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Transcriptional Organization and Regulation of a Polycistronic Cold Shock Operon in *Sinorhizobium meliloti* RM1021 Encoding Homologs of the *Escherichia coli* Major Cold Shock Gene *cspA* and Ribosomal Protein Gene *rpsU*

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A homolog of the major eubacterial cold shock gene *cspA* was identified in *Sinorhizobium meliloti* RM1021 by *luxAB* reporter transposon mutagenesis. Here we further characterize the organization and regulation of this locus. DNA sequence analysis indicated that the locus includes three open reading frames (ORFs) encoding homologs corresponding to CspA, a novel 10.6-kDa polypeptide designated ORF2, and a homolog of the *Escherichia coli* ribosomal protein S21. Transcription analysis indicated that this locus produced two different-sized *cspA*-hybridizing transcripts upon cold shock, a 400-nucleotide (nt) RNA encoding *cspA* alone and a 1,000-nt transcript encoding *cspA-ORF2-rpsU*. The sizes of the transcripts agreed with the location of the transcription start site determined by primer extension and the locations of two putative transcriptional terminators. The promoter of the *cspA-ORF2-rpsU* locus had −10 and −35 elements similar to the *E. coli* σ^{70} consensus promoter and, like the *cspA* locus of *E. coli*, included an AT-rich region upstream of the −35 hexamer. The promoter of the *S. meliloti cspA* locus was found to impart cold shock-induced mRNA accumulation. In addition, the 5′-untranslated region (5′ UTR) was found to increase the fold induction of *cspA* transcripts after cold shock and depressed the level of *luxAB* mRNA prior to cold shock, another feature similar to *cspA* regulation in *E. coli*. No "cold box" was identified upstream of the *S. meliloti cspA* gene, however, and there was no other obvious sequence identity between the *S. meliloti* 5′ UTR and that of *E. coli*. DNA hybridization analysis indicated that outside the *cspA-ORF2-rpsU* cold shock locus there are several additional *cspA*-like genes and a second *rpsU* homolog.

The cold shock response in eubacteria includes a number of adaptive changes ranging from alterations in membrane composition to changes in nucleoid structure (19, 37). Some of these changes involve the induction of cold shock genes. Indeed, a highly conserved feature of the cold shock response is the induction of one or more homologs of the major cold shock gene of *Escherichia coli*, *cspA* (25). CspA proteins are thought to act as RNA chaperones that bind to mRNAs and prevent the formation of secondary structures that prohibit their translation at low temperatures (23). Low-temperature regulation of the *E. coli cspA* gene has been shown to involve both transcriptional (18) and posttranscriptional (11, 22) mechanisms. Gene fusion studies indicate that the *E. coli cspA* promoter alone is responsive to cold shock, increasing transcription approximately sevenfold after temperature downshift (18). In addition, the 5′-untranslated region (5′ UTR) imparts dramatic temperature regulation of *cspA* mRNA accumulation which appears to result from temperature-influenced changes in mRNA secondary structure affecting mRNA stability (11, 22).

Sinorhizobium meliloti is a ubiquitous soil bacterium that forms nitrogen-fixing nodules on alfalfa and related plants (14). The ability of *S. meliloti* to form effective nodules, however, is adversely affected by a number of environmental conditions, including low temperature. This led Cloutier et al. (8) to initiate studies on the cold shock response in *S. meliloti* (and other temperate rhizobia) and also in arctic *Rhizobium* species. Their results established that rhizobia, like other bacteria, alter gene expression in response to low temperature. Beyond this, however, nothing is known about the function and regulation of cold shock genes in rhizobia. Thus, as a first step toward a better understanding of the cold shock response in *S. meliloti*, we conducted transposon mutagenesis by using a *luxAB* reporter gene to identify cold shock loci (31). Several *luxAB* reporter transposon recipients displayed higher LuxAB activity after cold shock. Two transposons were found to have been inserted near a homolog of the *E. coli cspA* gene. Here we further characterize the organization and regulation of this locus.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. *S. meliloti* strains (Table 1) were grown in tryptone-yeast extract (TY) broth medium (6) at 30°C. Cultures were maintained on solid TY medium containing 1.5% agar or frozen to −80°C in TY broth containing 10% glycerol. Cultures of *E. coli* were grown at 37°C in Luria-Bertani (LB) broth (33) and maintained on solid LB medium containing 1.5% agar. Antibiotics for *S. meliloti* were added to solid medium at the following concentrations: streptomycin (SM), 200 μg/ml (50 μg/ml in broth); spectinomycin (SP), 50 μg/ml; gentamicin (GM), 25 μg/ml; kanamycin (KM), 200 μg/ml (50 μg/ml in broth). KM and ampicillin (AP) were added to media for *E. coli* at 50 μg/ml. Tetracycline (TC) was added to media at a concentration of 10 μg/ml.

DNA cloning, sequencing, and hybridization analysis. Cloned DNA was prepared for sequencing from cells of *E. coli* by using Qiagen Maxi-Columns and cut with restriction enzymes according to the manufacturer's instructions (New England Biolabs). Manual double-stranded DNA sequencing reactions were performed with the Sequenase 2.0 Kit (Amersham) and [^{35}S]dATP. Oligonucleotide primers were synthesized by the Macromolecular Synthesis Facility, Michigan State University. Automated cycle sequencing reactions (fluorescent dye-terminator) were performed at the Michigan State University DNA Sequencing Fa-

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
Sinorhizobium meliloti		
RM1021	Wild type; Str^r	F. de Bruijn
RM509	RM1021 containing Tn*5*-1062 between *cspA* and ORF2	31
RM11	RM1021; ORF2::Tn*5*-1062	31
RM37	Ino^-; RM1021 with *cspA* promoter-*luxAB* fusion replacing part of the *ino* locus	This study
RM39	Ino^-; RM1021 with *cspA* promoter-UTR-*luxAB* fusion replacing part of the *ino* locus	This study
Escherichia coli DH5α	*supE44 hsdR17 recA1 thi-1 lacU169*(ϕ80*lacZ*M15) *endA1 gyrA96 relA1*	20
Plasmids		
pBluescript KS(−) Ap^r		Stratagene
pMW193	Tc^r SP-Str^r; pRK290-based vector for replacing the *ino* locus of RM1021 with promoter-reporter gene fusions	7
pRK2013	Km^r Tra^+; *mob*; helper plasmid for triparental matings	13
pJB251	Gm^r; incompatible with pRK290-based vectors	7
pRL1062a	Tc^r Km^r; vector for Tn*5*-1062 mutagenesis and source of 2.4-kb *Xba*I *luxAB* fragment	9, 41
pH2	Km^r; template for amplification of *cspA* promoter and coding region	31
pEco1	Km^r; template for amplification of *rpsU*	31
pJJ23	Km^r; template for amplification of *ORF2*	31
pKO11	2.4-kb *Xba*I fragment with promoterless *luxAB* genes in pBluescript KS(−)	This study
pKO23	*cspA* promoter (−188 to +1) on *Sac*I-*Not*I fragment in pKO11	This study
pKO26	*cspA* promoter and 5′ UTR (−188 to +110) on *Sac*I-*Not*I fragment in pKO11	This study
pKO37	*cspA* promoter-*luxAB* fusion from pKO23 cloned into *Kpn*I site of pMW193	This study
pKO39	*cspA* promoter-UTR-*luxAB* fusion from pKO26 cloned into *Kpn*I site of pMW193	This study

cility by using an ABI Catalyst 8000 Molecular Workstation (Applied Biosystems, Inc.). Additional sequencing and primer synthesis were performed at the Biotechnology Resource Laboratory, Yale University. Total genomic DNA was isolated from *S. meliloti* essentially as described earlier (3), omitting the NaCl-CTAB (cetyltrimethylammonium bromide) extraction step. Probe DNA was amplified from *cspA*, *ORF2*, and *rpsU* sequences by standard methods (21) by using the templates and primers listed in Table 1. Labelling of probe DNA with [α-^{32}P]dCTP, agarose gel electrophoresis, Southern blotting, and autoradiography were performed as described elsewhere (33).

Construction and integration of promoter-*luxAB* fusions. Promoterless *luxAB* genes were excised from pRL1062 on a 2.4-kb *Xba*I fragment and cloned into the *Xba*I site of pBluescript KS(−), generating plasmid pKO11. Fragments of DNA encoding the *cspA* promoter alone (−188 to +1) or the *cspA* promoter and 5′ UTR (+188 to +110) were amplified by standard methods (21) by using plasmid pH2 as template and the primers listed in Table 1. The promoter fragments (including linkers added during primer synthesis) were cut with *Sac*I and *Not*I and ligated into the *Sac*I and *Not*I sites of pKO11 to generate plasmids pKO23 and pKO26. The $_p$*cspA-luxAB* fusion (in pKO23) and the $_p$*cspA*-UTR-*luxAB* fusion (in pKO26) were then excised with *Kpn*I and ligated into the unique *Kpn*I site of pMW193, creating plasmids pKO37 and pKO39, respectively. Plasmid pMW193 is designed to allow replacement of the inositol utilization locus (*ino*) with cloned novel DNA by homologous recombination, while isolating the integrated DNA from transcriptional activity in the *S. meliloti* chromosome (7).

To integrate the promoter-reporter fusions into the *ino* locus in RM1021, plasmids pKO37 and pKO39 were mobilized separately into *S. meliloti* RM1021 from *E. coli* DH5α by triparental mating. Plasmid pJB251 (Gm^r), which is incompatible with pKO37 and pKO39, was then introduced, and transconjugants were selected for Tc^r, Sp^r, and Gm^r. The resulting strains, containing single recombinations between *ino* DNA on pKO37 or pKO39 and the chromosomal *ino* locus, were cultured in TY medium containing GM and SP but not TC. In subsequent screening we obtained isolates that were Gm^r and Sp^r but Tc^s and lacked the ability to use inositol as a sole carbon source. Replacement of chromosomal *ino* DNA with the promoter-reporter fusions, giving rise to strains RM37 and RM39, was confirmed by hybridization (not shown).

RNA isolation and analysis. Total RNA was isolated from bacteria essentially as described earlier (3), except that rifamicin (150 μg/ml) was added to the bacteria immediately upon harvesting to prevent further transcription. RNA was precipitated with 2 volumes of cold 100% ethanol, washed twice with 70% ethanol, dried, resuspended in water, and quantified spectrophotometrically. Northern and slot blot hybridization experiments were performed as described elsewhere (3, 33). The intensity of radioactive bands on slot blot filters was quantified with a Molecular Dynamics PhosphorImager and analyzed with ImageQuant 3.3 software. The fold induction of mRNAs was calculated by using the 0-h (30°C, pre-cold shock) level as the reference level. Primer extension experiments were carried out as described previously (33) with enzymes supplied with the Primer Extension System (Promega) and with 10 μg of *S. meliloti* RNA per reaction. The sequences of the oligonucleotide primers are given in Table 2.

Sequence analysis and accession number. Sequence data were compared to known nucleotide and protein sequences by using the BLAST e-mail server (National Center for Biotechnology Information, Bethesda, Md. [1]). Additional analyses were performed by using the GCG (10) and DNASTAR sequence analysis packages. Potential open reading frames (ORFs) were identified, and codon usage was assessed with CodonUse 3.1 software (window size, 33; logarithmic range, 3), written by Conrad Halling, University of Chicago. The sequence reported here has been assigned GenBank accession number AF030523.

RESULTS

Sequence analysis of the *cspA* cold shock locus of *S. meliloti*. The transposon insertions in two of these mutants, RM509 and RM11, were determined to be located downstream of a homolog of *cspA*. DNA sequencing of this region (Fig. 1) (GenBank accession number AF030523) revealed the presence of three tandem ORFs with codon usage typical for *S. meliloti* proteins. The 5′ ORF encodes a polypeptide of 69 amino acids with a predicted molecular size of 7.4 kDa and an isoelectric point of 8.6. A BLAST search of the GenBank-EMBL database revealed significant similarity between the 5′ ORF and the major cold shock protein of *E. coli*, CspA (Fig. 2A) (25). The best match, however, was with a putative CspA homolog encoded by an ORF on the recently sequenced symbiotic plasmid of *Rhizobium* sp. strain NGR234 (Fig. 2B) (16). The predicted *S. meliloti* CspA protein contains the consensus cold shock domain amino acids conserved among CspA homologs, the eukaryotic Y-box-binding proteins and certain glycine-rich proteins of *Arabidopsis thaliana* (40). Based on sequence similarity and induction by cold shock, we designated the 5′ ORF as the *cspA* gene of *S. meliloti*.

Just downstream of *cspA* was a second ORF that was preceded by a consensus ribosome binding site (Fig. 1A). The ORF was deduced to encode a polypeptide of 95 amino acids

TABLE 2. Oligonucleotides

Oligonucleotide	Sequence	Function and features
MT171	5′-CCGGAATTCAAACGCTCCCTGCCAGTACATCCG-3′	Amplification of *cspA* DNA; *Eco*RI
MT172	5′-CCGGAATTCCGTCCGGGAACTCTAGCGATTGAA-3′	Amplification of *cspA* DNA; *Eco*RI
MT173	5′-CCGGAATTCGACCAGGCTCTTCGCGTTCTCAAG-3′	Amplification of *rpsU* DNA; *Eco*RI
MT174	5′-CCGGAATTCCTACAGCGGCAGACGTGCAAAGA-3′	Amplification of *rpsU* DNA; *Eco*RI
MT176	5′-ATTTCGGCGTCCTGATGCTCAAGG-3′	Amplification of *ORF2* DNA
MT177	5′-GGGTGGCATGGACGAAGGAGATGG-3′	Amplification of *ORF2* DNA
MT183	5′-CAATCGCTAGAGTTCCCG-3′	Primer extension
MT184	5′-TGAATGAAGCCGAAGCCC-3′	Primer extension
MT169	5′-TTTGAGCTCGGTACCAAGCCGGAAGAACCTCAGCTCGTCC-3′	5′ primer for promoter amplification; *Sac*I and *Kpn*I sites
MT170	5′-TTTGCGGCCGCTTCAATCGCTAGAGTTCCCGGACG-3′	3′ primer for amplifying bases −188 to +110; *Not*I site
MT208	5′-TTTGCGGCCGCTGAGCTATATATAAACGCTGCTTCGAGAT-3′	3′ primer for amplifying bases −188 to +1; *Not*I site

having a molecular size of 10.6 kDa and an isoelectric point of 7.4 (Fig. 1). The predicted polypeptide displayed no significant similarities with previously described polypeptides, nor did it contain sequences similar to known functional motifs. The putative cold shock gene encoding the novel polypeptide was designated *ORF2*.

A third ORF located just downstream of *ORF2* (Fig. 1) was deduced to encode a polypeptide of 78 amino acids with a predicted molecular size of 9.3 kDa and an isoelectric point of 11.6. The predicted polypeptide was similar to ribosomal protein S21 from several species of bacteria, including that encoded by *E. coli rpsU* (Fig. 3). Thus, the ORF was designated *rpsU*. Translation of the *S. meliloti rpsU* homolog presumably begins at the unusual initiation codon TTG since the first in-frame ATG codon is downstream of several codons that specify amino acids that are conserved among S21 proteins (Fig. 1A). The use of TTG as an initiation codon has been documented in other bacteria, including members of the *Rhizobiaceae* (2). Also consistent with the TTG serving as the initiation codon is that immediately upstream of it there is a canonical ribosome-binding site.

Sequencing of the region upstream of *cspA* revealed the presence of a divergently oriented ORF that encodes a protein similar to AfuA, a putative iron-binding transport protein (39). The sequence of the entire ORF was determined (GenBank accession number AF030523) but was not studied further.

Transcription of the *cspA* cold shock locus. An inspection of the region upstream of the *cspA* gene indicated that there was a sequence resembling an *E. coli* σ^{70} consensus promoter and an AT-rich region similar to that found in the *cspA* promoter of *E. coli* (36). In addition, analysis of the entire *cspA* locus by using the TERMINATOR (10) program revealed two putative rho-independent termination signals, T1 and T2, downstream of *cspA* (Fig. 1). T1 and T2 were located, respectively, just upstream of *ORF2* and just downstream of *rpsU*. The locations of the putative promoter and terminator sequences suggested that *S. meliloti* might produce two cold shock-inducible *cspA* mRNAs, one approximately 400 nucleotides (nt) long encoding *cspA* alone and another one approximately 1,000 nt long encoding *cspA*, *ORF2*, and *rspU* (Fig. 1B). To determine whether this was the case, total RNA was isolated from strain RM1021 before and after a temperature downshift from 30 to 15°C, and Northern blots were prepared and hybridized with probes for *cspA*, *ORF2*, and *rpsU*. The results indicated that *cspA*-hybridizing transcripts of approximately 400 and 1,000 nt did indeed accumulate upon a cold shock of 1 h (Fig. 4). Moreover, the results indicated that the 1,000-nt transcripts hybridized with the *ORF2* and *rpsU* gene probes but that the 400-nt transcripts did not. Thus, it appears that the *cspA*, *ORF2* and *rspU* genes are organized into an operon that produces transcripts of two different lengths as diagrammed in Fig. 1B.

The results presented in Fig. 4 indicate that expression of the *cspA* operon was transient. At 1 h after temperature downshift, the 400- and 1,000-nt transcripts were approximately 20- and 125-fold, respectively, higher in abundance than before the cold shock (Fig. 4, left panel [phosphorimager quantification not shown]). By 3 h, however, the 1,000-nt transcripts had returned to preshock levels. At this time transcripts of 400 nt were present a low levels that hybridized with *cspA*. In addition, transcripts of 400 nt were present that hybridized with the *ORF2* and *rpsU* probes. Presumably, the faint hybridization observed with the ORF2 probe was due to degradation products generated from the 1,000-nt transcript. This could also be the case with the *rpsU* probes. Alternatively, this species (~400 nt, right panel) may represent the cold shock induction of another *rpsU*-like gene (see below).

Identification of the *cspA* promoter. We located the 5′ end of the *cspA* mRNAs and confirmed the cold shock induction of *cspA* mRNA by primer extension analysis (Fig. 5). Cold shock induced the accumulation of mRNA that hybridized to oligomer MT183, which is complementary to sequences 5′ to the *cspA* ORF (Table 2). In this experiment the increase in *cspA* mRNA after cold shock was more gradual than in the Northern blot experiments. While there was a significant increase in *cspA* mRNA, the intensity of the signal increased to a higher steady-state level without a large transient increase. We believe this pattern of increased *cspA* expression was likely due to the use of a larger volume of culture for RNA isolation in this experiment (680- versus 220-ml cultures used to harvest RNA for Northern blots), resulting in a more gradual cooling of the cells and therefore a more gradual accumulation of *cspA* mRNA. The 5′ end of *cspA* mRNAs terminate at one of two adjacent bases, 120 and 121 nucleotides 5′ to the beginning of the *cspA* start codon (Fig. 5). As previously mentioned, upstream of the start of transcription is a sequence resembling an *E. coli* σ^{70} consensus promoter; there are several overlapping TATA boxes ca. 10 bp upstream of +1, preceded by a hexamer that resembles the *E. coli* σ^{70} −35 consensus sequence. The distances between the 5′ end of the *cspA* transcripts and the two predicted terminators agree well with the sizes of the 400- and 1,000-nt transcripts determined by Northern hybridization, providing additional support for the proposed operon structure. Primer extension experiments with oligonucleotide

A

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GTTTGCCCTCGGTCGGAGAAAGATCGCGCCGAGGGATAGAGCGGGTGTTTTACAGGGATGTGACAGCGAAAAGCCGGAAGAACCTCAGCTCGTCCGCCGG  100

CCGGAGCATTCCCCCGAATGTTTTCGAACGATCCCTCGAGAATATTATTTGCGATTCAAATCAGCGGCTTGATCGTATTTTCCGGACGGGGTGAGGCCCT  200
<--------afuA
                        -35                          -10     +1---------->
CGCACAATTAAATTCGGTTCATCCATTGCATATCTCGAAGCAGCGTTTATATATAGCTCATCGAAACTTGCTTGTGACCTTTGTCTACGCGCCGAAGCGC  300
                                                                          SD         cspA----->
TTCGTCAGATTTCCTCTCAATATCGATCATACCGGACTGATATTCGTCCGGGAACTCTAGCGATTGAAAGGAAATCGTTATGAACTCAGGCACCGTAAAG  400
                                                                                  M  N  S  G  T  V  K
TGGTTCAACAGCACCAAGGGCTTCGGCTTCATTCAGCCCGACGATGGCGCTACGGACGTATTCGTGCACGCCTCCGCCGTTGAACGCGCTGGAATGCGTT  500
W  F  N  S  T  K  G  F  G  F  I  Q  P  D  D  G  A  T  D  V  F  V  H  A  S  A  V  E  R  A  G  M  R  S
CGCTCGTAGAAGGCCAGAAGGTTACCTACGACATCGTCCGCGACACCAAGTCCGGCAAGAGCTCGGCAGACAATCTGCGGGCCGCATAAATTCGTCATTC  600
  L  V  E  G  Q  K  V  T  Y  D  I  V  R  D  T  K  S  G  K  S  S  A  D  N  L  R  A  A  *
                                                  ↓ RM509
GCCTCCGCTGACCACGGATGTACTGGCAGGGAGCGTTTGAATGACGGGGGCGCCCTAGCGCGTCTGAAAAGACGCGCGGCGCTCAAGAGGAGGTCGGGGT  700
                                                                                    T1: >>>>>>>>
                                                  SD        ORF2----->
AACGCCCGGCCTTTTTTGCTTGTGCCGCCCTCCGGGTGGCATGGACGAAGGAGATGGCCATGGGCCGGAACACCTACAGCGTTGGCGACAGTGTCGTCCT  800
    <<<<<<<<                                      M  G  R  N  T  Y  S  V  G  D  S  V  V  L
                                  ↓ RM11
GAGAGCCGATCTCACGCGCACGGCTGCTGCGGACCGGACCTGCCGCATCGTGTCGATCCTGCCCGCGGCCGAACATGGGGAAGCGCAGTATCGGGTGCGG  900
 R  A  D  L  T  R  T  A  A  A  D  R  T  C  R  I  V  S  I  L  P  A  A  E  H  G  E  A  Q  Y  R  V  R
                                     ClaI
TTCGGAACGGAGAACTTCGAGCGGCGCATCTTCGAACACGACATCGATCCTTCGGAAACGGCTTCGCCGGTTCAGGAACATGGCCAGGCGGCATCGCTCG 1000
F  G  T  E  N  F  E  R  R  I  F  E  H  D  I  D  P  S  E  T  A  S  P  V  Q  E  H  G  Q  A  A  S  L  D
                                                                                SD        rpsU2--->
ACGGTAAGCCCTGGTTCAAACCCTTGAGCATCAGGACGCCGAAATAAAGAACCCAATCAGCAATCCCATAAATACGGAAAGGATCTACTTTGCAGGTACT 1100
  G  K  P  W  F  K  P  L  S  I  R  T  P  K  *                                    L  Q  V  L
CGTCAGGGACAACAACATCGACCAGGCTCTTCGCGTTCTCAAGAAGAAGATGCAGCGTGAAGGCGTCTTCCGCGAAATGAAGATGCGCAGCGCCTACGAA 1200
 V  R  D  N  N  I  D  Q  A  L  R  V  L  K  K  K  M  Q  R  E  G  V  F  R  E  M  K  M  R  S  A  Y  E
AAGCCGTCCGAAAAGCGCGTCCGCGAAAAGGCCGAAGCTGTCCGCCGCACCCGCAAGCTGGCGCGCAAGAAGCTTCAGCGAGAAGGCCTGCTGCCGTCGC 1300
K  P  S  E  K  R  V  R  E  K  A  E  A  V  R  R  T  R  K  L  A  R  K  K  L  Q  R  E  G  L  L  P  S  P
CGAAGAAAGTCGCCCGCGCGCGCTGACGCGCCGCTCCGGCTGCACAACAGCTGCGGCGATCTTTGCACGTCTGCCGCTGTAGGCTGGTACCACTTTGCGA 1400
  K  K  V  A  R  A  R  *                                                        T2: >>>
CGCGCCCTTGCGCGTCGTTTTCATGCGTGAAGTCGCTCTGTCCCCTCTGCGGCGTTTCCTTCGACACTGCGATCCCTTTTCCTGCGCTG             1489
>>>>>    <<<<<<<<
```

B

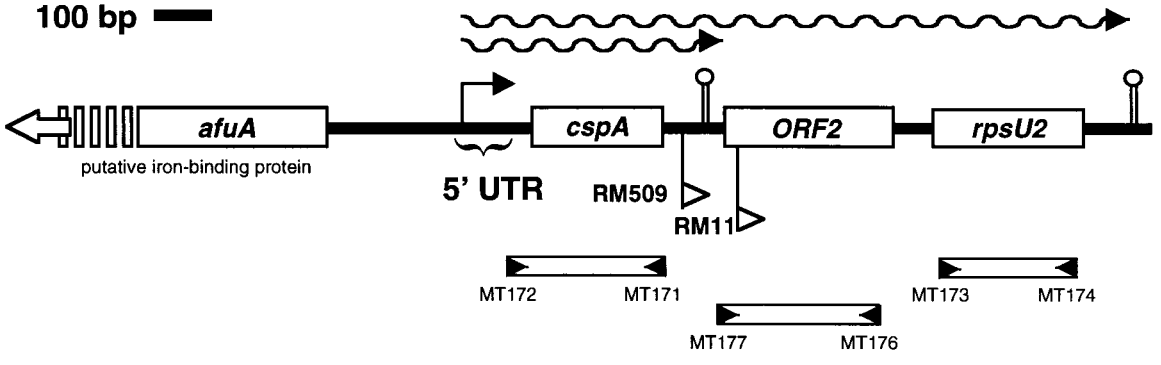

FIG. 1. Cold shock operon in *S. meliloti* RM1021. (A) Nucleotide sequence of the cold shock operon (bases 1001 to 2489 of accession number AF030523). The deduced amino acid sequences and putative gene identifications are given underneath the nucleotide sequence for ORFs which display codon usage typical of *S. meliloti*. Promoter sequences are printed in boldface and are underlined, and the location of the transcriptional start sites as determined by primer extension are double-underlined. Putative ribosome-binding sites (SD) are underlined. Rho-independent transcriptional terminators (T1 and T2) are indicated by chevrons. Inverted arrows indicate the location of transposon Tn*5*-1062 insertions in mutants RM11 and RM509. (B) Diagram of cold shock gene region in *S. meliloti*. The bent arrow indicates the transcriptional start site as determined by primer extension. Flags below the map indicate the location of Tn*5*-1062 insertions and the direction of transcription of the *luxAB* reporter genes. Bars below the map represent the amplified fragments used as probes in Southern and Northern blot experiments. Small arrows in the bars represent the oligonucleotide primers listed in Table 2. The complement of the start codon of the putative *afuA* homolog (bases 107 to 109) is underlined, and the direction of transcription (divergent from *cspA*) is indicated by the arrow. Part of the *afuA* sequence is omitted for brevity; however, it is included in GenBank in its entirety.

MT184, which binds inside the *cspA* ORF, gave an identical location for the 5′ end of the *cspA* mRNA (data not shown). Upstream of the −35 hexamer there is an AT-rich region that may serve as a UP element (32). The region surrounding +1 includes the sequence GCTCATCG, which is similar to the GCACATCA sequence that is found in the cold shock-stimulated P1 promoter of phage lambda (17). No CCAAT box was found in the entire region sequenced. We also did not find a region similar to the "cold box," an 11-bp motif that is conserved in the 5′ UTR of *cspA*, *cspB*, and *csdA* of *E. coli* and is proposed to play a negative regulatory role in the transcription of *cspA* (12).

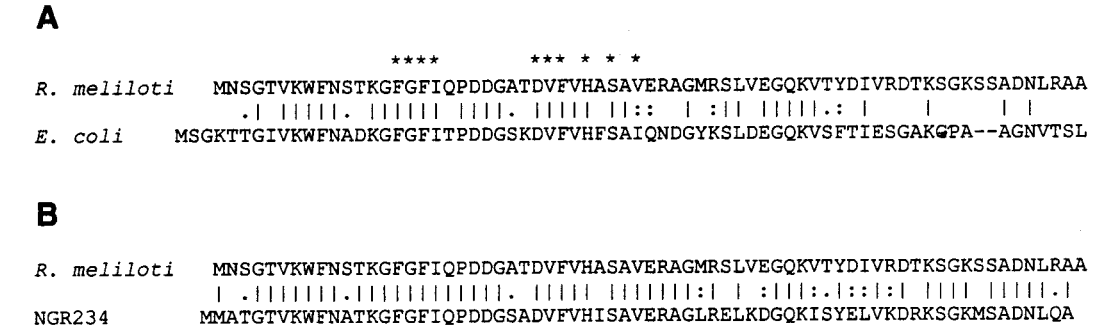

FIG. 2. Similarities between the predicted CspA amino acid sequence from *S. meliloti* and CspA sequences from other bacteria by using the BESTFIT program (10). Asterisks indicate the conserved cold shock domain residues, as listed in PROSITE (5): [FY]-GFI-X(6,7)-[DER]-[LIVM]-FXHX-[STK]-X-[LIVMFY]. (A) Comparison with the CspA protein from *E. coli*. (B) Comparison with the closest known match, a predicted cold shock protein from the symbiotic plasmid of *Rhizobium* sp. strain NGR234 (16).

Regulation of the *cspA* operon. Two reporter gene constructs were made to determine whether the *cspA* promoter and/or the 5′ UTR were involved in regulating the accumulation of the *cspA* transcripts in response to cold shock. One construct, pKO23, had the promoter region of *cspA*—nt −188 to +1—fused to the *luxAB* reporter genes (Fig. 6A). The other, pKO26, had both the promoter region and most of the 5′ UTR of *cspA*—nt −188 to +110—fused to the *luxAB* reporter gene (Fig. 6A). To avoid potential high background luminescence caused by multiple copies of the fusions in plasmid vectors, the constructs were cloned into the *Kpn*I site of pMW193, a vector designed to allow the integration of single copies of genes into the chromosome of *S. meliloti*. The fusions were then recombined into the inositol utilization (*ino*) locus (Fig. 6B), generating strains RM37 and RM39. Insertion of the promoter fusions into the *ino* locus was verified by Southern hybridization analysis (not shown).

The levels of *luxAB* mRNA in strains RM37 (*cspA* promoter alone fused to *luxAB*) and RM39 (*cspA* promoter and 5′ UTR fused to *luxAB*) before and after a temperature downshift from 30 to 15°C were determined by slot blot hybridization (Fig. 7). Strain RM509, which contains a *luxAB* reporter transposon (Tn*5*-1062) insertion between *cspA* and terminator T1 (Fig. 1) was used as a reference strain. In cells grown at 30°C, the level of *luxAB* mRNA in strain RM37 was approximately sixfold higher than in strain RM39 (Fig. 7). Therefore, the presence of the 5′ UTR upstream of *luxAB* in strain RM39 resulted in a lower steady-state level of *luxAB* mRNA prior to cold shock. The pre-cold shock level of *luxAB* mRNA in strain RM509, in which the intact 5′ UTR and the entire *cspA* ORF are present upstream of *luxAB* on the mRNA molecule (Fig. 1B) (30) was comparable to the pre-cold shock *luxAB* mRNA level in strain RM39.

Upon cold shock (2 to 4 h), the level of *luxAB* mRNA increased approximately sevenfold in strain RM37 (Fig. 7), suggesting that part of the cold shock accumulation of *cspA* mRNA is due to an increase in promoter activity. At this same time, the level of *luxAB* mRNA in strain RM39 increased approximately 25-fold and, in absolute levels, approached that attained in RM37. Thus, the negative effect on transcript accumulation caused by the 5′ UTR of the *cspA* operon appeared to be relieved by cold shock. Upon cold shock, the level of *luxAB* mRNA in RM509 was induced nearly 50-fold and was about the same as that observed in RM37.

Other sequences similar to *cspA* and *rpsU* are present in *S. meliloti*. Many species of bacteria contain several genes homologous to *cspA*. *E. coli* contains nine copies of *cspA*-like genes, only some of which are induced by cold shock (26, 29, 42). To determine whether *S. meliloti* also contains multiple homologs of *cspA*, the amplified *cspA* DNA used as probe in the Northern blot experiments was hybridized to genomic DNA of *S. meliloti* digested with *Cla*I, *Xho*I, or *Eco*RI. Even under stringent conditions (0.05× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), multiple sequences that hybridize to the amplified *S. meliloti cspA* were detected (Fig. 8, top panels). Washing under less-stringent conditions (0.2× to 6× SSC) revealed even more sequences that hybridized with the *cspA* probe, suggesting that *cspA* is part of a multigene family in *S. meliloti* with perhaps as many as five members. Similar experiments with the amplified *ORF2* sequence as a probe indicated that only one sequence hybridized strongly to the ORF2 probe under the least stringent condition (6× SSC), suggesting that a single copy of ORF2 is present in *S. meliloti* (Fig. 8, center panels).

Duplicate Southern blots of *S. meliloti* genomic DNA were also probed with a DNA fragment amplified from the *rpsU* homolog (Fig. 8, bottom panels). The Southern blot washed at high stringency (0.05× SSC) showed the cloned *rpsU* homolog and another faintly hybridizing signal, based on the sizes of the restriction fragments. Washing identical blots under less-stringent conditions allowed stronger hybridization of the second sequence to the *rpsU* probe. As mentioned above, we have not

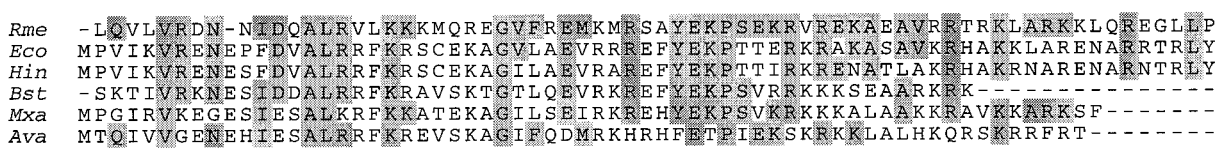

FIG. 3. Comparison of the predicted ribosomal protein S21 amino acid sequence from *S. meliloti* with S21 proteins from other prokaryotes made by using the BESTFIT program (10). Amino acid residues identical to the predicted *S. meliloti* protein are shaded. The last nine residues of the *S. meliloti* S21 sequence (Fig. 1) are omitted for brevity and display no matches to the other sequences. Rme, *S. meliloti*; Eco, *E. coli*; Hin, *Haemophilus influenzae*; Bst, *Bacillus stearothermophilus*; Mxa, *Myxococcus xanthus*; Ava, *Anabaena variabilis*.

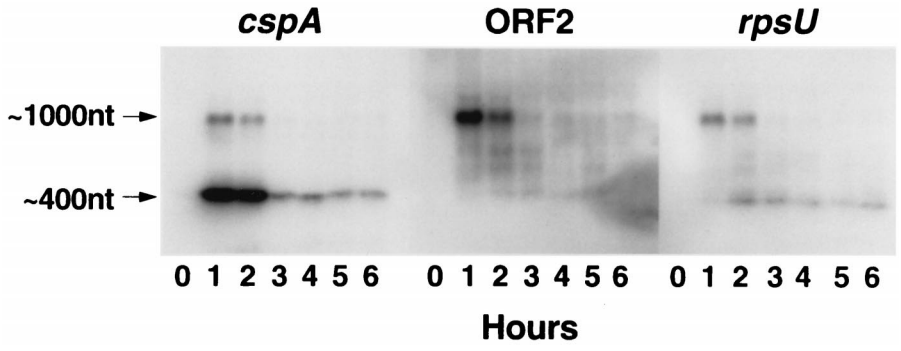

FIG. 4. Northern blot analysis of *S. meliloti* RM1021 total RNA isolated before and at 1-h intervals after a cold shock from 30 to 15°C. The filters were probed, from left to right, with the amplified *cspA*, *ORF2*, and *rpsU* genes shown in Fig. 1B.

ruled out the possibility that this other *rpsU*-like gene may be the source of the smaller *rpsU*-like transcript observed after cold shock (Fig. 4, right panel). Because both genes are visible under the most stringent wash condition (0.05× SSC), it is possible that the amplified *rpsU* DNA might have hybridized to transcripts of both *rpsU*-like genes under our standard Northern blot wash conditions (0.25× SSC).

DISCUSSION

We have identified a cold shock operon in *S. meliloti* that encodes a homolog of the *E. coli* CspA major cold shock protein, a small novel polypeptide (ORF2), and a homolog of *E. coli* ribosomal protein S21. To our knowledge, this is the first example of a *cspA* gene being organized into an operon with other cold shock genes. The significance of this grouping of genes is uncertain. The role of the *S. meliloti* CspA protein in cold shock is presumably the same as that proposed for other CspA proteins: to act as an RNA chaperone that aids in the translation of mRNAs at low temperature. The roles of the other two genes in the cold shock response, however, are less clear. Indeed, the ORF2 polypeptide has no sequence homology with any previously described protein or functional motif, and a transposon insertion mutation within the ORF2 coding sequence does not have any obvious effect on the growth of cells upon shift to low temperature (31). Thus, at this point, all that can be concluded is that the *ORF2* gene appears to be a novel single-copy gene that is transiently induced upon cold shock but does not have an essential role in low temperature growth.

As for the *rpsU* gene, in vitro studies of *E. coli* ribosome function suggest that the *rpsU*-encoded ribosomal protein S21 is required for initiation of translation (38); it possibly facilitates the binding of mRNAs to the ribosome (4) or stimulates the association of ribosomal subunits (27). Thus, increased synthesis of S21-like proteins in *S. meliloti* might function to alleviate an inhibition of translation initiation caused by low temperatures, as other known cold shock proteins, such as RbfA, are believed to do (24). Interestingly, a homolog of *rpsU* in *Anabaena variabilis* has also been shown to be induced by cold shock (34, 35), suggesting that the induction of *rpsU* homologs by cold shock is an important adaptive response in some bacteria. Whether this is the case in *S. meliloti* remains to be determined. Mutants RM509 and RM11, which have transposon insertions that are likely to exert polar effects and block the expression of the *rpsU* gene, do not display any obvious cold-sensitive phenotype when grown at 15°C (31). These results suggest that the *rpsU* gene is not required for cold shock adaptation. However, our Southern analysis indicates that *S. meliloti* has a second *rpsU* homolog (Fig. 8). Expression of this gene could mask a role of the cold shock-inducible *rpsU* gene in cold shock adaptation under the conditions tested. Of course, it is also possible that protein S21 is not required for ribosomal function under some conditions; at least one prokaryote, *Mycoplasma genitalium*, lacks this gene entirely (15).

Our results indicate that there are a number of similarities in the regulation of the *S. meliloti cspA* cold shock operon and the *E. coli cspA* cold shock gene. The promoter regions of the *cspA-ORF2-rpsU* operon and the *E. coli cspA* gene both have −10 and −35 elements similar to the *E. coli* σ^{70} consensus promoter and have AT-rich regions upstream of the −35 hexamer (this study; see also reference 36). One study found that the presence of the AT-rich region upstream of the *cspA* gene of *E. coli* is required for the expression of *cspA* (28); other

FIG. 5. Primer extension mapping of the 5′ end of *S. meliloti cspA* mRNA. Total RNA from RM1021 cells was isolated before and at several 1-h intervals after a cold shock from 30 to 15°C and then hybridized to oligonucleotide MT183. Arrows indicate the 5′ ends of *cspA* transcripts.

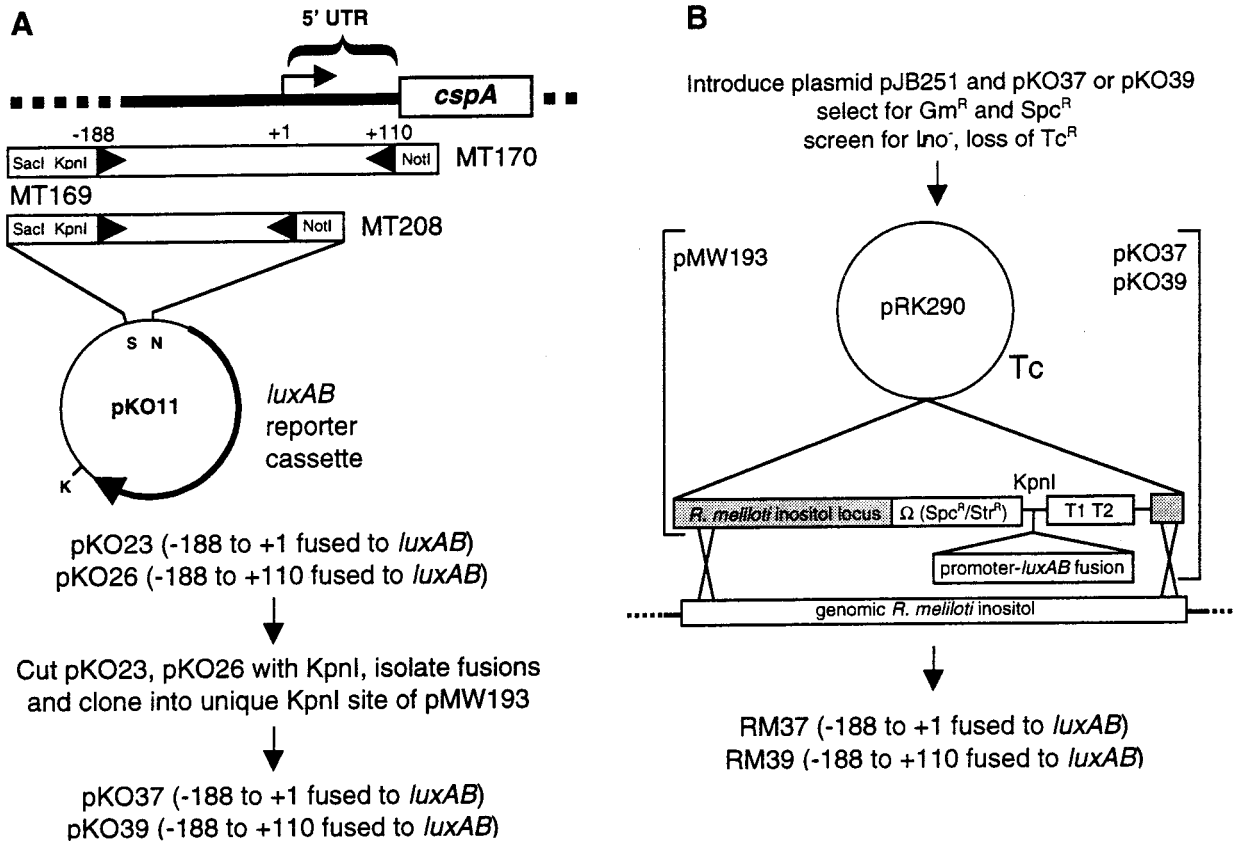

FIG. 6. Construction of $_{p}$*cspA-luxAB* fusions and integration into the *S. meliloti* genome. (A) Amplification and fusion of $_{p}$*cspA* with or without UTR sequences to promoterless *luxAB*. Promoter fragments were amplified by using the primers shown (boxes with arrowheads), with plasmid pH2 as template. Primer sequences are given in Table 2. *Sac*I, *Kpn*I, and *Not*I sites were added during primer synthesis. (B) Integration of promoter-*luxAB* fusions into the genomic inositol utilization (*ino*) locus of *S. meliloti*. The *ino* sequences in pMW193 flank the cloning site, omega fragment, and *rrnB* terminators, allowing replacement of the genomic *ino* locus with the promoter-*luxAB* fusion. Symbols: K, *Kpn*I; N, *Not*I; T1 T2, tandem *E. coli rrnB* terminators in pMW193.

workers have seen a positive but less dramatic effect (18). The role of this sequence in the expression of the *S. meliloti cspA* gene has not been determined. Our gene fusion studies indicate that the promoter of the *S. meliloti cspA* operon is induced approximately sevenfold in response to cold shock (Fig. 7), which is similar to that reported for the promoter of the *E. coli cspA* gene (18). Moreover, our results indicate that the 5′ UTR of the *S. meliloti cspA* operon, like the 5′ UTR of the *E. coli cspA* gene (11), has a substantial effect on the accumulation of transcripts. We found that at non-cold shock temperatures, the 5′ UTR of the *S. meliloti cspA* operon decreases the level of transcript accumulation by about sixfold; this effect seems likely to be due to the sequence causing an increased rate of degradation of the transcripts at the non-cold shock temperature. Upon cold shock, however, the effect of the 5′ UTR appeared to be eliminated. In *E. coli*, the 5′ UTR of the *cspA* gene is sufficient to regulate much of the accumulation of *cspA* mRNA in response to cold shock (11, 22). The current model posits that the presence of the 5′ UTR causes *cspA* mRNA to be rapidly degraded at 37°C but that it assumes a secondary structure at lower temperatures that facilitates its translation when other mRNAs are inaccessible. This is borne out by a study in which mutations of the *E. coli* 5′ UTR which alter its secondary structure cause *cspA* mRNA to accumulate at 37°C (11). This effect is at least partly due to the influence of RNase E (see below). The presence of a "cold box" (a sequence in the 5′ end of the UTR that influences *cspA* transcription) (12) in *S. meliloti* was not confirmed, since only one such gene was analyzed; however, no sequence in the *S. meliloti* UTR matches the cold box consensus of *E. coli*. This may simply indicate that *S. meliloti* either does not have such an element or that its sequence is different, since the entire UTR seems to have little similarity to those of other species.

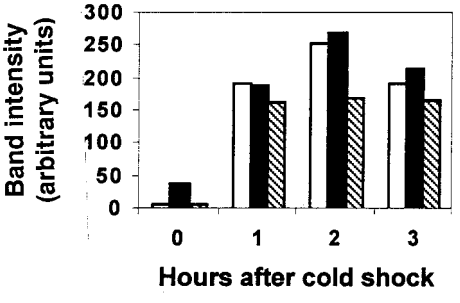

FIG. 7. Induction of $_{p}$*cspA-luxAB* fusions by cold shock. Total RNA from *S. meliloti* strains RM37, RM39, and RM509 was isolated before and at 1-h intervals after cold shock and analyzed by slot blot hybridization to *luxAB* mRNA. The intensity of the bands was determined with a phosphorimager as described in Materials and Methods. White columns, RM509; black columns, RM37; hatched columns, RM39. Data are representative of two experiments.

Lastly, there is preliminary evidence that an RNase E-like

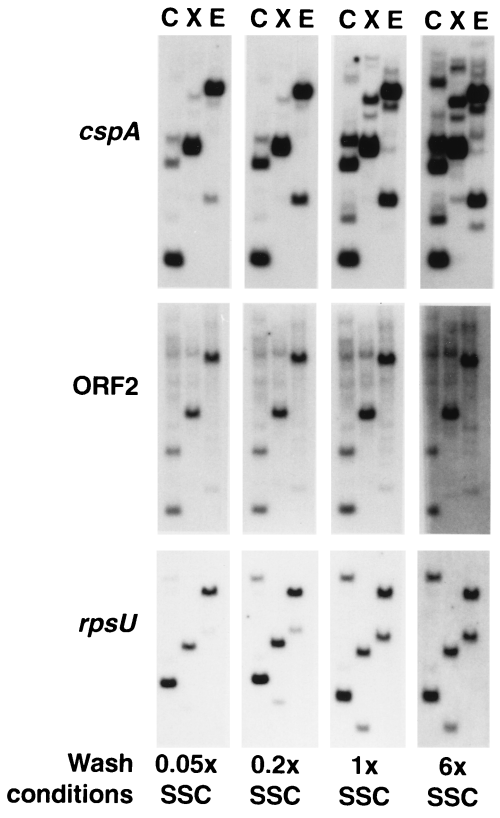

FIG. 8. Southern blots of *S. meliloti* RM1021 genomic DNA cut with *Cla*I (C), *Xho*I (X), or *Eco*RI (E). Filters were probed with the amplified *S. meliloti cspA*, *ORF2*, or *rpsU* DNA fragments shown in Fig. 1B. One filter from each hybridization was washed in one of four aqueous SSC solutions (all include 0.1% sodium dodecyl sulfate) at 60°C and then exposed to film. *Eco*RI and *Xho*I do not cleave within the cold shock operon; *Cla*I cleaves *ORF2* once (Fig. 1).

enzyme also regulates *cspA* accumulation in *S. meliloti*. In some Northern blot experiments we have observed an RNA species of approximately 250 nt that hybridizes to a *cspA* probe, appearing approximately 1 h after accumulation of the 400- and 1,000-nt transcripts after cold shock (unpublished data). A prediction of the secondary structure of the *S. meliloti* 400-nt *cspA* transcript (bases +1 to +458) by the MFOLD program (10) revealed two putative RNase E recognition sites (single-stranded A/U-rich regions flanked by stable secondary structures [30]) spaced about 250 nt apart. The first sequence, AUAUU (nucleotides +82 to +85), is located in the loop of a potential hairpin (bases +73 to +92; part of a larger stem interrupted by an unpaired region of about eight bases on each side). Bases +324 to +332 (CGCAUAAA) form a second potential RNase E site between two stem-loops (+302 to +323 and +333 to +388). Cleavage of these potential RNase E sites would produce an RNA species of approximately 250 bases. Fang et al. (11) found that eliminating a putative RNase E recognition site in the *E. coli cspA* transcript (+135 to +146) lengthens the half-life of the transcript and that the half-life of *cspA* mRNA is longer in a conditional *rne* mutant at the nonpermissive temperature. Thus, it is possible that an RNase E-like enzyme might play a role in the regulation of *cspA* mRNA stability in *S. meliloti*. Mutants with transposon insertions in an *rne*-like gene were recently isolated (A. M. Gustafson and M. F. Thomashow, unpublished data), and their analysis may reveal whether RNase E plays a role in regulating the accumulation of the *S. meliloti cspA* transcript.

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