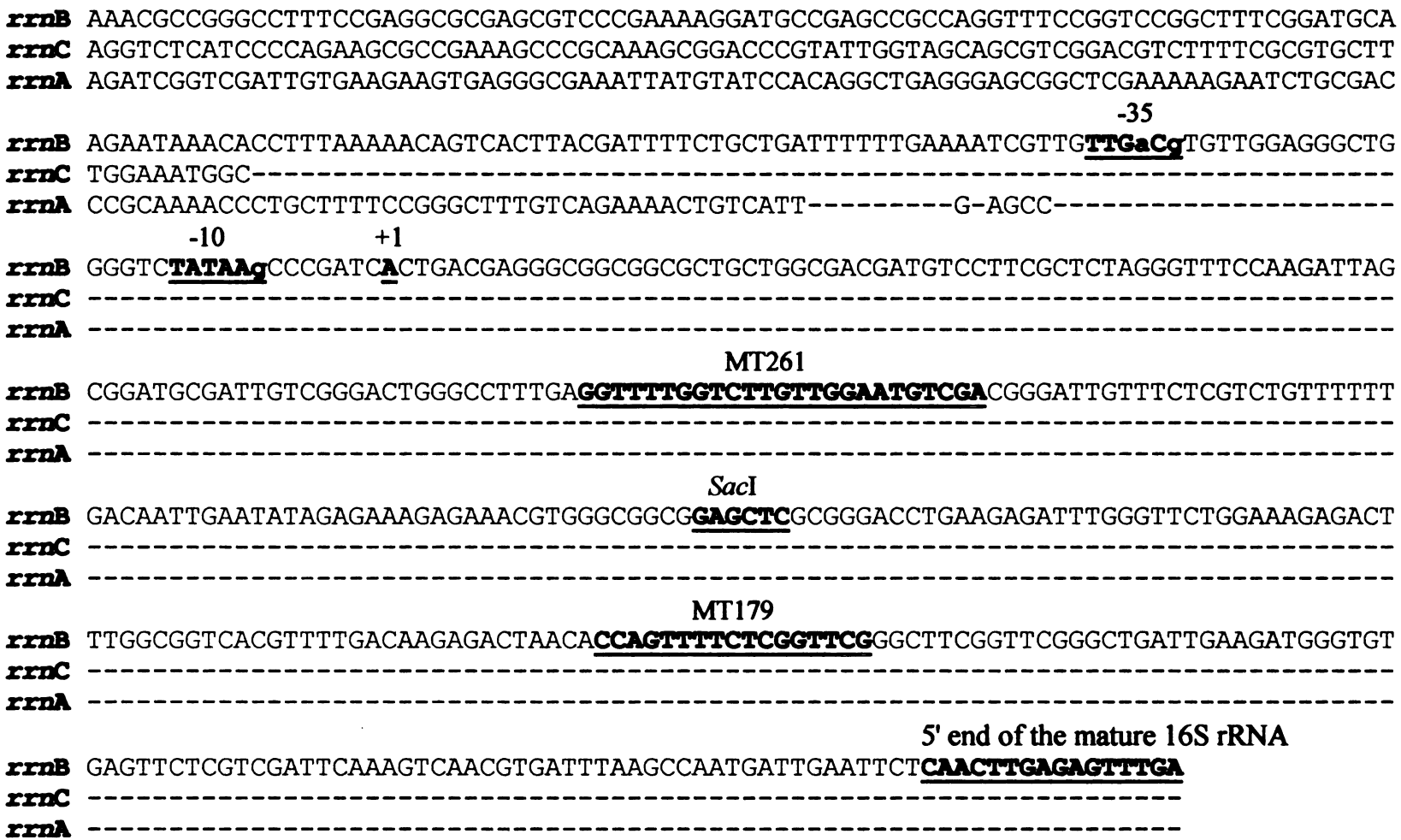

rrnB AAACGCCGGGCCTTTCCGAGGCGCGAGCGTCCCGAAAAGGATGCCGAGCCGCCAGGTTTCCGGTCCGGCTTTCGGATGCA
rrnC AGGTCTCATCCCCAGAAGCGCCGAAAGCCCGCAAAGCGGACCCGTATTGGTAGCAGCGTCGGACGTCTTTTCGCGTGCTT
rrnA AGATCGGTCGATTGTGAAGAAGTGAGGGCGAAATTATGTATCCACAGGCTGAGGGAGCGGCTCGAAAAAGAATCTGCGAC
-35
rrnB AGAATAAACACCTTTAAAAACAGTCACTTACGATTTTCTGCTGATTTTTTGAAAATCGTTGTTGaCgTGTTGGAGGGCTG
rrnC TGGAAATGGC----------
rrnA CCGCAAAACCCTGCTTTTCCGGGCTTTGTCAGAAAACTGTCATT---------G-AGCC----------
-10 +1
rrnB GGGTCTATAAgCCCGATCACTGACGAGGGCGGCGGCGCTGCTGGCGACGATGTCCTTCGCTCTAGGGTTTCCAAGATTAG
rrnC ----------
rrnA ----------
MT261
rrnB CGGATGCGATTGTCGGGACTGGGCCTTTGAGGTTTTGGTCTTGTTGGAATGTCGACGGGATTGTTTCTCGTCTGTTTTTT
rrnC ----------
rrnA ----------
SacI
rrnB GACAATTGAATATAGAGAAAGAGAAACGTGGGCGGCGGAGCTCGCGGGACCTGAAGAGATTTGGGTTCTGGAAAGAGACT
rrnC ----------
rrnA ----------
MT179
rrnB TTGGCGGTCACGTTTTGACAAGAGACTAACACCAGTTTTCTCGGTTCGGGCTTCGGTTCGGGCTGATTGAAGATGGGTGT
rrnC ----------
rrnA ----------
5' end of the mature 16S rRNA
rrnB GAGTTCTCGTCGATTCAAAGTCAACGTGATTTAAGCCAATGATTGAATTCTCAACTTGAGAGTTTGA
rrnC ----------
rrnA ----------

Figure 3.1

temperature-dependent expression of the *luxAB* genes in *S. meliloti* 1021 (Figure 2.7). The primer extension assay of the *rrn* transcripts isolated from RM1021 at 30°C gave two major products of 100 bp and 272 bp (Figure 3.2A, lane 1). These products were also present when RNA isolated from RM1021 at 15°C was used (data not shown). These results indicated that there might be two promoters driving the transcription of the *rrn* genes (Figure 3.2B).

Pausing of reverse transcriptase sometimes produces prematurely terminated extension products that can be mistaken for transcriptional start sites. Consequently, ribonuclease protection assays were performed to determine whether either of the two extension products resulted from premature termination. The ribonuclease protection assay identifies transcriptional start sites without the use of reverse transcriptase. A strain carrying the *rrn*A promoter region fused to the *luxAB* genes was constructed so that a single *rrn* promoter could be analyzed. The *rrn-luxAB* fusion produces a transcript that can be distinguished from those produced by the three endogenous *rrn* promoters. The resulting strain, RM311, (Figure 3.3A) contains a fragment of the *rrn*A promoter region beginning with the +1 nucleotide of P2 and extending to the *Bgl*II site used in the construction of pAG1.3. This fragment was cloned in front of the *luxAB* genes in the plasmid pMW193 (Bosworth *et al.*, 1994) and recombined into the inositol locus of RM1021 (Figure 3.5) such that the fusion was present in single copy.

A

B

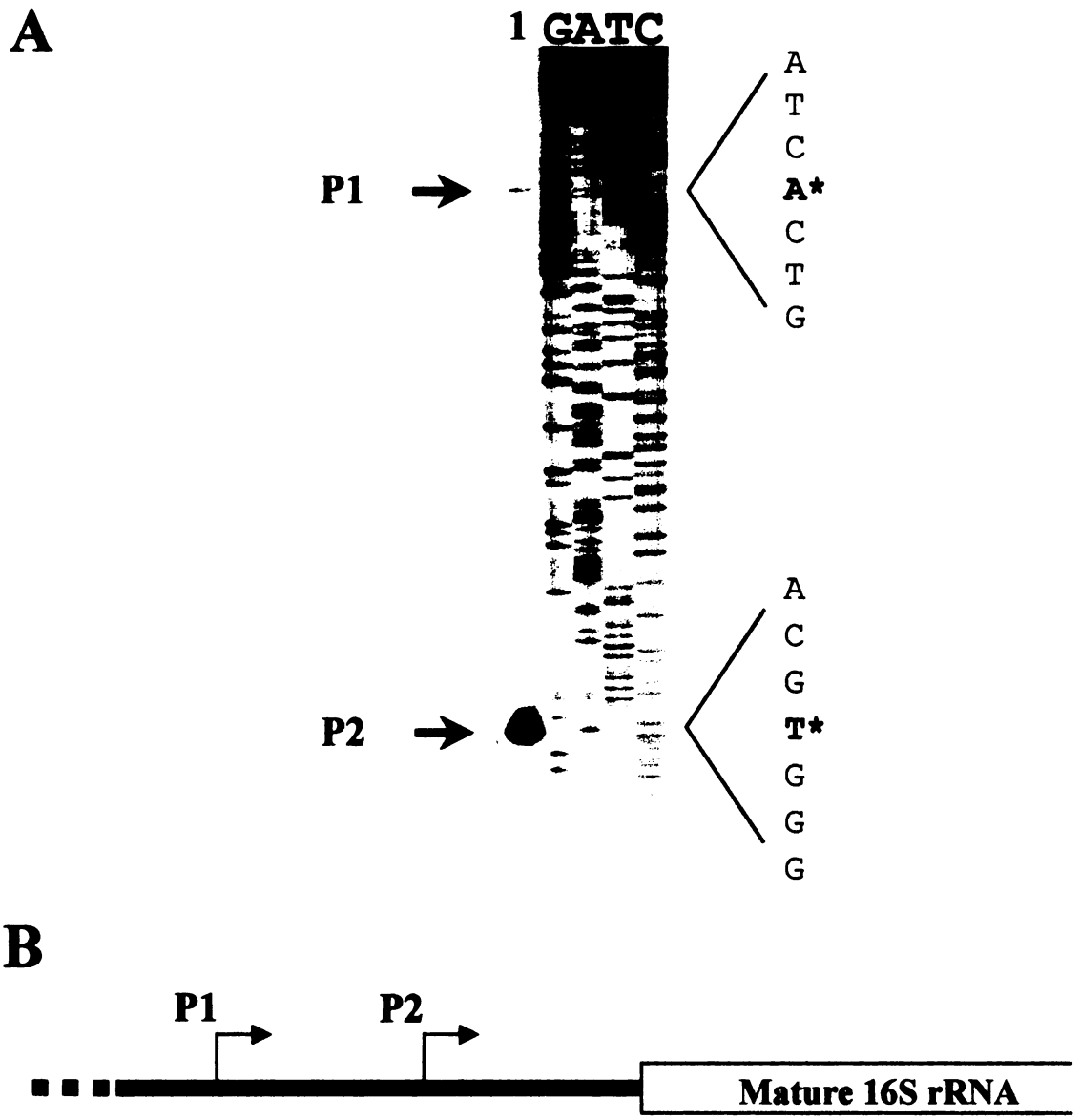


Figure 3.2. Primer extension assay of the *rrn* transcripts using MT179. (A) RNA was isolated from RM1021 grown at 30°C. 20 μg of RNA was added to 500 fmoles of MT179 (see Figure 3.1 for primer sequence) and annealed overnight at 65°C as described in Materials and Methods. The primer extension products (lane 1) were compared to a sequence ladder generated from MT179 priming off of pAG1X7. The 5' ends of the rRNA transcripts are shown in bold with an asterisk. (B) Schematic of the *rrn* promoter based on the primer extension products generated with MT179.

Figure 3.3. RNase protection assay of the *rrn* promoter regions. (A) A schematic of the *rrn* promoter-*luxAB* fusion in RM311 is shown (1). The transcripts that would be produced by the putative promoters, P1 (2) and P2 (3), are depicted and the dotted lines show the portion that would be complementary to the probe. A map of the RNA probe used in the experiment (4) is included as well. (B) RM311 was grown up in TY broth at 30°C then shifted to 15°C for six hours before RNA was isolated. 1μg of RNA was hybridized to 4 x 10^6 CPM of radiolabeled probe overnight at 55°C. The samples were then treated with RNase as described in Materials and Methods. In lane 1 are the radiolabeled RNA standards with sizes in base pairs. Lane 2 contains radioactive probe hybridized to 1μg tRNA and treated in parallel with the experimental sample. Lane 3 contains the RNA probe fragments protected by the complementary RM311 RNA .

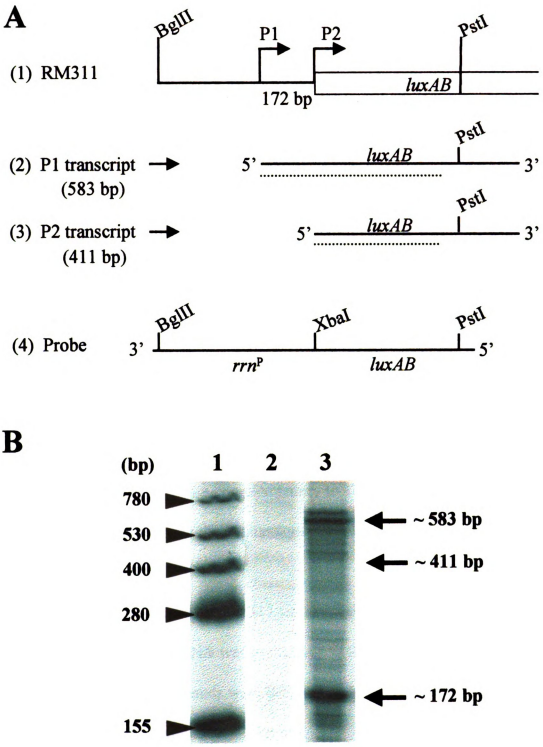

A
(1) RM311
BglII
P1
P2
PstI
luxAB
172 bp
(2) P1 transcript
(583 bp)
5'
3'
(3) P2 transcript
(411 bp)
(4) Probe
XbaI
rrnP
B
(bp)
1
2
3
780
530
400
280
155
~ 583 bp
~ 411 bp
~ 172 bp

Figure 3.3

The single-stranded RNA probe used in this assay to detect transcripts originating from the *rrn* promoter-*luxAB* gene fusion in RM311 was synthesized from the plasmid, pAG311-1 (Table 3.2). This probe (Figure 3.3A) is complementary to 399 bp of the *luxAB* transcript and the entire *rrn* upstream region in RM311. The probe was hybridized to RNA isolated from RM311 and then digested with ribonucleases T1 and A. These ribonucleases do not degrade double-stranded RNA; therefore, any transcript produced that is complementary to a portion of the ^{32}P-labeled, single-stranded probe will protect the probe from degradation. The transcript originating from P1 should protect 583 bp of the probe while the transcript originating from P2 should protect a 411 bp fragment (Figure 3.3A). The transcripts synthesized by the endogenous *rrn* promoters will also protect a portion of the probe. The 5' end of the transcripts originating from the endogenous P1 promoters are complementary to 172 bp of the probe but the transcripts originating from the endogenous P2 promoters have no region of complementarity to the probe.

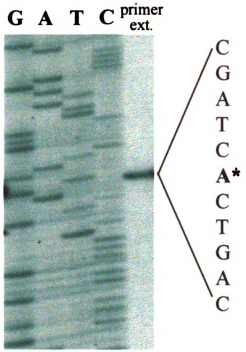
RNA was isolated from RM311 six hours after a temperature shift from 30°C to 15°C. After the RNA was hybridized to the probe and digested with the ribonucleases, two major bands were detected on the gel (Figure 3.3B). The smallest band of approximately 172 bp represents the region of the probe protected by the endogenous P1 transcript. The band of approximately 583 bp is the portion of the probe protected by the P1-*luxAB* transcript. The slightly larger band is most

likely the product of a partial digestion of the P1-*luxAB* transcript. The probe has 70 bp at its 5' end that do not hybridize with the transcript. This short segment may be removed with less efficiency by the ribonucleases. Of primary importance is the absence of a band in the vicinity of 411 bp indicating the lack of any transcript originating from P2. These results suggest that P2 is not an active promoter under the conditions tested and that the 100 bp primer extension product was most likely the product of pausing and premature termination by reverse transcriptase.

A second primer extension assay was performed using the primer MT261 (Figure 3.1). The purpose of this experiment was to confirm the +1 nucleotide determined for the P1 transcript in the earlier experiments. A single major band was produced in this primer extension assay when RNA isolated from RM1021 at both 30°C and 15°C was used (Figure 3.4A). This assay confirmed that adenine was the first base in the *rrn* transcript with no additional promoters present further upstream (Figure 3.4B). Based on the above data, we propose that each of the *rrn* operons in *S. meliloti* 1021 is controlled by a single promoter.

Expression of the *rrn* promoter-*luxAB* fusions. To begin to characterize the regulatory mechanisms acting on the *rrn* operons in response to low temperature, two fragments of the promoter region were amplified and cloned in front of the *luxAB* genes (Figure 3.5). Fragment A extends from -419 to the +1 nucleotide, thus containing only the promoter and upstream regions. Fragment B

A



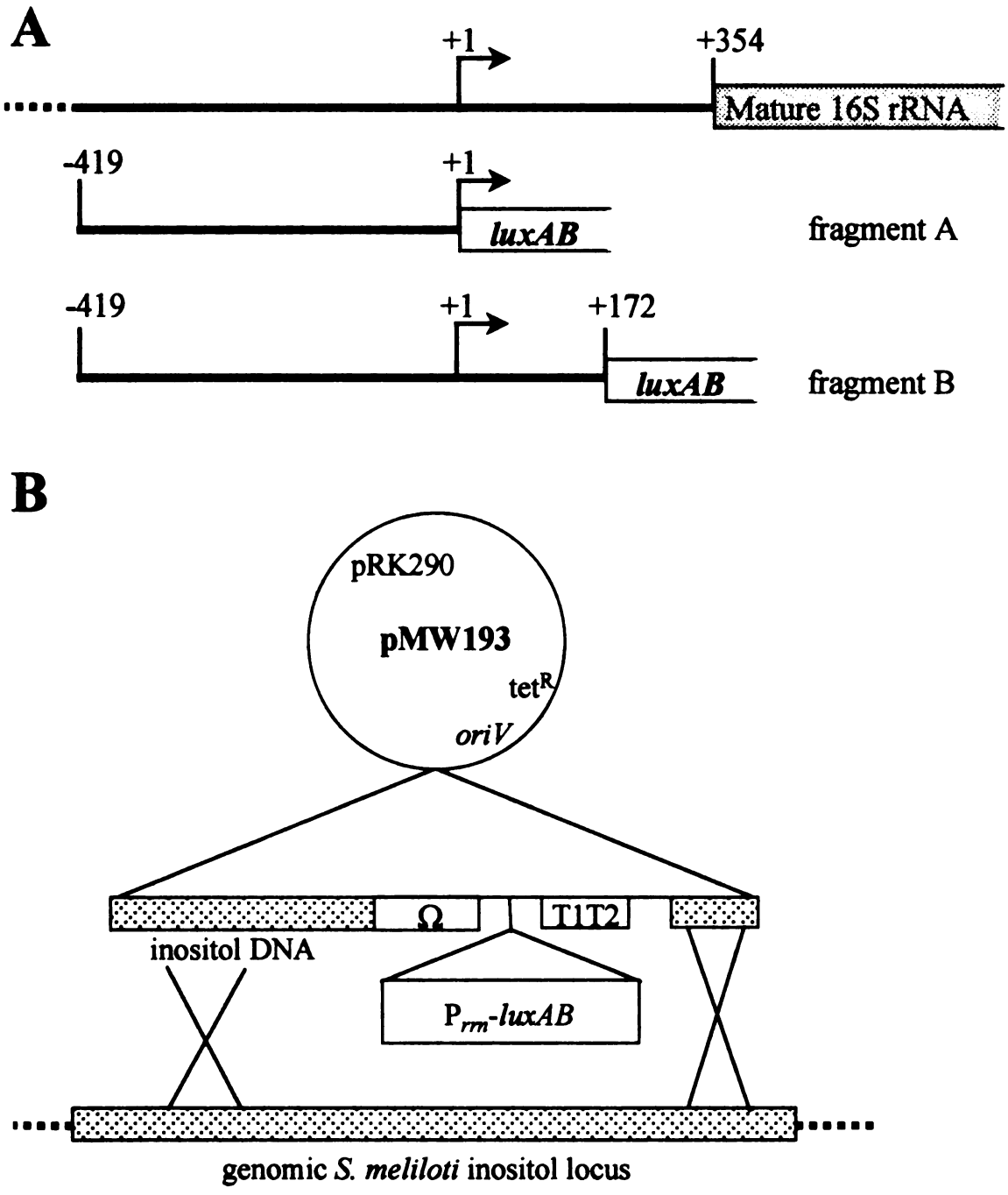
B

-35 -10 +1

TG**TTGaCg**TGTTGGAGGGCTGGGGTC**TATAAg**CCCGATC**A**CTGA

Figure 3.4. Mapping the 5' ends of the *rrn* transcripts by primer extension using MT261. (A) RNA was isolated from RM1021 grown at 30°C. 100 fmoles of MT261 (see Figure 3.1 for description of primer) were added to 10μg of RNA and annealed overnight at 60°C as described in Materials and Methods. The primer extension products were compared to a sequence ladder primed from MT261 using pAG1X7b as a template. The 5' end of the rRNA transcript is shown in bold with an asterisk. (B) Sequence of the *rrn* promoter region. The putative -35 and -10 hexamers are underlined. Nucleotides not in agreement with the *E. coli rrn* P1 consensus sequences are in lowercase letters. The *E. coli rrn* P1 consensus: (-35) TTGTc/gA (-10) TATAAT.

Figure 3.5. Construction of *rrn* promoter-*luxAB* fusions. (A) Two fragments were amplified from the *S. meliloti* 1021 *rrn* promoter region using three different primers: MT215, MT213, and MT214. The left primer, MT215, incorporates the *Sac*I and *Kpn*I restriction sites 5' to position -419. The two right primers contain the *Not*I site. The 3' end of fragment A begins with the first transcribed nucleotide and continues 5' to -419. Fragment B begins at the 3'end with base +172 of the *rrn* transcript and continues to -419. The amplified fragments were cloned into pBluescript utilizing the *Sac*I and *Not*I sites. The *luxAB* genes were cloned into the *Xba*I site downstream of the fragments. (B) The promoter-*luxAB* fusions were cloned into the *Kpn*I site of pMW193 between the **Ω** fragment containing a spectinomycin resistance gene flanked by the T4 translational and transcriptional terminators and two tandemly arranged *rrn* terminators (T1T2) (Bosworth *et al*, 1994). The resulting plasmids were introduced into RM1021 along with an incompatible plasmid, pJB251. Single recombinants were obtained by selecting for Sp^R and Gm^R colonies. Double recombinants were isolated by screening for Ino-, Sp^R and Tet^S colonies. RM111 and RM311 contain fragment A and fragment B, respectively, directing transcription of the *luxAB* genes.



*RM111 (-419 to +1 fused to *luxAB*)

*RM311 (-419 to +172 fused to *luxAB*)

Figure 3.5

extends from -419 to +172 so that it includes the 5' end of the *rrn* transcript but no structural 16S rRNA. This fragment encompasses all but 12 bp at the 3' end of the *rrn*A promoter fragment subcloned into plasmid pAG1.3 (Figure 2.7). The promoter-*luxAB* fusions were cloned into pMW193 between the omega fragment and two tandemly arranged *rrn* terminators (Bosworth *et al.*, 1994). The omega fragment confers spectinomycin resistance and contains T4 transcriptional and translational terminators. The vector also contains portions of the inositol locus of *S. meliloti* allowing the promoter-*luxAB* fusions to be recombined into the wild type strain and maintained in single copy in the genomic DNA. The fusions constructed from fragments A and B were recombined into the inositol locus forming strains RM111 and RM311, respectively.

The luminescence of the fusion strains was compared to that of the original 16S *rrn*A::Tn*5*-1062 insertion mutant, RM603, at both 30°C and 15°C (Figure 3.6A). The luminescence of RM311 (-419 to +172) closely resembled that of RM603 at both 30°C and 15°C. The luminescence of RM111 (-419 to +1), however, was significantly greater than that of either RM311 or RM603 at 30°C. After incubation at 15°C, the difference in luminescence between the three strains was no longer evident. To more accurately quantitate luminescence, cultures were grown in TY broth at 30°C and then subjected to a temperature downshift to 15°C (Figure 3.6B). At 30°C, RM111 produced approximately 7.8-fold more light than

Figure 3.6. Luminescence of *rrn* promoter-*luxAB* fusion strains. (A) The two fusion strains, RM311 and RM111, were streaked onto TY agar along with RM603 and grown for 2 days at 30°C. The plates were exposed to aldehyde and the luminescence monitored with the photonic camera. The plates were then incubated for approximately 5 1/2 hours at 15°C and re-exposed to aldehyde. The images recorded by the photonic camera are shown. (B) The two fusion strains and RM603 were cultured in TY broth at 30°C until reaching an A_{600} between 0.4 and 0.5. At this point 5µl aliquots were measured for luminescence as described in Materials and Methods. The cultures were then shifted to a 15°C shaking water bath. The measurement reported for 15°C was taken 3 hours after the temperature shift. Luminescence is reported in relative light units or RLU divided by the A_{600} of the culture to correct for culture density. Each bar represents the average luminescence from two experiments with a total of four samples per strain. The error bars represent $\pm$ the standard deviation. (C) The two fusion strains and RM603 were cultured in TY broth at 30°C until reaching an A_{600} between 0.4 and 0.5. At this point, samples were taken and luminescence measured as described in Materials and Methods. The cultures were then shifted to a 15°C shaking water bath and luminescence measured 1, 3, 5, and 7 hours after the temperature shift. Each bar represents the average fold induction determined in two experiments with a total of five flasks measured for each strain type. The standard deviation is shown with error bars.

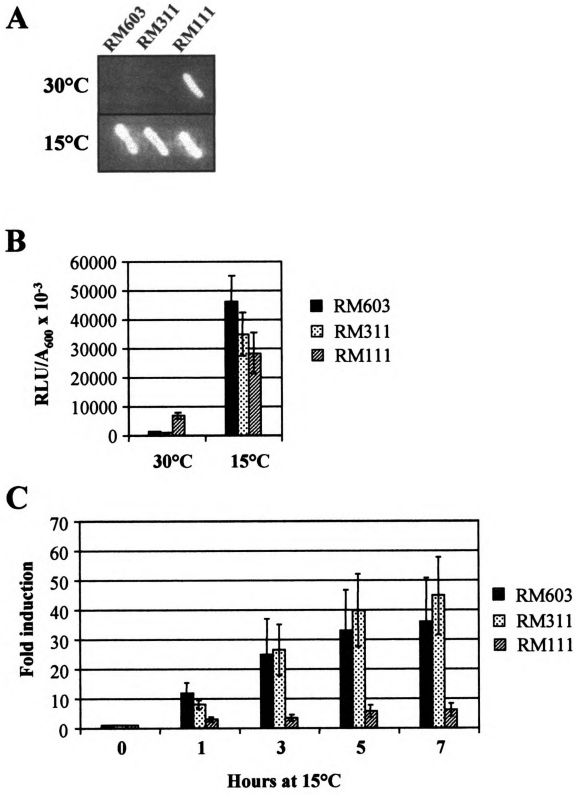

A
RM603
RM311
RM111
30°C
15°C
B
RLU/A600 x 10-3
60000
50000
40000
30000
20000
10000
0
30°C
15°C
RM603
RM311
RM111
C
Fold induction
70
60
50
40
30
20
10
0
0
1
3
5
7
Hours at 15°C
RM603
RM311
RM111

Figure 3.6

RM311 and 5.4-fold more than RM603. After three hours at 15°C, the difference in the luminescence of the strains had decreased to less than 2-fold with RM603 being brighter than RM111. In Figure 3.6 C, luminescence is expressed in terms of fold induction after a temperature downshift from 30°C to 15°C. Luminescence in RM311 and RM603 increased approximately 40-fold as compared to the 6-fold increase by RM111. Since RM311 and RM603 achieve levels of luminescence comparable to RM111 at 15°C, the difference in induction is primarily due to their relatively reduced luminescence at 30°C (Figure 3.6 B).

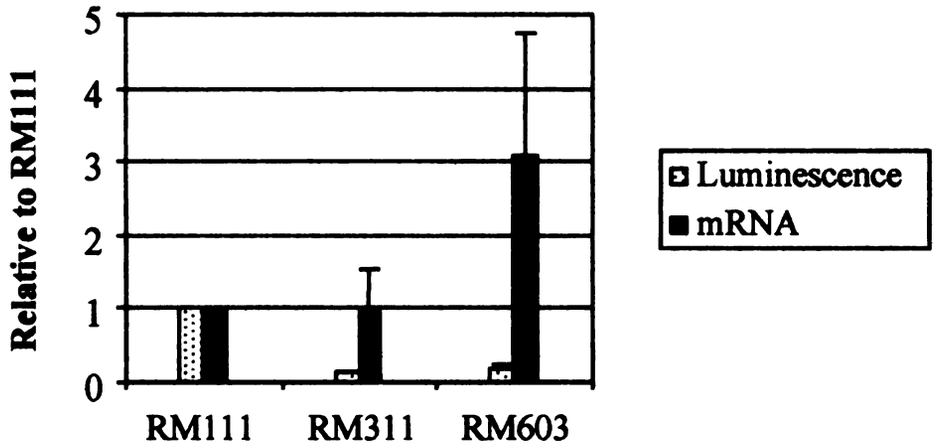
RNA analysis of the *rrn* promoter-*luxAB* fusions. The reduction in luminescence at 30°C observed in RM311 and RM603 relative to RM111 may have resulted from the destabilization of the *luxAB* mRNA by the 5' end of the *rrn* transcript. A similar mechanism has been observed in the regulation of the *S. meliloti* 1021 *cspA* gene (O'Connell and Thomashow, 1999). To determine whether the *luxAB* genes were in fact being destabilized in RM311 and RM603 at 30°C, the *luxAB* transcript and luminescence levels were assayed. RNA was isolated from RM111, RM311, and RM603 grown in TY broth at 30°C and three hours after being shifted to 15°C. RNA samples were probed with ^{32}P-labeled *luxAB* DNA. Luminescence and *luxAB* transcript levels at 30°C are compared in Figure 3.7A. The values measured for each strain are reported relative to those in RM111. Surprisingly, the level of *luxAB* transcript in RM311 and RM603 was not reduced relative to RM111 as the low level of luminescence suggested. While

RM111 contained two-thirds less *luxAB* mRNA than RM603, RM111 produced 5-fold more light. As in RM603, the amount of light produced by strain RM311 was less than expected considering the amount of *luxAB* mRNA present. The lack of correlation between the levels of *luxAB* transcript and luminescence in RM311 and RM603 suggests that translation of the *luxAB* genes is inhibited in these strains at 30°C.

As seen in Figure 3.6B, RM111, RM311 and RM603 reached comparable levels of luminescence following a temperature downshift. Since the low level of luminescence at 30°C appeared to result from inhibition of translation, the relief of this inhibition at 15°C may have accounted for the observed increase in luminescence. Alternatively, the *luxAB* transcripts produced by RM311 and RM603, while continuing to be translated less efficiently at 15°C, may have been more stable and accumulated to higher levels than those produced by RM111. In order to distinguish between these possibilities, the levels of *luxAB* transcript and the corresponding levels of luminescence at 15°C were examined. Figure 3.7B compares the luminescence and *luxAB* transcript levels present in strains RM111, RM311 and RM603 three hours after a shift to 15°C. All quantities are expressed relative to strain RM111 at 30°C. It can be seen that the luminescence of RM111 increased approximately 4-fold at 15°C and that the luminescence of RM311 and RM603 reached levels comparable to RM111 at 15°C (Figure 3.7B, light bars).

Figure 3.7. Induction of luminescence and *luxAB* mRNA in *rrn* promoter-*luxAB* fusions after a temperature downshift. The two fusion strains and RM603 were cultured in TY broth at 30°C until reaching an A_{600} between 0.4 and 0.5. At this point, samples were taken for RNA isolation and luminescence measured as described in Materials and Methods. The cultures were then shifted to a 15°C shaking water bath and sampled 3 hours after the temperature shift. Panel A is a graph of the measurements taken at 30°C and panel B is a graph of the measurements taken after 3 hours of incubation at 15°C. The light bars represent the average luminescence determined in two experiments with a total of four flasks measured for each strain type. The values expressed are normalized to the luminescence of RM111 at 30°C. Each solid bar represents the average levels of *luxAB* mRNA normalized to the levels in RM111 at 30°C. The *luxAB* mRNA levels were measured from Northern blots using a phosphoimager. The error bars show the standard deviations.

A


Relative to RM111
5
4
3
2
1
0
RM111
RM311
RM603
Luminescence
mRNA

B

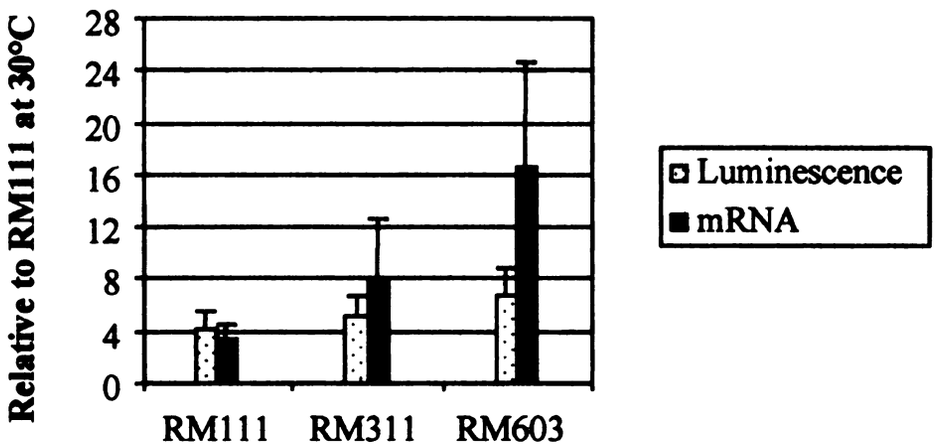

Relative to RM111 at 30°C
28
24
20
16
12
8
4
0
RM111
RM311
RM603
Luminescence
mRNA

Figure 3.7

The levels of *luxAB* mRNA and luminescence in RM111 remained consistent following the temperature downshift. As message levels increased approximately 4-fold at 15°C, luminescence also increased approximately 4-fold. If the increase in luminescence at 15°C in RM311 and RM603 was solely due to an increase in *luxAB* mRNA stability, the ratio of luminescence and transcript level should have remained constant at each time point. This was not the case, however, as the level of light production began to approach the level of *luxAB* transcript at 15°C in RM311 and RM603. While the ratio of transcript to luminescence in RM311 at 30°C was approximately 7.3, the ratio was reduced to 1.6 after three hours at 15°C. The same pattern was seen in RM603 as the ratio of transcript to luminescence was reduced from 16.4 to 2.5 after the shift to 15°C. The translation of the *luxAB* genes in RM311 and RM603 appeared to be preferentially inhibited at 30°C with the efficiency of translation increasing at 15°C. However, the increased stability of the *luxAB* message at 15°C in strains RM311 and RM603 may also have contributed to the increased luminescence in these strains as the *luxAB* transcript did accumulate more at 15°C in these two strains than in RM111. Evidently, the increased luminescence observed in the original *rrn*::Tn*5*-1062 strains resulted from the combined effect of multiple factors including the altered stability and translation of the *luxAB* transcript (Figure 3.7; RM603). Since the *rrn* promoter-*luxAB* fusion strain RM311 behaves similarly to RM603, a significant amount of the increased luminescence may be

caused by the 172 bp sequence located at the 5' end of the *rrn* transcript.

The induction of *luxAB* transcript levels was examined more thoroughly in RM111 and RM311 following a temperature downshift from 30°C to 15°C (Figure 3.8). The results confirmed the earlier observation that the presence of the 5' end of the *rrn* transcript in RM311 resulted in the greater accumulation of *luxAB* transcript at 15°C. Although the induction of *luxAB* mRNA was higher in both RM311 and RM111 in this experiment, the level of *luxAB* mRNA in RM311 was approximately 2-fold greater than that in RM111 after 3 hours at 15°C. This is the same ratio seen in Figure 3.7B. After 4 hours at 15°C, the levels of *luxAB* transcript began to drop in both RM111 and RM311 (Figure 3.8), indicating that the increased accumulation of *luxAB* transcript following a temperature downshift may be transient. Increased promoter activity appeared to contribute to the increase in *luxAB* expression in RM311 as *luxAB* transcript increased 6-fold in RM111 following the temperature downshift. The precise increase in promoter activity cannot be determined from these experiments, however, as both *luxAB* message stability and *rrn* promoter activity contributed to *luxAB* expression in RM111.

Expression of *rrn* promoter-*uidA* fusions. Promoter fragments A and B were also fused to the reporter gene, *uidA*, to confirm the results obtained with the *rrn* promoter-*luxAB* fusions. The fusions were cloned into the pMW193 plasmid and recombined in RM1021 in the same way as the *luxAB* fusions with one

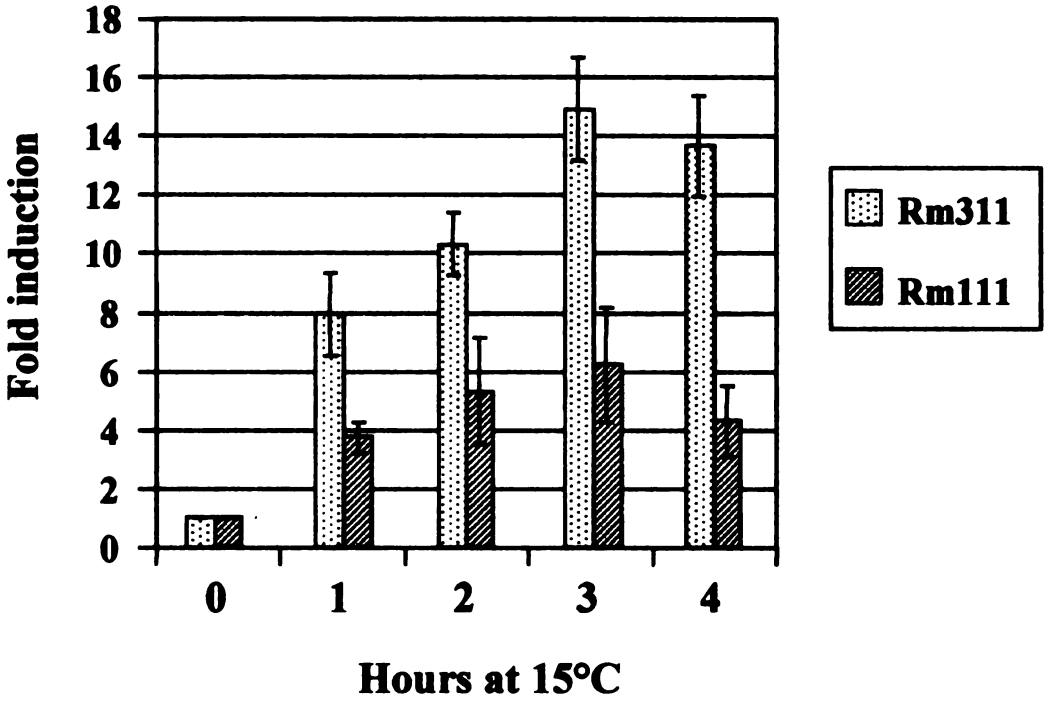


Figure 3.8. Comparison of *luxAB* mRNA levels. The *rrn* promoter-*luxAB* fusion strains, RM311 and RM111, were grown in TY broth supplemented with Sp. RNA was isolated from aliquots taken at 30°C and 1, 2, 3 and 4 hours following a temperature downshift to 15°C. Northern blots were performed and probed with a ^{32}P-labeled *Xba*I fragment containing the *luxAB* genes. The Northern blots were quantitated using a phosphoimager and the fold induction calculated. Each bar represents the average fold induction of 3-4 flasks from two different experiments. The standard deviation of the fold induction is indicated by the error bars.

exception (Figure 3.9A). The promoter-*uidA* fusions were cloned into the *Kpn*I site of pMW193 such that transcription initiating from the *rrn* promoter proceeds toward the omega fragment. Fragment A (-419 to +1) is cloned in front of *uidA* in RM112 and fragment B (-419 to +172) is cloned in front of *uidA* in RM312. Levels of the *uidA* transcript were examined before and 1, 2, 3, 4, and 8 hours following a temperature downshift from 30°C to 15°C (Figure 3.9B).

The *rrn* promoter appeared to be transiently induced at 15°C as the levels of *uidA* message increased within one hour and then declined by four hours in RM112. This general pattern was also observed in RM312 although the 5' end of the rRNA transcript (+1 to +172) appeared to either stabilize or increase the expression of the *uidA* transcript at both 30°C and 15°C. While the induction of *uidA* appears to be slightly prolonged in RM312, the effect was still transient.

Interestingly, two fragments with complementarity to *uidA* were transcribed in RM312 while only the smaller fragment was produced in RM112. Since the only difference between RM312 and RM112 is the presence of the 5' end of the *rrn* transcript, the larger transcript must result from the transcription of both the *uidA* gene and the omega cassette. The omega cassette consists of the spectinomycin resistance gene flanked by an inverted repeat containing a T4 transcriptional terminator. Apparently, sequences within the first 172 bp of the *rrn* transcript allow some transcription to proceed through the first terminator. Therefore, it appears that the first 172 bp of the *rrn* transcript has antitermination

Figure 3.9. Induction of the *rrn-uidA* fusions following a temperature downshift. (A) Schematic of the *rrn* promoter-*uidA* fusions is shown. Promoter fragments A and B were cloned in front of the *uidA* gene within pMW193 in the opposite orientation as the *luxAB* fusions (see Figure 3.5). Transcription from the *rrn* promoter proceeds toward the omega cassette. The inverted repeats containing the T4 transcriptional terminator are depicted by the shaded boxes. (B) RM112 (-419 to +1) and RM312 (-419 to +172) were grown in TY broth at 30°C then shifted to 15°C. RNA was isolated from each culture prior to shifting the cultures to 15°C and then 1, 2, 3, 4, and 8 hours after the temperature shift. 4μg RNA from each sample was electrophoresed and transferred to a nylon membrane. The membrane was probed with ^{32}P-labeled *uidA* DNA. Two exposures of the RM112 portion of the Northern blot are shown: 2.5 hours and 30 hours. The blot was subsequently stripped of radioactive probe and hybridized with ^{32}P-labeled 23S rDNA to check loading.

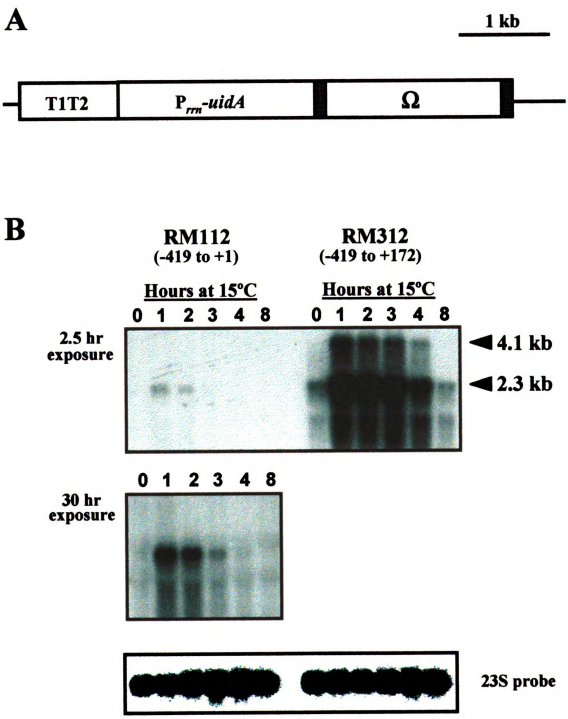

A
1 kb
T1T2
P$_{rrn}$-*uidA*
Ω
B
RM112
(-419 to +1)
RM312
(-419 to +172)
Hours at 15°C
Hours at 15°C
0 1 2 3 4 8
0 1 2 3 4 8
2.5 hr exposure
4.1 kb
2.3 kb
0 1 2 3 4 8
30 hr exposure
23S probe

Figure 3.9

activity. In fact, this region contains a putative *boxA* element and associated structures similar to those involved with the transcriptional antitermination of the *rrn* operons in *E. coli* (Figure 3.10).

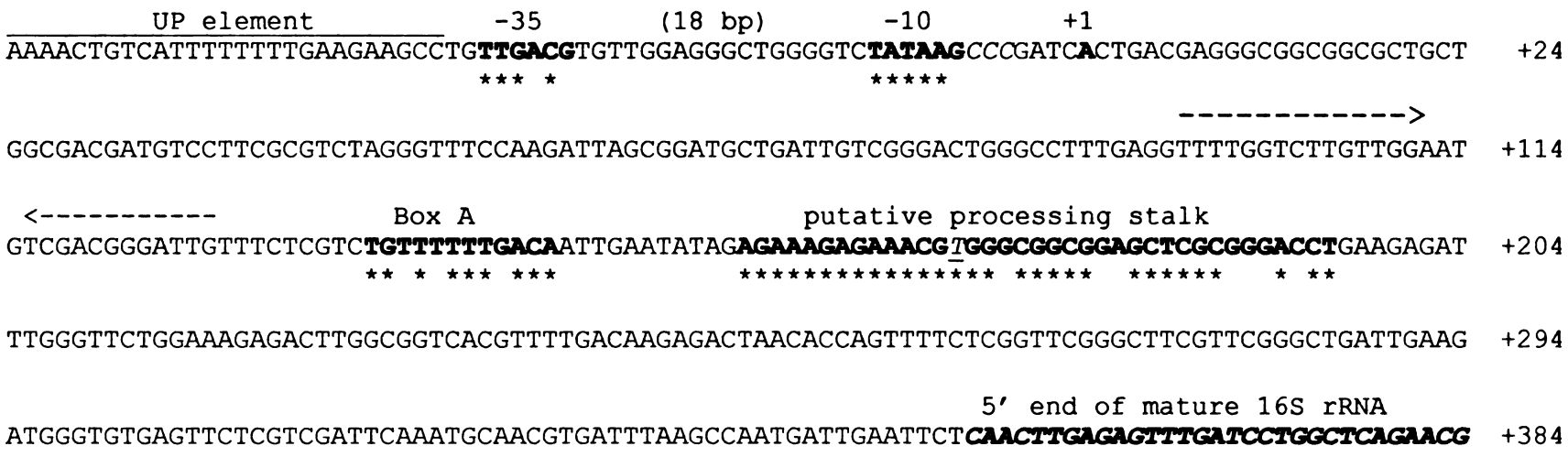


Figure 3.10. The promoter region of the *S. meliloti* 1021 *rrn*A operon. The sequence of the promoter region of the ribosomal RNA operon *rrn*A is shown above. The fully processed 5' end of the 16S rRNA is shown in bold italics. The left half of the inverted repeat thought to form the processing stalk of the 16S rRNA is shown in bold letters. The asterisks indicate the bases with identity to sequences 3' of the 16S rRNA. The 3' end of fragment B cloned into RM311 and RM312 is shown within the processing stalk sequence underlined and in italics. The *boxA* sequence is shown in bold with asterisks indicating bases with identity to the *E. coli boxA* sequence. Dashed arrows indicate sequences predicted to form a stem loop upstream of *boxA*. The predicted -35 and -10 sequences are shown upstream of the initiating nucleotide in bold with asterisks indicating identity with the *E. coli rrn* P1 promoter consensus. The putative UP element is shown as well.

Table 3.1. Bacterial strains

Strains	Description	Reference or source
Sinorhizobium meliloti		
RM1021	Str^R; derivative of SU47	Meade *et al*, 1982
RM603	Km^R; RM1021 containing Tn*5*-1062 insert in the 16S *rrn*A gene	This study
RM111	Ino^-,Sp^R; RM1021 containing *rrn* promoter-*luxAB* fusion (-419 to +1) recombined into inositol locus	This study
RM311	Ino^-,Sp^R; RM1021 containing *rrn* promoter-*luxAB* fusion (-419 to +172) recombined into inositol locus	This study
RM112	Ino^-,Sp^R; RM1021 containing *rrn* promoter-*uidA* fusion (-419 to +1) recombined into inositol locus	This study
RM312	Ino^-,Sp^R; RM1021 containing *rrn* promoter-*uidA* fusion (-419 to +172) recombined into inositol locus	This study
Escherichia coli		
DH5α	*supE44 hsdR17 recA1 thi-1* Δ*lacU169*(ϕ 80*lacZ*Δ*M15*) *endA1 gyrA96 relA1*	Hanahan, 1983

Table 3.2. Plasmids

Plasmids	Description	Reference or source
6H7	Tet^R; cosmid carrying the promoter region of the *rrn*A operon	This study
pAG1X7	Ap^R; *BamH*I fragment containing the *rrn* promoter subcloned from pAG1X and cloned into *BamH*I site of pBluescript SK-	This study
pAG1X7b	Ap^R; *Bgl*II/*Xba*I fragment subcloned from pAG1X7 and cloned into pBluescript SK-	This study
pAG1.3	Km^R; 605 bp *Bgl*II/*Sac*I fragment containing the *rrn* promoter, subcloned from pAG1X7, and cloned in front of the *luxAB* genes in pRK290	This study
pKO11	Ap^R; 2.5 kb *Xba*I fragment carrying the *luxAB* genes subcloned from pRL1062a cloned into the *Xba*I site of pBluescript KS	This study
pKO12	Ap^R; 2.5 kb *Xba*I fragment carrying the *uidA* cloned into the *Eco*RI site of pBluescript KS	This study
pAG111	Ap^R; fragment A (-419 to +1) of the *rrn*A promoter cloned into the *Sac*I/*Not*I sites of pKO11	This study
pAG111rec	Tet^R,Sp^R; *Kpn*I fragment containing the *rrn* promoter-*luxAB* fusion (-419 to +1) cloned into pMW193	This study
pAG112	Ap^R; fragment A (-419 to +1) of the *rrn*A promoter cloned into the *Sac*I/*Not*I sites of pKO12	This study

Table 3.2. Plasmids (cont'd)

Plasmids	Description	Reference or source
pAG112rec	Tet^R,Sp^R; *Kpn*I fragment containing the *rrn* promoter-*uidA* fusion (-419 to +1) cloned into pMW193	This study
pAG311	Ap^R; fragment B (-419 to +172) of the *rrn*A promoter cloned into the *Sac*I/*Not*I sites of pKO11	This study
pAG311rec	Tet^R,Sp^R; *Kpn*I fragment containing the *rrn* promoter-*luxAB* fusion (-419 to +172) cloned into pMW193	This study
pAG311-1	Ap^R; a derivative of pAG311, following the digestion of pAG311 with *Pst*I, this plasmid contains *rrn* promoter fragment B fused to the 399 bp *Xba*I-*Pst*I fragment of the *luxAB* genes	This study
pAG312	Ap^R; fragment B (-419 to +172) of the *rrn*A promoter cloned into the *Sac*I/*Not*I sites of pKO12	This study
pAG312rec	Tet^R,Sp^R; *Kpn*I fragment containing the *rrn* promoter-*uidA* fusion (-419 to +172) cloned into pMW193	This study
pMW193	Sp^R,Tet^R; pRK290-based vector designed to recombine promoter-reporter gene fusions into the inositol locus of RM1021	Bosworth *et al.*, 1994
pRK2013	Km^R; helper plasmid used to mobilize plasmids into RM1021 strains	Figurski *et al.*, 1979
pJB251	Gm^R; this plasmid incompatible with RK2 vectors	Bosworth *et al.*, 1994

Table 3.3. Oligonucleotides

Oligonucleotide	Sequence	Function and features
MT119	5' AATGTTAGCCGTCGGGCAGT 3'	complementary to central region of 16S *rrn* gene; used to probe *S. meliloti* cosmid library
MT179	5' CCAGTTTTCTCGGTTCG 3'	complementary to the *rrn* transcript from +93 to +117; used for primer extension
MT213	5' CTCGCGGCCGCACGTTTCTCTTTCTC -TATATTCAATTGTC 3'	right primer for amplification of fragment B of the *rrn* promoter region beginning at +172; includes the *Not*I site for cloning
MT214	5' CTCGCGGCCGCTGATCGGGCTTATA -GACCCCAGCCCTCCAA 3'	right primer for amplification of fragment A of the *rrn* promoter region beginning with +1; includes the *Not*I site for cloning
MT215	5' CTCGAGCTCGGTACCGATCTCGCGC -CGGTCCGGTG 3'	left primer for amplification of fragments A and B of the *rrn* promoter region beginning at -419 and including *Sac*I and *Kpn*I sites for cloning
MT261	5' GGTTTTGGTCTTGTTGGAATGTCGA 3'	complementary to the *rrn* transcript from +254 to +270;used for primer extension

Discussion

In a previous study it was shown that *Sinorhizobium meliloti* 1021 contained three operons encoding *rrn* genes (Honeycutt *et al.*, 1993). Here we report the sequence and regulation of the promoters controlling the expression of these operons (Figure 3.10). The sequence upstream of the predicted 5' end of the mature 16S rRNA transcript is essentially identical in each operon suggesting that the three operons are coordinately regulated (Figure 3.1). Two methods were used to map the transcriptional start sites of the three operons. While the primer extension analysis suggested that there might be two transcription start sites and, therefore, two promoters (Figure 3.2), the ribonuclease protection assay ruled out the existence of one of the start sites (Figure 3.3). Although neither assay can distinguish between the original 5' end of a transcript and a processed end, the primer extension assay can produce artefactual bands due to the premature termination of reverse transcriptase. Thus, we conclude that a single promoter controls the expression of each of the three operons. The banding patterns obtained from samples taken before and after a temperature downshift were the same, indicating that the same promoter sequences were used at both temperatures (data not shown).

The predicted -10 and -35 hexamers in the *S. meliloti* 1021 promoter regions are very similar to those of the *E. coli* P1 *rrn* promoters (Figures 3.10).

There is however, a larger separation between the two hexamers in *S. meliloti* 1021, 18 bp as opposed to the 16 bp found in *E. coli*. The rRNA transcript in all three *S. meliloti* 1021 *rrn* operons begins with adenine as do most of the rRNA transcripts in *E. coli*. This similarity suggests that *S. meliloti* and *E. coli* may share a similar mechanism for growth rate-dependent control. In *E. coli*, the efficiency of transcriptional initiation at the *rrn* promoters is sensitive to the availability of the initiating nucleotide, usually ATP (Gaal *et al.*, 1997). The level of ATP has been positively correlated with increasing growth rates. The efficiency of transcription initiation, therefore, increases with growth rate leading to higher expression of the *rrn* genes.

The UP element also contributes to *rrn* promoter activity in *E. coli* (Ross *et al.*, 1993). This element located between -60 and -40 is rich in adenines and thymines and is thought to interact with the α subunit of RNA polymerase. The region between -67 and -40 in the *S. meliloti rrn* promoter regions also contains a large number of adenines and thymines suggesting that this may be an activation sequence as well (Figure 3.10). It is interesting to note that while the sequence of *rrn*A begins to diverge in this region, the high frequency of adenines and thymines is maintained (Figure 3.1).

The leader regions of rRNA transcripts typically contain conserved elements important in the processing of the 16S rRNA and transcriptional antitermination. In *E. coli*, inverted repeats on either side of the mature 16S and

23S rRNA sequences anneal to form processing stalks which are then cleaved by RNase III to form the pre-16S and pre-23S rRNAs (see review Srivastava and Schlessinger, 1990). In *S. meliloti* 1021, inverted repeat sequences were also found on either side of the 16S rRNA sequence (Figure 3.10). It is important to note that the second transcriptional start site predicted by the first primer extension assay lies within the upstream repeat, (+158 to +194), further supporting the existence of only one *rrn* promoter. Consequently, the 5' end of the repeat is contained within promoter fragment B (-419 to +172).

A second conserved element, *boxA*, was located within the leader region and also contained in promoter fragment B cloned into RM311 and RM312. This element is important for the expression of the rRNA genes through a transcriptional antitermination mechanism. The *boxA* element in *E. coli* consists of the 12 bp sequence TGCTCTTTAACA. Point mutations made in this sequence showed that nucleotides 2 to 8 (GCTCTTT) were functionally important for antitermination (Berg *et al.*, 1989). This element appears to be highly conserved among many diverse bacteria including *Caulobacter crescentus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Mycoplasma capricolum*, *Mycoplasma hypopneumoniae*, *Thermus thermophilus*, *Thermatoga maratima*, and *Halobacterium halobium* (Berg *et al.*, 1989). These sequences were flanked by a stem loop and a short sequence high in adenines. An element with 75% identity to the 12 bp *E. coli boxA* was found in the *rrn* leader in *S. meliloti* 1021 (Figure

3.10). Five of the seven key nucleotides were identical to the *E. coli* sequence. This was the same criteria used in locating *boxA* in the bacteria listed above. The sequence immediately following the *boxA* sequence was reasonably high in adenines and a putative stem loop was present in the upstream sequence. A further characteristic found in the bacteria examined by Berg *et al.* (1989) was the placement of the *boxA* element adjacent to or within the processing stalk of the 16S rRNA. The *boxA* element identified in the *S. meliloti* 1021 *rrn* promoter is only 11 bp 5' to the inverted repeat predicted to form the processing stem of the 16S rRNA (Figure 3.10).

Previously, Tn*5*-1062 insertions in the *S. meliloti* 1021 16S and 23S *rrn* genes were observed to increase in luminescence following a temperature shift from 30°C to 15°C (Chapter 2). The level of *luxAB* transcript was induced as well, suggesting that the *rrn* promoters might be activated by the shift to low temperature (Figure 2.9). To determine whether the *rrn* promoters were, in fact, activated by a temperature downshift, two promoter fragments were fused to the *luxAB* reporter genes and integrated into the *S. meliloti* genome. Fragment A (-419 to +1) contained only the upstream and promoter regions thus reflecting promoter activity. Since fragment B (-419 to +172) contained a portion of the rRNA leader region, this promoter fragment fused to the *luxAB* genes would reflect any additional promoter-independent regulation.

The pattern of luminescence in the *luxAB* fusion strain, RM311 (-419 to +172), while similar to that observed in the Tn*5*-1062 insertion mutant, RM603, was quite different to that of RM111 (-419 to +1) (Figure 3.6). Luminescence in both RM311 and RM603 increased approximately 40-fold while luminescence in RM111 increased by only 6-fold (Figure 3.6C). Comparing levels of luminescence to levels of *luxAB* transcript revealed that while RM111, RM311 and RM603 contained comparable amounts of *luxAB* transcript, RM111 produced 5 to 8-fold more light than RM603 and RM311 at 30°C (Figure 3.7A). These data suggest that the *luxAB* transcript is translated less efficiently in RM603 and RM311. Since the *luxAB* genes are co-transcribed with a portion of the *rrn* transcript in RM603 and RM311, secondary structures formed in the rRNA portion of the transcript may interfere with the translation of the *luxAB* genes. Alternatively, proteins interacting with the rRNA transcript may also contribute to the inhibition of translation. When comparing RM311 and RM603, it is important to note that adding only 172 bp of the *rrn* transcript to the *luxAB* genes created a result comparable to the presence of over 1 kb of *rrn* transcript. While it is not clear what sequences at the 5' end of the *rrn* transcript caused the reduction in luminescence, it interesting to note that these sequences precede genes which are not translated.

While it is reasonable that a particular transcript may be translated less efficiently than another, it is unexpected that this effect would be lessened

following a temperature downshift. Although the ratio of *luxAB* transcript to luminescence in RM111 is comparable at both 30°C and 15°C, this ratio changes markedly in RM311 and RM603 (Figure 3.7B). At 15°C, it appears that the *luxAB* transcript is translated more efficiently in RM311 and RM603 than at 30°C. The mechanism causing this change is not clear although a possible explanation involves the nature of the cold shock response. In *E. coli*, adaptation of the ribosome to low temperature is thought to be central to the cold shock response (Jones and Inouye, 1996). Further, the cold shock proteins, CspA and CsdA, have been proposed to work together to remove secondary structure from mRNA thus improving translation initiation. If the inhibition of translation observed at 30°C in RM311 and RM603 is resulting from secondary structures in the *rrn* portion of the *luxAB* transcript, translation of the *luxAB* transcript may be improved following adaptation of the ribosome. Alternatively, the shift to low temperature may directly disrupt whatever inhibitory structures or interactions were affecting the *rrn-luxAB* transcript at 30°C.

The *rrn* promoter fused to the *luxAB* genes at +1 showed a modest induction in activity within an hour of the temperature downshift (Figures 3.6-3.8). In Figure 3.8, the levels of *luxAB* transcript began to decline after three hours at 15°C suggesting that the induction is temporary. A transient induction of promoter activity following an abrupt temperature downshift may be understood within the context of a sudden inhibition of translation following a shift to low temperature.

Since *rrn* promoter activity is modulated by the demand for protein synthesis, inhibition of protein synthesis may signal the need for more ribosomes. Jones *et al.* (1992) found that the stringent response and the cold shock response were opposing activities in *E. coli*. The stringent response occurs when the concentration of uncharged tRNAs increases relative to charged tRNAs resulting in an increase in ppGpp levels. Jones *et al.* (1992) found that triggering the stringent response prior to a cold shock delayed the cold shock response. In cells lacking the ability to synthesize ppGpp, several cold shock proteins were induced at higher temperatures. As one aspect of the stringent response involves the repression of rRNA transcription, the equivalent of a nutrient upshift may lead to the activation of *rrn* expression. Previous studies indicated that more ribosomes were not needed at low temperature as long as growth rate was allowed to decrease. Other regulatory mechanisms could then predominate resulting in the subsequent repression of rRNA transcription (Ryals *et al.*, 1982). Thus, there may be a transient increase in *rrn* expression immediately following a temperature downshift that is quickly moderated as the cell adapts to the new temperature. In fact, when stable RNA was measured in cells of *S. meliloti* 1021 grown at 30°C and 15°C, no significant difference was observed between cultures grown at the different temperatures (T. Schmidt, personal communication). This data indicates that there is no net increase in the number of ribosomes in cells grown at 15°C relative to those grown at 30°C.

Fusing the *rrn* promoter fragments to the *uidA* gene in RM112 and RM312 confirmed the transient induction of *rrn* promoter activity following a temperature downshift. The transient nature of the promoter activation can be seen more clearly in RM112 and RM312 as the *uidA* transcript appears to be less stable than the *luxAB* transcript. The peak of induction was between 1-2 hours in the experiment shown in Figure 3.9 as opposed to the peak at 3 hours shown in Figure 3.8. This difference is not necessarily characteristic of a particular reporter gene as some variation was seen within each set of *uidA* and *luxAB* fusions (data not shown). An interesting difference between the expression of the *uidA* and *luxAB* genes is the effect of fragment B on the level of gene expression. The level of *uidA* transcript is much higher at both temperatures in RM312 than in RM112 while the level of *luxAB* transcript differs by no more than two-fold between RM311 and RM111. The increased expression of the *uidA* transcript in RM312 is most likely due to antitermination activity originating from the 172 bp of *rrn* leader region. Apparently, transcription of the *uidA* gene in RM112 is sensitive to premature termination. Since the levels of *luxAB* transcript are comparable in RM311 and RM111, the transcription of the *luxAB* genes may not be affected by premature termination.

Taken together, our results indicate that each *rrn* operon in *S. meliloti* 1021 is controlled by a single promoter. As these promoters are very similar to the *rrn* P1 promoters of *E. coli*, they may be regulated by similar mechanisms. Contrary

to our previous studies of *rrn*::Tn*5*-1062 insertions, the *rrn*A promoter fused at +1 to the *luxAB* genes showed only a modest 4 to 6-fold induction in luminescence following a temperature downshift. However, fusing the *luxAB* genes to the *rrn*A promoter at +172 essentially recreated the induction of luminescence initially observed in the transposon insertion mutants. This induction appeared to be due primarily to the inhibition of *luxAB* translation at 30°C. Construction of *rrn* promoter fusions to *uidA* confirmed the transient induction of the *rrn* promoters and indicated the presence of antitermination activity in the 5' end of the *rrn* transcript.

Materials and Methods

Bacteria and growth conditions. Cultures of *Escherichia coli* DH5α were grown aerobically at 37°C in LB broth (Sambrook *et al.*, 1989) or on solid LB medium containing 1.4% agar. Cultures of *Sinorhizobium meliloti* were grown aerobically at 30°C in either tryptone-yeast extract medium, TY (Beringer, 1974), GTS medium (Kiss *et al.*, 1979), BSM medium (Bergersen, 1961), or BSI medium. BSI medium is modified BSM containing 1% inositol instead of 1% mannitol as the carbon source. Cultures were maintained on solid medium containing 1.4% agar, or frozen to -80°C in TY broth containing 15% glycerol. *S. meliloti* was cultured on solid media containing the following antibiotics when appropriate: gentamycin, Gm (50 μg/ml), kanamycin, Km (200 μg/ml), spectinomycin, Sp (100 μg/ml), streptomycin, Sm (250 μg/ml), and tetracycline, Tet (10 μg/ml). *E. coli* was grown on plates containing ampicillin, Ap (100 μg/ml), Gm (50 μg/ml), Km (100 μg/ml), Sp (100 μg/ml), or Tet (10 μg/ml) when appropriate. A lower concentration, 50 μg/ml, of Km and Sm was used in broth cultures. When used together in broth cultures, Gm and Sp were present at 20 μg/ml and 50 μg/ml, respectively.

DNA sequencing and analysis. Cosmid DNA was prepared for sequencing from *E. coli* bacteria using Qiagen columns (Qiagen Inc., Valencia, CA). The cosmids carrying the *rrn* promoter regions were sequenced at the Biotechnology Resource Laboratory, Yale University. Sequence alignments were performed using the Pileup program in the GCG sequence analysis package (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI).

Isolation of RNA. RNA was isolated from *S. meliloti* strains according to a modified protocol derived from that found in Ausubel *et al.*, 1987, for the isolation of RNA from gram negative bacteria. Bacteria were harvested from a 3-20 ml aliquot of cell culture to which rifampicin had been added to a final concentration of 0.2 mg/ml. The cell pellets were resuspended in 1.4 ml of protoplasting buffer and 80 µl of 50 mg/ml of lysozyme added. The tubes were incubated on ice 5-10 minutes. The protoplasts were collected by centrifugation for 2 minutes at 7,000 rpm. The pellets were resuspended in 0.5 ml of gram negative lysing buffer, mixed with 15 µl of diethylpyrocarbonate, DEPC, and incubated for 5 minutes at 37°C. Tubes were chilled briefly on ice, mixed with 250 µl of 5M NaCl, incubated 10 minutes on ice and centrifuged for 10 minutes. The supernatant was removed, added to a tube containing 1 ml ethanol and stored overnight at -20°C. The RNA was collected by centrifuging for 15 minutes at 4°C, dried under vacuum and resuspended in DEPC-treated water. Samples were quantified using a spectrophotometer and measuring A_{260} and A_{280}. Alternatively, the cell pellets

were frozen quickly in liquid nitrogen and the samples stored at -80°C. RNA was then isolated using Rneasy Mini Kits (Qiagen Inc., Valencia, CA).

Primer extension analysis. Primer extension assays were performed according to the manufacturer's recommendations using the AMV Reverse Transcriptase Primer Extension System (Promega, Madison, WI). The primers used in this experiment were synthesized by the Macromolecular Structure Facility located in the Department of Biochemistry at Michigan State University. The annealing temperatures used in the assay were specific to the primer, 65°C for MT179 and 60°C for MT261. Depending on the experiment, 100 or 500 fmoles of end-labeled primer were used in a reaction with 10 or 20 μg of *S. meliloti* RNA. The primer extension reaction products were compared to a sequencing ladder synthesized with the same primer used in the primer extension assay. Standard protocols were used as described in the T7 Sequenase v. 2 DNA Polymerase DNA sequencing kit (Amersham Life Science, Inc., Cleveland, OH). The plasmid template used with MT179 to produce a sequencing ladder was pAG1X7 (*BamH*I fragment of pAG1X cloned into the *Xba*I site of pBluescript SK-). The template used with MT261 was a smaller version of the above plasmid, pAG1X7b (*Bgl*II-*Xba*I fragment cloned into pBluescript SK-). Primer extension products and sequencing ladders were analyzed using standard gel electrophoresis and autoradiography techniques.

RNase protection assay. RNase protection assays were performed according to standard protocols (Ausubel *et al.*, 1987). The RNA probe was synthesized from the plasmid pAG311-1. The plasmid was digested with *Sac*I and the 3' overhang removed by incubation with T4 DNA polymerase. The cut plasmid was then treated with proteinase K to remove any contaminating ribonucleases. The template DNA was extracted once with phenol:chloroform:isoamyl alcohol, ethanol precipitated, and resuspended in water to a concentration of 500 ng/μl. The RNA probe was then synthesized from the template plasmid using the T3 DNA polymerase from Boehringer Mannheim. The Promega Riboprobe system components (Promega, Madison, WI) were used according to the manufacturer's suggested protocol to synthesize a ^{32}P-labeled RNA probe complementary to a 399 bp fragment of the *luxAB* gene cassette fused to a 500 bp fragment of the *S. meliloti rrn* promoter region. The RNeasy Miniprep columns (Qiagen, Inc., Valencia, CA) were used to separate the RNA probe from the unincorporated nucleotides according to the kit's RNA cleanup protocol. The radioactivity of the RNA probe was quantified using the Packard Tri-Carb® liquid scintillation analyzer model 1500 (Packard Instrument Company, Inc., Downers Grove, IL). 10 μg aliquots of *S. meliloti* RNA were briefly dried under vacuum and resuspended in 30μl of hybridization buffer containing 5 x 10^5 cpm of probe. Samples containing 10μg of tRNA were included with each assay as a control. After 5 minutes at 85°C samples were hybridized at 55°C overnight.

After ribonuclease digestion and treatment according to the standard protocol, the samples were separated on a 4% acrylamide/urea gel and visualized by autoradiography. The GibcoBRL 0.16-1.77 RNA ladder was end-labeled with [γ-^{32}P]ATP to act as a size standard for gel electrophoresis.

PCR cloning and integration of the resulting promoter-*luxAB* fusions. Two regions of the *rrn*A promoter region were amplified from the cosmid 6H7 (Figure 3.1) which carries the upstream region of the 16S *rrn* isolated on fragment 1 (Figure 2.6) and corresponds to the region cloned into pAG1.3 (Figures 2.7 and 2.8). The left primer used for both fragments was MT215 5'-CTCGAGCTCGGT ACCGATCTCGCGCCGGTCCGGTG-3' and includes the *Sac*I and *Kpn*I sites for cloning. The right primer used for the larger fragment B (Figure 3.5) was MT213 5'-CTCGCGGCCGCACGTTTCTCTTTCTCTATATTCAATTGTC-3' incorporating the *Not*I site. The right primer used for the smaller fragment A was MT214 5'-CTCGCGG CCGCTGATCGGGCTTATAGACCCCAGCCCTCCAA-3' with the *Not*I site included as well. Vent$_R$® DNA polymerase (New England Biolabs Inc., Beverly, MA) was used to amplify the fragments from 0.05 ng of 6H7 DNA with 20 pmoles of each primer. The PCR conditions were as follows: 94°C (5 minutes) then 30 cycles at 94°C (1 minute)/ 50°C (1 minute)/ 72°C (1 minute) followed by 5 minutes at 72°C. A second round of amplification was necessary to obtain enough of the fragments for cloning. The conditions of the second round were the same as the first with two exceptions: only 26 cycles were

used and the first PCR product was used as template. The PCR products were then digested with *Sac*I and *Not*I and band isolated. These fragments were cloned into the *Sac*I and *Not*I sites in the multiple cloning site of pKO11. The plasmid pKO11 is a derivative of pBluescript KS containing the *luxAB* genes cloned into the *Xba*I site. The resulting plasmids, pAG111 or pAG311, were isolated and digested with the enzyme *Kpn*I. The enzyme cut at the *Kpn*I site engineered into the left primer and the *Kpn*I site in the multiple cloning site from pKO11. The band isolated contained the promoter fragment in the correct orientation to control transcription of the *luxAB* genes. This fragment was then cloned into pMW193 forming the plasmids, pAG111rec and pAG311rec. The plasmids were moved into the wild type strain RM1021 via triparental matings using the plasmid pRK2013 as a helper plasmid. Transformed colonies were selected for on BSM plates containing Tet or Sp. The incompatible plasmid pJB251 (Gm^R) was moved into the RM1021 strains carrying pAG111rec or pAG311rec using the triparental mating method and pRK2013 (Km^R) as a helper plasmid. Mating spots were streaked on BSM plates containing Gm and Sp. The resulting colonies were grown up in TY broth containing Sp and Gm until cultures were dense and then diluted and spread on BSM with Sp and Gm. These colonies were then screened for the inability to grow on inositol and the loss of tetracycline resistance. Three replicate plates were made: BSI with Sp and Gm, TY with Sp, Gm, and Tet, and TY with Sp and Gm. Single recombinants should grow on both sets of TY plates

while double recombinants should grow only on TY with Sp and Gm. In the construction of these plasmids the second recombination event happened at a high enough frequency such that no single recombinants were isolated, only double recombinants.

Detection and quantification of bacterial luciferase activity. Luciferase activity was measured differently depending on the culture conditions used to grow the bacteria. If the bacteria were grown on a solid agar medium, luminescence was measured with the Hamamatsu Photonic System model C1966-20 (Photonic microscopy, Oak Brook, IL). The Berthold LUMAT luminometer LB 9501 (Wallac Inc., Gaithersburg, MD) was used to measure aliquots of liquid bacterial cultures. Bacteria grown on agar plates were exposed to the enzyme substrate, N-decyl aldehyde, for two minutes prior to being assayed for light production for 59 seconds. The aldehyde was spread on a glass petri dish which was then placed over the agar plate. The Hamamatsu system converts photon counts to a visual image displayed on a monitor. This system can also quantitate the amount of light produced by each colony.

Bacteria grown in broth culture were assayed quantitatively for light production. In this assay, the N-decyl aldehyde was dispersed in an aqueous solution of bovine serum albumin (20 mg/ml) at a concentration of 1 μl/ml. A 5-10 μl aliquot of the cell culture was added to 50-100 μl of the aldehyde solution, vortexed for 30 seconds, then assayed for 60 seconds. The measured

luminescence was reported in arbitrary units or RLU and adjusted for cell number by dividing this number by the A_{600} of the culture. All assays were performed at room temperature.

Northern blots. RNA samples were treated with formaldehyde and electrophoresed on formaldehyde/agarose gels according to standard protocols (Sambrook *et al.*, 1989). RNA was transferred from the gel to a nylon membrane in 10X or 20X SSC via capillary action according to the standard protocol (Sambrook *et al.*, 1989) with one modification. After transfer, the RNA was crosslinked to the membrane by exposure to 20 mjoules of light energy in the UV Stratalinker 2400 (Stratagene, La Jolla, CA). The membranes were pre-hybridized at 42°C in the following solution: 50% formamide, 5X SSC, 50mM potassium phosphate, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate, and 100μg/ml denatured DNA. After a minimum of 2 hours of pre-hybridization treatment, ^{32}P-labeled probe was added to the solution and incubated overnight at 42°C in a Robbins Hybridization Incubator Model 400 (Robbins Scientific Corp., Sunnyvale, CA). The probe was prepared from a band isolated fragment by a random priming method (Feinberg and Vogelstein, 1983). The membranes were treated first at room temperature with three washes of 2X SSC and 0.5% SDS. After one wash in 0.1X SSC and 0.5% SDS, the temperature was raised to 50°C for two more washes in the same solution. Washes were continued until the spent wash solution was no longer detectably radioactive.

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CHAPTER 4

A *Sinorhizobium meliloti* 1021 gene similar to the *Escherichia coli rne* gene is induced following a temperature downshift

Summary

Sinorhizobium meliloti 1021 was mutagenized with the transposon, Tn*5*-233, to identify genes whose products affected the expression of the cold-inducible *rrn*^{P}-*luxAB* fusion, pAG1.3. The transposon recipients were assayed for their ability to affect the light production of pAG1.3 which contains the *luxAB* gene cassette fused to the *rrn*A promoter at +187. Approximately 28,000 colonies were screened for increased luminescence at 30°C and 14,000 colonies for decreased luminescence at 15°C. Thirteen transposon inserts were found that increased luminescence from pAG1.3 at 30°C. Two of these inserts, RM26b1 and RM483 increased light production approximately 5-fold at 30°C when they were transduced into strain RM603 (16S *rrn*A::Tn*5*-1062). Both inserts were located within an ORF whose deduced protein product showed significant similarity to RNase E, an essential endoribonuclease involved in the processing and degradation of mRNA and rRNA. The transposon insertions are located near the

carboxy terminus of this protein. The insertion mutants, therefore, should produce a truncated form of RNase E. The inserts were shown to affect the accumulation and processing of the *luxAB* mRNA at 30°C in the double mutants RM603(26b1) and RM603(483). They also influenced the processing and accumulation of the *cspA* transcript following a temperature downshift. When expression of *rne*, the gene encoding RNase E, was examined before and after a temperature downshift, the levels of *rne* transcript were found to be transiently induced.

Introduction

While it is known that many bacteria induce a cold shock response following a temperature downshift, the mechanisms by which the change in temperature is sensed and the protein expression regulated are not fully understood. The cold shock response has been most thoroughly studied in *E. coli*. Following a shift in temperature from 37°C to 15°C, *E. coli* induces a set of 16 proteins despite a transient inhibition of overall protein synthesis (Thieringer, 1998). These cold shock proteins can be divided into two classes. The first class includes proteins that are primarily expressed at low temperature and the second comprises proteins that are expressed at the higher temperature and only moderately induced following a cold shock (Mitta *et al.*, 1997). While these proteins have a variety of cellular functions, several proteins including CsdA, RbfA and IF2 are specifically involved with the ribosome (Jones *et al.*, 1996; Jones and Inouye, 1996; Jones *et al.*, 1987). Because of this and the transient inhibition of protein synthesis that occurs in *E. coli* following a cold shock, the cold shock response has been viewed in terms of adapting the translational machinery to function at low temperature (Thieringer *et al.*, 1998). One aspect of this adaptation involves the action of proteins like CsdA and CspA in destabilizing RNA secondary structure (Jones *et al.*, 1996; Jiang *et al.*, 1997). Secondary structures formed in mRNA at low temperature are thought to inhibit translation.

According to the "Cold-Shock Ribosome Adaptation" model proposed by Jones and Inouye (1996), CsdA, a protein with RNA helix unwinding activity, and CspA, a putative RNA chaperone, are thought to interact to remove and prevent formation of secondary structures in mRNA.

Interestingly, the addition of low levels of antibiotics like chloramphenicol that inhibit ribosome function can induce the cold shock response at 37°C (VanBogelen and Neidhart, 1990). This observation and the effect of low temperature in inhibiting translation in *E. coli* led to the proposal of the ribosome as a sensor of temperature change. The relationship of the cold shock response and the stringent response was then investigated. The stringent response involves the changes in gene expression following amino acid starvation. While treatment with chloramphenicol, low temperature and amino acid starvation all result in the inhibition of protein synthesis, the immediate effect on the ribosome differs. Treatment with chloramphenicol and low temperature blocks the ribosome in such a way that charged tRNAs are in excess. Amino acid starvation results in the opposite situation. Indeed, the stringent response was found to work in opposition to the cold shock response (Jones *et al.*, 1992). The overexpression of ppGpp, the signal molecule for the stringent response, inhibited the cold shock response in *E. coli*.

The regulation of the major cold shock protein, CspA, has been studied in detail and has been found to occur at many levels. Promoter activity is only a

minor component in this regulation which affects message stability, translation and potentially transcription attenuation. The *cspA* transcript contains a 159 bp untranslated region at its 5' end that causes the transcript to be extremely unstable at 37°C (Brandi *et al.*, 1996). The message is then transiently stabilized at 15°C allowing the rapid induction of CspA protein. Further investigation of this region by Mitta *et al.* (1997) suggests that while the first 26 bp are sufficient to destabilize the transcript at 37°C, the remainder of the UTR is required to inhibit expression of the *cspA* gene. Contrary to its inhibitory function at 37°C, the larger fragment of the UTR was also found to be required to achieve the full level of mRNA stability at 15°C. Mitta *et al.* (1997) observed that the downstream box, located at the 5' end of the coding region, increased translation at 15°C. This downstream box was required for the full induction of the CspA protein and is thought to increase translation by interacting with the ribosome. Four other highly induced cold shock proteins have UTR sequences at their 5' ends and appear to be derepressed in a *cspA* mutant although the full interaction of these regulatory elements is not yet clear (Bae *et al.*, 1997).

In studying the cold shock response in *Sinorhizobium meliloti* 1021, we isolated several reporter transposon insertions that exhibited cold-inducible gene expression (Chapter 1). While two of these insertions were within an operon containing genes similar to *cspA* and *rpsU* (O'Connell and Thomashow, 1999), the five other insertions were in ribosomal RNA genes. The *rrn*A promoter was

isolated on a 605 bp DNA fragment and fused to the *luxAB* genes at +187 in the plasmid, pAG1.3. This construct showed temperature-dependent luminescence when propagated in *S. meliloti* 1021. In order to identify potential regulatory factors acting on the rrn^P-*luxAB* fusion, *S. meliloti* 1021 was mutagenized and the transposon mutants screened for their ability to alter the luminescence of the *luxAB* fusion. Two insertional mutants were isolated that caused increased luminescence of the rrn^P-*luxAB* fusion and a 16S *rrn* Tn*5*-1062 insertion at 30°C. These two insertions were located within an ORF encoding a protein with significant similarity to Ribonuclease E. The temperature-dependent expression of this locus is described as well as the effect of the insertional mutants on the 16S *rrn* Tn*5*-1062 insertion and on *cspA* expression.

Results

Transposon mutagenesis and screen for transposon inserts affecting the luminescence of pAG1.3. *Sinorhizobium meliloti* 1021 was mutagenized with the spectinomycin resistant Tn*5*-derivative, Tn*5*-233 (De Vos *et al.*, 1986), (Figure 4.1A). The *rrn*^{P}-*luxAB* fusion, pAG1.3, was mobilized into groups of transposon recipients (Figure 4.1B). Approximately 28,000 of the resulting colonies were screened for increased luminescence at 30°C and 14,000 colonies were screened for decreased luminescence at 15°C. No transposon inserts were found that affected luminescence at 15°C, but thirteen colonies showed increased luminescence at 30°C.

Characterization of the putative regulatory mutants. Southern blot analysis of the mutant strains showed that each contained a single Tn*5*-233 insertion (data not shown). Each of these insertions was reconstructed in strain RM603 using the transducing phage Φ-M12 (Finan *et al.*, 1984). RM603 contains a Tn*5*-1062 transposon inserted in the 16S *rrn*A gene that show cold-inducible luminescence. By transducing the Tn*5*-233 insertions into RM603, we hoped to correlate increased luminescence at 30°C with the presence of the transposon insertion. In addition, the number of *luxAB* genes present in the cell was reduced to a single chromosomal insertion, thus decreasing background luminescence at 30°C. All thirteen of the inserts affected the luminescence of RM603 at 30°C.

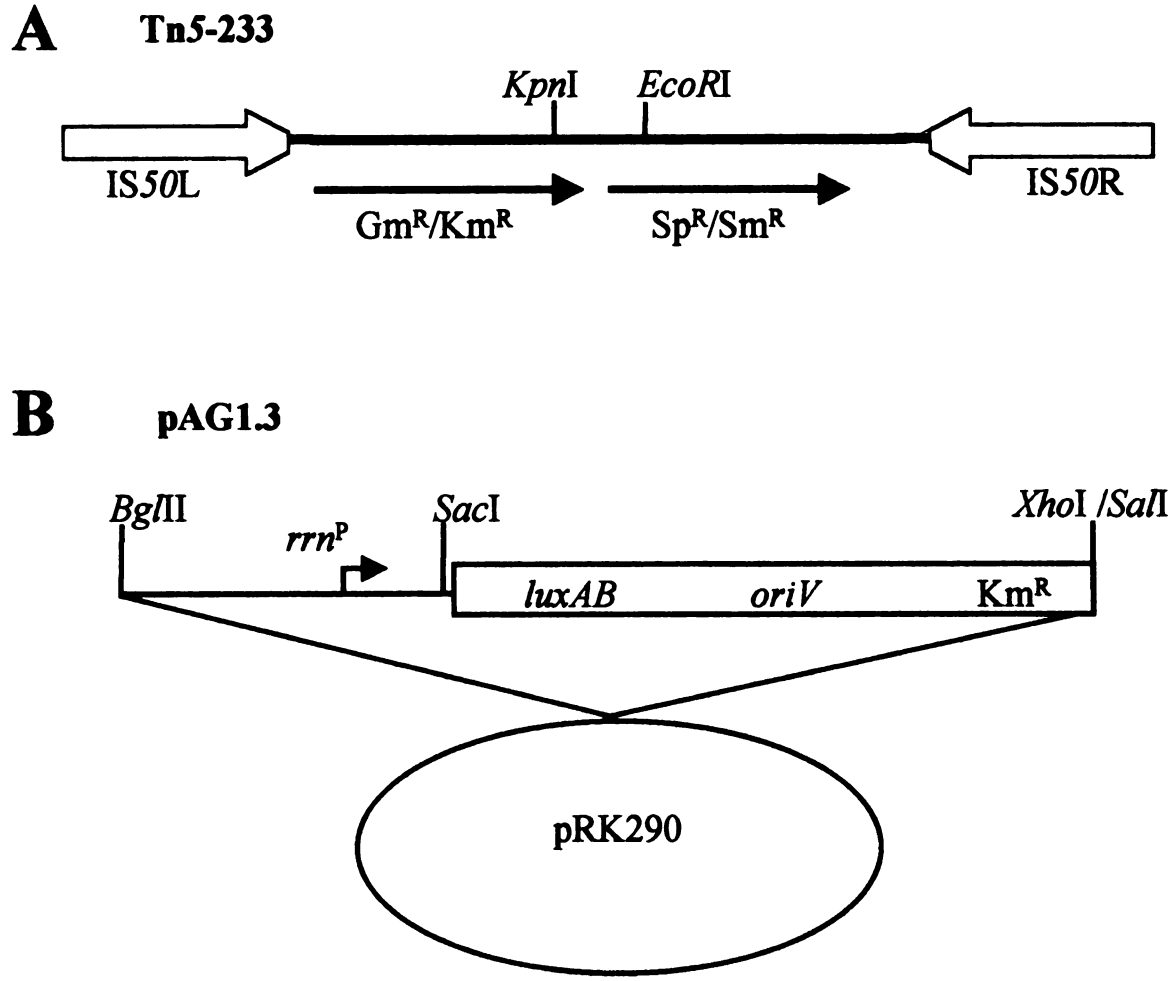

Figure 4.1. Structures of Tn*5*-233 and reporter plasmid, pAG1.3. (A) The structure of Tn*5*-233 (De Vos *et al*, 1986) is shown. Tn*5*-233 is a derivative of Tn*5* from which the central *Bgl*II fragment has been removed and replaced by genes encoding resistance to Gm, Km, Sp, and Sm. The two restriction enzyme sites used in cloning out the ends of the transposon are shown. (B) The structure of the *rrn*^{P}-*luxAB* plasmid, pAG1.3, is shown. The plasmid, pAG1.3, is described in detail in Figure 2.8.

Two of these insertions, RM26b1 and RM483, are described below.

Characterizing the luminescence of the double mutants RM603(26b1) and RM603(483). The Tn*5*-233 inserts, RM26b1 and RM483, were transduced into RM603 forming the double mutants, RM603(26b1) and RM603(483). When cultured on TY agar plates, the double mutants exhibited increased luminescence at 30°C relative to the original strain RM603 but showed little difference in luminescence at 15°C (Figure 4.2). The double mutants were then grown in TY broth with the original insertion mutant RM603 to better quantitate the effect of the Tn5-233 insertions on the luminescence of the Tn*5*-1062 insertion. At 30°C, RM603(26b1) and RM603(483) both produced approximately 5-fold more light than RM603 (Figure 4.3). However, within an hour of a temperature downshift to 15°C, the difference in luminescence between the double mutants and RM603 had declined significantly. After three hours at 15°C, the transduced strains produced only 17 to 20% more light than RM603. While the Tn*5*-233 insertions did increase luminescence at 30°C, luminescence was not fully induced until after the temperature downshift. In fact, luminescence in the double mutants still increased approximately 5-fold after the temperature downshift.

Identification of the insertion sites in RM26b1 and RM483. The insertion sites in RM26b1 and RM483 were identified by cloning and sequencing the DNA contiguous with either one or both ends of the transposon in each of the strains (Figure 4.4). Comparison of the sequences indicated that the two

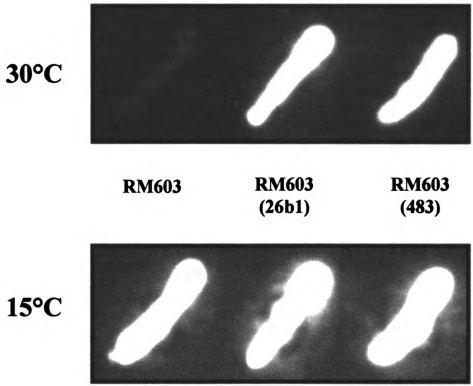

Figure 4.2. The Tn*5*-233 inserts in RM26b1 and RM483 cause increased luminescence at 30°C when transduced into RM603. RM603 (16S *rrn*::Tn*5*-1062) was compared to two double mutants RM603(26b1) and RM603(483). Each double mutant is a derivative of RM603 containing a Tn*5*-233 insert. Cultures were streaked on TY agar plates and incubated at 30°C for two days before being assayed for luminescence. After being assayed for luminescence at 30°C , the plates were moved to 15°C for 5-6 hours then assayed again.

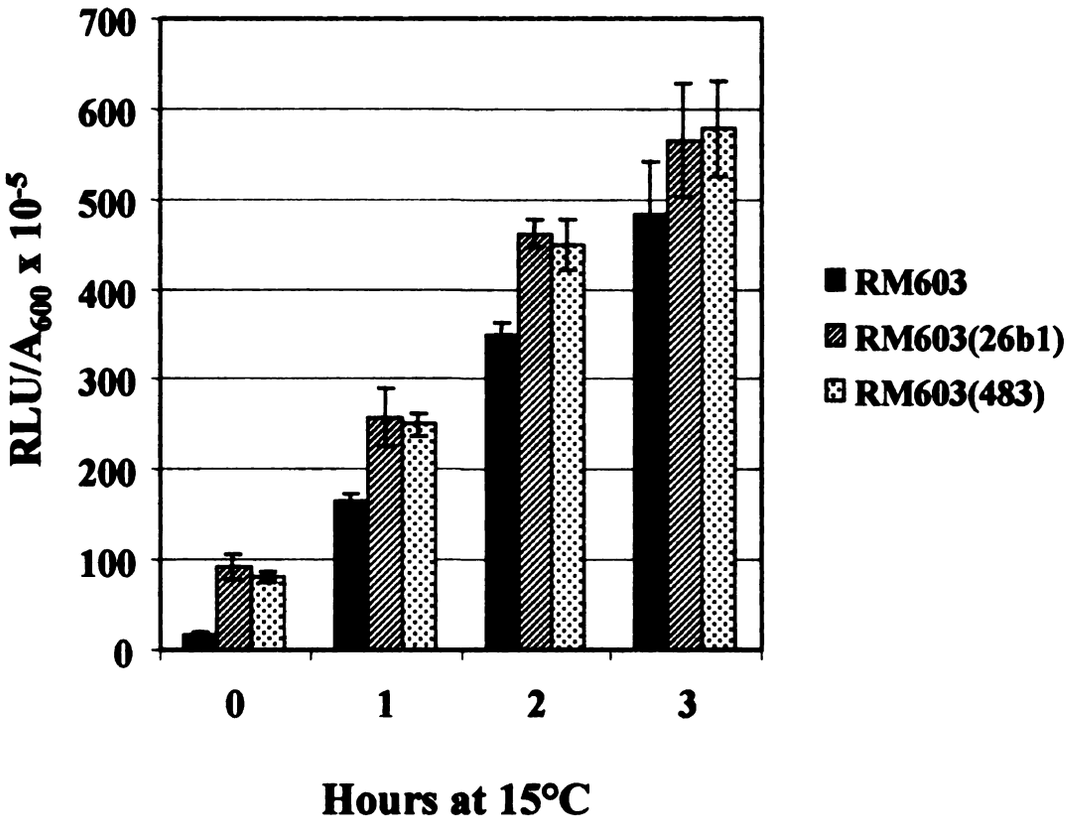

Figure 4.3. Luminescence of RM603(26b1) and RM603(483) at 30°C and 15°C. Luminescence of the two double mutants RM603(26b1) and Rm603(483) was compared to that of RM603 (16S *rrn*::Tn*5*-1062) during growth at 30°C and after a shift to 15°C. The double mutants are derivatives of RM603 containing Tn*5*-233 inserts. The strains were cultured in TY broth with Km (50 μg/ml). Cultures were grown at 30°C until they reached an A_{600} of approximately 0.3. At that point, samples were taken and the luminescence measured. The cultures were then shifted to 15°C and samples taken 1, 2, and 3 hours after the temperature downshift. Luminescence is measured in relative light units, RLU, and adjusted to culture density by dividing by the A_{600} of the culture. Each bar represents the average of measurements of three flasks and the error bars give the standard deviation of these measurements.

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GAATTCGATT TCGAATCGGA ACATAAGAAG CAGATCCGCG GCAATATCTA   50
CCTGGCGAAG GTAACCAGAG TGGAGCCGTC GCTCCAGGCG GCCTTCGTCG  100
ATTACGGCGG CAATCGCCAC GGGTTTCTGG CCTTCGCCGA AATCCATCCC  150
GACTACTACC AGATTCCCCT CGCCGATCGT CAGGCCCTGT TGAAGGCGGA  200
AGCCGAAGAG GCTCGCCGCG ACGATGACGA GCCGAACGAC ACCGTGGCGG  250
AACAGAAATT CGCGCCGGCT GGCGATACGG TTGCCGAAGT CGTGGCACAG  300
GCCGAAGCCG CCGAGGCAGC CGGAGAAGCC GCTGAGGCCG CTGCGGAGAA  350
GCCGAAGGCC AAGGCCAAGC CGAAGCGTAC CCGCCGCGCG AAGCCGAAGG  400
CCGGCGAAGA GGCCGTGGCG CCTGAAAGCG GCCCGTCGGA AGACGGCGAA  450
GGCGACAAGA ACGAAATGGC CGCGGTGGTC GATACCGACT CGATCTCCGA  500
GGATATCGAC GCGCGCCGTC AGCGTGACGA CGACGATGAC GACGACAATC  550
ACGACGGCGA AAAGGAAATC ATCGAATCCG TCGGCGCCGA AGATGCCATG  600
GAAGAGGTGG CGGATCGTCA TATCCGCAAG CCGCGCAAGC AATACCGCAT  650
CCAGGAAGTG ATCAAGCGCC GGCAGATCCT GCTGGTGCAG GTCGCAAAGG  700
AAGAGCGCGG CAACAAGGGT GCGGCACTCA CGACCTATCT TTCGCTCGCC  750
GGCCGCTACT CCGTTCTCAT GCCGAACACG GCGCGCGGCG GCGGTATTTC  800
GCGCAAGATC ACCAACCTGC AGGACCGCAA GCGTCTGAAG GAGATCGCCC  850
GCGGTCTCGA CGTGCCGCAG GGGATGGGTG TGATCCTGCG CACCGCCGGA  900
GCCAATCGCA CGAAAGTCGA GATCAAGCGC GACTTCGAAT ATCTCATGCG  950
CCTGTGGGAG AACGTCCGCA CGCTGACGCT GAACTCGACT GCCCCCTGCC 1000
TCGTCTACGA GGAGGGCAGC CTTATCAAGC GATCGATCCG CGACCTCTAC 1050
ACCAAGGACA TCAGCGAGAT CGTCGTTTCG GGCGAGGAAG GCTACAAGGA 1100
AGCCAAGGGC TTCATGAAGA TGCTGATGCC GAGCCACGCA AAGGTCGTGC 1150
AGCCCTATCG CGACGTGCAT CCGATCTTCT CCCGCTCCGG CATCGAAGCA 1200
CAGCTCGATC GCATGCTGCA GCCGCAGGTG ACGCTGAAGT CGGGCGGCTA 1250
CATCATCATC AACCAGACCG AAGCTCTGGT TTCCATCGAT GTGAACTCCG 1300
GTCGTTCGAC GCGCGAGCAC TCGATCGAGG ATACGGCTCT CCAGACGAAT 1350
CTCGAGGCAG CCGAGGAAGT AGCGCGGCAA TTGCGCCTTC GCGACCTTGC 1400
CGGCCTGATC GTCATCGACT TCATCGACAT GGAAGAGAAG CGGAACAACC 1450
GCTCCGTCGA GAAGAAGCTA AAGGATTGCC TGAAGAACGA TCGTGCCCGT 1500
ATCCAGGTCG GCCGCATCTC GCATTTCGGC CTGCTCGAAA TGTCTCGCCA 1550
GCGCATCCGC GCCTCGGTGC TGGAAAGCAC GATGCAGACC TGCCCGCACT 1600
GCAACGGCAC GGGCCACATT CGCTCGCAAT CCTCCGTTGC CCTGCATGTC 1650
CTGCGCGGCA TCGAGGAACA TCTGCTCAAG AACACCACGC ACGACATCAG 1700
CGTGCGGACG ACACCCGATA TCGCGCTCTA CCTGCTCAAT CAGAAGCGCA 1750
GTTCGATCAC GGACTACGAG CAGCGCTTCG GTGTCTCCAT CTTCATCGAA 1800
GCGGACGCCC ATGTCGGTGC GCAGCACTTC GCGATCGATC GCGGTGAACC 1850
GGTCGAAAAT CCGGTGAGGA TCGACCAGAT CCTGCAGTTC GAGCCGGAGC 1900
CGGAAGACGA AGAAGAGGTC CTCATCGAGG AGGAGTCCGA CGAAGAGGAA 1950
GTGGAAGAGA TTGCTGCCGA GCGTCAGGAC CCGAAAAAGG GGCAGACCGA 2000
CGAACAAGGC GGCCGCAAGC GCAAGCGTCG TCGCCGCCGG CGCGGCAAGG 2050
                RM483
GTGCGGGTCA GGGGGCGGAC GAGGCCTTCG CTGACGCCGC CGACGCGGCT 2100
GAGGACGAGA CCGGCGACGA AGCCGCCGTG GACGATGCCG AGGATGAAGT 2150
CGACGCTACG GATGCCTTGA ATGGCGATAG CGAGCAGAAG CGCAAGCGTC 2200
```

Figure 4.4

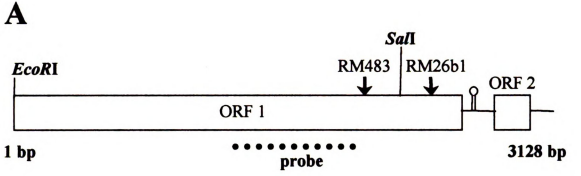
```
GCCGCCGCGG CAAGCGTGGC GGCCGCCGCA GCCGTCAGGA CGAACTGGCC 2250
GAAGCCGAGG GCGAGACCGG CATGGCTGCA GAAGCCGGCG AAGGAGCCGG 2300
CGACGACGCA GAGGCGGAAG TCGCCCCAGC CGCGGCAGCC GAGGAGACGT 2350
                          RM26b1
CACCGGATAT CGCGGCAGCC GCTGCCGTCG AGGCAGCAGC GGAGGAGCCG 2400
AAACCGGCAA AACCCCGGCG CAGCCGCAAG AAAGCAGTCA AGGCCGAGGA 2450
GCCGGCCGAG AGTGTCGATG AAGTAGCCGA GAAGACCGTC GAGGCCCAAC 2500
CCGAAGCAAC CGAAGCCGCC CCCGAGGCCG TGGAGGAAGC TGTTGCCGAT 2550
CTGGAAGGCG CAAAGCCCGC GCGCGCAAAC CGGGATCTGT CGGCGATCGC 2600
TACCGAGCCG GTCGTGACGT CCAGCAAGGG CGAGGGCGAA GAGGAGCCGA 2650
CAAAACCCAA AAAGGGCGGC TGGTGGCAGC GCCGCGGTTT CTTCTAAgaa 2700
      >>>>>>>>>>>>>>     <<<<<<<<<<<<<<
gaataggaaa aagcccggag cgatccgggc tttttcatgg ctggggccgc 2750
gtggcggctc agttgtgacc tgcctctttg aaaactccgc actttccgga 2800
cggaaagcgg cacacgcttt tccttatccc gctctatact ttcccgcagg 2850
gaggacgagg ccATGCGCAA GTTCACAGTC ATCGTTACGG AAGAGTTCGA 2900
AGCCGACACT GCCGAGGAGG CCGCCTTGCT CATGTATCAC CAACTGACCA 2950
ACGGGCCGGC ACCTCTGCAT TATTCGGTCA CGGATGAAAC GAAGATTGCA 3000
ACCAGCCTGA TACTGGACCG GAAAAAGGCA GATGAATTTG CCAGTGTCGA 3050
TCACACTGCA GACCCCGGCA ATTGGTAAgc cacgcgtaac cggccgatgt 3100
gcggataccc atgtctggag cagagcac 3128
```

Figure 4.4 (cont'd). DNA sequence analysis of the insertion sites in RM483 and RM26b1. The DNA contiguous with the transposons in RM483 and RM26b1 was cloned and sequenced. Putative coding sequences are capitalized. Non-coding sequence is typed in lowercase. Predicted terminator sequences are indicated by arrowheads above the relevant sequence. The insertion sites for each strain are labeled with the strain name and the relevant bases printed in bold.

transposon insertion sites were separated by only 310 bp. Two open-reading frames (ORFs) were predicted for this region using the codon preference program, CodonUse 3.1, developed by Conrad Halling (University of Chicago). A schematic of the region showing the predicted ORFs and the location of the Tn*5*-233 insertions in the two mutants is shown (Figure 4.5A). Both inserts are located within ORF1 which encodes a peptide 898 amino acids in size. This first ORF is followed by a putative rho-independent terminator and a second, smaller ORF encoding a 71 amino acid peptide (Figure 4.4). The presence of the terminator suggests that ORF1 may be the last ORF in an operon or is transcribed alone. Alternatively, ORF2 may be co-transcribed with ORF1. The insertions, then, may be exerting a polar effect on the transcription of this second ORF in addition to truncating the product of ORF1.

Southern blot analysis of strains RM26b1 and RM483. Since the transposons from RM26b1 and RM483 were isolated as two different clones, there existed the formal possibility that a gene similar to ORF1 existed and two different loci had been mutagenized. A Southern blot was performed to confirm that the transposons were, in fact, inserted in the same locus (Figure 4.5). RM1021, RM483, and RM26b1 were digested with *BamH*I, *Eco*RI, and *Sal*I and then probed with a ^{32}P-labeled DNA fragment complementary to ORF1 upstream of the RM483 insertion site (Figure 4.5A). As the enzyme *BamH*I does not cut within the transposon, the *BamH*I fragments in the mutant strains are larger than the

Figure 4.5. Southern blot analysis of RM26b1 and RM483. (A) A schematic of the predicted ORF's is shown with the insertion sites of the two transposons indicated by arrows. Pertinent restriction sites are noted and the size of the sequence shown in base pairs. The predicted rho-independent terminator is represented by a stem loop. The region of the sequence complementary to the double-stranded DNA probe used in the Southern blot analysis is shown by the dotted line. (B) DNA isolated from the reference strain, RM1021, and the mutant strains, RM483 and RM26b1, was digested with *BamH*I (B), *Eco*RI (E), and *Sal*I (S). The DNA fragments were separated by electrophoresis through an agarose gel and blotted to a nylon membrane. The membrane was probed with a ^{32}P-labeled *Xho*I-*Not*I fragment corresponding to a section of ORF1.


A
SalI
EcoRI
RM483
RM26b1
ORF 2
ORF 1
1 bp
probe
3128 bp

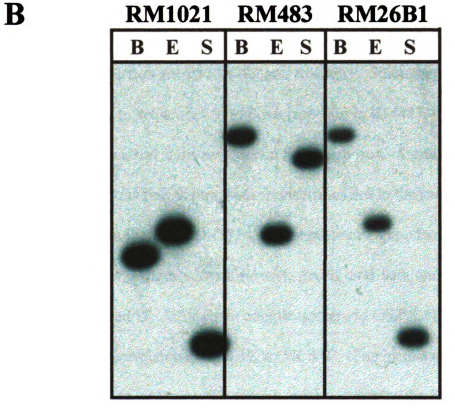

B
RM1021
RM483
RM26B1
B E S B E S B E S

Figure 4.5

corresponding fragment in RM1021 because of the transposon insertion. The restriction enzyme, *EcoR*I, cuts within Tn*5*-233 and produces a slightly larger fragment in RM26b1 as would be expected if that strain's insertion site were 3' to the insertion site in RM483. The restriction enzyme, *Sal*I, cuts between the insertion sites of RM483 and RM26b1. Because the DNA fragment used to probe the blot was complementary to sequences 5' of the transposon in RM483 and the enzyme cuts 3' to the transposon, (Figure 4.5A), the *Sal*I fragment in RM483 should be larger than the corresponding fragment in RM1021. The *Sal*I fragment in RM26b1 is the same size as the fragment in RM1021 as the enzyme cuts between the insertion site of RM483 and RM26b1. Since the probe is complementary to sequences 5' to the transposon in RM483, the *Sal*I fragment it anneals to in RM26b1 does not contain the transposon. Southern blot analysis, therefore, confirms that the transposon insertions are in the same locus.

To determine whether ORF1 is a member of a gene family, DNA isolated from RM1021 was digested with *BamH*I, *EcoR*I, and *Sal*I, and probed in 6X SSC with a ^{32}P-labeled DNA fragment complementary to ORF1. The blots were washed in solutions containing 0.1X to 5X SSC. The lowest stringency wash, 5X SSC, (data not shown), gave the same banding pattern as the highest stringency wash, 0.1X SSC, indicating that there is most likely a single copy of ORF1 in *S. meliloti* 1021 (Figure 4.5B, RM1021).

ORF1 encodes a protein similar to RNase E. The ORF containing the two insertion sites of RM26b1 and RM483 encodes a peptide with significant similarity to the RNase E proteins of *E. coli* and *H. influenzae*. In *E. coli*, RNase E has been shown to be an essential endoribonuclease involved in the processing and degradation of mRNA and rRNA (see review Cohen and McDowall, 1997; Besseraub *et al.*, 1998). Figure 4.6 shows an alignment of the *S. meliloti* RNase E protein sequence with the *E. coli* RNase E sequence. The *S. meliloti* protein is most similar to the *E. coli* RNase E protein in the region between residues 25 and 525 of the *E. coli* protein. This region is the most highly conserved portion of the protein, (Kaberdin *et al.* 1998), and has been shown to contain the enzyme's catalytic domain (McDowall and Cohen, 1996). Within this region, an S1-like RNA-binding domain was identified between residues 41 and 122 (Bycroft *et al.*, 1997). While the *S. meliloti* protein shows significant similarity to this domain, this region of sequence homology is interrupted by a non-homologous stretch of 163 residues (Figure 4.6). A second region of *E. coli* RNase E is required for site-specific cleavage of several RNA species (McDowall and Cohen, 1996). This region is located between residues 321 and 498 and is contained within the region of greatest similarity between the *E. coli* and *S. meliloti* RNase E proteins (Figures 4.6 and 4.7).

The carboxy terminal end of the *E. coli* protein was initially thought to be required for cell viability (Wang and Cohen, 1994), but has since been shown to

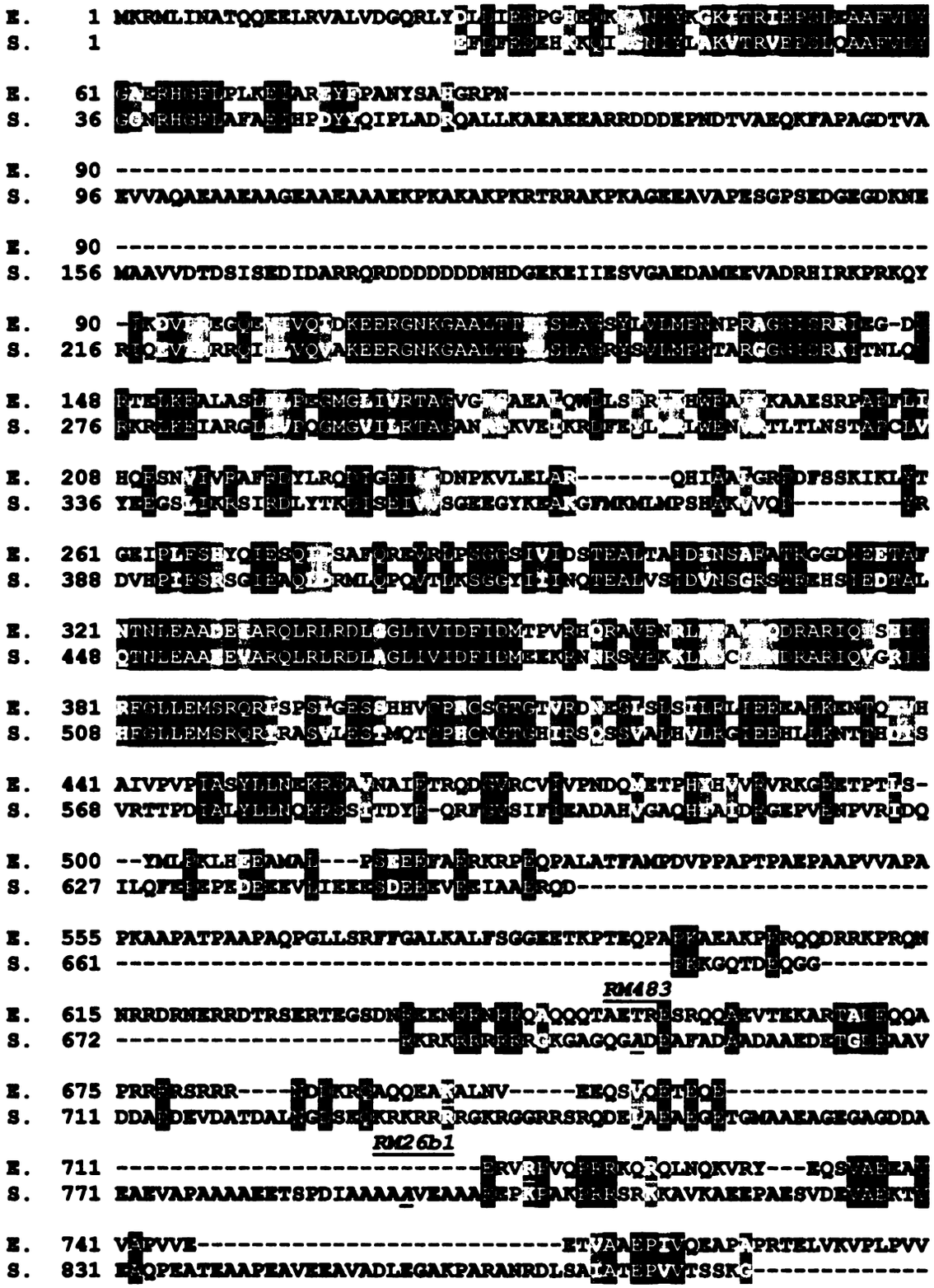


Figure 4.6

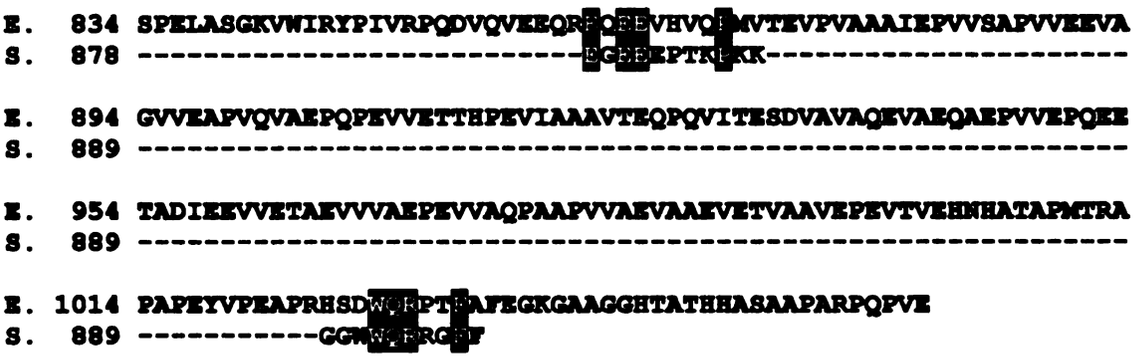


Figure 4.6 (cont'd). Alignment of the *E. coli* and *S. meliloti* RNase E protein sequences. The predicted peptide encoded by ORF 1 was compared to the database and found to be similar to *E. coli* RNase E (P21513). An alignment of the two proteins is shown above, E. = *E. coli* RNase E and S. = *S meliloti* RNase E. Identical regions are shaded in black and similar regions are shaded in gray. The insertion sites of the transposons in RM483 and RM26b1 are labeled and the affected residue is underlined.

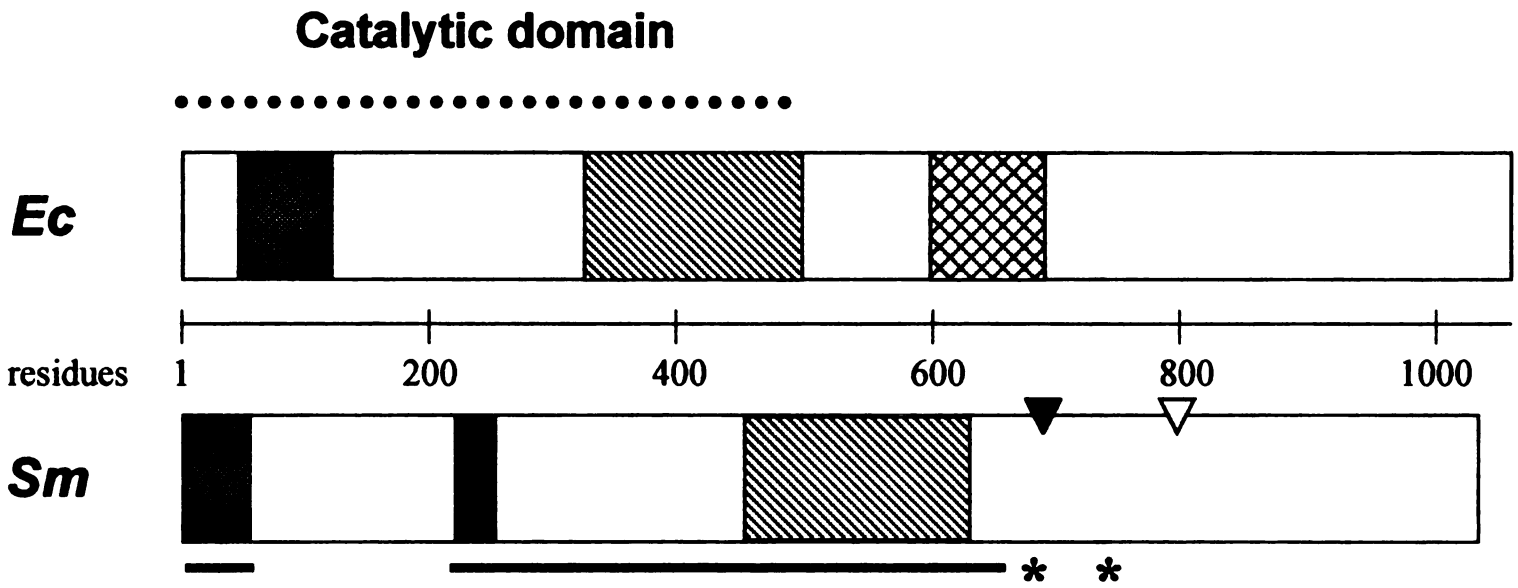


Figure 4.7. Comparison of domains in *E. coli* and *S. meliloti* RNase E proteins. The RNase E proteins from *E. coli* (Ec) and *S. meliloti* (Sm) are depicted. Regions of the *S. meliloti* protein with similarity to *E. coli* are underlined with a solid line. The S1-like RNA-binding region in *E. coli* is indicated by shading ■ with corresponding sequences in *R. meliloti*. The region in *E.coli* required for the endoribonuclease activity and the corresponding sequences in *S. meliloti* are indicated by diagonal stripes ▨ . The arginine-rich RNA-binding region in *E. coli* is indicated by the cross-hatching ▩ . Arginine-rich regions in *S. meliloti* are noted by asterisks *. The black and white triangles indicate the positions of the transposon insertions in RM483 and in RM26b1, respectively.

be dispensable. Kido *et al.* (1996) found mutants in *rne* expressing RNase E proteins truncated at residue 592 that were fully viable. The carboxy-terminal half is not well conserved and is thought to serve as a platform for protein-protein interactions in the assembly of the ribonucleolytic complex termed the degradosome (Kaberdin *et al.*, 1998). An arginine-rich RNA-binding site is located within the carboxy-terminal half of the protein between residues 597 and 684 (McDowall and Cohen, 1996). While there is not a corresponding domain in the *S. meliloti* RNase E protein, there are two arginine-rich regions, residues 670-683 and residues 730-745. The two Tn*5*-233 transposons in mutants RM483 and RM26b1 are located at residues 688 and 791, respectively.

ORF2 encodes a novel polypeptide. The second predicted ORF encodes a small hydrophilic protein 71 amino acids in length. This peptide showed no significant similarity to any other protein in the database. The pI of the deduced polypeptide is 4.51.

The *rne* transcript accumulates transiently after a temperature downshift. We examined the expression of the *rne* transcript before and after a temperature downshift. RNA was isolated from RM212, a derivative of RM1021, at 30°C and 1, 2, 3, 4, and 8 hours after the culture was shifted to 15°C (Figure 4.8). Samples were probed with a ^{32}P-labeled fragment of the *rne* gene. The largest band on the gel is approximately 3.5 kb in length. The sequence data predicted a transcript of approximately 2.7 kb if ORF1 is transcribed alone or 3.1

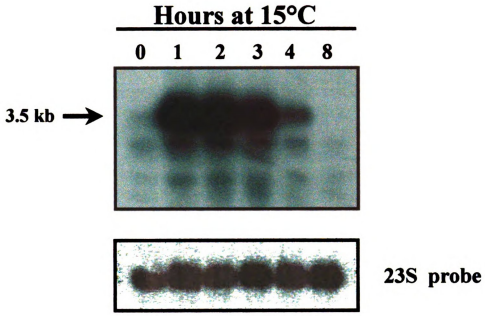


Figure 4.8. The *rne* transcript accumulates transiently following a temperature downshift from 30°C to 15°C. RNA was isolated from RM212, a derivative of RM1021 containing a promoterless-*uidA* fusion recombined into the inositol locus. The strain was grown in TY broth at 30°C then shifted to 15°C. Samples were taken immediately before the temperature shift and 1, 2, 3, 4, and 8 hours afterward. A *XhoI-NotI* DNA fragment corresponding to a portion of the *rne* gene was labeled with [α-^{32} P]CTP and used to probe the Northern blot. Subsequently, the blot was stripped of the original probe and hybridized to ^{32}P-labeled 23S rDNA to check sample loading.

kb if ORF2 is included in the same transcript. Alternatively, it is possible that ORF1 extends further upstream of the sequenced region. It is not clear from these data whether ORF1 and ORF2 are co-transcribed. While relatively low levels of the transcript were present at 30°C, the *rne* transcript levels increased dramatically within 1 hour of the temperature downshift. Levels remained high for 3 hours at 15°C and then decreased significantly at 4 hours. Transcript was barely detectable 8 hours after the temperature downshift indicating that the *rne* gene is expressed at a lower constitutive level at 15°C than at 30°C.

Effect of Tn*5*-233 insertions on *lux* mRNA. Since the Tn*5*-233 insertions caused increased luminescence at 30°C when transduced into RM603, the level of *luxAB* transcript was quantified in these strains. RM603, RM603(483),and RM603(26b1) were grown in TY broth and the luminescence and the *luxAB* mRNA levels measured for each time point. Samples were taken at an A_{600} of 0.5 and 0.9 during growth at 30°C. As expected, the double mutants, RM603(483) and RM603(26b1), produced 5 to 6-fold or 7 to 9-fold more light than RM603 at each time point. The level of *luxAB* transcript appeared to be elevated at each time point in RM603(483) and RM603(26b1) (Figure 4.9). Interestingly, the processing or degradation of the 16S *rrn-luxAB* message appeared to be inhibited by the Tn*5*-233 insertions. At both sampling times, more signal was present in the higher molecular weight bands in RM603(483) and RM603(26b1) than in RM603. The increase in luminescence in RM603(26b1) and RM603(483) appears to be due

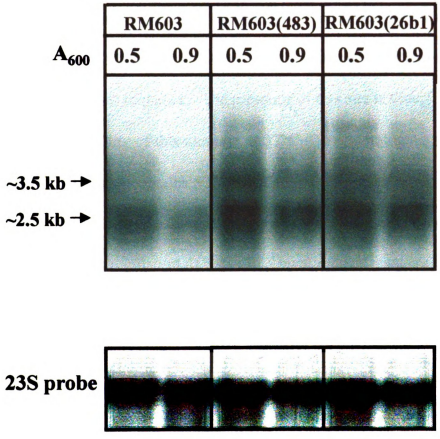


Figure 4.9. Effect of *rne* inserts on *luxAB* transcript levels at 30°C when transduced into RM603. The two double mutants, RM603(26b1) and RM603(483), carrying a Tn*5*-233 insert in *rne*, and RM603 (16S *rrn*::Tn*5*-1062) were grown in TY broth at 30°C. RNA was isolated from samples taken at the following A_{600}: 0.5 and 0.9. 2 μg of each sample was electrophoresed on a formaldehyde/agarose gel and blotted to a nylon membrane. The samples were probed with a ^{32}P-labeled *Xba*I-*Pst*I, *luxAB* DNA fragment. The blot was then stripped and reprobed with ^{32}P-labeled 23S rDNA to check sample loading.

in part to the increased stability of the *rrn-luxAB* transcript.

Effect of *rne* mutants on the *cspA* mRNA. RNase E has been implicated in the regulation of CspA, one of the major cold shock proteins in *E. coli* (Fang *et al.*, 1997). The *cspA* transcript contains a 5' untranslated region, UTR, containing a RNase E target sequence. The presence of this site is thought to contribute to the extreme instability of this message at 37°C. The homologous transcript in *S. meliloti* 1021 also contains a 5' UTR with a putative RNase E cleavage site (O'Connell and Thomashow, 1999). In order to determine whether RNase E is involved in the regulation of CspA in *S. meliloti*, the levels of *cspA* transcript in RM1021, RM26b1 and RM483 were examined during a temperature downshift from 30°C to 15°C (Figure 4.10). The *cspA* gene is the first ORF in an operon containing two additional genes. The lower molecular weight band seen in all samples represents the *cspA* gene while the larger molecular weight band seen immediately following the temperature downshift represents the entire operon (O'Connell and Thomashow, 1999). Interestingly, the *rne* inserts appeared to have no effect on the *cspA* message at 30°C. However, in RM483 and RM26b1 at 2-4 hours after the downshift, the *cspA* transcripts appeared to accumulate to a greater extent than in the wild type strain.

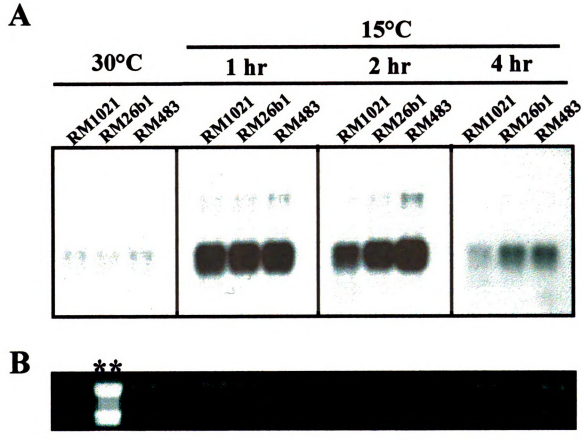


Figure 4.10. Effect of *rne* inserts on *cspA* levels before and after a temperature downshift. The three strains were grown at 30°C in TY broth supplemented with either Sm or Sp at 50μg/ml. At an A_{600} of 0.4-0.5, RNA was isolated and the cultures were shifted to 15°C. Samples were taken 1, 2, and 4 hours after the temperature shift. (A) 2μg of each RNA sample was run on a formaldehyde/MOPS/agarose gel and blotted to a nylon membrane. The membrane was probed with ^{32}P-labeled antisense *cspA* DNA and exposed to film. (B) Photograph of agarose gel prior to blotting. Equal amounts of ethidium bromide were added to each sample prior to loading on the gel with one exception. The sample indicated by the ** received excess ethidium bromide.

Table 4.1. Bacterial strains

Strains	Description	Reference or source
Sinorhizobium meliloti		
RM1021	Str^R; derivative of SU47	Meade *et al*, 1982
RM603	Km^R; RM1021 containing Tn*5*-1062 insert in the 16S *rrn*A gene	This study
RM26b1	Sp^R, Gm^R/Km^R ; RM1021 containing Tn*5*-233 inserted in the *rne* gene	This study
RM483	Sp^R,Gm^R/Km^R; RM1021 containing Tn*5*-233 inserted in the *rne* gene	This study
RM603(26b1)	Sp^R,$Gm^R/Km^R/Nm^R$; Rm1021 containing 16S *rrn*A::Tn*5*-1062 and *rne*::Tn*5*-233	This study
RM603(483)	Sp^R,$Gm^R/Km^R/Nm^R$; Rm1021 containing 16S *rrn*A::Tn*5*-1062 and *rne*::Tn*5*-233	This study
RM212	Sp^R; RM1021 containing the +1 to +172 fragment of the *rrn*A promoter region fused to the *luxAB* genes and recombined into the inositol locus	This study
Escherichia coli		
DH5α	*supE44 hsdR17 recA1 thi-1 ΔlacU169*(ϕ 80*lacZΔM15*) *endA1 gyrA96 relA1*	Hanahan, 1983

Table 4.2. Plasmids

Plasmids	Description	Reference or source
pRK607	Sp^R,Gm^R/Km^R; plasmid used to perform Tn*5*-233 mutagenesis	De Vos *et al*, 1986
pAG1.3	Km^R; 605 bp *Bgl*II/*Sac*I fragment containing the *rrn* promoter, subcloned from pAG1X7, and cloned in front of the *luxAB* genes in pRK290	This study
pRK2073	Sp^R; helper plasmid used to mobilize pAG1.3 into RM1021 strains	Better *et al*, 1983

Discussion

In this study, we have identified a *Sinorhizobium meliloti* 1021 cold-inducible transcript that encodes a protein with significant similarity to Ribonuclease E (RNase E) (Figures 4.4 and 4.6). RNase E is encoded by the gene, *rne*, and has been identified in *Escherichia coli*, *Haemophilus influenzae Rd*, *Mycobacterium tuberculosis*, *Synechocystis* sp., and *Porphyra purpurea* (Kaberdin *et al.*, 1998). The enzyme has been studied most thoroughly in *E. coli* and is thought to be a key enzyme in the degradation and processing of mRNA and rRNA (see review Cohen and McDowall, 1997; Besseraub *et al.*, 1998). RNase E has been isolated as a member of a multiprotein complex known as the degradosome which includes polynucleotide phosphorylase (PNP), DnaK, *rhlB* RNA helicase, and enolase (Miczak *et al.*, 1996). Interestingly, PNP is a cold-inducible protein in the psychrotroph *Yersinia enterocolitica* and is required for growth at 5°C (Goverde *et al.*, 1998). PNP has also been identified as a cold shock protein in *E. coli* (Jones *et al.*, 1987). The *rhlB* RNA helicase is a member of the DEAD-box family of RNA helicases and has been shown to assist in RNA degradation through the removal of secondary structures (Py *et al.*, 1996). Another member of the DEAD-box family of RNA helicases, CsdA, is an *E. coli* cold shock protein although this enzyme is associated with the ribosome (Jones *et al.*, 1996). In *S. meliloti* 1021, we have shown that the *rne* transcript levels transiently increase

following a temperature downshift from 30°C to 15°C (Figure 4.8). This is an interesting observation considering the cold-inducible expression of related proteins in other organisms.

Although the *rne* transcript levels are cold-inducible, the level of RNase E protein must be examined directly. In *E. coli*, the translation of mRNA has been shown to be differentially affected by cold shock. The induction of three cold shock proteins, CspA, CsdA and IF2, was compared to that of the reporter gene *cat* encoding the protein chloramphenicol acetyltransferase (CAT), (Goldenberg *et al.*, 1997). When *cat* was fused to the *cspA* promoter and UTR, the transcript levels were induced maximally at 1 hour following a temperature downshift, similarly to the *cspA* transcript. The synthesis of the CAT protein, however, was delayed and did not reflect the levels of *cat* transcript in the cell. In contrast, the three cold shock proteins showed peak induction within three hours of the cold shock. To accurately assess the role that RNase E may play at low temperature, the level of the protein must be monitored following a temperature downshift.

The identification of RNase E as a cold shock protein would emphasize another facet of low temperature's effect on cellular physiology and the cold shock response. While much attention has been paid to the effect low temperature may have on the secondary structure of mRNA and its effect on translation, less attention has been paid to its potential effect on RNA processing and degradation. In *E. coli*, the understanding of the cold shock response has centered around the

adaptation of the ribosome to low temperature (Jones and Inouye, 1996). Interestingly, the major cold shock proteins, CspA and CsdA, have been shown to interact with RNA and can destabilize secondary structures (Jiang *et al.*, 1997; Jones *et al.*, 1996). While destabilizing secondary structure could impact translation of mRNA, it would also influence the degradation of mRNA. The identification of PNP as not only cold-inducible but essential for growth at low temperature in *Y. enterocolitica* emphasizes the importance of message degradation to cell viability and adaptation to low temperature (Goverde *et al.*, 1998). The mechanisms required for RNA degradation are also related to ribosomal RNA processing. Ribosomal RNA processing is inhibited by a cold shock in yeast and deletion of the cold shock protein, NSR1, has been shown to further inhibit processing, especially at low temperature (Kondo *et al.*, 1992).

The two *rne*::Tn*5*-233 insertion mutants, RM26b1 and RM483, were identified in a screen of Tn5-233 mutagenized *S. meliloti* 1021. The two inserts caused increased luminescence from the *rrn-luxAB* fusion, pAG1.3 and the 16S *rrn*::Tn*5*-1062 strain, RM603, at 30°C (Figures 4.2 and 4.3). The transposon insertions are located downstream of the putative S1-like RNA-binding and endoribonucleolytic domains of RNase E and should lead to the production of truncated proteins (Figure 4.7). Although RNase E is an essential enzyme in *E. coli* , Kido *et al.* (1996) found that RNase E proteins truncated at residue 592, though exhibiting reduced activity, were still able to support cell growth. A

similar situation likely exists in RM483 and RM26b1. Southern blots indicated that while there is only one *rne* gene in *S. meliloti*, the insertions in RM483 and RM26b1 are not lethal. In RM603(483) and in RM603(26b1), the 16S *rrn-luxAB* transcript appears to be processed more slowly and to be generally more stable (Figure 4.9). These data suggest that the truncated RNAse E proteins, while retaining essential nucleolytic activities, are functioning at a reduced level.

RNase E has been implicated in the regulation of the *cspA* transcript in *E. coli* (Fang *et al.*, 1997). At the normal incubation temperature of 37°C, the *cspA* transcript is very unstable. Mutation of a putative RNase E site in the untranslated leader region or UTR of the *cspA* gene resulted in the dramatic stabilization of the message at 37°C. The *cspA* transcript was also somewhat stabilized in a temperature-sensitive RNase E mutant at the non-permissive temperature of 44°C. A similar mechanism may contribute to the regulation of *cspA* in *S. meliloti* 1021. Fusing the *cspA* UTR to the *luxAB* genes lowered expression of the *luxAB* genes at 30°C relative to a direct *cspA* promoter-*luxAB* fusion (O'Connell and Thomashow, 1999). As the *S. meliloti cspA* UTR contains a putative RNase E site, the levels of *cspA* were examined in the *rne* insertion mutants. Interestingly, an effect was detectable at 15°C but not at 30°C (Figure 4.10); the stabilization of the *cspA* message appeared to be prolonged in the *rne* insertion mutants. It must be noted that the insertions only partially affect the function of RNase E and the lack of an effect at 30°C does not mean that full length RNase E does not interact with the

cspA mRNA at that temperature.

In sum, we have identified a *S. meliloti* 1021 homolog of RNase E, an enzyme involved in mRNA degradation and processing. The *rne* transcript accumulates significantly following a temperature downshift suggesting that RNase E is a cold shock protein in *S. meliloti* 1021. Although the *rne* gene is present as a single copy, two transposon insertions 3' to residue 688 were not lethal. The truncated RNase E protein appeared to be affected in its processing of the *cspA* transcript immediately following a temperature downshift while having no effect on its expression at 30°C. Finally, the identification of RNase E as a cold shock protein suggests that cold shock may have an important influence on mRNA degradation and processing.

Materials and Methods

Bacteria and growth conditions. Cultures of *Escherichia coli* DH5α were grown aerobically at 37°C in LB broth (Sambrook *et al.*, 1989) or on solid LB medium containing 1.4% agar. Cultures of *Sinorhizobium meliloti* were grown aerobically at 30°C in either tryptone-yeast extract medium, TY (Beringer, 1974), GTS medium (Kiss *et al.*, 1979), BSM medium (Bergersen, 1961), or M9 medium (Sambrook *et al.*, 1989). Cultures were maintained on solid medium containing 1.4% agar, or frozen to -80°C in TY broth containing 15% glycerol. *S. meliloti* was cultured on solid media containing the following antibiotics when appropriate: kanamycin, Km (200 μg/ml), neomycin, Nm (200 μg/ml), spectinomycin, Sp (100 μg/ml), streptomycin, Sm (250 μg/ml), and tetracycline, Tet (10 μg/ml). *E. coli* was grown on plates containing ampicillin, Ap (100 μg/ml), Km (100 μg/ml), Sp (100 μg/ml), or Tet (10 μg/ml) when appropriate. A lower concentration, 50 μg/ml, of Km and Sm was used in broth cultures.

Transposon mutagenesis and screen. *Sinorhizobium meliloti* 1021 was mutagenized with the transposon Tn*5*-233 via biparental conjugation. The transposon was carried on the plasmid pRK607. DH5α containing the plasmid pRK607 was spotted with *S. meliloti* 1021 on TY agar plates. After 12-24 hours, these spots were resuspended in 0.5 ml of TY broth, diluted 1 to 10 and 100μl

spread onto TY agar plates containing Sp (100 μg/ml) and Sm (250 μg/ml). After three days of incubation at 30°C, 2 mls of 0.85% NaCl was applied to each plate and the colonies resuspended. The cells were harvested by a brief centrifugation and resuspended in 1.0 ml of TY broth. 0.5 ml of this suspension was used to inoculate 2.5 mls of TY broth containing Sp and Sm at 50 μg/ml. After 3-5 hours of incubation at 30°C, 1.5 mls of the culture was harvested and rinsed with 1 ml of TY broth. The cells were finally resuspended in only 150 μl of TY broth. 20-25 μl of this suspension was spotted onto a TY agar plate with an equal volume of *Escherichia coli* DH5α containing both the reporter plasmid, pAG1.3, and the helper plasmid, pRK2073. After incubating overnight at 30°C, the spots were each resuspended in 1.0 ml of TY broth, diluted to various concentrations and spread on selective TY agar plates containing Nm (200 μg/ml) and Sm (250 μg/ml). These plates were incubated for 3-5 days until colonies were large enough to be assayed for luciferase activity. Plates were either exposed to aldehyde immediately and screened for those showing increased luminescence at 30°C or they were incubated at 15°C for 4-6 hours before screening for colonies showing decreased luminescence.

Measurement of luciferase activity. Luminescence was measured with the Hamamatsu Photonic System model C1966-20. Bacteria grown on agar plates were exposed to the enzyme substrate, N-decyl aldehyde, for two minutes prior to

being assayed for light production for 59 seconds. The aldehyde was spread on a glass petri dish which was placed over the agar plate. The Hamamatsu system converts the photon counts to a visual image on a monitor which can be used to quantitate the amount of light produced by each colony.

Bacteria grown in broth culture were assayed quantitatively for light production. In this assay, the N-decyl aldehyde was dispersed in an aqueous solution of bovine serum albumin (20 mg/ml) at a concentration of 1 μl/ml. A 5-10 μl aliquot of the cell culture was added to 50-100 μl of the aldehyde solution, vortexed for 30 seconds, then assayed for 60 seconds. The measured luminescence was reported in arbitrary units, RLU, and adjusted for cell number by dividing this number by the A_{600} of the culture. All assays were performed at room temperature.

Φ-M12 transduction. The Tn*5*-233 transposons were introduced into strains already carrying the Tn*5*-1062 transposon by phage transduction using the phage Φ-M12 (Finan *et al.*, 1984). Preparations of Φ-M12 were prepared from dense cultures of the donor strain. Cultures of the donor strains were grown to late log phase in LB broth supplemented with 2.5 mM of $MgSO_4$ and $CaCl_2$. The cultures were then inoculated with Φ-M12 to a final concentration of 10^8 PFU/ml. After 6-8 hours, the cultures were checked for evidence of lysis and centrifuged to remove remaining intact cells and debris. The supernatant was collected and 0.05 ml chloroform added per 1.0 ml lysate. The mixtures were vortexed thoroughly

and stored overnight at 4°C. The tubes were spun again to pellet the chloroform and remaining debris. The supernatant was removed and stored at 4°C.

In order to determine the titer of the phage lysates, the lysate was diluted and mixed 1:10 with a 1-2 day old culture of *S. meliloti* cultured in LB broth supplemented with 2.5 mM $MgSO_4$ and $CaCl_2$. After incubating for 20 minutes at 30°C, the phage/bacteria mixtures were added to 3 mls of top agar [Nutrient Broth (Difco Laboratories, Detroit, MI) containing 0.65% agar with 2.5 mM $MgSO_4$ and $CaCl_2$] and spread on LB agar plates. Plaques were easily visible after incubating the plates overnight at 30°C.

The phage lysates prepared from the donor strains were used to transduce various strains containing Tn*5*-1062. The recipient strain was cultured in LB broth supplemented with 2.5 mM $MgSO_4$ and $CaCl_2$. After 1-2 days of growth, the CFU/ml were estimated by measuring the optical density of the culture and an aliquot mixed with phage lysate such that a ratio of 1 PFU/ 2 CFU was achieved. 0.5 ml of cells was added to a volume of 0.5 ml of phage lysate. Supplemented LB broth was used to adjust the volume of the phage lysate to achieve the correct ratio. After incubation at 30°C for 20 minutes, 0.1 ml of 0.2M sodium citrate was added to each tube in order to chelate the Mg^{+} and Ca^{++} ions and, therefore, interrupt the infection of the bacterial cells. The cells were then rinsed with a sterile solution consisting of 0.85% NaCl and 0.01 M sodium citrate. The cells were resuspended in the saline solution and plated on selective plates, LB agar

with Sp (100 μg/ml) and Nm (200 μg/ml).

Cloning, DNA sequencing, and sequence analysis. Genomic DNA was isolated from 3-5 ml cultures of *S. meliloti* according to a standard CTAB extraction protocol (Ausubel *et al.*, 1987) with one modification. Prior to extraction with CTAB, the bacterial lysate was passed several times through an 18-gauge needle using a sterile syringe in order to partially shear the DNA. This step reduced sample viscosity and facilitated extraction of the samples with phenol and chloroform.

Genomic DNA was digested with either *EcoR*I or *Xma*I and *Kpn*I. *EcoR*I and *Kpn*I cut within the transposon allowing the isolation of either the left end or the right end of the transposon. Since Tn*5*-233 has identical ends, this facilitated sequencing directly out of the transposon. Digested genomic DNA was cloned into pBluescript SK- and transformed into *E. coli* DH5α. Colonies resistant to either kanamycin or spectinomycin were selected depending on which end of the transposon was cloned.

The resulting plasmids were isolated using a Qiagen column (Qiagen, Inc., Valencia, CA) or a standard alkaline lysis method (Sambrook *et al.*, 1989). The plasmids were sequenced manually using the T7 Sequenase v.2 kit according to the recommended protocol (Amersham Life Science, Inc., Cleveland, OH) or at the MSU-DOE-PRL DNA Sequencing facility using the ABI Catalyst 800 for Taq cycle sequencing and the 373A Sequencer for the analysis of the products. Initial

sequences were obtained using primers complementary to the end of Tn*5*. Either the primer, "Tn5 right", 5'-CACA TGGAATATCAG-3' or the primer, MT93, 5'-CACGATGAAGAGCAGAA GTTAT-3' were used for the first round of sequencing. Subsequent primers were chosen using PrimerSelect 3.11 (DNASTAR Inc., Madison, WI). Primers were synthesized by the Macromolecular Structure Facility located in the Department of Biochemistry at Michigan State University. Sequence data alignments were carried out using SeqMan II 3.61 (DNASTAR Inc., Madison, WI).

Potential open reading frames were identified using a codon preference program, CodonUse 3.1, developed by Conrad Halling (University of Chicago). Sequences encoding proteins similar to the predicted peptides were identified using the BLAST algorithms (Altschul *et al.*, 1997). Possible terminator sequences were located using the Terminator program, (Brendel and Trifonov, 1984), in the GCG sequence analysis package (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI). Protein sequence alignments were created by using the Match-Box Web Server 1.2 at http://www.fundp.ac.be/sciences/biologie/bms/matchbox_submit.html

Southern blotting. DNA fragments were separated on an agarose gel and transferred to a nylon membrane in 20X SSC via capillary action according to the standard protocol (Sambrook *et al.*, 1989) with two modifications. Instead of the acid depurination step, the gel was first exposed to UV light for 10 minutes in the

UV Stratalinker 2400 (Stratagene, La Jolla, CA). After transfer, the DNA was crosslinked to the nylon membrane by exposure to 20 mjoules of light energy in the UV Stratalinker. The blot was hybridized to a ^{32}P-labeled probe following a protocol based on the one described in Sambrook *et al.*, 1989. The pre-hybridization and hybridization solutions contained 6X SSC, 0.5% SDS, and 0.25% nonfat dry milk. Hybridization was performed at 60-65°C in a Robbins Hybridization Incubator Model 400 (Robbins Scientific Corp., Sunnyvale, CA). The probe was prepared from a band isolated fragment by a random priming method (Feinberg and Vogelstein, 1983). The blots were washed under standard conditions four times in a solution of 0.1X SSC and 0.1% SDS at 60-65°C. The blot was then exposed to film. For the low stringency washes, the concentration of SDS was held constant and SSC added to a final concentration of 0.5X, 2.5X or 5.0X. The washes were performed at 60-65°C.

Isolation of RNA. RNA was isolated from *S. meliloti* strains according to a modified protocol derived from that found in Ausubel *et al.* (1987) for the isolation of RNA from gram negative bacteria. Bacteria were harvested from a 3-20 ml aliquot of cell culture. In all but one experiment, the assay of *cspA* message levels, rifampicin was added to culture samples to a final concentration of 0.2 mg/ml just after harvesting. The cell pellets were resuspended in 1.4 ml of protoplasting buffer and 80 µl of 50 mg/ml of lysozyme added. The tubes were incubated on ice 5-10 minutes. The protoplasts were collected by centrifugation

for 2 minutes at 7,000 rpm. The pellets were resuspended in 0.5 ml of gram negative lysing buffer, mixed with 15 µl of diethylpyrocarbonate, DEPC, and incubated for 5 minutes at 37°C. Tubes were chilled briefly on ice, mixed with 250 µl of 5M NaCl, incubated 10 minutes on ice and centrifuged for 10 minutes. The supernatant was removed, added to a tube containing 1 ml ethanol and stored overnight at -20°C. The RNA was collected by centrifugation for 15 minutes at 4°C, dried under a vacuum and resuspended in DEPC-treated water. Samples were quantified using a spectrophotometer and measuring the A_{260} and A_{280}. Alternatively, the cell pellets were frozen quickly in liquid nitrogen and the samples stored at -80°C. RNA was then isolated using RNeasy Mini Kits (Qiagen Inc., Valencia, CA).

Northern blots. RNA samples were treated with formaldehyde and electrophoresed on formaldehyde/agarose gels according to standard protocols (Sambrook *et al.*, 1989). RNA was transferred from the gel to a nylon membrane in 10X or 20X SSC via capillary action according to the standard protocol (Sambrook *et al.*, 1989) with one modification. After transfer, the RNA was crosslinked to the membrane by exposure to 20 mjoules of light energy in the UV Stratalinker 2400 (Stratagene, La Jolla, CA). The membranes were pre-hybridized at 42°C in the following solution: 50% formamide, 5X SSC, 50mM potassium phosphate, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate, and 100µg/ml denatured DNA. After a minimum of 2 hours of pre-hybridization treatment, ^{32}P-

labeled probe was added to the solution and incubated overnight at 42°C in a Robbins Hybridization Incubator Model 400 (Robbins Scientific Corp., Sunnyvale, CA). The membranes were treated first at room temperature with three washes of 2X SSC and 0.5% SDS. After one wash in 0.1X SSC and 0.5% SDS, the temperature was raised to 50°C for two more washes in the same solution. Washes were continued until the spent wash solution was no longer detectably radioactive. The probe was prepared from a band isolated fragment by a random priming method with one exception.(Feinberg and Vogelstein, 1983). The *cspA* antisense probe was prepared from a PCR-amplified fragment using one of the primers, MT171, 5'-CCGGAATTCAAACGCTCCCTGCCAGTA CATCCG-3'. This primer is complementary to the 3' end of *cspA* and therefore will amplify the non-coding strand of the *cspA* gene. The protocol for random labeling was used with one modification. The randomized oligo was replaced by 11.4 pmoles of the primer MT171.

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APPENDICES

Appendix A

Auxotrophic mutants cause increased luminescence of *rrn-luxAB* fusions at 30°C

Introduction

Tn*5*-233 insertion mutants were tested for their ability to alter the luminescence of plasmid, pAG1.3, at 30°C and 15°C. The plasmid, pAG1.3, contains the *luxAB* genes of *Vibrio harveyi* fused to the *rrn*A promoter at +187 and exhibits increased luminescence following a temperature downshift from 30°C to 15°C. Thirteen Tn*5*-233 insertion mutants that caused increased luminescence at 30°C were isolated. Initial characterization of the mutants revealed that nine mutants were unable to grow on minimal medium. Further analysis of the nine auxotrophic mutants is described below.

Results and Discussion

The nine auxotrophic mutants causing increased luminescence from pAG1.3 required addition of either uracil or arginine, or both, for growth on minimal medium (Table A.1). The biosynthetic pathways for uracil and arginine are connected through the use of carbamoyl phosphate as a substrate (Figure A.1). Carbamoyl phosphate is synthesized for each pathway by a single enzyme,

Table A.1. List of auxotrophic Tn*5*-233 insertional mutants.

Mutant strains	Auxotrophy	Location of Tn*5*-233 insertion
RM75	uracil	*pyrB*
RM65a	uracil/arginine	*carAB*
RM945	uracil/arginine	*carAB*
RM15a	uracil	---
RM35	uracil	---
RM835	uracil	---
RM26a	uracil	---
RM522	uracil	---
RM65b	arginine	---

Figure A.1. Arginine and pyrimidine biosynthesis in *Escherichia coli.* The synthesis of L-arginine from L-glutamate is shown on the left of the figure. Synthesis of the pyrimidine nucleotides from carbamoyl phosphate and aspartate is shown on the right. The reaction of carbamoyl phosphate synthetase is shown at the top of the figure as carbamoyl phosphate is used in both pathways. The genes affected by Tn*5*-233 inserts are shown in italics at the step in biosynthesis performed by the enymes they encode. PRPP = 5-phosphoribosyl-1-pyrophosphate. This figure is adapted from G. Gottschalk, Bacterial Metabolism , 2nd Edition, Springer-Verlag, New York, 1986.

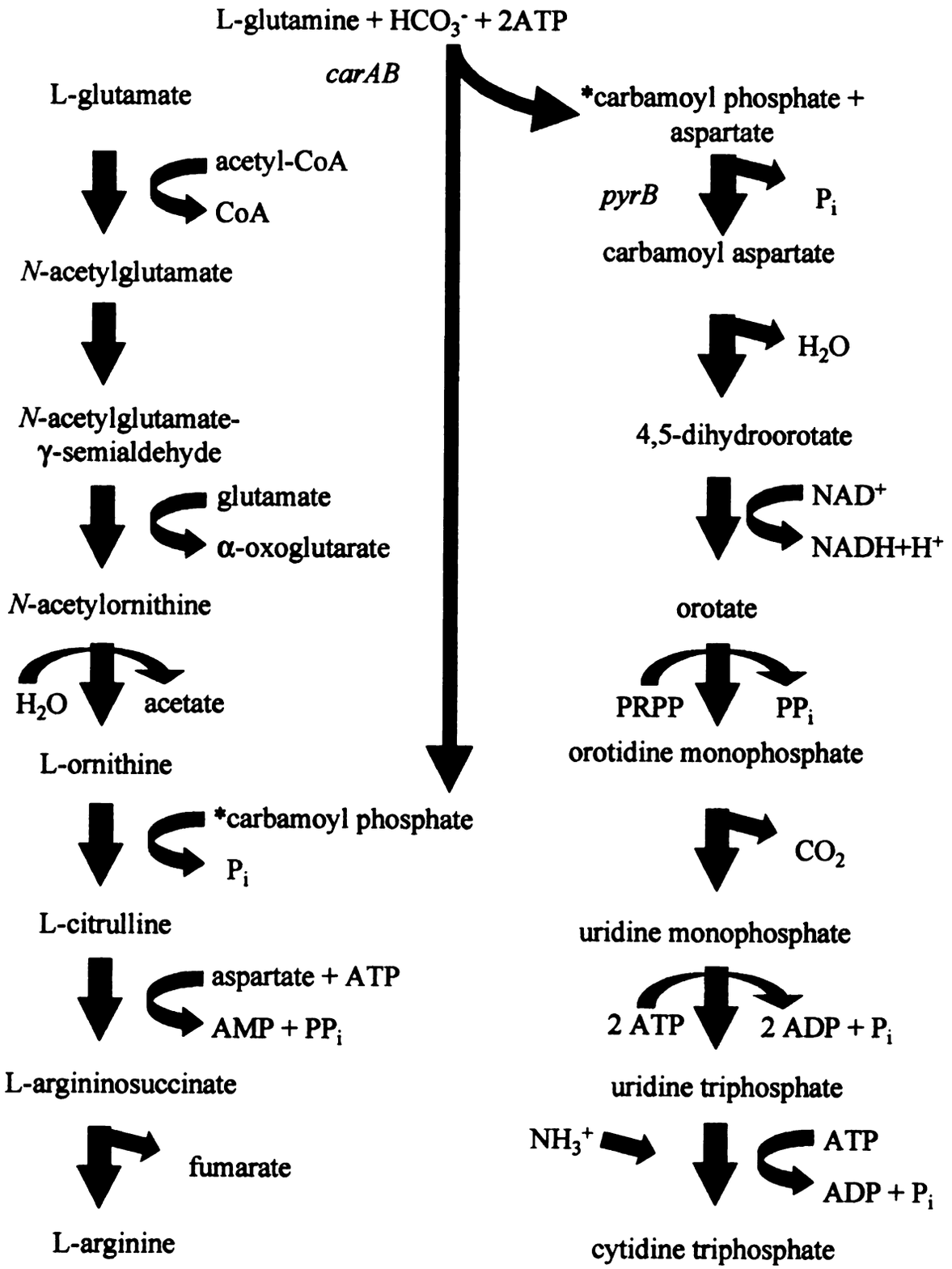
L-glutamine + HCO_3^- + 2ATP
carAB
L-glutamate
*carbamoyl phosphate +
aspartate
acetyl-CoA
CoA
pyrB
P_i
N-acetylglutamate
carbamoyl aspartate
H_2O
N-acetylglutamate-
γ-semialdehyde
4,5-dihydroorotate
glutamate
α-oxoglutarate
NAD^+
$NADH+H^+$
N-acetylornithine
orotate
H_2O
acetate
PRPP
PP_i
orotidine monophosphate
L-ornithine
*carbamoyl phosphate
P_i
CO_2
L-citrulline
uridine monophosphate
aspartate + ATP
AMP + PP_i
2 ATP
2 ADP + P_i
L-argininosuccinate
uridine triphosphate
NH_3^+
ATP
fumarate
ADP + P_i
L-arginine
cytidine triphosphate

Figure A.1

carbamoyl phosphate synthetase. The transposon insertion site in the uracil auxotroph, RM75, was identified by cloning out one end of the transposon and the neighboring genomic sequences. Sequence analysis indicated that the transposon was located within an ORF encoding a protein showing significant similarity to aspartate carbamoyltransferase in *Synechocystis* PCC6803 (Figure A.2). In *E. coli*, this protein is the second enzyme in the pyrimidine biosynthetic pathway and is encoded by the *pyrB* gene (Figure A.1). Two other mutants, RM65a and RM945, required both arginine and uracil for growth on a minimal medium. In *S. meliloti*, auxotrophs requiring both arginine and uracil for growth have been complemented by either the *carA* or the *carB* loci (Kerppola and Kahn, 1988). These loci encode the two subunits of the enzyme carbamoylphosphate synthetase (Figure A.2).

In order to correlate the presence of the Tn*5*-233 transposon with the observed increase in luminescence, each insert was transduced into strain RM603 (16S *rrn*A::Tn*5*-1062) using the phage Φ-M12. Luminescence was increased in all of the double mutants relative to RM603 when the strains were cultured on TY agar plates (Figure A.3). When cultured in TY broth, the relative increase in luminescence by the double mutants was not constant over the growth curve but became more pronounced as the cultures approached stationary phase. Figure A.4 shows the relative luminescence of six double mutants during growth in TY broth at 30°C. Five of the strains showed a greater than 8-fold increase in luminescence relative to RM603 at the final time point as opposed to a less than 4-fold increase

Figure A.2. The Tn*5*-233 insert in RM75 is located in a gene similar to the *pyrB* gene of *Synechocystis* PCC6803. (A) The DNA continuous with one end of the transposon in RM75 was cloned and sequenced. The DNA sequence is shown and the one letter amino acid codes of the predicted peptide are shown in italics above. (B) An alignment of the *S. meliloti* peptide, Sm, and the *Synechocystis* PCC6803 aspartate transcarbamoylase (P74163), Sy, is shown. Identical residues are shaded in black and similar residues in gray.

A

```
1                                                                  57
 T  Q  I  N  L  F  F  E  A  S  T  R  T  Q  S  S  F  E  L
ACGCAGATCAATCTGTTCTTTGAAGCCTCGACCCGTACCCAGTCCTCATTCGAGCTC
58                                                                 114
 A  G  K  R  L  G  A  D  V  M  N  M  S  V  G  N  S  S  V
GCCGGAAAGCGGCTCGGCGCCGACGTCATGAACATGTCCGTCGGCAATTCCTCGGTG
115                                                                171
 X  K  G  E  T  L  I  D  T  A  M  T  L  N  A  M  X  P  D
AANAAAGGCGAGACGCTGATCGATACGGCGATGACGCTGAACGCCATGCNCCCGGAC
172                                                                228
 V  L  V  V  R  H  S  S  A  G  A  A  S  L  L  A  X  K  V
GTGCTGGTCGTTCGCCACTCGTCGGCGGGGGCTGCCTCGCTGCTTGCCCANAAGGTC
229                                                                285
 S  C  S  V  V  N  A  G  D  X  X  X  E  H  P  T  Q  A  L
TCCTGCTCGGTCGTCAACGCCGGCGACNGCCANCNCGAGCACCCGACNCAGGCGCTG
286                                                                339
 L  X  X  X  D  X  P  A  R  P  X  A  S  S  X  R  D  H
CTCNACNCCNCTGACNATCCGGCGCGCCCTANGGCNAGCTCTCNGCGGGATCAT
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B

```
Sy    1 MTMVASWTRNHILDLTDWRGEELDIVLQTATTFQQVLSGQTKKVPALQGQ

Sy   51 VVTNLFFEPSTRTRSSFELAAKRLSADVMNFSPGTSSLTKGETLEDTAKT
Sm    1 TQINLFFEASTRTQSSFELAGKRLGADVMNMSVGNSS-VKGETLEDTAMT

Sy  101 YLAMGSDIFVIRHQQAGVPHFIASQMDRLQTGVKVLNAGDGQHEHPSQGL
Sm   50 LNAMPDV-LVVRHSSAGAASLLAK------VSCSVVNAG---DEHPTQAL

Sy  151 LDLFTICSQFAPDNPAIQCLQGKKIAVVGDILHSRVARSNLWSLTTAGAD
Sm   90 LDPARPASSRDH

Sy  201 VHLAGPPTLLPKEFQQLTLAPGSGKLHCHWQLQPALEGADIVMTLRLQKE

Sy  251 SMTAHLLPSLREYHHYFGITHDRLKVCQPGVKVLHPGPVNRGVEISSELM

Sy  301 DDPDISLIQDQVTSGVAISMALLYLLGTVQE
```

Figure A.2

Figure A.3. Set of Tn*5*-233 inserts that caused auxotrophy and increased luminescence when transduced into RM603. RM603 (16S *rrn*::Tn*5*-1062) was compared to a collection of double mutants. Each double mutant is a derivative of RM603 containing a Tn*5*-233 insert. Cultures were streaked on TY agar plates and incubated at 30°C for two days before being assayed for luminescence. After being assayed for luminescence at 30°C , the plates were moved to 15°C for 5-6 hours then assayed again.

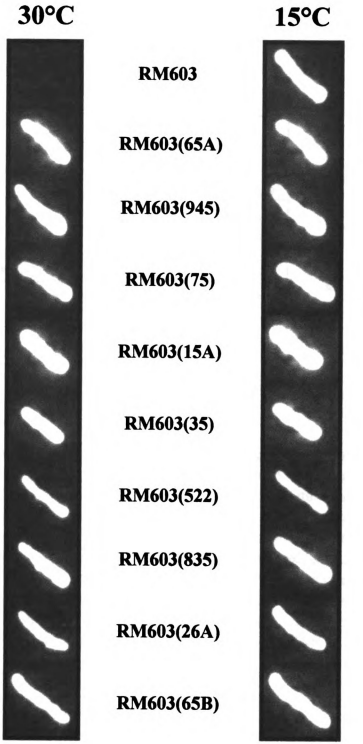
30°C
15°C
RM603
RM603(65A)
RM603(945)
RM603(75)
RM603(15A)
RM603(35)
RM603(522)
RM603(835)
RM603(26A)
RM603(65B)

Figure A.3

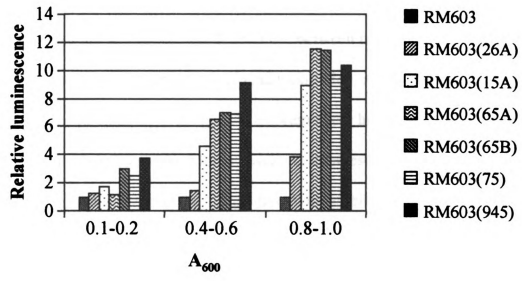

Figure A.4. Luminescence of auxotrophic double mutants increases with culture density. The luminescence of RM603 (16S *rrn*::Tn*5*-1062) was compared to that of six double mutants. These double mutants are derivatives of RM603 containing an additional Tn*5*-233 insert that disrupts the arginine and/or pyrimidine biosynthetic pathways. All strains were cultured at 30°C in TY broth. Samples were taken when the cultures reached an A_{600} of 0.1-0.2, 0.4-0.6, and 0.8-1.0 and assayed for luminescence. Luminescence was reported as RLU or relative light units and adjusted for cell density by dividing the RLU by the A_{600} of the sample. Relative luminescence was calculated by dividing the RLU/A_{600} of each double mutant by the RLU/A_{600} of RM603.

at the earlier time point. RM603(26A) showed a smaller effect on luminescence overall; however, luminescence of this strain relative to RM603 increased from 1.3 to 3.9-fold at an A_{600} of approximately 1.0 (Figure A.4).

Because the difference in luminescence between the double mutants and RM603 increased as the cultures approached saturation, it was thought that uracil may have become limiting in the growth medium at the higher cell density and that this limitation might be required for an effect on luminescence to be observed. If this were true, supplementing the TY medium with uracil should delay the effect on luminescence in the double mutants. To test this idea the *pyrB* mutant double mutant, RM603(75), and RM603 were grown in TY broth with and without added uracil at 30°C (Figure A.5). When the cultures not supplemented with uracil were sampled at an A_{600} of 0.1-0.2, RM603(75) produced 2.7 times more light than RM603. At an A_{600} of 0.8-0.9, the difference in luminescence had increased to 8.2-fold. In cultures supplemented with uracil, RM603(75) produced only 2.7 times more light than strain RM603 at the higher culture density. These results indicate that the amount of uracil in the growth medium influences luminescence in *pyrB* mutant, RM603(75).

The results obtained with this mutagenesis are quite interesting in relation to the model for growth rate-dependent regulation of the *rrn* promoters proposed by Gaal *et al.* (1997). In *Escherichia coli*, the open complexes formed by RNA polymerase at the *rrn* P1 promoters are unusually sensitive to the concentration of

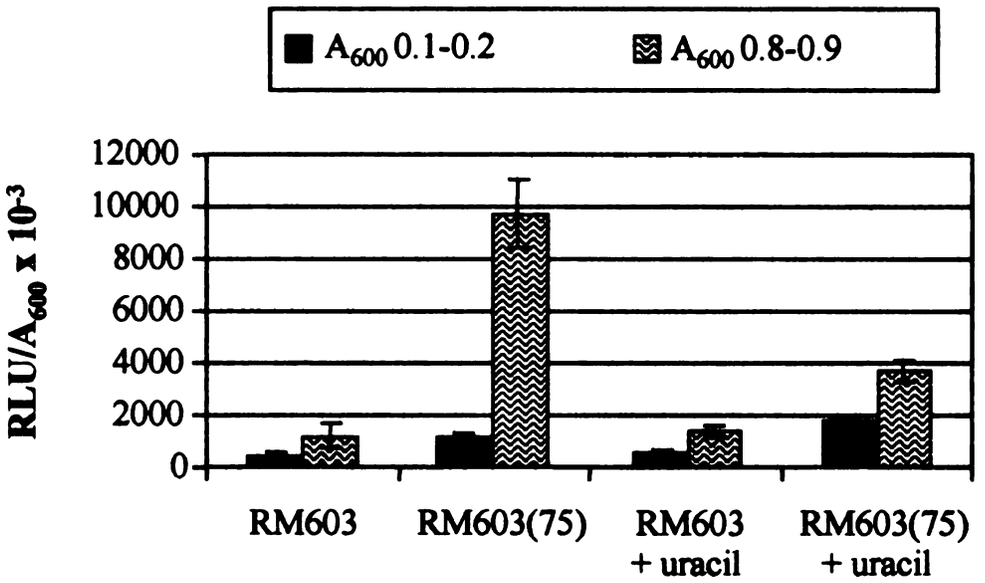

Figure A.5. Effect of added uracil on luminescence in RM603(75). The luminescence of RM603 (16S *rrn*::Tn*5*-1062) was compared to that of RM603(75), a derivative of RM603 containing a Tn*5*-233 insert in *pyrB*. The strains were grown at 30°C in either TY broth or TY supplemented with uracil (20 µg/ml). Samples were taken at A_{600} of 0.1-0.2 and again at A_{600} of 0.8-0.9 and luminescence measured. Luminescence is reported as relative light units, RLU, and is adjusted to cell density by dividing RLU by the A_{600} of the culture. Each bar represents the average of three flasks and error bars show the standard deviation.

the initiating nucleotide. In *E. coli* all but one of the *rrn* promoters initiates transcription with ATP. Because the concentration of ATP varies depending on nutrient conditions and its rate of consumption within the cell, it could function as a signal molecule. Gaal *et al.* (1997) demonstrated *in vivo* and *in vitro* that *rrn* P1 promoter activity varies positively with ATP concentration. Further, the *rrn* promoters were shown to respond specifically to ATP concentration through the use of mutants defective in pyrimidine biosynthesis. When these mutants were grown under partial pyrimidine limiting conditions, ATP concentration was no longer correlated with growth rate. P1 *rrn* promoter activity increased with higher levels of ATP although growth rate was decreasing. While sensitivity of the *S. meliloti* 1021 *rrn* promoters to the initiating nucleotide has not been described, it is interesting that all three *rrn* transcripts begin with adenine. Thus, the *S. meliloti* 1021 pyrimidine auxotrophs might be expected to increase the activity of the *rrn-luxAB* fusions when uracil becomes limiting in the growth medium.

In order to further investigate the apparent increase in *rrn* expression in a pyrimidine auxotroph, RM603(75) and RM603 were grown in TY broth at 30°C and RNA isolated at A_{600} of 0.5 and 0.9. Surprisingly, the *luxAB* transcript accumulated only slightly more in RM603(75) than in RM603 (Figure A.6). While this result could indicate that the pyrimidine auxotrophy simply has a general effect on luciferase activity, previous observations of the *pyrB* insertion do not support this conclusion. The Tn*5*-233 insertion in RM75 was transduced into

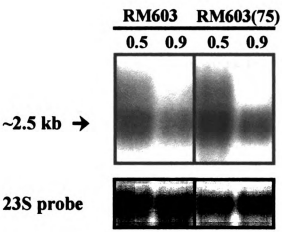

Figure A.6 Effect of *pyrB*::Tn*5*-233 insert on *luxAB* transcript levels at 30°C when transduced into RM603. The double mutant, RM603(75), and RM603 were grown in TY broth at 30C. RNA was isolated from samples taken at an A_{600} of 0.5 and 0.9. 2 µg of each sample was electrophoresed on a formaldehyde/agarose gel and blotted to a nylon membrane. The samples were probed with a ^{32}P-labeled *XbaI-PstI, luxAB* DNA fragment. The blot was then stripped and reprobed with ^{32}P-labeled 23S rDNA to check for sample loading.

three different Tn*5*-1062 insertion mutants to test the specificity of its effect on luminescence (data not shown). Although the insertion did increase the luminescence in these strains, the increase in luminescence was 2-3 fold as opposed to the 12-fold increase observed in RM603(75) in the same experiment. Apparently, the *pyrB* insertion is affecting some post-transcriptional step in the expression of the RM603 Tn*5*-1062 insertion.

The experiments presented here do not directly address the growth rate-dependent regulation of the *rrn* promoters in *S. meliloti* 1021. Growth rate-dependent regulation of *rrn* expression in *E. coli* was studied using only the core promoter sequences of the *rrn* P1 promoter (-41 to +1) while the *rrn-luxAB* fusions in this study included the full promoter and over 1 kb of 16S *rrn*. While the initiation of *rrn* promoter activity was shown to be influenced by the ATP concentration alone, other factors could negate this activity resulting in no net increase in rRNA. If this regulation is to be examined in *S. meliloti*, *rrn* promoter fusions at +1 must be used instead of the Tn*5*-1062 insertions used in this study.

Materials and Methods

Bacteria and growth conditions. Cultures of *Escherichia coli* DH5α were grown aerobically at 37°C in LB broth (Sambrook *et al.*, 1989) or on solid LB medium containing 1.4% agar. Cultures of *Sinorhizobium meliloti* were grown aerobically at 30°C in either tryptone-yeast extract medium, TY (Beringer, 1974),

GTS medium (Kiss *et al.*, 1979), BSM medium (Bergersen, 1961), or M9 medium (Sambrook *et al.*, 1989). Cultures were maintained on solid medium containing 1.4% agar, or frozen to -80°C in TY broth containing 15% glycerol. *S. meliloti* was cultured on solid media containing the following antibiotics when appropriate: kanamycin, Km (200 μg/ml), neomycin, Nm (200 μg/ml), spectinomycin, Sp (100 μg/ml), streptomycin, Sm (250 μg/ml), and tetracycline, Tet (10 μg/ml). *E. coli* was grown on plates containing ampicillin, Ap (100 μg/ml), Km (100 μg/ml), Sp (100 μg/ml), or Tet (10 μg/ml) when appropriate. A lower concentration, 50 μg/ml, of Km and Sm was used in broth cultures.

Determination of auxotrophy. Each mutant strain was initially tested for auxotrophy by streaking on a minimal medium agar plate containing Sp (100μg/ml). Two different minimal media were used, M9 or BSM. If the mutant strain was not capable of growth on a minimal medium, the strain was complemented with various combinations of amino acids, purines, pyrimidines and vitamins. Ten different pools of supplements were added to agar plates of the minimal medium, M9. Each component was present in two of the pools in different combinations so that if growth occurred in the presence of two pools, those pools should have only one component in common. The concentrations of amino acids used to prepare the media were as recommended, (Cutting and Youngman, 1994), with two exceptions: cysteine, 100μg/ml, and aspartic acid, 100 μg/ml. The pyrimidines and purines were added as recommended with two

exceptions: uracil, 40 μg/ml, and guanine was not tested. Thiamin was added to a final concentration of 2μg/ml. The mutants, 75 and 15A, were tested only with cytosine and uracil. Because these compounds were able to support growth of these strains in a minimal medium, no other factors were tested. The mutant strains were tested either as the original mutant strain or as RM603 transductants. Because RM603 is not an auxotroph, any auxotrophy of the transduced strain could be attributed to the Tn*5*-233 insert.

Φ-M12 transduction. The Tn*5*-233 transposons were introduced into strains already carrying the Tn*5*-1062 transposon by phage transduction using the phage Φ-M12 (Finan *et al.*, 1984). Preparations of Φ-M12 were prepared from dense cultures of the donor strain. Cultures of the donor strains were grown to late log phase in LB broth supplemented with 2.5 mM of $MgSO_4$ and $CaCl_2$. The cultures were then inoculated with Φ-M12 to a final concentration of 10^8 PFU/ml. After 6-8 hours, the cultures were checked for evidence of lysis and centrifuged to remove remaining intact cells and debris. The supernatant was collected and 0.05 ml chloroform added per 1.0 ml lysate. The mixtures were vortexed thoroughly and stored overnight at 4°C. The tubes were spun again to pellet the chloroform and remaining debris. The supernatant was removed and stored at 4°C.

In order to determine the titer of the phage lysates, the lysate was diluted and mixed 1:10 with a 1-2 day old culture of *S. meliloti* cultured in LB broth supplemented with 2.5 mM $MgSO_4$ and $CaCl_2$. After incubating for 20 minutes at

30°C, the phage/bacteria mixtures were added to 3 mls of top agar [Nutrient Broth (Difco Laboratories, Detroit, MI) containing 0.65% agar with 2.5 mM $MgSO_4$ and $CaCl_2$] and spread on LB agar plates. Plaques were easily visible after incubating the plates overnight at 30°C.

The phage lysates prepared from the donor strains were used to transduce various strains containing Tn*5*-1062. The recipient strain was cultured in LB broth supplemented with 2.5 mM $MgSO_4$ and $CaCl_2$. After 1-2 days of growth, the CFU/ml were estimated by measuring the optical density of the culture and an aliquot mixed with phage lysate such that a ratio of 1 PFU/ 2 CFU was achieved. 0.5 ml of cells was added to a volume of 0.5 ml of phage lysate. Supplemented LB broth was used to adjust the volume of the phage lysate to achieve the correct ratio. After incubation at 30°C for 20 minutes, 0.1 ml of 0.2M sodium citrate was added to each tube in order to chelate the Mg^{+} and Ca^{++} ions and, therefore, interrupt the infection of the bacterial cells. The cells were then rinsed with a sterile solution consisting of 0.85% NaCl and 0.01 M sodium citrate. The cells were resuspended in the saline solution and plated on selective plates, LB agar with Sp (100 μg/ml) and Nm (200 μg/ml).

Measurement of luciferase activity. Luminescence was measured with the Hamamatsu Photonic System model C1966-20. Bacteria grown on agar plates were exposed to the enzyme substrate, N-decyl aldehyde, for two minutes prior to being assayed for light production for 59 seconds. The aldehyde was spread on a

glass petri dish which was placed over the agar plate. The Hamamatsu system converts the photon counts to a visual image on a monitor which can be used to quantitate the amount of light produced by each colony.

Bacteria grown in broth culture were assayed quantitatively for light production. In this assay, the N-decyl aldehyde was dispersed in an aqueous solution of bovine serum albumin (20 mg/ml) at a concentration of 1 μl/ml. A 5-10 μl aliquot of the cell culture was added to 50-100 μl of the aldehyde solution, vortexed for 30 seconds, then assayed for 60 seconds. The measured luminescence was reported in arbitrary units, RLU, and adjusted for cell number by dividing this number by the A_{600} of the culture. All assays were performed at room temperature.

Cloning, DNA sequencing, and sequence analysis. Genomic DNA was isolated from 3-5 ml cultures of *S. meliloti* according to a standard CTAB extraction protocol (Ausubel *et al.*, 1987) with one modification. Prior to extraction with CTAB, the bacterial lysate was passed several times through an 18-gauge needle using a sterile syringe in order to partially shear the DNA. This step reduced sample viscosity and facilitated extraction of the samples with phenol and chloroform.

Genomic DNA was digested with either *EcoR*I or *Xma*I and *Kpn*I. *EcoR*I and *Kpn*I cut within the transposon allowing the isolation of either the left end or the right end of the transposon. Since Tn*5*-233 has identical ends, this facilitated

sequencing directly out of the transposon. Digested genomic DNA was cloned into pBluescript SK- and transformed into *E. coli* DH5α. Colonies resistant to either kanamycin or spectinomycin were selected depending on which end of the transposon was cloned.

The resulting plasmid was isolated using a Qiagen column (Qiagen, Inc., Valencia, CA) or a standard alkaline lysis method (Sambrook *et al.*, 1989). The plasmids were sequenced manually using the T7 Sequenase v.2 kit according to the recommended protocol (Amersham Life Science, Inc., Cleveland, OH) or at the MSU-DOE-PRL DNA Sequencing facility using the ABI Catalyst 800 for Taq cycle sequencing and the 373A Sequencer for the analysis of the products. Initial sequences were obtained using primers complementary to the end of Tn*5*. Either the primer, "Tn5 right", 5'-CACA TGGAATATCAG-3' or the primer, MT93, 5'-CACGATGAAGAGCAGAA GTTAT-3' were used for the first round of sequencing. Subsequent primers were chosen using PrimerSelect 3.11 (DNASTAR Inc., Madison, WI). Primers were synthesized by the Macromolecular Structure Facility located in the Department of Biochemistry at Michigan State University. Sequence data alignments were carried out using SeqMan II 3.61 (DNASTAR Inc., Madison, WI).

Potential open reading frames were identified using a codon preference program, CodonUse 3.1, developed by Conrad Halling (University of Chicago). Sequences encoding proteins similar to the predicted peptides were identified

using the BLAST algorithms (Altschul *et al.*, 1997). Protein sequence alignments were created by using the Match-Box Web Server 1.2 at http://www.fundp.ac.be/sciences/biologie/bms/matchbox_submit.html

Uracil complementation experiment. RM603(75) contains a Tn*5*-233 insert in a gene homologous to *pyrB* and is auxotrophic for pyrimidine synthesis. This auxotrophy can be complemented by supplementing the growth medium with uracil. In order to examine the influence of uracil limitation on luminescence in RM603(75), RM603 and RM603(75) were grown at 30°C in TY broth with and without added uracil. TY broth containing streptomycin at 250 μg/ml was inoculated with a single colony of either RM603 or RM603(75). After two days of incubation at 30°C, each culture was used to inoculate six flasks of TY broth. Three of the six flasks were supplemented with uracil at a concentration of 20 μg/ml. The cultures were grown with shaking at 30°C. Luciferase activity was assayed when the cultures reached an A_{600} of approximately 0.1 and again at an A_{600} of 0.8 to 0.9. Three measurements were taken at each time point from each flask. These values were averaged and adjusted for culture density by dividing by the A_{600}. The measurements from each set of flasks were then averaged and represented on the graph.

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Appendix B

Preliminary characterization of Tn*5*-233 insertions causing decreased luminescence of a *rrn-luxAB* fusion at 15°C

Introduction

Sinorhizobium meliloti 1021 was mutagenized with the transposon, Tn*5*-233, in order to identify factors involved with the low temperature regulation of cold shock loci. Transposon insertion mutants were tested for their ability to alter the luminescence of one of two *luxAB* reporter plasmids, pAG1.3 and pKOC1.1, at 30°C and 15°C. The plasmid, pAG1.3, contains the *luxAB* genes of *Vibrio harveyi* fused to the *rrn*A promoter at +187 and shows increased luminescence following a temperature downshift. The plasmid, pKOC1.1, contains the *luxAB* genes inserted downstream of the *S. meliloti* cold-induced gene, *cspA* (O'Connell and Thomashow, 1999). The preliminary characterization of the six Tn*5*-233 insertion mutants that decreased luminescence from either of the reporter plasmids, pAG1.3 or pKOC1.1, is described below.

Results and Discussion

Six Tn*5*-233 insertion mutants that decreased the luminescence from pAG1.3 or pKOC1.1 at 15°C were isolated. To correlate the Tn*5*-233 insertions

with the observed decrease in luminescence and to examine the effect of all the inserts on a *rrn*::Tn*5*-1062 insertion, each Tn*5*-233 insertion was transduced into strain RM603 (16S *rrn*A::Tn*5*-1062) using phage Φ-M12 (Figure B.1). While luminescence was less in each of the double mutants relative to RM603 at 15°C, two of the Tn*5*-233 insertions, RMD11 and RMAE5, also caused decreased luminescence at 30°C (Figure B.1). Interestingly, the insertion in RM603(AE5) showed a temperature-specific effect on growth rate. The growth of RMAE5 was compared to RM1021 at 30°C and 15°C in TY broth. While RMAE5 grew only 4% slower than RM1021 at 30°C, the difference in growth rates increased to 43% at 15°C. This result suggests that the transposon insertion has interfered with a cellular process that becomes more important at low temperature.

Although the inserts in RM42 and RM546 cause decreased luminescence at 15°C (Figure B.1), the mutants were originally isolated because they increased the luminescence from pAG1.3 at 30°C. These Tn*5*-233 inserts also increased luminescence when transduced into RM603 and grown on TY agar (data not shown). However, this relative increase in light production at 30°C was only observed under the original assay conditions and not when the colonies were exposed continuously to aldehyde as in the assay shown in Figure B.1. When RM603(42) and RM603(546), were grown in TY broth at 30°C, no significant increase in light production was observed relative to the original strain RM603. In fact, when RM603(42) was compared to RM603 during growth at 30°C in TY

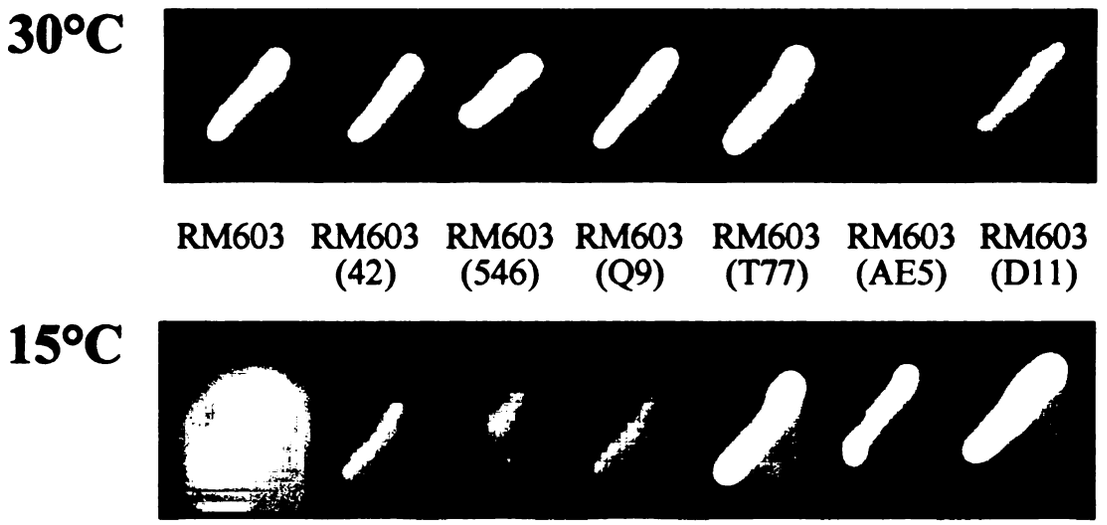
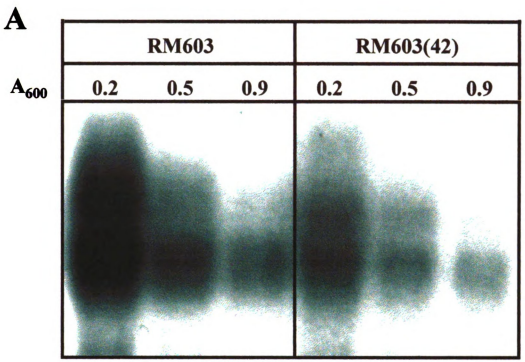


Figure B.1. Set of Tn*5*-233 inserts that decreased luminescence at 15°C when transduced into RM603. RM603 (16S *rrn*::Tn*5*-1062) was compared to a collection of double mutants. Each double mutant is a derivative of RM603 containing an additional Tn*5*-233 insert. Cultures were streaked on TY agar plates and incubated at 30°C for two days before being assayed for luminescence. After being assayed for luminescence at 30°C, the plates were moved to 15°C for 5-6 hours then assayed again.

broth, *luxAB* mRNA levels were reduced in the double mutant at the three culture densities measured, A_{600} of 0.2, 0.5 and 0.9 (Figure B.2).

The Tn*5*-233 insertion mutants, RMQ-9 and RMT-77, were isolated because of their effect on the luminescence of the plasmid pKOC1.1 at 15°C. When transduced into RM603, these inserts also reduced luminescence of the 16S *rrn*A::Tn*5*-1062 insert at 15°C (Figure B.1). Interestingly, RM603(Q9) produced light at levels comparable to that produced by RM603(42) and RM603(546) at both 30°C and 15°C (Figure B.1). The double mutant RM603(T77) also showed decreased luminescence at 15°C but not to the same extent as the other three strains. When RM603(Q9) and RM603(T77) were grown in TY broth culture and exposed to a temperature downshift, the Tn*5*-233 inserts' effect on luminescence was different than that observed during growth on agar plates (Figure B.1 and B.3). In RM603(Q9), the Tn*5*-233 insert had no effect on luminescence at either temperature. The Tn*5*-233 insert in RM603(T77) appeared to decrease luminescence at 30°C while luminescence gradually increased after the temperature downshift approaching the levels observed in RM603 after 3 hours. The effect of this insert on luminescence during growth on plates was essentially opposite to that observed in broth culture. Although RM42, RM546, RMQ9, and RMT77, showed consistent effects on luminescence when transduced into RM603 and assayed on plates, further study of these mutants would be difficult without reproducing these effects in broth cultures.

Figure B.2. Effect of RM42 insert on luminescence of 16S *rrn*::Tn*5*-1062 in RM603. (A) RM603 and RM603(42) were grown in TY broth at 30°C and sampled at A_{600} of 0.2, 0.5, and 0.9. RNA was isolated, separated on a denaturing formaldehyde/agarose gel, transferred to a nylon membrane and probed with a radiolabeled DNA fragment complementary to the *luxAB* message. (B) Luminescence was measured at the same time as the above samples were taken. Relative luminescence was calculated by dividing the RLU/A_{600} of RM603(42) by the RLU/A_{600} of RM603. Each bar represents the average relative luminescence calculated from two experiments.


A
RM603
RM603(42)
A_{600}
0.2
0.5
0.9
0.2
0.5
0.9

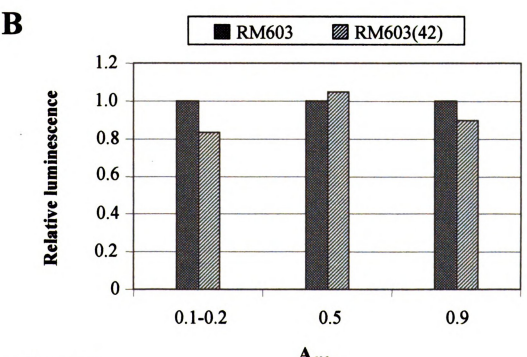
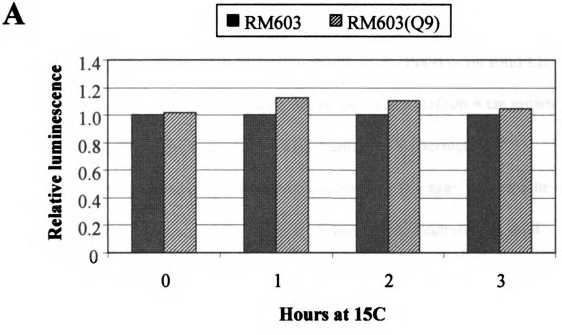

B
RM603
RM603(42)
Relative luminescence
1.2
1.0
0.8
0.6
0.4
0.2
0
0.1-0.2
0.5
0.9
A_{600}

Figure B.2

Figure B.3. Effect of Tn*5*-233 inserts in RMQ9 and RMT77 on luminescence of 16S *rrn*::Tn*5*-1062 in RM603. (A) RM603 and RM603(Q9) were grown in TY broth at 30°C then shifted to 15°C. Luminescence was measured immediately before the shift and 1, 2, and 3 hours afterward. Relative luminescence was calculated by dividing the RLU/A_{600} of RM603(Q9) by the RLU/A_{600} of RM603. Each bar represents the average relative luminescence from two experiments. (B) RM603 and RM603(T77) were grown in TY broth at 30°C then shifted to 15°C. Luminescence was measured immediately before the shift and 1, 2, and 3 hours afterward. Relative luminescence was calculated by dividing the RLU/A_{600} of RM603(T77) by the RLU/A_{600} of RM603.


A
RM603
RM603(Q9)
Relative luminescence
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
Hours at 15C

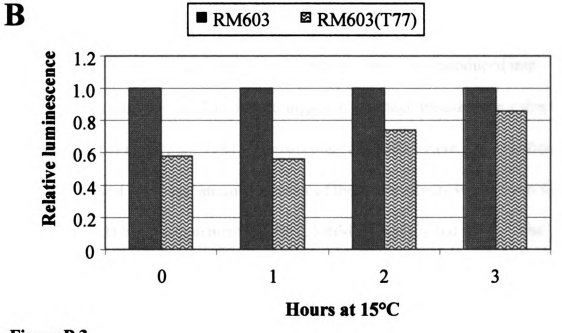

B
RM603
RM603(T77)
Relative luminescence
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
Hours at 15°C

Figure B.3

Materials and Methods

Bacteria and growth conditions. Cultures of *Escherichia coli* DH5α were grown aerobically at 37°C in LB broth (Sambrook *et al.*, 1989) or on solid LB medium containing 1.4% agar. Cultures of *Sinorhizobium meliloti* were grown aerobically at 30°C in tryptone-yeast extract medium, TY (Beringer, 1974). Cultures were maintained on solid medium containing 1.4% agar, or frozen to -80°C in TY broth containing 15% glycerol. *S. meliloti* was cultured on solid media containing the following antibiotics when appropriate: kanamycin, Km (200 μg/ml), neomycin, Nm (200 μg/ml), spectinomycin, Sp (100 μg/ml), streptomycin, Sm (250 μg/ml), and tetracycline, Tet (10 μg/ml). *E. coli* was grown on plates containing ampicillin, Ap (100 μg/ml), Km (100 μg/ml), Sp (100 μg/ml), or Tet (10 μg/ml) when appropriate. A lower concentration, 50 μg/ml, of Km and Sm was used in broth cultures.

Φ-M12 transduction. The Tn*5*-233 transposons were introduced into strains already carrying the Tn*5*-1062 transposon by phage transduction using the phage Φ-M12 (Finan *et al.*, 1984). Preparations of Φ-M12 were prepared from dense cultures of the donor strain. Cultures of the donor strains were grown to late log phase in LB broth supplemented with 2.5 mM of $MgSO_4$ and $CaCl_2$. The cultures were then inoculated with Φ-M12 to a final concentration of 10^8 PFU/ml. After 6-8 hours, the cultures were checked for evidence of lysis and centrifuged to

remove remaining intact cells and debris. The supernatant was collected and 0.05 ml chloroform added per 1.0 ml lysate. The mixtures were vortexed thoroughly and stored overnight at 4°C. The tubes were spun again to pellet the chloroform and remaining debris. The supernatant was removed and stored at 4°C.

In order to determine the titer of the phage lysates, the lysate was diluted and mixed 1:10 with a 1-2 day old culture of *S. meliloti* cultured in LB broth supplemented with 2.5 mM $MgSO_4$ and $CaCl_2$. After incubating for 20 minutes at 30°C, the phage/bacteria mixtures were added to 3 mls of top agar [Nutrient Broth (Difco Laboratories, Detroit, MI) containing 0.65% agar with 2.5 mM $MgSO_4$ and $CaCl_2$] and spread on LB agar plates. Plaques were easily visible after incubating the plates overnight at 30°C.

The phage lysates prepared from the donor strains were used to transduce various strains containing Tn*5*-1062. The recipient strain was cultured in LB broth supplemented with 2.5 mM $MgSO_4$ and $CaCl_2$. After 1-2 days of growth, the CFU/ml were estimated by measuring the optical density of the culture and an aliquot mixed with phage lysate such that a ratio of 1 PFU/ 2 CFU was achieved. 0.5 ml of cells was added to a volume of 0.5 ml of phage lysate. Supplemented LB broth was used to adjust the volume of the phage lysate to achieve the correct ratio. After incubation at 30°C for 20 minutes, 0.1 ml of 0.2M sodium citrate was added to each tube in order to chelate the Mg^{+} and Ca^{++} ions and, therefore, interrupt the infection of the bacterial cells. The cells were then rinsed with a

sterile solution consisting of 0.85% NaCl and 0.01 M sodium citrate. The cells were resuspended in the saline solution and plated on selective plates, LB agar with Sp (100 μg/ml) and Nm (200 μg/ml).

Measurement of luciferase activity. Luminescence was measured with the Hamamatsu Photonic System model C1966-20. Bacteria grown on agar plates were exposed to the enzyme substrate, N-decyl aldehyde, for two minutes prior to being assayed for light production for 59 seconds. The aldehyde was spread on a glass petri dish which was placed over the agar plate. The Hamamatsu system converts the photon counts to a visual image on a monitor which can be used to quantitate the amount of light produced by each colony.

Bacteria grown in broth culture were assayed quantitatively for light production. In this assay, the N-decyl aldehyde was dispersed in an aqueous solution of bovine serum albumin (20 mg/ml) at a concentration of 1 μl/ml. A 5-10 μl aliquot of the cell culture was added to 50-100 μl of the aldehyde solution, vortexed for 30 seconds, then assayed for 60 seconds. The measured luminescence was reported in arbitrary units, RLU, and adjusted for cell number by dividing this number by the A_{600} of the culture. All assays were performed at room temperature.

Cloning, DNA sequencing, and sequence analysis. Genomic DNA was isolated from 3-5 ml cultures of *S. meliloti* according to a standard CTAB extraction protocol (Ausubel *et al.*, 1987) with one modification. Prior to

extraction with CTAB, the bacterial lysate was passed several times through an 18-gauge needle using a sterile syringe in order to partially shear the DNA. This step reduced sample viscosity and facilitated extraction of the samples with phenol and chloroform.

Genomic DNA was digested with *Xma*I and *Kpn*I. *Kpn*I cuts within the transposon allowing the isolation of the right end of the transposon. Since Tn*5*-233 has identical ends, this facilitated sequencing directly out of the transposon. Digested genomic DNA was cloned into pBluescript SK- and transformed into *E. coli* DH5α. Colonies resistant to spectinomycin were selected.

The resulting plasmid was isolated using a Qiagen column (Qiagen, Inc., Valencia, CA) or a standard alkaline lysis method (Sambrook *et al.*, 1989). The plasmids were sequenced manually using the T7 Sequenase v.2 kit according to the recommended protocol (Amersham Life Science, Inc., Cleveland, OH) or at the MSU-DOE-PRL DNA Sequencing facility using the ABI Catalyst 800 for Taq cycle sequencing and the 373A Sequencer for the analysis of the products. Initial sequences were obtained using primers complementary to the end of Tn*5*. Either the primer, "Tn5 right", 5'-CACA TGGAATATCAG-3' or the primer, MT93, 5'-CACGATGAAGAGCAGAA GTTAT-3' were used for the first round of sequencing. Subsequent primers were chosen using PrimerSelect 3.11 (DNASTAR Inc., Madison, WI). Primers were synthesized by the Macromolecular Structure Facility located in the Department of Biochemistry at

Michigan State University. Sequence data alignments were carried out using SeqMan II 3.61 (DNASTAR Inc., Madison, WI).

Potential open reading frames were identified using a codon preference program, CodonUse 3.1, developed by Conrad Halling (University of Chicago). Sequences encoding proteins similar to the predicted peptides were identified using the BLAST algorithms (Altschul *et al.*, 1997). Protein sequence alignments were created by using the Match-Box Web Server 1.2 at http://www.fundp.ac.be/sciences/biologie/bms/matchbox_submit.html

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Appendix C

Nucleotide sequence of the promoter and upstream regions of the three *rrn* operons in *Sinrhizobium meliloti* 1021

Sequence of *rrn*A upstream region obtained from cosmid 6H7.

A putative ORF is indicated by the capitalized sequences. Translation is predicted to proceed from nucleotide 390 toward nucleotide 1. The deduced polypeptide shows no significant similarity to any protein in the database. The -10 and -35 *rrn* promoter sequences are shown in bold type.

```
GGCAAGCGCN ATCATGACAT GCACGAAGGG CTGGCGGCGC ACCACCTCCT  50
TCTCGCCGTC ANGGATCATG GTCGAGACTT CCATCACGGT CTTGTCGGCT 100
GGCTTGGCGA CGATGTTGGG GGAAAGAACG CGATCTCCCC GTTTGGCGGC 150
GCCGACCGGC GCCGACGGAT CGAGCGCGGC AAAGGCTTCC GCCGGAATCG 200
CCAGTTGCTG CCGGCCGTCG AGCGCCGCAA AGAGCGCGAC GCCCATCAGA 250
AGACTTGAGG TGATGCCGGT CAAGAACGTG CCCGAGAGCC AGCGCATCGA 300
GATCTCGCGC CGGTCCGGTG CGCGACGGCC GTCCGCTAGG ATCGGCGGGT 350
GGTCGCCAAG CGAACGAAGC ATGTTCTTAT CGCTGCTCAT gcaggctgga 400
ggtcccctaa atgctctgcc gtatgcttat cgtcgatacg ttgcgcgaag 450
gtgtgcatct tccgtatacg gaagtcaaat cgcagcgcag ggcaggcccc 500
gacgccgcgc ctccggaacg aagcggcgat gatctgggtt agatcggtcg 550
attgtgaaga agtgagggcg aaattatgta tccacaggct gagggagcgg 600
ctcgaaaaag aatctgcgac ccgcaaaacc ctgcttttcc gggctttgtc 650
agaaaactgt catttttttt gaagaagcct gttgacgtgt tggagggctg 700
gggtctataa gcccgatca                                   719
```

Sequence of *rrn*B upstream region obtained from cosmid 8D2.

A putative ORF is indicated by the capitalized sequences. Translation is predicted to proceed from nucleotide 224 to nucleotide 577. The deduced polypeptide sequence shows limited similarity to several transcriptional regulators. The -10 and -35 *rrn* promoter sequences are shown in bold type.

```
tctcgcttga gctcgtggcg atccaacaga tcatgtcttt gtcgcgcaag  50
gaggcggata tcgcggtagc gctcgccgca ccgaaagccg gagcttacca 100
tgccgaagtg ctgacgccct atacgctgca tgtctatggg cacgcagcta 150
tctcgccggt cagccgccga tccgcacacg cagcgatctc ggctcccacc 200
gcttcgtcgg ttatatcgac gacATGATCT TCACGCCGGG GCTCGATTAT 250
CTCGGCGAGA TACAGCCGGG GCTGCGCGCC CATTTTCAAA GCTCGAGCAT 300
CCTCACCCAG TTGAAGGCGG TGCGCCAGGG ATTAGGGCTT TGCGTGCTCC 350
CGCACTTCAT GGCGAAGGAC GAACCCAACC TCGAGATAGT GCTGCCGGAT 400
GAAATCGAGC TGAAACGGAC CTATTGGCTG ATCTGCCACC GCGATCTGAT 450
CGCGATACCC AGGGTGAGGG CCGTACGGGA TTTCCTGGTG GATGCCGCCG 500
TCGAAAACCG CGGCTGCTTC CTGCGTGAGA CGCCGTTCGC TTTGACCGGT 550
CCTCGGCGGG TTCTTGAACG TCAATAGggc gttaaccata taggcgaggc 600
aaacgccggg cctttccgag gcgcgagcgt cccgaaaagg atgccgagcc 650
gccaggtttc cggtccggct ttcggatgca agaataaaca cctttaaaaa 700
cagtcactta cgattttctg ctgatttttt gaaaatcgtt gttgacgtgt 750
tggagggctg gggtctataa gcccgatca                        779
```

Sequence of *rrn*C upstream region obtained from cosmid 5C4.

A putative ORF is indicated by the capitalized sequences. Translation is predicted to proceed from nucleotide 372 toward nucleotide 1. The deduced polypeptide sequence shows 61% similarity over 97 amino acids to a predicted ORF in *Escherichia coli* (AE000270). The -10 and -35 *rrn* promoter sequences are shown in bold type.

```
GCCGGGCGNG TAAAGCAGCC CTNGGCGTTT NATCGCGGCG AAGTTCTCGC  50
CGTTGATCCA CACGAGATCG ACCGATCCGC CCTCGTCCTT TCCTGCGGAT 100
TTTTCCGCGA GCACGGTCGC GGTCGCCTTA GCGGTGTCGT CGAGCTTCAC 150
ATGGACGACG GTGACGCCGT AGCGCGCCTT CATTTCATCG CCGGCCCATC 200
TGATATAGGC ATTGACGTTC TCGGAGCCGC CCCAGGCGTT GAAATAGACC 250
GTCTGGCCGC TCGCCTCCGC GGCGACCGCC TCCCAGTTCC CAGCGTCCGT 300
GGCGGACGCC CTGCCGGCAA GGCCGAGCGC CAGCAGCGCT GCGGCGATGA 350
TCTTCCCTGC CCGCCTGATC ATgacgctcc tctacccccg ccacatgccg 400
ttgccgccga gggtaagaca agggcggtct gcgtcaatgc acgcggccgc 450
ctgctgccgt cacgtttcgg tgaaacctgc tctggtcgcc tctttcgcgg 500
gagcggcaat gggcttccag agctaggaat tcgcgtcacg cagggccctt 550
gaaaggcgga actgcgttaa ccatataggt ctcatcccca gaagcgccga 600
aagcccgcaa agcggacccg tattggtagc agcgtcggac gtcttttcgc 650
gtgctttgga aatggccctt taaaaacagt cacttacgat tttctgctga 700
ttttttgaaa atcgttgttg acgtgttgga gggctggggt ctataagccc 750
gatca                                                  755
```