Final Report (7/26/2006)

Covering 9/2001 to 5/2006
DE-FG02-01ER15175 "Molecular characterization of catabolite repression by succinate in the nodulating bacterium Sinorhizobium meliloti" PI: Daniel J. Gage, University of Connecticut, Dept. of Molecular and Cell Biology

Our initial Energy Biosciences grant from the DOE, was designed to generate information into how the progress of succinate-mediated catabolite repression (SMCR) operates in the nitrogen-fixing symbiont *Sinorhizobium meliloti*. The rational for studying catabolite repression in *S. meliloti* was twofold. First, the genes and proteins that affect catabolite repression are often extremely important in controlling processes that are among the most fundamental in bacteria. These include control of central metabolic pathways, and control of growth, cell division and differentiation. Secondly, *S. meliloti* offered a unique opportunity to gain insight into catabolite repression in response to succinate and other dicarboxylic acids. While, catabolite repression in response to compounds like succinate had been documented in several important groups of bacteria, the molecular mechanisms involved in its operation were obscure. Because most work done on catabolite repression involved organisms that preferred glucose and related sugars, we anticipated that study of succinate-mediated catabolite repression in *S. meliloti* would lead to unique insights into the molecular physiology of this organism, and perhaps lead to insights into the metabolic, and gene-regulatory affairs of other organisms that use TCA-cycle intermediates as preferred carbon sources.

In order to better understand SMCR in *S. meliloti* we proposed the following two specific aims:

A. To characterize the succinate-repressed promoter region of *melA* using biochemical and genetic approaches.

B. To identify *S. meliloti* genes needed to establish succinate mediated repression.

The progress we made on these two aims is described below:

A. Characterization of the succinate-repressed promoter region of *melA* using biochemical and genetic approaches.

We had shown, before submission of the original DOE proposal, that *S. meliloti* has an operon encoding an α-galactosidase and four proteins that make up a periplasmic-binding-protein dependent ABC α-galactoside transport system. We had shown that these genes were required for the uptake and utilization of a-galactosides such as melibiose, raffinose and stachyose. We had also shown that the expression of these genes is repressed by succinate, even when inducers such as melibiose or raffinose are present. It was this operon (the *melA-agp* = melibiase, α-gal transport) that we suggested would make a good model for studying SMCR. The following was accomplished terms of characterizing this operon in terms of its induction by a-galactosides and repression by succinate.

1. We showed that the *melA-agp* operon requires an AraC-type transcriptional activator for induction. This gene encoding this activator, *agpT*, is immediately upstream *melA* and is transcribed in the opposite direction. Mutation of *agpT* results in cells that are unable to grow on a-galactosides.

2. We cloned, and over-expressed, the AgpT gene in *E. coli*, and showed that crude extracts made from the over-expressing strain were able to bind to the promoter region of the *melA-agp* operon.

3. We made transcriptional fusions between the *melA-agp* promoter region and *gfp*. These fusions are convenient tools that allowed us to easily monitor expression of the *melA-agp* promoter region.

4. Because of its useful characteristics (high levels of expression induced by α-galactosides, and low levels of expression when succinate is present and a-galactosides are absent) the *melA-agp* promoter is being used by the rhizobium research community as a promoter for heterologous expression is *S. meliloti*.

5. *S. meliloti* carrying *melA-gfp* reporter plasmids were used as whole-cell biosensors to study the release of galactosides from plant roots that were grown in non-sterilized soil. This was the first time that anyone was able to non-destructively, and at micrometer spatial scales, monitor the release of a specific class of sugars from roots growing in non-sterilized soil. The fluorescent reporters also allowed visualization of trophic interactions because protists that consumed fluorescent bacteria, became fluorescent themselves.

6. Studies of *melA-agp* promoter control revealed that a gene down stream of *agpT*, that encoded a LacI-type repressor, affected expression of the *melA-agp* operon. This gene was subsequently shown by us to be LacR, which controls the expression of the *lac* genes which are next to the *melA-agp* operon. These genes are required for the transport and utilization of β-galactoside, and they are also downregulated by SMCR.

7. Biochemical and genetic studies utilizing the *lac* and *melA-agp* genes showed that SMCR occurs, at least in part through inducer exclusion. That is succinate blocks the transport of secondary carbon sources, such as raffinose, when both are present together. This was the first time that anyone had elucidated any of the molecular mechanisms by which SMCR works in rhizobia or other α-proteobacteria.

8. Epistatic analysis of HprK, ManX(EIIA) and Hpr. Our current model postulates that the enhanced SMCR phenotypes of the Δ*hprK* and Δ*manX(EIIA)* mutants are brought about because they cause Hpr to exist mainly as Hpr-His22-P. If this is true, then Δ*hprK*/Δ*hpr* and Δ*manX(EIIA)*/ Δ*hpr* double mutants should exhibit the same phenotype as a Δ*hpr* mutant -- they should show weakened SMCR rather than enhanced SMCR (Δ*hprK* and Δ*manX(EIIA)* phenotypes). This set of experiments was thought to be particularly important because it should reveal whether ManX (EIIA) and HprK affect SMCR only through their effects on Hpr, or whether they can affect SMCR independently of Hpr. The double, unmarked, Δ*manX(EIIA)*/Δ*hpr* mutant has been constructed using *sacB*-gene replacement methods similar to those used to make the in-frame mutans described in the main proposal. This strain, CAP32(Δ*manX(EIIA)*Δ*hpr*), showed weak SMCR, similar to the Δ*hpr* mutant upon growth on XGal-succinate + lactose + Xgal plates. Our hypothesis that SMCR phenotype of the Δ*manX(EIIA)* mutation was caused by its effect on Hpr is consistent with the observation described above that the Δ*hpr* mutation is epistatic to the Δ*manX (EIIA*) mutation. Construction of the Δ*hprK*/Δ*hpr* double mutant is in progress.

9. We have not identified the protein responsible for phosphorylating Hpr. In other

phosphotransferase systems Hpr is phosphorylated on histidine by an EI protein. In *S. meliloti* the EI^{Ntr}, is the only EI-like protein so far identified in the genome. Because of the possibility that the EI^{Ntr} is involved in SMCR we proposed to construct an unmarked in-frame knockout of the gene encoding EI^{Ntr} study its effects on SMCR. If EI^{Ntr} does phosphorylate Hpr, and if our current model is correct, cells lacking EI^{Ntr} should exhibit weak SMCR because Hpr will not be phosphorylated on His22. A *sacB*-based suicide plasmid, pCAP51, which contains an in-frame deletion that removes much of the *ptsP*(encoding EI^{Ntr}) open reading frame has been constructed and is currently being introduced into Rm1021. This vector should recombine twice in the genome, replace the wild type copy of *ptsP* with the deleted copy, and resulting a new strain with an in-frame deletion of *ptsP*. SMCR phenotypes, as well as nodulation phenotypes, will be evaluated in the resulting strain.

10 Structure of nodules induced by mutant strains RB111(Δ*hpr*), CAP14(Δ*hprK*) and CAP16 (Δ*hprK-suppressor2*). Internal organization of alfalfa nodules was investigated by fixing, embedding, slicing and staining nodules induced by *S. meliloti* wild type and mutant strains. Wild type induced nodules presented clear meristematic, infection and nitrogen fixation zones with high-density infection in the nitrogen fixation zone, characterized by elongated bacteroids, In contrast, most nodules from CAP14(Δ*hprK*) infected plants showed very narrow infection and nitrogen-fixing zones with low-density infection, many empty plant cells, and bacteroids that were short in comparison with wild type-induced nodules. The plant cells in these nodules contained many starch granules. Pink nodules were induced by RB111(Δ*hpr*) and CAP16(Δ*hprK-suppressor2*). These showed internal structures similar to wild type-induced nodules, except for the high number of starch granules present. Strains R105(Δ*manX*) and CAP15(Δ*hprK-suppressor1*) induced pink nodules with normal internal organization. These results indicate that the *hpr* and *hprK* mutations can affect nodule development and function. Suppressors of the Δ*hprK* mutation can either partially (*suppressor2*) or completely (*suppressor1*) fix the Δ*hprK* nodule phenotypes. At present it is not clear whether the nodule defects of the Δ*hpr* and Δ*hprK* strains are caused directly by the mutations, or whether the nodule defects result from the indirect effects arising from the pleotropic nature of the mutations.

B. The identification of *S. meliloti* genes needed to establish succinate mediated repression

In order to fulfill this aim, we carried out random mutagenesis to identify genes involved in SMCR, and we also carried out targeted mutagenesis of gene we thought were likely to be involved in SMCR. The results of these, and the behavior of the resulting mutant strains, are outlined below.

1. *S. meliloti* has an incomplete phosphotransferase system (PTS). Lacking are genes that encode the integral membrane permease parts of typical PTS. In order to determine if the genes that are present are involved in SMCR, we made in-frame deletions of *hpr* and *manX*. These encode a Hpr protein and EIIA protein respectively.

2. We have shown that the Δ*hpr* mutant has weakened SMCR, and that the Δ*manX*(*EIIA*) has enhanced SMCR. To our knowledge, this is the first time it has shown that an abbreviated PTS plays an important role in cellular physiology.

3. The Δ*hpr* and Δ*manX*(*EIIA*) mutants also displayed other phenotypes that suggesting that other

aspects of carbon metabolism were altered. Growth yields, growth rates, and survival in stationary phase differed from wild-type.

4. We have shown that neither the Δ*hpr* and Δ*manX*(*EIIA*) mutations affect nodulation, or nitrogen fixation, on alfalfa or *M. truncatula* plants.

5. *S. meliloti* also contains a gene that encodes a protein similar to Hpr kinase/phosphatase. In *B. subtilis* this protein is involved in the signal transduction pathway that leads to glucose-mediated catabolite repression. We constructed an in-frame deletion of the *hprK* gene. The Δ*hprK* mutant was pleotropic, displayed slow growth, low yields and extremely strong SMCR.

6. We have collected, and begun to characterize, suppressor mutants of the Δ*hprK* strain that show increased colony sizes on lab media along varying degrees of SMCR.

7. We have shown that the Δ*hprK* mutant nodulates alfalfa and *M. truncatula* plants, however the nodules fail to fix nitrogen.

8. Analysis of mutations that suppress the Δ*hprK* growth and carbon utilization defects to varying degrees have shown that role of HprK in control of SMCR, and its role in the development of functioning nodules can be separated. That is, it appears that the very strong SMCR seen in Δ*hprK* mutants is not responsible for the Fix- phenotype of the mutant.

9. We have constructed His-tagged versions of HprK and Hpr.

10. We conducted a genetic screen and found a two component system that is involved in regulation of SMCR. This histidine kinase, Sma0113, contains 5 tandem PAS domains and a HWE kinase domain. The response regulator Sma0114 is a single domain CheY-like protein. These may be involved in sensing succinate, or the metabolic or energetic consequences of transporting or catabolizing succinate.

11. We have made unmarked, in-frame, deletions of *sma0113* and *sma0114*. These mutants show weakened SMCR, and our data indicates that the phosphorylated form of Sma0114 either enhances SMCR, or inhibits release from SMCR.

12. The *sma0113* and *sma0114* mutations also affect growth yields, and confer the ability to catabolize glucose-1-phosphate and glucose-6-phosphate.

13. We have shown that the *sma0113* and *sma0114* mutations do not appreciably alter the ability of *S. meliloti* to nodulate plants, and fix nitrogen.

14. Recent progress has been in constructing in-frame mutants and covering plasmids for the mutants. Some of the new mutants are to be used to determine the relationships between the Sma0113/0114 system and the PTS proteins in terms of modulating SMCR. In addition, site-directed mutant genes encoding Sma0114-D57A(inactive) and Sma0114-D13K(constitutively active?) have been made and their SMCR phenotypes are being investigated. Strains and plasmids constructed since submission of the proposal are:

A). New in-frame, unmark deletion strains (purified and confirmed):

Strain	**Genotype**	**Plasmid carries**
PG26	Δ*sma0113* Δ*sma0114*	
PG28	Δ*sma0113* Δ*manX*	
PG30	Δ*sma0113* Δ*hpr*	
PG32	Δ*sma0114* Δ*manX*	
PG34	Δ*sma0114* Δ*hpr*	
PG24/pPG65	Δ*sma0114*	*sma0114*
PG24/pPG71	Δ*sma0114*	*sma0114*-D13K (constitutively active Sma0114?)
PG24/pPG73	Δ*sma0114*	*sma0114*-D57A

B.) Constructed and currently purifying:

Strain	**Genotype**	**Plasmid carries**
Rm1021/pPG67	wt	no insert control plasmid
PG22/pPG67	Δ*sma0113*	no insert plasmid control
PG24/pPG67	Δ*sma0114*	no insert plasmid control
PG26/pPG65	Δ*sma0113* Δ*sma0114/*	*sma0114*
PG26/pPG67	Δ*sma0113* Δ*sma0114*	no insert plasmid control
PG26/pPG71	Δ*sma0113* Δ*sma0114/*	*sma0114*-D13K

C.) Plasmids (purified and confirmed by sequencing)

Plasmid	**Plasmid carries**
pPG67	*trp* terminator insertion into *Cla*I of pHC41, used for construction of all plasmids below
pPG65	*sma0114* expressed from native promoter
pPG71	*sma0114*-D13K expressed from native promoter
pPG73	*sma0114*-D57A expressed from native promoter
pPG72	*sma0114*-D13K in pJQ200SK (*sacB*) for chromosomal insertion of *sma0114*-D13K if needed
pPG74	*sma0114*-D57A in pJQ200SK (*sacB*) for chromosomal insertion of *sma0114*-D57A if needed

15. Epistatic analysis of HprK, ManX(EIIA) and Hpr. We are in the process of using double, *sma*/*PTS* mutants, in analyses of epistasis. This is being done to determine if the Sma0113/0114 two component system affects SMCR by signaling upstream of the PTS proteins, downstream of the PTS proteins or independently of the PTS proteins. On succinate + lactose + Xgal plates the strains have the following phenotypes:

Strain	**Genotype**	**SMCR phenotype[a]**
Rm1021	w.t	+
RB105	Δ*manX*	++
RB111	Δ*hpr*	+/-

PG22	Δ*sma0113*	+/-
PG24	Δ*sma0114*	+/-
*PG28	Δ*sma0113* Δ*manX*	++
*PG32	Δ*sma0114* Δ*manX*	++
PG30	Δ*sma0113* Δ*hpr*	+/-
PG34	Δ*sma0114* Δ*hpr*	+/-

[a] += light blue, ++= very light blue, +/- dark blue

In those cases where single *sma0113 or sma0114* mutations and PTS mutations differ in their effects on SMCR, the double mutants showed the phenotype of the PTS mutation (see strains marked "*" above). This indicates that the Sma0113/0114 two component system likely signals upstream of the PTS proteins when modulating SMCR, and such modulation may happen in one of two ways:

•The Sma0113/0114 proteins may integrate information about the catabolism of succinate, or the general metabolic state of the cell and pass that information to the PTS proteins, which may then influence SMCR.

•The Sma0113/0114 proteins may regulate succinate catabolism, or metabolism in general, and the lack of such regulation in the Δ*sma0113* and Δ*sma0114* mutants may impact the PTS proteins and how they influence SMCR.

To differentiate between these two possibilities we will be isolating and characterizing protein partners that interact with Sma0114. The identity and function of the partners may indicate which of the possibilities is most likely. We will also investigate the effects of the Sma0114 mutants (both inactive and constitutively active) to see if they affect electron transport, the composition of the electron transport chain, or the redox state of *S. meliloti* cells.

Publications resulting from DOE funding

C. Arango and **D. J. Gage** (2006) Hpr kinase (HrpK) is required for the formation of nitrogen-fixing root nodules by Sinorhizobium meliloti. In progress.

C. Arango and **D. J. Gage** (2006) A truncated PTS system plays a key role controlling succinate-mediated-catabolite-repression in *Sinorhizobium meliloti*. Submitted.

M. Rosado and **D. J. Gage**. (2003) Transcriptional control of a rRNA promoter of the nodulating symbiont *Sinorhizobium meliloti.* FEMS Microbiol. Letters **226**:15-22

Bringhurst, R. M. and **D. J. Gage** (2002) Inducer exclusion/expulsion plays a key role in succinate-mediated catabolite repression in *Sinorhizobium meliloti*. J. Bacteriology 184:5385-5392

Bringhurst, R. M., Cardon, Z., and **D. J. Gage** (2000). Galactosides in the rhizosphere: utilization by *Sinorhizobium meliloti* and development of a biosensor. Proc. Nat. Acad. Sci. **98:**4540-4545

Bringhurst, R. M., and **D. J. Gage** (2000) An AraC-like transcriptional activator is required for induction of genes needed for - galactoside utilization in *Sinorhizobium meliloti.* FEMS Microbiol. Letters **188:**23-27

Abstracts

P. M. Herron, **D. J. Gage**, and Z. G. Cardon. (2005) Patterns of water potential gradients around roots in soil as reported by microbiosensors. Annual Meeting, Ecological Society of America

P. M. Herron, **D. J. Gage**, and Z. G. Cardon. (2003) Divining Rods: *Pseudomonas putida* as a microbiosensor of fine-scale osmotic potentials in soil. Annual Meeting, Ecological Society of America. (winner best poster)

P. M. Herron, M. Rosado, R. M Bringhurst, **D. J. Gage**, and Z. G. Cardon. (2002) Speedometer bacteria: a proxy for carbon availability in the rhizosphere. Annual Meeting, Ecological Society of America.

R. M. Bringhurst, **D. J. Gage.** (2002) Inducer exclusion plays a key role in succinate-mediated catabolite repression in *Sinorhizobium meliloti*. Annual Meeting, American Society for Microbiology.

R. M. Bringhurst, Z. G. Cardon, **D. J. Gage.** (2001) A biosensing strain of *Sinorhizobium meliloti* used to monitor release of -galactosides into the rhizosphere. Annual Meeting, American Society for Microbiology.