Identification of Cold Shock Gene Loci in *Sinorhizobium meliloti* by Using a luxAB Reporter Transposon

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Using a luxAB reporter transposon, seven mutants of *Sinorhizobium meliloti* were identified as containing insertions in four cold shock loci. LuxAB activity was strongly induced (25- to 160-fold) after a temperature shift from 30 to 15°C. The transposon and flanking host DNA from each mutant was cloned, and the nucleic acid sequence of the insertion site was determined. Unexpectedly, five of the seven luxAB mutants contained transposon insertions in the 16S and 23S rRNA genes of two of the three rrn operons of *S. meliloti*. Directed insertion of luxAB genes into each of the three rrn operons revealed that all three operons were similarly affected by cold shock. Two other insertions were found to be located downstream of a homolog of the major *Escherichia coli* cold shock gene, *cspA*. Although the cold shock loci were highly induced in response to a shift to low temperature, none of the insertions resulted in a statistically significant decrease in growth rate at 15°C.

In most environments, bacteria experience fluctuations in temperature that are both regular (diurnal and seasonal) and random (as a result of physical disturbances of the environment). Because temperature has wide-ranging effects on growth and survival, bacteria have developed responses that allow them to adapt to changes in temperature. The evolution of cold shock genes is one such response. Many species of bacteria have been shown to alter gene expression in response to a shift to low temperature (3, 5, 12, 16, 17, 19). The synthesis of many cellular proteins ceases and several other proteins, called cold shock proteins (Csps), accumulate. In the cyanobacterium *Synechococcus* sp. strain PCC7002 (20), fatty acid desaturases are induced by cold shock and appear to be involved in maintaining proper membrane fluidity. In *Bacillus subtilis*, the levels of at least 37 proteins increase after cold shock, including enzymes in central metabolism, SpoVG, ribosomal proteins, and several proteins of unknown function (12).

In *Escherichia coli*, where the induction of cold shock genes has been most extensively studied (23), Csps have been classified based on their level of increased expression after a temperature downshift. Class I Csps, which are at low levels at 37°C, appear to influence the structure of the nucleoid at low temperature (24). Paramount among these are CspA, the major cold shock protein of *E. coli*, and its homologs. These proteins, which are referred to as RNA chaperones, bind to RNA and are thought to “open up” mRNA secondary structures that form at low temperatures, preventing initiation of translation (14). The inhibition of translation after cold shock is evidently problematic for many species since most of those studied possess one or more *cspA* homologs that are upregulated in response to a shift to low temperature.

A low temperature has been reported to limit the efficiency of the *Rhizobium*-legume symbiosis (22). To better understand the responses of *Rhizobium* spp. to low temperatures, Cloutier et al. (7) examined protein synthesis in temperate and arctic isolates that had been subjected to a cold shock. It was found that cold shock induced changes in protein synthesis in all of the species tested, including *Sinorhizobium meliloti*. However, the proteins induced by cold shock in *S. meliloti* were not identified and a protein the size of CspA was not observed, raising the question of whether *S. meliloti* encodes a *cspA* homolog. Thus, to further understand the cold shock response in *S. meliloti*, we used reporter transposon mutagenesis to tag, clone, and identify genes in *S. meliloti* that are induced by a temperature downshift.

**Mutagenesis with the luxAB reporter transposon.** *S. meliloti* RM1021 was grown in tryptone-yeast extract (TY) broth medium (4) at 30°C on a rotary shaker. Streptomycin (SM) and kanamycin (KM) were both added to solid medium at 200 μg/ml (50 μg/ml in broth). To mutagenize strain RM1021 with the luciferase reporter transposon, plasmid pRL1062a was transferred to *S. meliloti* by triparental mating by using pRK2013 as the helper plasmid (11). Plasmid pRL1062a carries Tn5-1062, a Tn5-based reporter transposon containing the luxAB genes of *Vibrio harveyi* (8). Transposon recipients were selected by plating undiluted mating mixes on solid TY medium containing SM and KM, followed by incubation at 30°C.

**Identification of mutants with cold shock loci fused to luxAB.** One hundred transposon recipients per mating (>10,000 total) were transferred to TY SM KM plates and grown for 2 days at 30°C. Bacteria were exposed to n-decanal (Sigma Co., St. Louis, Mo.) spread on the inside of a glass petri dish cover for 2 min and then visualized with the Hamamatsu Photonic system essentially as described earlier (8). Colonies of transposon recipients were transferred to a 10 or 15°C growth chamber for 5 h, reexposed to n-decanal, and then observed again under the photonic camera. Isolates that appeared brighter after temperature downshift after two or more assays were selected for further study.

Examining the light emission from transposon recipients
grown at 30°C revealed a variety of levels of light emission (Fig. 1A). While the pattern of light emission from the majority of transposon recipients did not vary greatly after cold shock (Fig. 1B), we identified seven mutants whose light emission was substantially increased by cold shock in repeated experiments (Fig. 1B; Fig. 2A). To quantify the light emission from each mutant strain, bacteria were grown to an optical density at 600 nm of approximately 0.4 in TY broth at 30°C. Then, 10 μl of each culture was mixed with 100 μl of n-decanal solution (5 ml of distilled water, 100 mg of bovine serum albumin, 10 μl of n-decanal) and vortexed for 30 s. Light emission was measured for 60 s by using a Berthold Lumat luminometer (model LB-9501; Wallac Co., Gaithersburg, Md.). After we measured the light emission and culture density, the cultures were transferred to a 15°C shaking water bath. Light emission and culture density were measured again 5 h after temperature downshift. Light emission from the seven mutants increased 25- to 160-fold over a 5-h period post-cold shock (Fig. 2B). Southern analysis indicated that, with the exception of RM3166, each mutant contained a single transposon insertion; RM3166 had two inserts (data not shown).

**Identification of cold shock loci.** The transposon and flanking host DNA were cloned from each mutant that displayed cold shock-induced light emission. Transposon Tn5-1062 contains a p15A origin of replication, allowing the direct cloning of flanking DNA sequences from *S. meliloti* genomic DNA. Total genomic DNA was isolated from RM1021 and mutant strains essentially as described earlier (2); the NaCl-CTAB (cetyltrimethylammonium bromide) extraction step was omitted from some preparations. Genomic DNA that had been digested with SacI, EcoRI, HindIII, or ApaI (New England Biolabs) was circularized by using T4 ligase and transformed into *E. coli* DH5α by electroporation. Plasmid-containing transformants were selected by plating electroporated cells onto solid Luria-Bertani medium (19) containing KM (50 μg/ml) and incubating them overnight at 37°C. After cloning the reporter transposon and flanking DNA from each strain, cloned DNA was prepared for sequencing from cells of *E. coli* by using Qiacells (Valencia, Calif.) Maxi-Columns. Manual double-stranded DNA sequencing reactions were performed with the Amersham (Arlington Heights, Ill.) Sequenase 2.0 Kit and [35S]dATP with primers unique to each end of Tn5-1062. Additional sequencing reactions were performed at the Michigan State University Sequencing Facility. The DNA sequences flanking each transposon insertion mutation were compared to existing known nucleotide and protein sequences by using the BLAST 2.0 program (National Center for Biotechnology Information, Bethesda, Md. [1]) and the GCG (10) and DNAStar (Madison, Wis.) sequence analysis packages. Potential open reading frames (ORFs) were identified by using CodonUse 3.1 (win-
TABLE 1. Putative identification of interrupted genes in mutants of RM1021 demonstrating cold-inducible light emission

| Strain         | Flanking DNA sequenced (bp) | Best gene match | Organism                  | BLAST score | E value* |
|----------------|----------------------------|-----------------|---------------------------|-------------|----------|
| RM11           | See text                   | NA              | NA                        | NA          | NA       |
| RM509          | See text                   | Y4CH, similar to cspA | *Sinorhizobium meliloti* | 112         | 9 x 10^{-24} |
| RM3166         | 565                        | 16S rRNA        | *Sinorhizobium meliloti* | 789         | 0        |
| RM603          | 255                        | 16S rRNA        | *Sinorhizobium meliloti* | 500         | 1 x 10^{-10} |
| RM518          | 150                        | 16S rRNA        | *Azospirillum sp.*        | 291         | 2 x 10^{-77} |
| RM523          | 297                        | 23S rRNA        | *Agrobacterium vitis*     | 589         | 1 x 10^{-166} |
| RM73           | 520                        | 23S rRNA        | *Agrobacterium vitis*     | 739         | 0        |

* E values represent the probability that the query sequence matches the database sequence by chance. A value of “0” indicates a very low probability of matching by chance.

NA; not applicable. The transposon interrupted an ORF that matches no known sequence.

dow size, 33; logarithmic range, 3) for the Apple Macintosh, written by Conrad Halling (University of Chicago).

Based on sequence similarities, putative gene identifications were assigned to each cold shock locus (Table 1). Unexpectedly, five of the seven mutants had insertions in rRNA genes; three in 16S genes (RM3166, RM603, and RM518) and two in 23S genes (RM523 and RM73) (Fig. 3). Three rrn operons exist in *S. meliloti* RM1021 (A. G. Gustafson, K. P. O’Connell, and M. F. Thomashow, unpublished data). To determine whether we had isolated insertion mutations in each of the three operons, we digested the genomic DNA of the five mutants and RM1021 with *SacI*, an enzyme that does not cut within Tn5-1062, and separated the three wild-type rrn operons on DNA fragments of different sizes (Fig. 3B). Hybridization with radiolabelled 23S ribosomal DNA (rDNA) from *S. meliloti* revealed that the insertions were located in two of the three rrn operons (Fig. 3A). Probing with 16S rDNA from *S. meliloti* gave nearly identical results (as alluded to above, RM3166 was found to contain a second transposon). The positions of each insertion within the two operons were determined by using the sequence of the DNA flanking each insertion (Fig. 3B). The results indicated that cold shock induced expression of at least two of the three rrn operons of *S. meliloti*. The characterization of rrn-luxAB fusions and the effect of cold shock on their expression will be described elsewhere (Gustafson et al., unpublished data).

The transposon inserts in RM11 and RM509 were found to be located adjacent to each other just downstream from a homolog of the major *E. coli* cold shock gene, cspA (Table 1). The transposon in mutant RM11 was inserted in the 5’ end of the novel ORF; the insert in RM509 was in the intergenic region between the cspA homolog and the novel ORF. Presumably, cold shock-induced expression of the reporter genes in these mutants resulted from upregulation of the cspA homolog in response to the low temperature.

**Insertion of luxAB into all three rrn operons.** Only two of the three rrn operons were identified as putative cold shock loci by transposon mutagenesis. Therefore, directed luxAB insertions in all three operons were constructed to determine whether luxAB insertions in the remaining operon could also be affected by cold shock. As described above, a portion of the 16S gene from mutant RM603 was cloned by circularization of Tn5-1062 and flanking DNA. A DNA fragment containing a portion of the 16S gene and the luxAB and kanamycin resistance gene of the transposon was cloned into the suicide vector pSUP202, giving plasmid pKO4 (Fig. 4A). Conjugal transfer of pKO4 into RM1021 and selection for kanamycin resistance yielded mutants in which single homologous recombination events had occurred between the 16S rDNA fragment on pKO4 and each of the three chromosomal 16S rDNA genes. The insertion of pKO4 into each of the three rrn operons resulted in a fusion of luxAB with each 16S rRNA gene at the same position. The insertions were verified by restriction of genomic DNA with *SacI*, blotting, and probing with 16S rDNA sequences (Fig. 4B). Mutants X76 and X78 contained luxAB fusions in the rrn operon not found in the initial mutant screen (Fig. 3). LuxAB activity increased in strains containing luxAB fusions in each of the three rrn operons after a temperature downshift from 30 to 15°C (Fig. 4C), suggesting that all three rrn operons respond similarly to cold shock.

**Effect of mutations on growth rate at 15°C.** To determine whether mutations in the novel ORF and rrn operons affected the growth of *S. meliloti* at a low temperature, we compared the generation times of the wild-type and mutant strains in liquid TY medium at 30 and 15°C. Triplicate cultures were
grown in TY broth, and cell growth was measured spectrophotometrically at 600 nm. Although the results hint that there might be a small effect of the insertion mutations on the growth rate at 15°C, the differences between the wild-type and mutant strains were not statistically significant (Student’s t test). The doubling times of RM1021 were 2.1 h at 30°C and 13.1 h at 15°C. The doubling times of the mutants ranged from 2.1 to 2.3 h at 30°C and from 14.3 to 15.5 h at 15°C.

**Conclusions.** Our results suggest that all three of the *S. meliloti* rrn operons are upregulated in response to a low-temperature downshift. Finding rrn operons in a screen for cold shock loci was unexpected; indeed, we are unaware of rrn operons having been described as cold shock loci in any organism. These results are intriguing given that many of the known cold shock proteins in other species have functions associated with translation. However, the results are counterintuitive since the rate of RNA transcription is generally tied to the growth rate of the bacterium during balanced growth (6, 9), and the growth rate of *S. meliloti* at 15°C is only about a sixth of the rate at 30°C. One interesting possibility is that the rrn operons might be induced in response to a perceived lack of translational capacity (feedback response) (16) caused in some manner by cold shock. Clearly, additional study of this phenomenon will be required to understand its significance and possible role in low-temperature adaptation. The finding that reporter insertions in all three rrn operons respond similarly to cold shock is consistent with the observation that rrn operons are coordinately regulated in response to other stimuli (9).

*S. meliloti* also encodes a homolog of the *E. coli cspA* cold shock gene. The observation that a 10.6-kDa protein is encoded by a downstream putative cold shock gene is consistent with the finding of Cloutier et al. (7) that a protein of about 11.1 kDa accumulated after cold shock in all rhizobial strains studied. A more complete characterization of this cold shock locus is reported elsewhere (18).

The isolation of multiple cold shock mutants with insertions in the *cspA* and *rrn* operons indicates that our mutagenesis was beginning to reach saturation. Yet the results of Cloutier et al. (7) indicate that a number of *S. meliloti* proteins accumulate in response to cold shock. Why didn’t our screen result in the identification of more cold shock loci? One possible explanation is that the regulation of many of these cold shock proteins might not occur at the transcriptional level. Moreover, a likely contributing reason is that our screen was largely qualitative in nature; only strains that produced low levels of LuxAB activity at 30°C and high levels at 15°C were identified as cold shock mutants in the screen. Thus, the screen was biased toward identifying class I-type (over 10-fold induced) cold shock loci; indeed, we identified a *cspA* homolog. The identification of class II-type loci, genes that are upregulated only a few fold by low temperature, would likely have been missed in the screen due in part to the fact that most inserts that resulted in the production of luminescence were upregulated about two- to fourfold in response to cold shock (presumably resulting from a general increase in LuxAB transcript and/or LuxAB protein stability at the cold shock temperature). To identify class II-type genes in such a background would require the screen to be conducted in a more quantitative manner. This, however, is possible with digital charge-coupled device imaging systems and can be incorporated into future screens.

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