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in Rhizobium meliloti

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By

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ABSTRACT

STUDIES ON COLD-REGULATED GENE EXPRESSION IN *RHIZOBIUM MELILOTI*

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Rhizobium meliloti strain 1021 (Rm 1021) was grown at various temperatures; growth rate decreased with a decrease in temperature. SDS-PAGE analysis of protein synthesis showed 8 up-regulated and 8 down-regulated proteins after a shift from 30°C to 15°C. Rm 1021 was mutagenized using Tn*5-luxAB* to identify cold-induced genes. Temperature shift from 30°C to 10°C revealed two mutants, Rm 5-5-6 and Rm 3-16-6, which exhibited an increase in light emission. Timing and degree of induction was characterized at various temperatures. Both Rm 5-5-6 and Rm 3-16-6 were induced by a shift from 30°C to 20°C or 15°C, but not 25°C. A shift to 10°C resulted in delayed induction. Cloning and sequencing of genomic regions flanking the Tn*5-luxAB* insertion in Rm 3-16-6 identified the cold-regulated gene as a 16S rRNA gene.

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INTRODUCTION

Prokaryotes experience many environmental stresses, including extremes in temperature, pH, osmolarity, and nutrient-limitation. Bacteria are often able to adapt to harsh conditions and the mechanism can involve changes in gene expression. The general subject of this thesis regards microbial acclimation to low temperature.

Cold shock response in *Escherichia coli*.

E. coli exhibits an ability to rapidly adjust to shifts up or down in temperature between 37°C and 20°C. Cultures shifted within this range assume a new steady state growth rate almost immediately with reported periods of adjustment varying from zero to ten minutes (10, 15, 17). Total protein synthesis reaches its new rate almost immediately; however, synthesis rates of individual proteins exhibit a transient increase or decrease then reach a steady state synthesis rate after approximately 20 minutes (15).

Temperature downshifts outside the 37°C to 20°C range require a much longer period of adjustment before resuming growth. For example, *E.coli* experiencing a shift from 37°C to 10°C generally exhibits a 4 hour lag before assuming the steady state growth rate for 10°C (12). An earlier study

(17) reported a slightly more complex situation in which the 4 hour lag was followed by a period of rapid growth which gradually declined to the new steady state rate of growth. After a shift from 37^{o}C to 5^{o}C, the bacteria are unable to initiate protein synthesis and the culture no longer doubles (2, 4, 6).

Cold shock proteins in *E. coli*.

Overall, the rate of protein synthesis decreases when a culture is cold shocked, but some proteins are produced, at least transiently, at a higher level. These upregulated proteins are known as cold shock proteins (CSPs). CSPs were first identified by comparing the quantitative levels of proteins at different temperatures using 2-D gel electrophoresis (9). Thirteen CSPs were identified, 12 of which were found to be upregulated 2- to 10-fold (12). Nine of the 12 have been identified as polynucleotide phosphorylase (PNP), NusA, initiation factor 2α, initiation factor 2β, RecA, dihydrolipoamide acetyltransferase, pyruvate dehydrogenase (lipoamide), H-NS, and DNA gyrase subunit A (11, 12, 13). Together these proteins have a wide range of activity, such as formation of acetyl CoA for the tricarboxylic acid cycle (dihydrolipoamide acetyltransferase and pyruvate dehydrogenase), recombination and induction of the SOS response (RecA), DNA supercoiling (GyrA), degradation of single stranded RNA (PNP) thermoregulation of transcription of the pilin genes (H-NS), termination of transcription (NusA), and binding $tRNA^{fmet}$ to the 30S

ribosomal subunit for initiation of translation (initiation factors α and β) (8, 11, 12, 13).

CspA and CspA-like proteins.

The major CSP in *E. coli*, CspA (CS7.4), is induced 100-fold in response to cold shock; it is undectable at 37°C, but is the most abundantly synthesized protein at 10°C (7, 11, 12). Within 30 minutes of temperature shift, CspA is detectable (7). CspA levels increase during the cold shock-induced lag period (11), then decrease to a level approximately 20% of the maximum level (7). No temperature has been determined as the threshold for induction. Fourteen degree temperature shifts from 42°C to 28°C, 37°C to 23°C, and 24°C to 10°C all resulted in the induction of CspA (10), but shifting from 24°C to 10°C resulted in the strongest induction. Shifting from 37°C to 10°C resulted in even higher levels of CspA than the shift from 24°C to 10°C (10). Together these results indicate that the level of induction is a factor of both the magnitude of the temperature shift and the final temperature itself.

CspA appears to be a DNA-binding protein based on sequence identity to the human protein YB-1; YB-1 binds to the CCAAT-containing "Y box" (5, 23). Binding of a Y box factor to the *cis*-acting Y box in a promoter has been shown to activate transcription (20). CspA has been shown to bind to the CCAAT sequence and *hns*, *cspA*, *recA*, *nusA*, *pnp*, and *gyrA* contain at least one ATTGG sequence (11, 13). Thus, it

is possible that CspA is a transcriptional activator which is auto-regulated (11, 13, 19).

Recently, three other genes have been identified in *E. coli* that encode proteins with 79, 70, and 45% amino acid identity to CspA (14). These proteins have been designated CspB, CspC, and CspD, respectively. Curiously, while CspB is induced by cold shock, CspC exhibits no response to cold and CspD is actually inhibited by cold shock (14).

CspA-like proteins exist in other prokaryotes as well. For example, *Bacillus subtilus* contains a protein, CspB, which is induced by a cold shock (22). CspB protein demonstrates 61% amino acid identity to CspA, while the DNA sequences show 60% identity (22). CspB demonstrates 43% identity to the human Y-box factor binding site (22, 23). The Gram positive bacterium *Streptomyces clavuligerus* has a 7.0 kDa protein which is 56% identical and 80% similar to CspA (1).

Cold shock proteins in *Rhizobium* spp.

Three arctic strains of *Rhizobium* spp. were isolated from arctic legumes and compared to three temperate strains, (one being *R. meliloti* A2), in regard to their heat and cold shock responses (3). After a shift from 25^{o}C to -2^{o}C for arctic strains and 30^{o}C to -2^{o}C for temperate strains, 17-24 cold shock proteins were present in arctic strains, while temperate strains had 18-22 cold shock proteins. Quantification was not reported. An 11.1 kDa protein was found to be abundant after temperature downshifts, with

levels increasing with decreasing temperature, similar to CspA. Whether this protein is a CspA-like protein is not yet known.

Regulation of the cold shock response in *E. coli*.

The mechanism(s) responsible for regulating the cold shock response has yet to be defined. There is evidence, however, that the cold shock response may involve the action of (p)ppGpp. (p)ppGpp levels have been shown to decrease with a decrease in temperature (10, 16, 18). The fact that induction of the stringent response prior to temperature downshift resulted in the repression of CspA, NusA, RecA, PNP, dihydrolipoamide acetyltransferase and pyruvate dehydrogenase (10), suggests that (p)ppGpp may negatively regulate CspA, which in turn regulates the other ppGpp-repressed cold shock proteins. A mutant unable to synthesize detectable levels of ppGpp (*relA spoT*), had a much higher steady state level of CspA at 24^{o}C than the wild type (12). At 30^{o}C, however, CspA levels in the mutant did not differ from the wild type (21). Thus, CspA is not regulated by (p)ppGpp alone.

Ribosomes have been proposed to act as sensors of heat and cold shock based on the upregulation of heat shock or cold shock proteins after addition of antibiotics which target the ribosome (21). In particular, heat shock proteins were found to be induced by kanamycin, puromycin and streptomycin. Ten of the fourteen CSPs, including CspA, were induced by a second set of antibiotics which target the A

site of the ribosome, including chloramphenicol, erythromycin, fusidic acid, spiramycin, and tetracycline (21). Although (p)ppGpp and/or ribosomes may be involved in regulation of the cold shock response, the mechanism(s) remains to be elucidated.

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CHAPTER 2

Studies on cold-regulated gene expression in *Rhizobium meliloti*

ABSTRACT

Rhizobium meliloti strain 1021 (Rm 1021) was grown at various temperatures to determine generation time. Growth rate was found to be a linear function of temperature between 15 and 30°C. Above 30°C and below 15°C, however, growth was "restricted". Protein synthesis at 30°C and 15°C was evaluated by SDS-PAGE. Analysis indicated that 8 proteins were up-regulated and another 8 down-regulated in response to a temperature downshift of 30°C to 15°C. To identify genes induced by low temperature, Rm 1021 was mutagenized using a Tn*5-luxAB* reporter transposon containing a kanamycin resistance gene for selection of mutants. The *luxAB* gene cassette encodes the light-producing luciferase enzyme of *Vibrio harveyi*. Mutants were shifted from 30°C to 10°C for six hours and screened for increased light emission. Two mutants, designated Rm 5-5-6 and Rm 3-16-6, exhibited 20- and 100-fold increase in light emission at low temperature and were selected for further study. The timing and degree of induction was characterized at various temperatures between 30°C and 10°C. Overall patterns of induction were the same; both Rm 5-5-6 and Rm 3-16-6 were induced by a shift from 30°C to 20°C or 15°C, but not 25°C. A shift to 10°C resulted in

delayed induction. Cloning and sequencing of the genomic regions flanking the Tn*5-luxAB* insertion in Rm 3-16-6 identified the cold-regulated gene as a 16S rRNA gene.

INTRODUCTION

When *E. coli* experiences a change in temperature within a range in which growth is not restricted (37°C to 20°C), the culture immediately assumes the steady-state growth rate of the post-shift temperature (17). In contrast, cold shocks from 37°C to 10°C exibit a four hour lag before assuming the new growth rate, implying a change in cellular composition when growing outside the "normal" range (21, 22). At 5°C, *E. coli* is unable to initiate protein synthesis, ribosomal subunits accumulate, and cellular growth stops (5, 9, 14).

The proteins involved in response to cold shock have been best characterized in *E. coli*. The major cold shock protein, CspA, demonstrates 100-fold induction after a thirteen degree temperature downshift. (15). CspA shows sequence identity to known transcriptional activators which bind to a CCAAT-containing "Y box" within a promoter (12, 38). CspA also has CCAAT binding activity (21). Thus, CspA may be a transcriptional activator. CspA-like proteins have been identified in *Bacillus subtilus* and *Streptomyces clavuligerus* (2, 37). 2D gel analysis of the cold shock response has shown the presence of an additional twelve proteins that are induced some 2- to 10-fold in response to low temperature (15, 24). Ten of these proteins have been

identified (23, 24, 26), some of which are involved in either transcription or translation.

Little is known about the cold shock response in *Rhizobium* spp. A study of three arctic and three temperate strains of *Rhizobium* spp. revealed the presence of 17-24 and 18-22 cold shock proteins, respectively (6). The identity of the genes and the mechanism for the regulation is unknown.

The overall goals of my research project were to describe more fully *R. meliloti* growth at low temperature and to isolate, identify, and characterize the expression of one or more cold-regulated genes using *R. meliloti* strain 1021 (Rm 1021). The data indicate that the growth rate of Rm 1021 is a linear function of temperature between 15 and 30^{o}C, but that below 15^{o}C, growth is "restricted." The upregulation of 8 polypeptides, and downregulation of 8 polypeptides, was found to occur when Rm 1021 cells were shifted from 30^{o}C to 15^{o}C. Two genes that were cold-regulated were tagged by transposon mutagenesis and the patterns of induction were examined. Both Rm 3-16-6 and Rm 5-5-6 were induced by temperature shifts from 30^{o}C to 20, 15, or 10^{o}C, although induction at 10^{o}C was delayed. The identity of one was determined to be a 16S rRNA gene. This appears to be the first description of a cold-regulated *rrn* gene in bacteria.

MATERIALS AND METHODS

Bacteria and plasmids. Table I describes the bacterial strains and plasmids used in this study.

Media and Culture Conditions. *R. meliloti* strains, (Rm 1021, Rm 3-16-6, Rm 5-5-6 and Rm C-30) were grown at 30°C in either TY (4) medium adapted to 0.5 g/l $CaCl_2$-$2H_2O$, or GTS (25) medium, generally supplemented with 50 ug/ml streptomycin. *E. coli* strains were grown at 37°C in LB medium (34). Strains containing plasmids were generally supplemented with 50 ug/ml kanamycin. (Tryptone and yeast extract were purchased from Difco, St. Louis, MO.)

Tri-Parental Matings. Transposon mutagenesis was accomplished via tri-parental matings, adapted from the method of De Bruijn and Rossbach (10). Rm 1021, *E. coli* containing pRL1062a and *E. coli* containing pRK2013 were grown to late log phase. 100 ul of each culture was placed in the center of a TY plate, allowed to dry, and incubated at 30°C overnight. The resulting cell mass was scraped off with a loop and resuspended in 1 ml of TY medium and the dilutions plated on TY plates containing 200 ug/ml kanamycin and 250 ug/ml streptomycin. Plates were incubated at 30°C for 3-4 days and screened for light emission.

Table 1 Strains and plasmids used.

Strain or plasmid	Description	Source or reference
E. coli DH5α	*supE*44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA96thi*-1 *relA*1	34
R. meliloti 1021	Sm^R, Mos^-, Moc^- derivative of SU47	29
R. meliloti 3-16-6	Rm 1021 mutagenized with pRL1062a	This study
R. meliloti 5-5-6	Rm 1021 mutagenized with pRL1062a	This study
R. meliloti C-30	Rm 1021 mutagenized with pRL1062a	This study
pRL1063a	Suicide vector carrying Tn*5* with promoterless *luxAB* from *Vibrio fischeri*	40
pRL1062a	Suicide vector carrying Tn*5* with promoterless *luxAB* from *Vibrio harveyi*	C.P. Wolk
pRK2013	ColE1 Km^R *tra*(RK2)	13
pAG1a	Tn*5*-*luxAB* and flanking DNA from Rm 3-16-6 (*Apa* I digest)	A.M. Gustafson

Screening of Mutants. Mutants obtained by triparental mating were screened for light emission at 30°C and 10°C. After exposure to n-decanal for two minutes, light emission was quantitated for one minute with a Hamamatsu photonic camera (model c1966-20, Photonic microscopy, Oak Brook, IL). Light emission was measured at 30°C, the aldehyde allowed to dissipate in a sterile environment, and the plates incubated at 10°C for 6 hours before being screened again. Mutants that emitted light at 10°C, but not 30°C, were picked and rescreened using the same method.

Stability of *V. fischeri* and *V. harveyi luxAB* proteins. 2 ul of overnight cultures were pipetted onto filter squares on TY plates. Plates were incubated at 30°C overnight and filters were transferred to fresh plates and incubated at 10°C for 6 hours. Filters were then transferred to fresh plates at room temperature for 0, 15, 30, and 45 minutes. The plates were exposed to aldehyde for 2 minutes then screened for light emission for 1 minute using a Hamamatsu photonic camera.

Genomic DNA Analysis. Total DNA from Rm 1021, Rm 5-5-6 and Rm 3-16-6 was isolated by a method adapted from Rossbach (32). One and a half ml of an overnight culture was centrifuged for 3 min. The pellet was washed with 1 volume 1M NaCl, 25mM potassium phosphate and centrifuged 3 minutes. The pellet was resuspended in 350 ul TE, 25 ul 20% SDS, 25 ul pronase (10 mg/ml) and 100 ul sterile distilled H_2O. After incubating for 1 hour at 37°C, the DNA was passed three times

through a sterile syringe with an 18 gauge needle. DNA was purified by 2 phenol extractions with a ten minute centrifugation, followed by 2 phenol/chloroform extractions with five minute centrifugations and 1 chloroform extraction followed by a 2 minute centrifugation. Two volumes of 100% ethanol were added and mixed by careful shaking. The DNA was spooled out with a pasteur pipette and dissolved overnight in 100 ul sterile distilled water.

Kpn I and *Sma* I (New England Biolabs, Beverly, MA) were used separately to digest Rm 1021, Rm 5-5-6 and Rm 3-16-6. Digests were incubated according to manufacturer's specifications. DNA fragments were separated on a 0.7% agarose gel. DNA was transferred to a nylon filter (nytran) using a posiblotter (Stratagene, La Jolla, CA). Hybridization was accomplished using ^{32}P-radiolabeled *luxAB* fragments from pRL1062 or the 5' 16S DNA flanking genomic fragment from pAG1a as a probe for quantitating to number of Tn*5-luxAB* insertions or confirming the 16S copy number, respectively.

Protein synthesis. Rm 1021 was grown at 30^{o}C to an OD_{600} of ~0.3. Before shifting to 15^{o}C, 1 ml of culture was added to a disposable 15 ml culture tube and labeled with ^{35}S methionine (5 ul/ml) for 10 minutes at 30^{o}C. 500 ul of 10 mg/ml l-methionine was added to stop labeling. Cells were centrifuged for 15 minutes, washed with 1 ml of 25 mM Tris/ 1 M NaCl, centrifuged for 5 minutes and resuspended in 100 ul 25 mM Tris/10 mM $MgCl_2$, 5 ul 6 mg/ml lysozyme, 5 ul 1 mg/ml

DNase I. Cells were incubated at 30°C for 30 minutes, 110 ul of a cracking buffer containing 100 mM NaOH, 1% SDS and 10 mM EDTA was added, cells were boiled for 2 minutes then centrifuged for 30 minutes. To precipitate proteins, 1.3 ml of acetone was added to the supernatant, placed at -20°C for 2 hours then centrifuged for 30 minutes. The resulting pellet was resuspended in SDS sample buffer. After the remaining culture was incubated at 15°C for one hour, 1 ml was removed, labeled with ^{35}S methionine for 30 minutes at 15°C and labeled proteins were purified as above. Proteins were separated by SDS-PAGE (27).

Induction in TY medium. Light emission was measured using a Berthold luminometer (Lumat LB9501, Wallac Inc., Gaithersburg, MD). 0.1% n-decyl aldehyde (D-7384, Sigma, St. Louis, MO) was buffered with 2% BSA (initial fractionation by heat shock, 98-99%, A3803, Sigma, St. Louis, MO) and 50 ul was added to 5 ul of culture. Light emission was measured in triplicate and the values for each set were averaged and adjusted for background and O.D. Fold induction was defined as light emission at low temperature divided by light emission at 30°C.

Growth rates. Culture growth rates were determined for wild type and mutant strains Rm 3-16-6 and Rm C-30 in triplicate. Twenty-four hour old cultures grown at 30°C were diluted into fresh medium to give a final $O.D._{600}$ of 0.1 before incubation at lower temperatures. Growth rates were established by measurement of increased O.D. over time.

RESULTS

Growth rates of Rm 1021 at low temperatures.

Steady state growth rates at various temperatures were determined for the *R. meliloti* wild type, Rm 1021, in TY and GTS media (Table 2). As expected, growth rate decreased with a decrease in temperature. Interestingly, when compared to growth in TY medium, Rm 1021 in GTS medium exhibited a slower growth rate at 30°C, but a faster growth rate at 10°C.

Growth lags were not observed upon shift of Rm 1021 cultures from 30°C to 10°C, or any of the higher temperatures tested in either TY (Figure 1) or GTS media (not shown).

An Arrhenius plot is often used to show the relationship between the specific growth rate constant (k) and temperature (19). Prokaryotes typically have a "normal" range in which the growth rate decreases linearly with a decrease in temperature. Growth outside this range is "restricted" (17, 19). An Arrhenius plot of Rm 1021 from 37°C to 10°C indicated a linear relationship between 30°C and 15°C with a calculated temperature characteristic (u) of about 102,800 J/mol (~24,600 cal/mol) (Figure 2). Outside of the 30 to 15°C range, growth became restricted; i.e., the k value fell

Table 2. Generation times of Rm 1021 in TY and GTS media at various temperatures.

Temperature	TY medium	GTS medium
37°C	2.0 hr ± 0.7 hr	
30°C	2.4 hr ± 0.1 hr	3.6 hr ± 0.6 hr
25°C	5.5 hr ± 0.6 hr	
20°C	9.5 hr ± 0.7 hr	8.0 hr ± 0.4 hr
15°C	21.5 hr ± 0.9 hr	
10°C	80.3 hr ± 10.5 hr	32.3 hr ± 2.4 hr

Flasks of media were inoculated with overnight cultures of Rm 1021 to an $O.D._{600}$ of ~ 0.1. In each case, three replicate cultures were used. Generation times were calculated from growth rate during exponential phase.

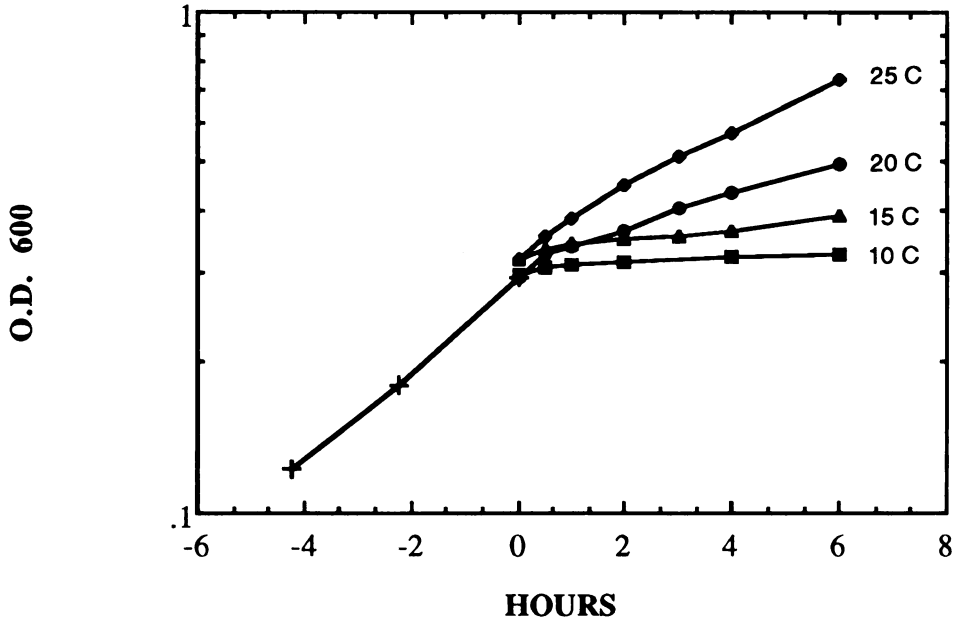

Figure 1. Growth after shift of Rm 1021 from 30°C to lower temperature. Overnight cultures of Rm 1021 grown in TY medium at 30°C were diluted into fresh TY medium to an O.D.600 of ~0.1 and incubated at 30°C until the cultures reached an optical density of ~0.3. The cultures were transferred to either 25, 20, 15, or 10°C and O.D.600 measurements were taken at the indicated times. Plus, 30°C. Diamond, 25°C. Circle, 20°C. Triangle, 15°C. Square, 10°C.

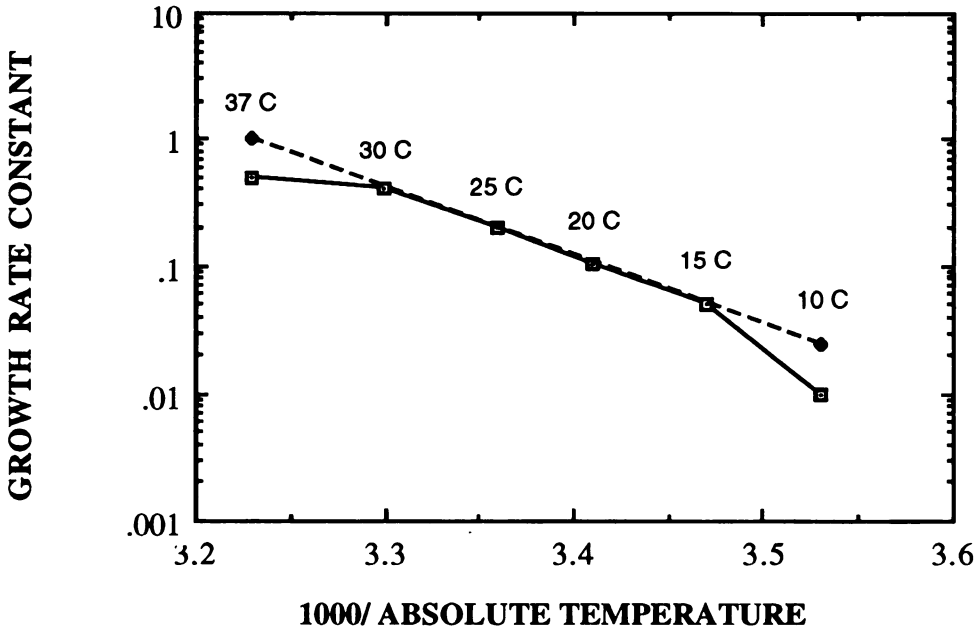

Figure 2. Arrhenius plot of Rm 1021 steady state growth rates at decreasing temperatures. Steady state growth rates at each temperature were measured and used to calculate the growth rate constant, k (h^{-1}), which is the inverse of the generation time. Each data point is labeled with the growth temperature, in Celsius. Dashed line indicates the expected growth rate constant if growth was not restricted.

below that predicted by extrapolation from the linear region of the Arrhenius plot.

Protein synthesis.

Changes in gene expression were found to occur in Rm 1021 upon a shift from 30°C to 15°C (Figure 3). In particular, the synthesis of at least 8 polypeptides was upregulated in response to the temperature shift and the synthesis of at least another 8 proteins was downregulated by temperature downshift.

Comparison of *V. fisheri luxAB* and *V. harveyi luxAB* as reporter genes to "tag" cold-regulated genes.

Rm 1021 was mutagenized with Tn*5* derivatives carrying *luxAB* genes from either *V. fischeri* or *V. harveyi* (Figure 4) (39). Mutants were screened at 30°C and 10°C and any that appeared to be upregulated in response to low temperature were isolated and rescreened.

Initially Rm1021 was mutagenized using *luxAB* from *V. fischeri*. Unexpectedly, the majority of the mutants demonstrated a dramatic increase in light emission upon shift from 30°C to 10°C. Five such isolates are shown in Figure 5 (spots 1-5); light emission of cells grown at 30°C (panel B) was much less than that of cells incubated at 10°C for 15 minutes (panel C). This result, however, was in apparent conflict with the protein synthesis study indicating only modest changes in gene expression in response to low temperature (Figure 3). Thus, it was suspected that the increase in light emission might be due to an increase in

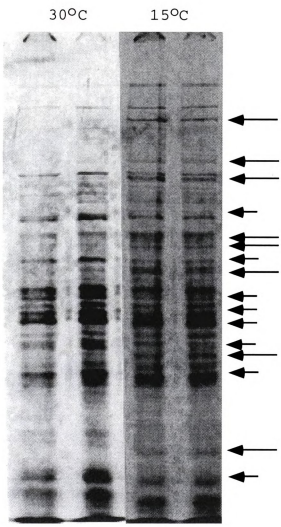

Figure 3. 1D gel analysis of Rm 1021 ^{35}S-labeled proteins at 30°C and 15°C. An Rm 1021 culture was grown in TY medium at 30°C. At an O.D.$_{600}$ of ~0.3, half the culture was ^{35}S-labeled at 30°C. The other half was transferred to 15°C for 2 hours, then ^{35}S-labeled. ^{35}S-labeled proteins were extracted from the cells and run on a polyacrylamide gel. Exposure of the 15°C lanes was increased to allow comparison of overall protein expression. Long arrows indicate upregulated proteins. Short arrows indicate downregulated proteins.

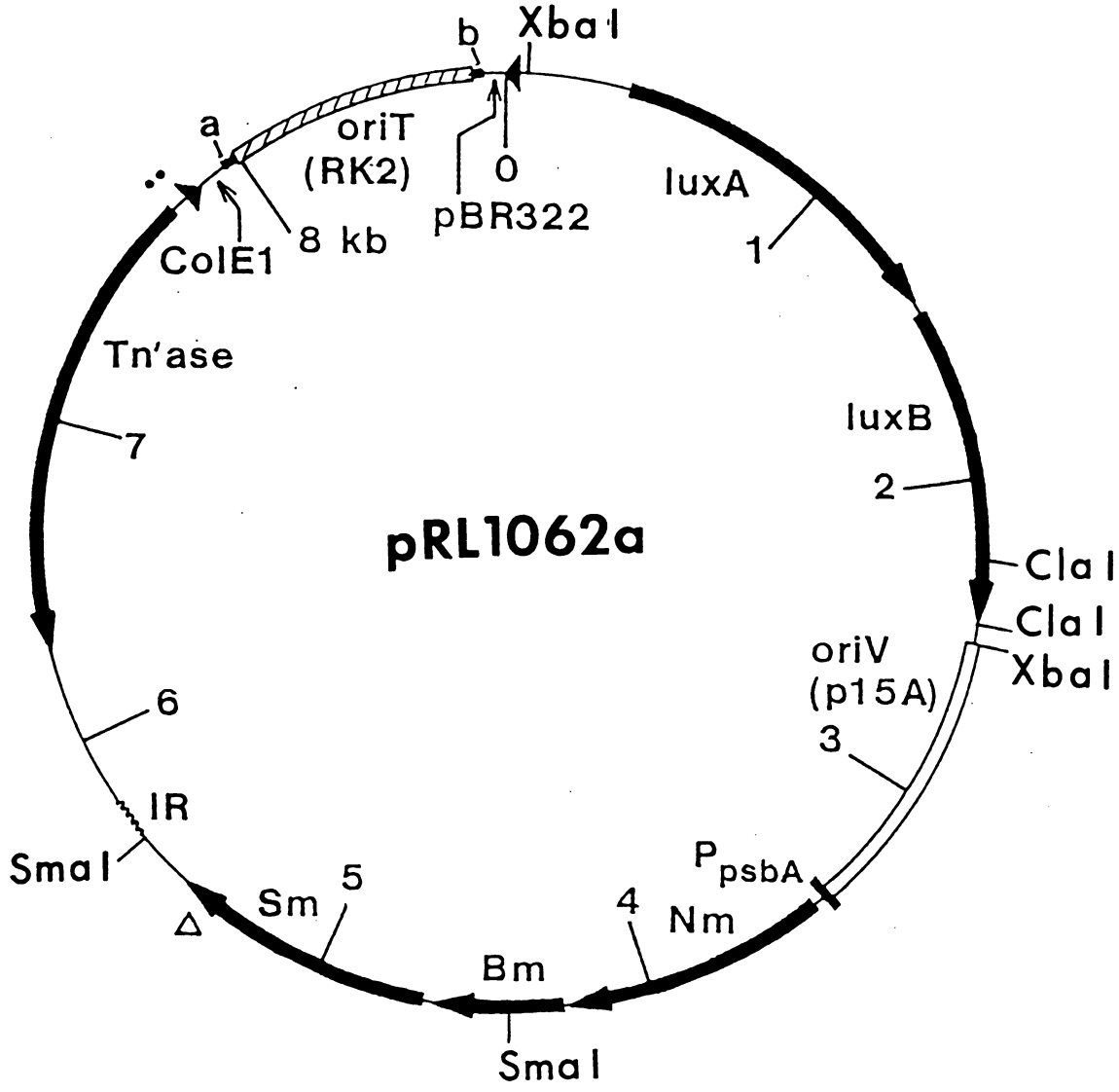

Figure 4. Map of the suicide vector, pRL1062a. pRL1062a carries the *luxAB* genes from *V. harveyi*. pRL1063a (not shown), which carries *luxAB* from *V. fischeri*, varies only in the *luxAB* region. Enzyme sites of interest are indicated.

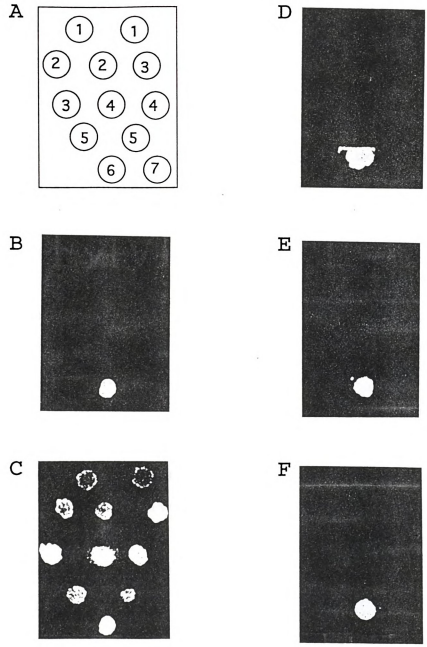

Figure 5. Stability of *V. fischeri* and *V. harveyi luxAB*. Mutants containing *luxAB* from *V. fischeri* or *V. harveyi* were compared to determine the relative stability of *luxAB*. (A) 1, 2, and 3 are *V. fischeri* mutants with putative constitutive expression; 4 and 5 are *V. fischeri* mutants which were apparently induced by a shift to 10°C; 6 and 7 are *V. harveyi* mutants which demonstrated no change in light emission upon temperature shift. Plates were incubated overnight at 30°C (B), shifted to 10°C, then placed at room temperature for 0 min (C), 15 min (D), 30 min (E), or 45 min (F).

LuxAB protein stability at low temperature as opposed to cold-induced expression of the genes. The *V. harveyi* LuxAB protein is known to be considerably more stable than the *V. fischeri* LuxAB protein (30). Indeed, the half life of the *V. fischeri* protein in Rm 1021 at 30°C must be less than 3 minutes as the light emission decreased more than 10 fold within 15 minutes of returning cold-treated cells to 30°C (Figure 5d).

If protein instability accounted for the results obtained with the *V. fischeri luxAB* transposon, then it was possible that the transposon carrying the *luxAB* gene from *V. harveyi* might alleviate the problem. Indeed, when the *V. harveyi luxAB* transposon was used, most of the inserts gave constitutive light emission at 30°C and 15°C. An example of such a mutant is shown in Figure 5 (spot 6).

Transposon tagging of cold-regulated genes.

The Tn*5* derivative carrying the *luxAB* from *V. harveyi* was used for transposon mutagenesis. Mutants were grown at 30°C, then transferred to 10°C for 5 to 7 hours. The screening of approximately 17,000 mutants resulted in the isolation of two mutants, designated Rm 5-5-6 and Rm 3-16-6, carrying a transposon insert that displayed cold regulation (Figure 6). Rm 3-16-6 and Rm 5-5-6 exhibited an approximate 100- and 20-fold induction, respectively, upon a temperature shift from 30°C to 10°C for 6 hours (Table 3). Rm C-30 was selected as a control due to its constitutive expression. Approximately 4,500 mutants were shifted from 30°C to 15°C

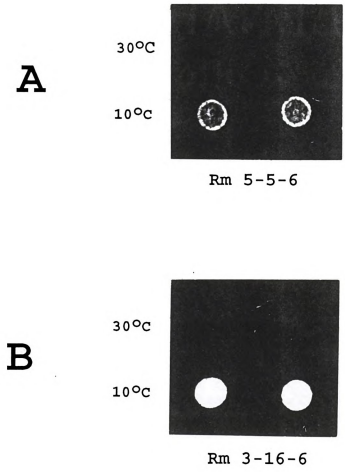

Figure 6. Light emission from cold-regulated mutants at 30°C and 10°C. Overnight cultures of Rm 5-5-6 (A) and Rm 3-16-6 (B) were spotted in replicate onto TY plates and incubated overnight. The following day plates were split in half with one half placed at 10°C and the other half returned to 30°C. After 6 hours the halves were placed together, exposed to n-decanal and screened for light emission.

Table 3. Light emission at 30°C and 10°C in mutant carrying *Tn5-luxAB* insert.

Mutant	Average Light Emitted (relative counts) 30°C	10°C	Fold Increase at 10°C
Rm 3-16-6	245 ± 74 (4)	29620 ± 9525 (4)	~ 120
Rm 5-5-6	265 ± 75 (4)	5340 ± 145 (4)	~ 20
Rm C-30	1640 ± 265 (2)	1880 ± 190 (2)	~ 1

Overnight cultures were spotted in replicate onto TY plates and incubated overnight at 30°C. Plates were split, one half was placed at 10°C, the other half placed at 30°C, then placed together after 6 hours and exposed to n-decanal. Relative light emission was measured from each culture spot. Reported relative light emission from Rm 5-5-6 and Rm 3-16-6 is the average from four replicate spots; relative light emission reported for Rm C-30 is the average of two replicate spots.

for 24 hours but screening did not yield any additional cold-regulated mutants.

Number of Tn*5-luxAB* inserts.

Southern analysis was used to determine the number of Tn*5-luxAB* insertions into the chromosome in Rm 5-5-6 and Rm 3-16-6. DNA from the mutants and wild type, Rm 1021, was digested with either *Kpn* I, which has no sites within the transposon, or *Sma* I, which cuts twice within the transposon (see Figure 4). After separation of the DNA fragments by agarose gel electrophoresis and transfer to nitrocellulose, a ^{32}P-labeled *Xba* I fragment containing *luxAB* from pRL1062a (see Figure 4) was used to probe for the Tn*5-luxAB* insertion(s). As expected for a single insert, the probe hybridized to only one band in each of the digests for each mutant, (Figure 7). No hybridization was detected with the wild type strain, Rm 1021.

Low temperature induction in TY medium.

Rm 3-16-6, Rm 5-5-6, and Rm C-30 were grown in TY liquid culture to further examine the level of low temperature induction. Light emission upon shifts from 30^{o}C to various lower temperatures was measured (Figure 8). Little to no induction was evident after a shift to 25^{o}C. Both Rm 3-16-6 and Rm 5-5-5 were induced by a shift to 20^{o}C, 15^{o}C or 10^{o}C, but the level of induction at 20^{o}C and 15^{o}C was much greater over the time course of the experiment than at 10^{o}C.

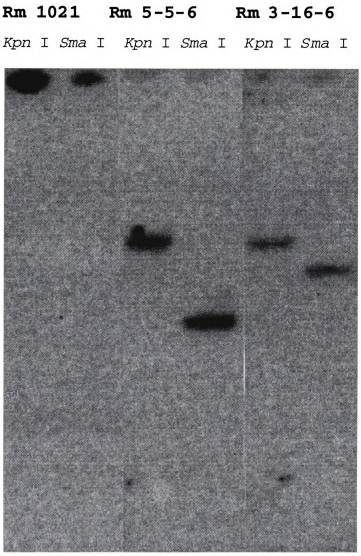

Figure 7. Detection of Tn-*luxAB* insertions in Rm 5-5-6 and Rm 3-16-6. DNA from Rm 1021, Rm 5-5-6, and Rm 3-16-6 was digested with *Kpn* I or *Sma* I and separated on an agarose gel. DNA was transferred to nitrocellulose and probed with ^{32}P-labeled *luxAB* from pRL1062a.

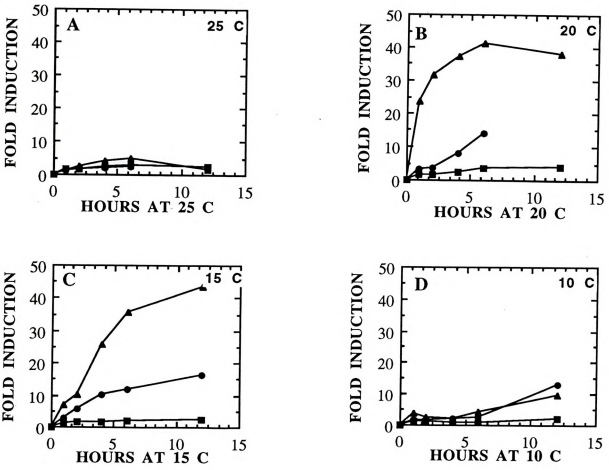

Figure 8. Induction of *luxAB* insertion in Rm 5-5-6 and Rm 3-16-6 upon shift from 30°C to lower temperatures. TY medium was inoculated from overnight cultures grown at 30°C to an $O.D._{600}$ of ~0.1, incubated at 30°C until an $O.D._{600}$ of ~0.3 was reached, then shifted to 25°C, 20°C, 15°C, or 10°C. Relative light emission was measured and adjusted for optical density. Results are the average from one experiment run in triplicate. (A) 25°C. (B) 20°C. (C) 15°C. (D) 10°C. Squares, Rm C-30. Triangles, Rm 3-16-6. Circles, Rm 5-5-6.

Expression in response to heat shock.

To determine if Rm 3-16-6 was also responsive to a temperature upshift, light emission after heat shock was examined. Rm 3-16-6 and Rm C-30 were grown at 30°C in TY medium to an optical density (O.D.) of ~0.3 then shifted to 37°C. When light emission was measured at 37°C, then adjusted for O.D., a decrease in light emission was seen (Figure 9).

Identification of cold-regulated gene.

The gene into which the Tn*5-luxAB* inserted in Rm 3-16-6 was identified by cloning and sequencing. Total DNA from Rm 3-16-6 was digested with restriction enzymes that have no sites in the transposon. The fragments were recircularized using T4 ligase and the molecules were transformed into *E. coli*. An *Apa* I digest yielded a 9 Kb clone, designated pAG1a, which was used for sequencing the *R. meliloti* genomic DNA flanking the transposon. A BLAST search of Genbank (1) indicated that the cloned gene had 99% sequence identity to *R. meliloti* 16S rRNA (accession number D12783) (Figure 10).

Southern blots used to quantitate the number of Tn*5-luxAB* insertions (see Figure 7) were used to determine the number of 16S rRNA genes present in Rm 1021. The *luxAB* probe was removed from the blot and the blot was re-probed with ^{32}P-labeled fragment of genomic DNA flanking the transposon in pAG1a. Three bands were revealed in the *Sma* I digest of Rm 1021 (Figure 11) which is consistent with the conclusions of Honeycutt, *et al.* that *R. meliloti* has three *rrn* operons

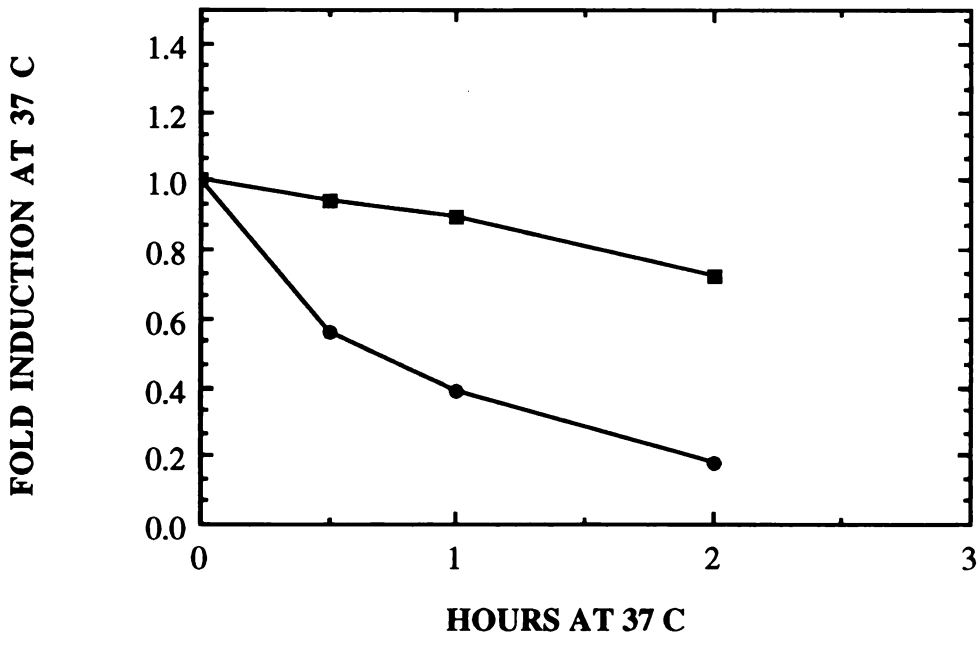

Figure 9. Fold induction after shift from 30°C to 37°C. Rm 3-16-6 and Rm C-30 overnight cultures grown at 30°C were used to inoculate TY medium to an O.D.$_{600}$ of ~0.1. After incubation at 30°C until an O.D.$_{600}$ of ~0.3 was reached, cultures were shifted to 37°C. Light emission was measured at 0, 0.5, 1, and 2 hours and adjusted for optical density. Adjusted light emission at 37°C was divided by adjusted light emission at 30°C to give relative light emission. Results shown are the average of three replicates in one experiment. Squares, Rm C-30. Circles, Rm 3-16-6.

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A:    1 GGGCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTA 56
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B:  842 GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTA 897

A:   57 CCAGCCCTTGACATCCCGATCGCGGATACGAGAGATCGTATCCTTCAGTTCGGCTG 112
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B:  898 CCAGCCCTTGACATCCCGATCGCGGATACGAGAGATCGTATCCTTCAGTTCGGCTG 953
                                                              *
A:  113 GATCGGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT 168
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B:  954 GATCGGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT 1009

A:  169 AAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTCAGTTGGGCACT 224
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B: 1010 AAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTCAGTTGGGCACT 1065

A:  225 CTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTC 280
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B: 1066 CTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTC 1121

A:  281 ATGGCCCTTACGGGCTGGGCTACACACGTGCTACA TGGTGGTGACAGTGGGCAGC 335
        |||||||||||||||||||||||||||||||||| ||||||||||||||||||||
B: 1122 ATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGC 1177

A:  336 GAGACCGCGAGGTCGACTGAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTG 391
        ||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
B: 1178 GAGACCGCGAGGTCGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTG 1233

A:  392 CAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCATGCTGCG 447
        |||||||||| ||||||||||||||||||||||||||||||||||||||||||
B: 1234 CAACTCGAGTNCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCATGCTGCG 1287
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Figure 10. Sequence comparison between Tn*5-luxAB* insertion site in Rm 3-16-6 and *R. meliloti* 16S rRNA. Regions flanking the Tn-*luxAB* insertion in Rm 3-16-6 were sequenced. A BLAST search (1) revealed 99% identity to *R. meliloti* 16S rRNA. Line A indicates sequence from pAG1a. Line B indicates sequence from *R. meliloti* 16S rRNA (accession number D12783). Asterick indicates site of Tn-*luxAB* insertion.

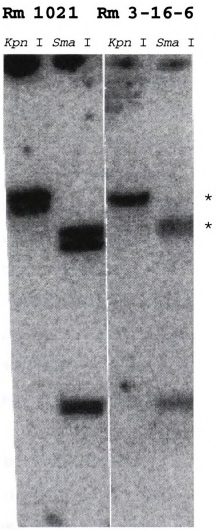

Figure 11. Detection of 16S rRNA copies. DNA from Rm 1021 and Rm 3-16-6 was digested with either *Kpn* I or *Sma* I and fragments were separated by agarose gel electrophoresis. DNA fragments were transferred to nitrocellulose and probed with ^{32}P-labeled genomic DNA from pAG1a. Astericks indicate bands which hybridized to the *luxAB* probe (see Figure 7). One of the three 16S rRNA fragments present in Rm 1021 was shifted in Rm 3-16-6, due to the Tn*5-luxAB* insertion.

(18). One of the three 16S rRNA fragments present in Rm 1021 was shifted in Rm 3-16-6, due to the Tn*5-luxAB* insertion.

rRNA operon structure in Rm 1021.

There have been no reports on the structure of rRNA operons in *R. meliloti*. It was therefore of interest to determine whether the 16S gene in Rm 1021 was followed by tRNA, 23S and 5S genes as is found in the rRNA operons of *E. coli* and other prokaryotes such as *Bacillus subtilus* and *Halobacterium thermophilus* (for review, see ref. 35). *Cla* I, which has a restriction site within *luxAB* (Figure 4), was used to clone a fragment containing 9 Kb of sequence downstream of Tn*5-luxAB*. This clone was then subcloned and sections of it were sequenced. Comparison of DNA sequence (11) indicated that the Rm 1021 16S rRNA gene was upstream of a 23S rRNA gene, separated by approximately 1.2 Kb of spacer region containing an alanine tRNA gene (Figure 12) (41). Sequence obtained from regions further downstream did not conclusively demonstrate whether there was or was not a 5S rRNA gene.

Comparison of growth rates.

Rm 3-16-6 growth rates were compared to Rm 1021 to determine whether the presumed change in the number of functional rRNA operons affected the growth rate at either high or low temperature. Since Rm 3-16-6 contained a Tn*5-luxAB* insertion into a cold-regulated gene, it was possible that growth at lower temperatures might have been compromised. No significant difference in growth rate was

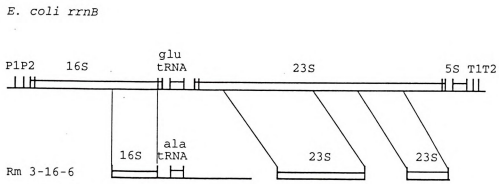

Figure 12. Sequence identity between *E. coli rrnB* and Rm 3-16-6 rRNA operons. Rm 3-16-6 fragments were cloned, subcloned and sequenced 5' and 3' of the Tn-*luxAB* insertion site. The resulting sequences were compared to *E. coli rrnB* by GenBank searches and manual searches. Rm 3-16-6 boxed regions indicate identity to *E. coli rrnB*.

seen between Rm 1021 and Rm 3-16-6 at 30°C, 25°C, 20°C, or 10°C when grown in TY medium (Figure 13). However, repetition of the experiment demonstrated a reproducible, albeit slight, difference in growth rates at 15°C.

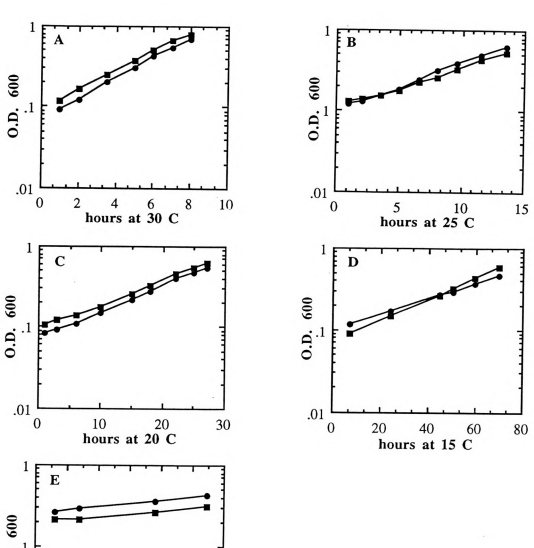

Figure 13. Steady state growth rates of Rm 1021 and Rm 3-16-6 at decreasing temperatures. Rm 1021 and Rm 3-16-6 cultures grown at 30°C were used to inoculate TY medium to an O.D.600 of ~0.1. Growth rate was determined during exponential growth. Results shown are the average from triplicate cultures in one experiment. (A), 30°C. (B), 25°C. (C), 20°C. (D), 15°C. (E), 10°C. Squares, Rm 1021. Circles, Rm 3-16-6.

DISCUSSION

Temperate strains of *R. meliloti*, such as Rm 1021, exhibit optimal growth between 28°C and 30°C. In the northern regions of the U.S., however, soil bacteria are exposed to temperature ranges from 10 to -5°C nearly half the year. How bacteria survive and adapt to low temperatures is largely unknown. To address this question, several aspects of response to low temperature were examined in Rm 1021 and two mutants containing Tn*5-luxAB* inserts in cold-regulated genes.

Growth in Rm 1021 between 15°C and 30°C is a linear function (Figure 2). At temperatures lower than 15°C or higher than 30°C, growth is restricted. In *E. coli*, an Arrhenius plot shows a linear relationship between 37°C and 21°C (17). The molecular basis for restricted growth at low temperature is not well understood. It is known that *E. coli* can not initiate protein synthesis after a shift from 37°C to 5°C (5, 9, 14). However, it is unknown whether problems in translation are responsible for restricted growth at higher temperatures.

As has been shown with other organisms, *R. meliloti* alters gene expression in response to low temperature. Previous reports have shown the presence 18-22 cold shock

proteins in *Rhizobium meliloti* A2 and other temperate rhizobia (6). Similarly, our SDS-PAGE analysis indicated 8 proteins are upregulated by a shift to a lower temperature (Figure 3). Additionally, certain proteins were downregulated. The upregulation of only eight proteins on first consideration seems like a small number but only the most abundant proteins are visible by SDS-PAGE analysis. The total number of upregulated proteins in *R. meliloti*, therefore, may be much higher.

Presumably the changes in gene expression that occur in *R. meliloti* in response to low temperature are involved in cold acclimation. The identity and functions of cold-regulated genes in Rhizobia, and their relationship to cold-regulated genes in *E. coli* are unknown. Towards an understanding of the roles of *R. meliloti* cold-regulated genes in low temperature growth and survival, we used the Tn*luxAB* transposon from *V. harveyi* to tag cold-regulated genes. In screening > 17,000 colonies, two mutants with insertions into cold-regulated genes were obtained.

Although Rm 5-5-6 was not induced as strongly as Rm 3-16-6, the overall induction patterns are similar. Both Rm 3-16-6 and Rm 5-5-6 were induced by a shift from 30^{o}C to 20, 15, or 10^{o}C, but not 25^{o}C. Interestingly, induction after a shift to 10^{o}C was weak and delayed compared to induction after a shift to 20 or 15^{o}C. The delay may be related to restricted growth at 10^{o}C (Figure 2).

The cold-regulated gene tagged in Rm 3-16-6 was identified as a 16S rRNA gene (Figure 10). This result is somewhat surprising. Indeed, upregulation of an rRNA promoter at low temperature seems counter-intuitive since the promoter of *rrn* genes are subject to growth rate control. In nutritional growth rate control, rRNA promoter activity decreases with a decrease in growth rate (16). In our studies, however, fusion of the promoter region to *luxAB* showed increased promoter activity with a decrease in growth (data not shown). Although promoter activity was not directly measured, a previous study demonstrated a lack of correlation between stable rRNA and temperature-controlled growth rate (33).

Currently, two models for growth rate control of *rrn* gene promoters exist. One is the ppGpp-dependent RNA polymerase partitioning model in which σ^{70}-RNA polymerase has two forms. *rrn* promoter strength depends on the ratio of form I, which maintains full activity, to form II, which has greatly decreased activity due to ppGpp (3). The feedback regulation model states that an excess in ribosomes increases translation which is then responsible for feedback control (7, 16, 20, 36). Control is believed to be at both the level of *rrn* transcript initiation and the elongation rate of RNA polymerase (7, 8). The partitioning model requires ppGpp to regulate synthesis. The feedback regulation model does not necessarily involve ppGpp, but proponents of the feedback regulation model agree that ppGpp may be the signal molecule

produced by excess translation (31). After temperature downshift, ppGpp levels decrease and RNA synthesis increases, suggesting ppGpp may be responsible, to some degree, for control of rRNA synthesis after cold shock (28).

Overall, comparison of growth rates revealed little difference between Rm 1021 and Rm 3-16-6 despite the interruption of an rRNA operon in Rm 3-16-6 (Figure 13). This is not surprising since previous studies in *E. coli* have shown that the addition or deletion of rRNA operons does not change the number of ribosomes present (7, 8, 20). Rm 3-16-6, with 67% functional operons, is comparable to an *E. coli* strain which has 5 out of 7, or 71%, functional operons. This *E. coli* strain demonstrated little to no change in growth rates after deletion of two of its rRNA operons (8).

Interestingly, we did see a slight difference between Rm 1021 and Rm 3-16-6 growth rates at 15°C. The difference does not appear to be due to the effects of the reporter gene, since we see induction at 20°C without a difference in growth rate. At this time, the significant of this observation is unknown.

There is a discrepancy in our reported light emission from Rm 3-16-6 and Rm 5-5-6 between solid and liquid media after 6 hours at 10°C (Figure 6 vs. Figure 8). Initially mutants were screened for changes in light emission after a temperature downshift while growing on solid medium. When light emission was measured from mutants growing in liquid medium, the measured fold induction was dramatically reduced

for both Rm 3-16-6 and Rm 5-5-6. This reduction may be due to physiological differences between bacteria growing in liquid or on solid medium. In addition, the assay conditions vary between cells grown in liquid and on solid medium. For example, aldehyde is delivered as a vapor to colonies on plates but liquid culture samples are vortexed with a solution of aldehyde dissolved in 2% BSA. Although the BSA acts as a stabilizer, the aldehyde is not easily suspended in water, thus the amount of aldehyde immediately available to the cell may be limited. Attempts to simulate solid medium conditions, either by using agar plugs or liquid culture spotted onto filter paper, did not result in a higher fold induction (not shown). It is possible that either of these methods may have interfered with light detection.

Given the fact that 1D gel analysis indicated eight cold-regulated proteins, isolation of only two mutants seems low. There are a number of possible explanations. Some cold-regulated genes may be essential for survival at low temperature. Thus, an insertion into these genes would be lethal. Additionally, changes in light emission were visually detected, therefore, I may have overlooked mutants that weren't strongly induced. In *E. coli*, for example, most cold shock proteins are only upregulated 2- to 10-fold (15, 24). It is likely that I would have missed a 2- to 5-fold induction. Perhaps the most likely reason for obtaining only two mutants was due to the time and temperature used in the screen. Induction of Rm 3-16-6 and Rm 5-5-6 at 10°C is much

slower and less pronounced than at 15°C. Indeed, K. O'Connell (unpublished data), has screened for mutants using a shift from 30°C to 15°C for 4-6 hours and has obtained 8 cold-regulated mutants in screening a total of 4,000 mutants. Whether the Tn*5-luxAB* in the mutants inserted into new cold-regulated genes remains to be determined.

FUTURE DIRECTIONS

The data presented suggest many directions for future work. Induction after a temperature downshift was examined using 30°C as the preshift temperature. It is unknown, therefore, if induction after a shift to 20°C was a function of the absolute post-shift temperature (20°C), the change in temperature (10°C), or both. Use of lower preshift temperatures, such as 25°C and 20°C, would be a first step in addressing this question.

Rm 5-5-6 and Rm 3-16-6 displayed similar patterns of induction. It is unknown, however, if the similarity is due to control by the same regulatory mechanisms. Secondary transposon mutagenesis could be used to tag the regulatory elements, followed by cloning and sequencing of the tagged gene to reveal the identity of the regulatory gene.

Studies to date have been limited to TY or GTS media. We have not characterized response to low temperature in the soil. Also, we have not evaluated the effect of insertion upon survivability in the soil. Long term soil studies need to be done.

At this time it is not known if the response to low temperature is cold-specific or a general stress response. Response to other environmental stresses has not been

characterized in either Rm 3-16-6 or Rm 5-5-6. Measuring light emission before and after other stresses, such as osmotic shock or carbon limitation, will evaluate the specificity of the cold-regulated genes.

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