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Control of Inducer Accumulation Plays a Key Role in Succinate-Mediated Catabolite Repression in *Sinorhizobium meliloti*

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The symbiotic, nitrogen-fixing bacterium *Sinorhizobium meliloti* favors succinate and related dicarboxylic acids as carbon sources. As a preferred carbon source, succinate can exert catabolite repression upon genes needed for the utilization of many secondary carbon sources, including the α-galactosides raffinose and stachyose. We isolated *lacR* mutants in a genetic screen designed to find *S. meliloti* mutants that had abnormal succinate-mediated catabolite repression of the *melA-agp* genes, which are required for the utilization of raffinose and other α-galactosides. The loss of catabolite repression in *lacR* mutants was seen in cells grown in minimal medium containing succinate and raffinose and grown in succinate and lactose. For succinate and lactose, the loss of catabolite repression could be attributed to the constitutive expression of β-galactoside utilization genes in *lacR* mutants. However, the inactivation of *lacR* did not cause the constitutive expression of α-galactoside utilization genes but caused the aberrant expression of these genes only when succinate was present. To explain the loss of diauxie in succinate and raffinose, we propose a model in which *lacR* mutants overproduce β-galactoside transporters, thereby overwhelming the inducer exclusion mechanisms of succinate-mediated catabolite repression. Thus, some raffinose could be transported by the overproduced β-galactoside transporters and cause the induction of α-galactoside utilization genes in the presence of both succinate and raffinose. This model is supported by the restoration of diauxie in a *lacF lacR* double mutant (*lacF* encodes a β-galactoside transport protein) grown in medium containing succinate and raffinose. Biochemical support for the idea that succinate-mediated repression operates by preventing inducer accumulation also comes from uptake assays, which showed that cells grown in raffinose and exposed to succinate have a decreased rate of raffinose transport compared to control cells not exposed to succinate.

Bacteria belonging to the genera *Sinorhizobium*, *Rhizobium*, and *Bradyrhizobium* are members of the α-proteobacteria, a fascinating group of organisms, many of which are intracellular symbionts or pathogens. *Sinorhizobium meliloti* can grow in soil as free-living organisms but can also live as nitrogen-fixing symbionts inside root nodules of alfalfa and a few other plants belonging to the family Leguminosae (3, 9, 15, 19, 22, 34).

Free-living *S. meliloti*, like many heterotrophic bacteria, utilizes a wide variety of compounds as sources of carbon for growth. *S. meliloti* can utilize α-galactosides in laboratory medium and also when growing in the rhizospheres of host and nonhost plants (4). The utilization of α-galactosides requires genes which are part of an operon located on pSymB, a 1.7-Mb plasmid, in *S. meliloti* (Fig. 1) (7, 12). The *agpA* gene encodes a 77-kDa periplasmic protein that is required for α-galactoside transport and that is similar to periplasmic binding protein components of the oligopeptide family of permeases (Fig. 1) (12). Immediately downstream of the *agpA* gene is *agpB*, which encodes a protein similar to transmembrane proteins of the oligopeptide family of permeases. Downstream of *agpB* are genes encoding a second transmembrane permease and an ATP binding protein. Thus, this operon appears to encode a complete ATP binding cassette-type transport system for α-galactosides. The *agpA* gene is preceded by the *melA* gene, which encodes a 55-kDa α-galactosidase (12). The *melA* and *agp* genes are cotranscribed and are referred to in this study as the *melA-agp* operon. Induction of the *melA-agp* operon by α-galactosides requires the action of an AraC-type transcriptional activator, AgpT, which is encoded upstream of *melA* (5).

S. meliloti favors succinate and related dicarboxylic acids as carbon and energy sources. Succinate, fumarate, and malate are brought into the cell via membrane-bound permease DctA (28, 38). The expression of *dctA* is inducible by dicarboxylic acids and structurally related compounds and requires the action of a two-component signal transduction system composed of DctB, the sensor kinase, and DctD, the response regulator (25, 27, 29, 38). As a favored carbon source, succinate often exerts catabolite repression upon genes needed for the utilization of secondary carbon sources. Secondary carbon sources include compounds such as glucose, fructose, galactose, lactose, *myo*-inositol, and several pentoses and polyols (16, 17, 24, 37). This preference for succinate can manifest itself as diauxie when *S. meliloti* is grown on succinate plus a secondary carbon source (16, 37).

Succinate also exerts catabolite repression on the consumption of α-galactosides in *S. meliloti*. The expression of the *melA-agp* operon is repressed by succinate (12), and *S. meliloti* shows diauxie when grown on a combination of succinate and the α-galactoside raffinose or stachyose. While the phenomenon of succinate-mediated catabolite repression in *S. meliloti* is well documented, most of the molecular details of its operation

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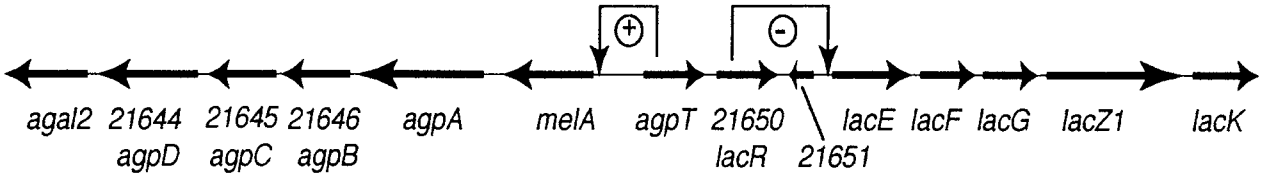

FIG. 1. Map of the region of pSymB involved with the transport and utilization of α-galactosides and β-galactosides. ORFs are indicated along with the official ORF names (top row) and names indicating the functions of the proteins encoded by the ORFs (bottom row). Regulatory roles of AgpT and LacR are indicated above the map.

remain to be elucidated. This is also true for organisms of other genera, such as *Pseudomonas*, which utilize succinate and other tricarboxylic acid cycle intermediates in preference to other carbon sources (8, 14, 23, 35).

It is known that succinate transport is important for the establishment of catabolite repression in *S. meliloti*. *S. meliloti dctA* mutants are unable to repress the expression of the *lac* operon in the presence of succinate plus lactose, indicating that succinate transport, or at least the interaction of succinate with the DctA permease, is required for the establishment of catabolite repression (17).

In order to find genes needed to establish succinate-mediated catabolite repression, we screened for mutants unable to repress the *melA-agp* operon when grown on a combination of succinate and raffinose. *lacR* mutants, which constitutively overexpress β-galactoside utilization genes, were isolated in this screen. We show that the effects of *lacR* mutations on the succinate-mediated catabolite repression of the *melA-agp* operon are due to the overexpression of β-galactoside transport genes, which likely overwhelms inducer exclusion or inducer expulsion mechanisms (26, 30, 36). The genetic and biochemical experiments presented in this study support this idea and support the conclusion that succinate-mediated catabolite repression acts, in part, through the regulation of intracellular inducer accumulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Bacterial strains, plasmids, and their relevant characteristics are listed in Table 1. Cells were grown in tryptone-yeast (TY), Luria-Bertani, or M9 minimal medium with various carbon sources (32). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 5 μg/ml for *S. meliloti* and 100 μg/ml for *Escherichia coli*; gentamicin, 30 μg/ml; neomycin, 200 μg/ml; spectinomycin, 100 μg/ml; and streptomycin, 500 μg/ml.

Construction of *S. meliloti* strains. Strain RB21 (*agpA*::Tn*phoA lacR*::Tn*5lacZ*) was isolated from the following genetic screen. *S. meliloti* strain SG2001 (*agpA*::Tn*phoA*) was mutagenized by triparental mating with *E. coli* strain S17-1 λpir/pB22 (Tn*5lac*) and *E. coli* helper strain MT616. The three strains were streaked together on a TY plate without antibiotics and incubated for 24 h at 30°C. Swaths of bacteria were picked from the plate, suspended in 100 μl of M9

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
S. meliloti		
Rm1021	Wild type (Sm^r)	21
Rm8002	*pho* derivative of Rm1021	18
DG73	Rm1021 *agpA*::Tn*phoA*-Sp^r; the Nm^r gene of *agpA*::Tn*phoA* was swapped with the Sp^r region of Tn*5-233*	Gage lab
RB21	Rm1021 *lacR*::Tn*5lacZ* (Sm^r Gm^r)	This study
RB33	Rm1021 *lacR*::Tn*5-233* (Sm^r Gm^r Sp^r)	This study
RB45	Rm1021 *lacF*::pRB69	This study
RB46	Rm1021 *lacF*::pRB69 *lacR*::Tn*5-233*	This study
RB47	Rm1021 *lacF*::pRB69 *agpA*::Tn*phoA*	This study
SG2001	Rm1021 *agpA*::Tn*phoA*	Gage lab
E. coli		
XL1Blue	Used for cloning (Tc^r)	Stratagene
MT616	Helper strain for conjugal transfer of plasmids (Cm^r)	13
MM294a	Used to deliver pRK607	13
Plasmids		
pRB27	p*melA*::*gfp* fusion in pMB393 (Sp^r Cm^r)	4
pRB69	Suicide vector pMB438 containing a 594-bp internal piece of *lacF* (Ap^r Nm^r/Km^r)	This study
pMB393	Broad-host-range plasmid	2
pMB438	Suicide vector (Ap^r Nm^r/Km^r)	2
pB22	Plasmid used to deliver Tn*5lacZ* (Gm^r)	33
pRK607	Plasmid used to deliver Tn*5-233* (Nm^r/Km^r Gm^r/Km^r Sp^r/Sm^r)	10
pJGJ54	*lacR*-complementing plasmid	17
pJGJ86	*lacR*-complementing plasmid	17
pGEM-T Easy	PCR product cloning vector	Promega

salts, and spread on M9 plates containing 0.2% succinate, 0.02% raffinose, 80 μg of 5-bromo-4-chloro-3-indolyl phosphate (X-Phos)/ml, neomycin, and gentamicin. Deep blue colonies were retained for further study; one of these was strain RB21.

Strain RB33 (*lacR*::Tn*5-233*) was also isolated from a genetic screen. *S. meliloti* wild-type strain Rm1021 was mutagenized by mating with MM294a/pRK607; pRK607 is a self-transmissible plasmid harboring Tn*5-233*. The strains were streaked together on a TY plate without antibiotics and incubated for 24 h at 30°C. Swaths of bacteria were picked from the plate, suspended in 100 μl of M9 salts, and spread on M9 plates containing 0.2% succinate, 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)/ml, streptomycin, and gentamicin. The *lacR* mutants were expected to be blue on this medium because they constitutively expressed the endogenous *S. meliloti lacZ* gene. One of the resultant deep blue colonies was strain RB33. Southern hybridization confirmed that the Tn*5-233* transposon was in the *lacR* gene of strain RB33 (data not shown).

Strain RB45 is a *lacF* mutant constructed by inserting suicide plasmid pRB69 into the *lacF* gene of *S. meliloti* wild-type strain Rm1021. Suicide plasmid pRB69 was constructed as follows. A 576-bp internal piece of the *lacF* gene was generated by PCR with primers 5′GCGTGGTCGCTCTGGATGT and 5′TCGTCG AAATGACTGTCGTGAA. The resulting amplification product was inserted into T/A cloning vector pGEM-T Easy. This piece was then removed by *Eco*RI digestion, and the 594-bp fragment was cloned into broad-host-range suicide vector pMB438, resulting in plasmid pRB69. pRB69 was mobilized from strain XL1Blue/pRB69 into strain Rm1021 by triple mating with *E. coli* helper strain MT616 and selected on Luria-Bertani medium plus streptomycin and neomycin.

Strain RB46 (*lacF lacR*) was constructed by transducing the *lacF* mutation from strain RB45 into strain RB33 (*lacR*::Tn*5-233*) with phage N3 (20). Strain RB47 (*lacF agpA*) was constructed by transducing the *lacF* mutation from strain RB45 into strain DG73 (*agpA*::Tn*phoA*-Sp^r) with phage N3.

Diauxic growth curves. *S. meliloti* strains were grown overnight in M9 minimal medium plus antibiotics and with succinate as the sole carbon source. A quantity (1 ml) of cell culture was pelleted, washed thee times with M9 salts to remove residual succinate, and resuspended in 100 μl of M9 salts. Ten microliters of this suspension was used to inoculate 10 ml of M9 minimal medium with 0.05% succinate or with 0.05% succinate plus 0.1% raffinose, maltose, or lactose. Cultures were incubated in 125-ml flasks at 30°C with shaking. Cell density was determined by measuring the absorbance of 100 μl of culture at 415 nm with a Bio-Rad 550 plate reader. Optical densities determined with the plate reader should be multiplied by three to approximate the optical density determined with a spectrophotometer with a standard 1-cm path length.

Quantitation of p*melA*::*gfp* expression. Bacterial strains were grown overnight in M9 minimal medium with succinate as the sole carbon source. Cells (25 ml) were pelleted, washed three times in M9 salts, and resuspended in 1 ml of M9 salts. Twenty microliters of this cell suspension was used to inoculate 200 ml of M9 minimal medium with 0.1% succinate plus 0.1% raffinose. Growth was monitored by measuring the absorbance of 100 μl of culture at 415 nm with a Bio-Rad 550 plate reader. Periodically, a quantity of culture (1 to 10 ml, depending on the culture density) was pelleted, resuspended in 15% glycerol, and stored at −80°C until all samples had been taken. After all samples were collected, the fluorescence of the pellets was measured by using a CytoFluor 4000 fluorimeter (PerSeptive Biosystems, Foster City, Calif.) with excitation at 485 nm and emission at 508 nm. Relative fluorescence was determined by dividing sample fluorescence by sample optical density at 415 nm. Optical density at 415 nm was determined by using a Bio-Rad 550 plate reader.

Catabolite repression of the p*melA*::*gfp* fusion was measured by growing test strains in tubes (18 by 150 mm) containing 2.5 ml of M9 minimal medium with 0.4% succinate plus 0.4% raffinose. Under these conditions, some succinate remains when cells enter stationary phase and can exert catabolite repression on the p*melA*::*gfp* fusion. After 72 h, 100 μl of culture was removed, and its relative fluorescence was determined as described above.

Raffinose uptake assays. Bacterial strains were grown overnight in M9 minimal medium with 0.4% succinate or 0.4% raffinose as the sole carbon source. Cells grown in succinate were used to inoculate 25 ml of M9 minimal medium containing 0.4% succinate plus 0.4% raffinose, and cells grown overnight in raffinose were used to inoculate 25 ml of M9 minimal medium containing 0.4% raffinose. Cultures were grown to mid-exponential phase at 30°C with shaking. The raffinose culture was split, and succinate was added to one of the portions to a final concentration of 0.4%. All cultures were incubated for an additional hour. One to 2 ml of culture containing raffinose plus succinate was washed once with M9 salts plus 0.4% succinate, resuspended in 100 μl of M9 salts plus 0.4% succinate, and incubated at room temperature for 5 min. Nine hundred microliters of M9 salts plus 0.4% succinate and 10 μl of 10 mM [^{3}H]raffinose (0.1 mCi/ml) (American Radiolabeled Chemicals, Inc.) were added to the cell suspension. Raffinose-grown cells were prepared in the same way, except that they were washed and suspended in M9 salts without succinate. Samples (100 μl) were removed every 4 min and filtered through 45-μm-pore-size nitrocellulose filters. The filters were immediately rinsed three times with 5 ml of M9 salts containing 10 mM raffinose, and counts were determined with a scintillation counter. Counts were normalized by dividing counts per minute by the optical density of the cell suspension.

RESULTS

Genetic screen for mutants unable to establish succinate-mediated catabolite repression of the *melA-agp* operon. To conduct a screen for catabolite repression mutants, we made use of the fact that an *agpA*::Tn*phoA* reporter is highly expressed when *S. meliloti* is grown on the α-galactoside raffinose but is repressed when the organism is grown on a combination of succinate and raffinose. *S. meliloti* strain SG2001 (*agpA*::Tn*phoA*) was mutagenized with Tn*5lac* and plated on M9 plates containing selective antibiotics plus succinate, raffinose, and X-Phos. Most colonies were pale blue, indicating that succinate-mediated repression had down-regulated the *agpA*::Tn*phoA* reporter in spite of the presence of raffinose. However, 12 colonies out of the 20,000 screened were deep blue and were candidates for mutants that had altered succinate-mediated catabolite repression. Transductional mapping and Southern analysis of the Tn*5lac* mutations showed that two were linked to *agpA* (data not shown). Sequencing showed that the insertions were in open reading frame (ORF) Y21650, which encodes a *lacI*-type repressor just downstream of *agpT* (Fig. 1) (data not shown).

The orientation of Tn*5lac* in the two ORF Y21650 mutants was such that the *lacZ* reporter should not have been transcribed. However, we believed that it was still possible for the heterologous *lacZ* gene, carried on Tn*5lac* in these strains, to confound the interpretation of experiments investigating galactoside utilization. For this reason, we reisolated an insertional mutation in ORF Y21650 by using Tn*5-233*. The resulting strain, RB33, was used in the following experiments.

ORF Y21650 encodes the *lac* repressor LacR. Jelesko and Leigh reported that LacZ activity in *S. meliloti* is controlled by a repressor protein, LacR, and that mutations in *lacR* result in the constitutive expression of β-galactosidase activity (17). The genome sequence of *S. meliloti* (1, 6, 11) indicates that ORF Y21650 encodes a LacI-type repressor and is about 1,000 bp upstream of the first gene in an operon containing five genes similar to the genes involved in β-galactoside transport and catabolism (http://sequence.toulouse.inra.fr/meliloti.html) (Fig. 1). We believed that it was likely that the ORF Y21650 gene was the same as the *lacR* gene characterized genetically, but not sequenced, by Jelesko and Leigh (17). The fact that the Tn*5-233* mutation in ORF Y21650 caused the constitutive expression of β-galactosidase activity (data not shown) lent weight to this idea. To confirm that ORF Y21650 was the same as *lacR*, we partially sequenced plasmids pJGJ54 and pJGJ86, which complemented *lacR* mutations (17), and found that they carried the ORF Y21650 gene. Because the phenotype of an ORF Y21650 mutant is the same as the phenotype of a *lacR* mutant and because ORF Y21650 is carried on plasmids which can complement *lacR*, it is likely that ORF Y21650 is the same as the *lacR* locus described first by Jelesko and Leigh (17).

Mutations in *lacR* cause aberrations in succinate-mediated catabolite repression of α-galactoside and β-galactoside utili-

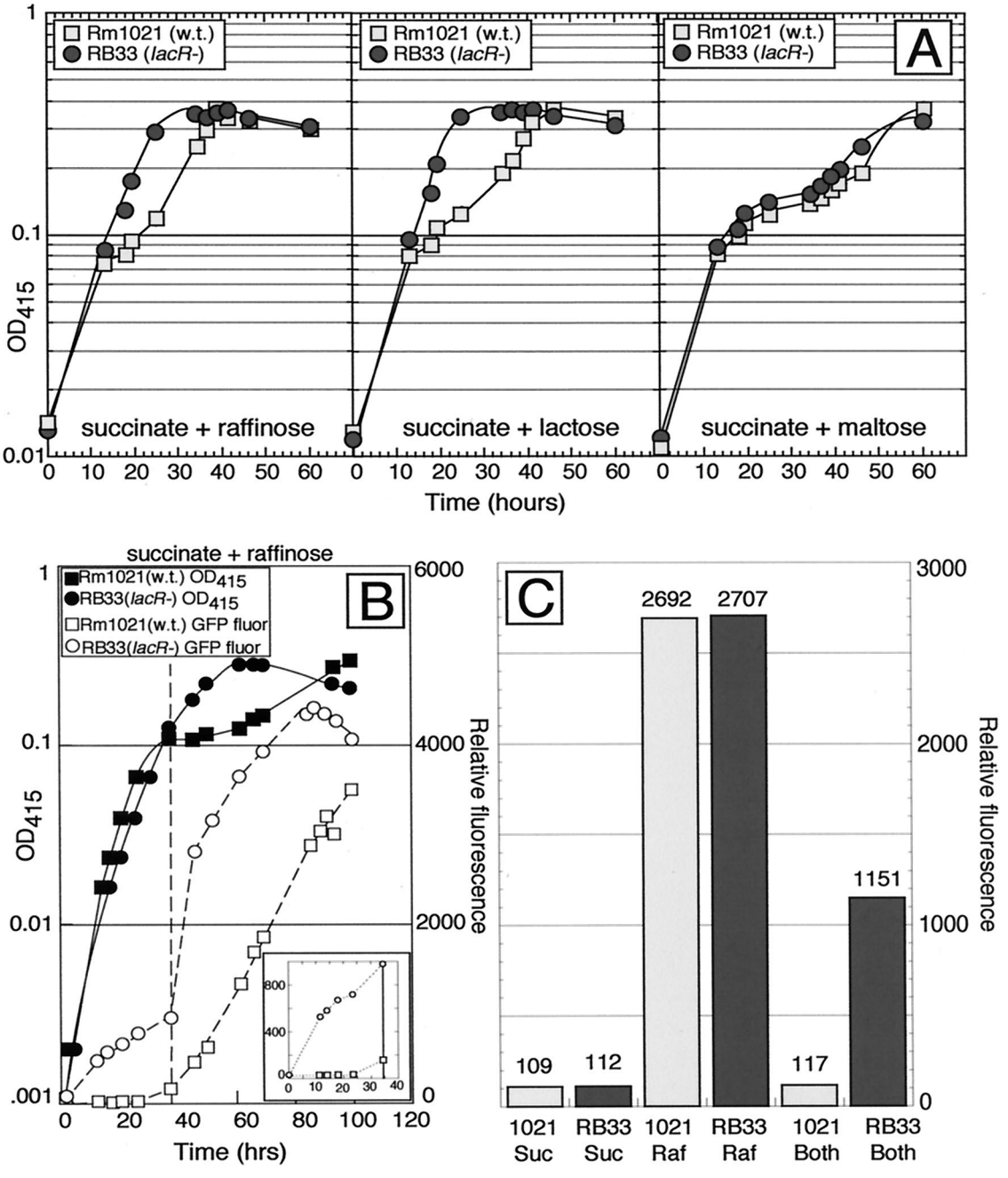

FIG. 2. The *lacR*::Tn*5-233* mutation abolishes succinate-mediated diauxic utilization of α- and β-galactosides. (A) Strains Rm1021 (wild type [w.t.]) and RB33 (*lacR*::Tn*5-233*) were grown in medium containing 0.05% succinate and 0.1% the indicated secondary carbon source. Growth was monitored by measuring the optical density at 415 nm (OD_{415}). (B). Strain RB33 (*lacR*::Tn*5-233*)/pRB27 (p*melA*::*gfp*) does not exhibit diauxie or repression of the *melA*::*gfp* reporter when grown in medium containing succinate plus raffinose. Strains Rm1021/pRB27 (p*melA*::*gfp*) and RB33 (*lacR*::Tn*5-233*)/pRB27 (p*melA*::*gfp*) were grown in M9 minimal medium containing 0.1% succinate plus 0.1% raffinose. The OD_{415} and relative green fluorescent protein (GFP) fluorescence were determined. The vertical broken line marks the time at which growth on succinate stopped in the wild-type strain. The inset shows the relative fluorescence of the strains during the first phase of diauxic growth. (C) The *lacR* mutation does not cause constitutive expression of the *melA-agp* promoter. Strains Rm1021/pRB27 (p*melA*::*gfp*) and RB33 (*lacR*::Tn*5-233*)/pRB27 (p*melA*::*gfp*) were grown in M9 minimal medium containing 0.4% succinate (Suc), 0.4% raffinose (Raf), or 0.4% succinate plus 0.4% raffinose (Both). After 72 h, cells were harvested, and the relative GFP fluorescence was determined. Numbers above the bars indicate the relative fluorescence value for each culture.

zation. *S. meliloti* wild-type strain Rm1021 showed diauxie when grown in M9 minimal medium containing a combination of succinate and either raffinose, lactose, or maltose (Fig. 2A). *lacR* mutant RB33 failed to show diauxie when grown on succinate plus raffinose or on succinate plus lactose. Diauxie on succinate plus maltose was normal, indicating that the abnormal diauxie seen in this strain was not a general phenomenon affecting the utilization of all secondary carbon sources.

Plasmid pRB27 (p*melA*::*gfp*) contains a transcriptional fusion of the *melA* promoter to *gfp*. This fusion was overexpressed in the *lacR* mutant, relative to the wild type, when succinate and raffinose were present together (Fig. 2B and C). In the experiments depicted in Fig. 2B, the fusion was induced in wild-type cells during the diauxic lag only after succinate had been consumed; however, the fusion was expressed throughout the growth curve for cells of strain RB33 (*lacR*::Tn*5-233*), including early times, when succinate was still present.

The *lacR*::Tn*5-233* mutation caused the constitutive expression of β-galactoside utilization genes. If it also caused the constitutive expression of α-galactoside utilization genes, then that would explain its ability to alleviate succinate-mediated catabolite repression in medium containing succinate plus raffinose. The data presented in Fig. 2C show relative fluorescence from the p*melA*::*gfp* fusion when strain Rm1021/pRB27 and strain RB33 (*lacR*::Tn*5-233*)/pRB27 were grown for 72 h in M9 minimal medium containing either 0.4% succinate, 0.4% raffinose, or 0.4% succinate plus 0.4% raffinose. In the wild-type strain, the p*melA*::*gfp* fusion was expressed when cells were grown in raffinose but not when cells were grown in succinate or in succinate plus raffinose. In strain RB33 (*lacR*), the fusion was not expressed when cells were grown in succinate, but it was expressed when cells were grown in raffinose or in succinate plus raffinose. In addition, the p*mel*::*gfp* fusion was not expressed in either strain when the medium contained glycerol as the sole carbon source (data not shown). Thus, the *lacR*::Tn*5-233* mutation does not cause merely constitutive expression of the *melA-agp* genes but causes aberrant expression of these genes when succinate and raffinose are present together.

Abnormal succinate-mediated catabolite repression of the *melA-agp* operon in a *lacR* mutant requires a functional β-galactoside transport system. If succinate-mediated catabolite repression acts, at least in part, by preventing the intracellular accumulation of raffinose, then the effect of *lacR* mutations on the catabolite repression of the *melA-agp* operon could be explained as follows (Fig. 3). In *lacR* mutants, β-galactoside transporters are overproduced (17). These may allow some α-galactoside molecules into the cell, where they can interact with AgpT and cause the induction of the *melA-agp* operon. The overproduction of the β-galactoside transporters may overwhelm the ability of the succinate-mediated catabolite repression system to inhibit the accumulation of α-galactoside inducers. If α-galactoside transport through overproduced β-galactoside transporters is key for the eradication of succinate-mediated control of the *melA-agp* operon in *lacR* mutants, then inactivating a β-galactoside transport gene in the *lacR*::Tn*5-233* mutant should alleviate the abnormal catabolite repression of the *melA-agp* operon caused by the *lacR* mutation.

The *lacF* gene, which is postulated to encode a permease portion of a lactose ATP binding cassette transport system, was mutated by insertion of a suicide plasmid. Strains carrying this mutation grew on lactose with a doubling time of 30 h, compared to 4 h for the wild-type parental strain (Fig. 4A). The *lacF* mutation was combined with a *lacR* mutation to produce the double-mutant strain RB46 (*lacR lacF*), which also grew slowly on lactose. Unlike strain RB33 (*lacR*), strain RB46 (*lacR lacF*) showed diauxie when grown in M9 minimal medium containing a combination of succinate and raffinose (Fig. 4B). Thus, the double mutant, with defects in *lacR* and β-galactoside transport, appears phenotypically normal with respect to succinate-mediated repression of α-galactoside utilization genes. This finding indicated that the transport of some raffinose through β-galactoside transporters is required for the abnormal catabolite repression phenotype seen when a *lacR* strain is grown on succinate plus raffinose. Even though enough raffinose appears to cross into the cell via the Lac transport system to trigger the induction of the *melA-agp* genes, the amount must be rather small, because *agpA* (12) or *agpB* mutants cannot grow on α-galactosides (data not shown). Even when the Lac transport system is fully induced, not much raffinose crosses into the cell via this system, because *lacR agpA* double mutants are also unable to grow on α-galactosides (data not shown).

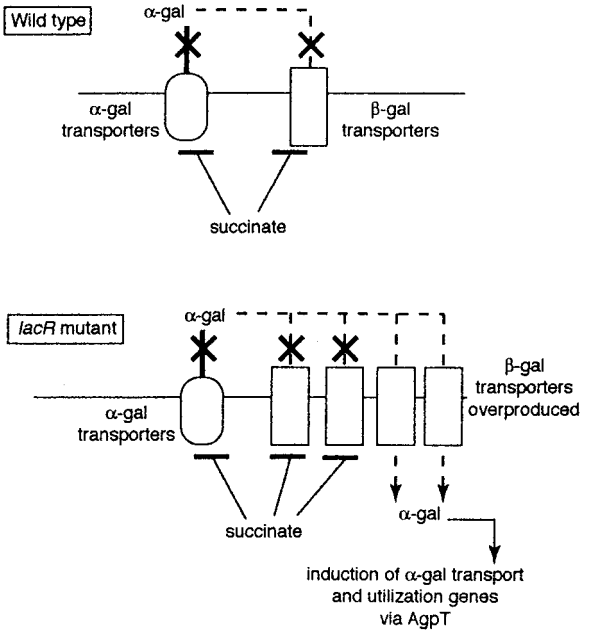

FIG. 3. Model to explain the effects of *lacR* mutations on succinate-mediated repression of α-galactoside utilization. The model depicts the α-galactoside (α-gal) and β-galactoside (β-gal) transport systems when wild-type (top) and *lacR* mutant (bottom) cells are growing on succinate plus an α-galactoside. The model postulates that succinate-mediated catabolite repression of the *melA-agp* operon is due, in part, to succinate causing inducer exclusion of α-galactosides. In the *lacR* mutant, the overproduction of β-galactoside transporters would allow some α-galactosides into the cell, thus short-circuiting succinate-mediated repression. The details of the model are described in the text.

α-Galactoside accumulation is inhibited by succinate. The experiments described above suggested that succinate repression of the *melA-agp* genes operates by preventing inducer accumulation. We tested this idea directly by conducting raffinose uptake assays with strain Rm1021 grown in M9 minimal medium containing 0.4% raffinose, 0.4% raffinose with 0.4% succinate present continuously, or 0.4% raffinose with 0.4% succinate added 1 h before the uptake assay was started. The results showed that cells grown in raffinose with succinate present continuously did not transport raffinose (Fig. 5B). Cells grown in raffinose and exposed to succinate for 1 h before

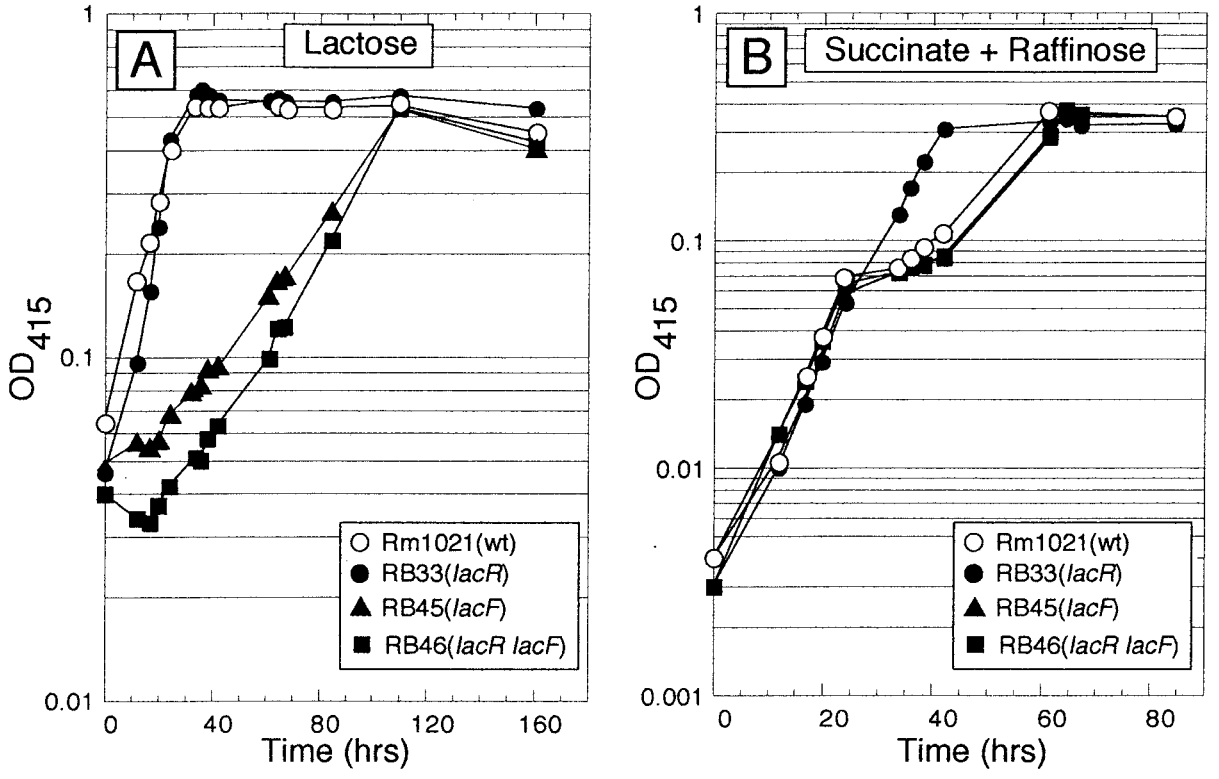

FIG. 4. The *lacR lacF* double mutant shows wild-type growth in succinate-plus-raffinose medium. (A) *lacF* mutants grow very slowly on lactose. The indicated strains were grown in M9 minimal medium containing lactose as the sole source of carbon. OD_{415}, optical density at 415 nm. (B) Succinate-mediated diauxie of α-galactoside utilization is restored in a *lacR lacF* double mutant. The indicated strains were grown in M9 minimal medium containing 0.05% succinate plus 0.1% raffinose. The *lacR* mutant failed to undergo diauxic growth, whereas the *lacR lacF* double mutant exhibited diauxic growth which was indistinguishable from that of wild-type (wt) strain Rm1021.

the uptake assay were restricted in their ability to transport raffinose compared to raffinose-grown cells not exposed to succinate. Such cultures showed a 45 to 80% reduction in the accumulation of raffinose compared to control cultures not exposed to succinate (Fig. 5A).

Unlike wild-type strain Rm1021 (Fig. 5A), *lacR* mutant strain RB33 was able to transport raffinose when grown in M9 minimal medium containing raffinose with succinate present continuously (Fig. 5B).

The biochemical data presented above do not indicate whether succinate decreased raffinose uptake by preventing raffinose from entering the cell or by accelerating its efflux from the cell. That is, succinate may decrease raffinose uptake through inducer exclusion, inducer expulsion, or a combination of the two.

DISCUSSION

Succinate is able to repress the utilization of many different carbon sources, including α-galactosides and β-galactosides in *S. meliloti*. In order to better understand this phenomenon, we conducted a screen to identify genes involved in the succinate-mediated repression of α-galactoside utilization. This screen revealed that *lacR* mutants no longer repressed the *melA-agp* operon when succinate was present along with raffinose. The mutations also eradicated the diauxie normally seen when cells are grown in M9 salts containing succinate plus raffinose. The elimination of succinate-mediated repression of the *melA-agp* operon in *lacR* mutant strains was not caused by a general derepression of the *melA-agp* operon, because these mutants did not exhibit constitutive expression of the *melA-agp* operon when grown on single carbon sources such as glycerol or succinate.

Experiments were done to determine whether the altered succinate-mediated catabolite repression seen in *lacR* mutants was general or restricted to the *melA-agp* operon. These experiments showed that the *lacR* mutation also eradicated succinate-mediated catabolite repression in a medium containing succinate plus lactose but did not do so in a medium containing succinate plus maltose. The *lacR* mutant strains constitutively expressed LacZ activity in all media tested. Thus, with succinate plus lactose, the altered succinate-mediated repression seen in the *lacR* mutants was likely due to the fact that the genes needed for lactose transport and utilization are always overexpressed.

While it is easy to understand how the *lacR* mutations gave rise to a loss of succinate-mediated repression with respect to lactose, it is not so easy to explain the effects of these mutations on the succinate-mediated repression of α-galactoside utilization. This is because the *melA-agp* operon is aberrantly expressed only when an α-galactoside is present along with succinate. Our data best support the idea that succinate normally prevents raffinose from accumulating in the cell when both are

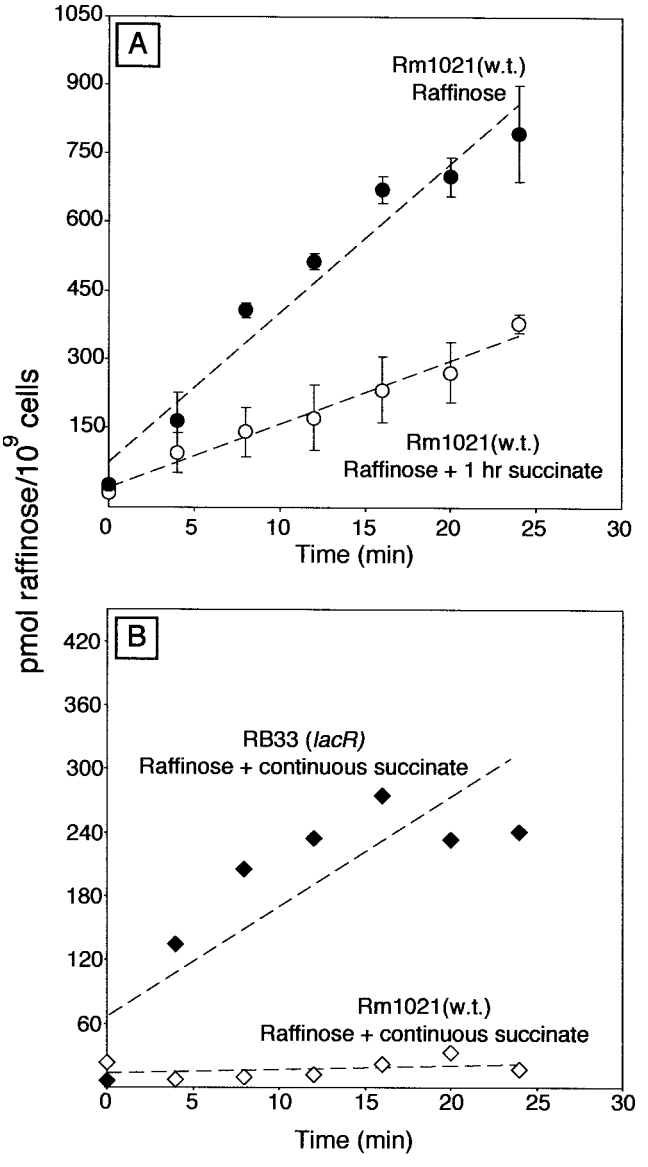

FIG. 5. Succinate inhibits the accumulation of raffinose. (A) Strain Rm1021 (wild type [w.t.]) was grown in M9 minimal medium plus 0.4% raffinose. A portion of the culture was exposed to 0.4% succinate for 1 h, and then the uptake of [^{3}H]raffinose was measured in both the raffinose and the raffinose-plus-succinate cultures. Error bars indicate one standard error. (B) Strains Rm1021 and RB33 (*lacR*::Tn*5-233*) were grown in M9 minimal medium containing 0.4% raffinose plus 0.4% succinate. Succinate was present continuously as cells were grown in preparation for uptake measurements.

present together. The overproduction of β-galactoside transporters in the *lacR* mutants (17) may overwhelm the ability of the succinate-mediated catabolite repression system to inhibit some Lac transporter-dependent accumulation of α-galactoside inducers, even though the succinate-mediated repression system itself is fully operational. The fact that *lacR lacF* double mutants are normal with respect to the succinate-mediated repression of the *melA-agp* genes indicates that the overexpression of the Lac transport system is critical for the aberrant succinate-mediated repression of the *melA-agp* genes observed in the *lacR* mutants.

Raffinose uptake assays supported the hypothesis that succinate prevents raffinose from accumulating when cells are grown in the presence of both succinate and raffinose. Wild-type cells growing in raffinose and exposed to succinate for 1 h exhibited a 45 to 85% decrease in their rate of raffinose accumulation. Cells grown in raffinose and succinate have a doubling time of about 6 h; thus, the decrease in raffinose uptake seen following the addition of succinate for 1 h cannot be accounted for by a halt of α-galactoside transporter synthesis followed by dilution of the remaining transporters by cell growth. If synthesis of the transporters were shut off completely for 1 h, then one would expect a one-sixth (16%) decline in the rate of raffinose accumulation, because the number of active transporters per culture mass would decline 16%.

Wild-type cells grown in raffinose with succinate continually present did not accumulate raffinose to any significant extent (Fig. 5). The difference in α-galactoside transport between cells grown in raffinose with succinate present continuously and those grown in raffinose and exposed to succinate for 1 h is similar to the effect of preinduction on catabolite repression of the *lac* system by glucose in *E. coli* (7a). The more complete shutdown of raffinose transport seen in the cells exposed to raffinose and succinate continuously may have resulted from one or more of the following: (i) active down-regulation of *melA-agp* expression by transcriptional factors, (ii) down-regulation of *melA-agp* expression caused by lower internal concentrations of inducer resulting from nearly complete inhibition of α-galactoside transporter activity, or (iii) succinate-dependent degradation of proteins required for α-galactoside transport.

In bacterial species which exhibit glucose-mediated catabolite repression, glucose status is linked to gene repression and/or inducer exclusion by the Hpr or GlcIIA proteins. These two proteins, which are directly involved in glucose transport, also mediate inducer exclusion, cyclic AMP synthesis, and repression of catabolic genes (31, 36). How the information concerning succinate status is transmitted to the regulated transport systems in rhizobia is not yet known. Jelesko and Leigh showed that the dicarboxylic acid transporter DctA was required for the succinate-mediated catabolite repression of the Lac system (17). It is possible that DctA bound to succinate can meditate inducer exclusion directly or that DctA is needed only to bring succinate into the cell, where the presence of succinate then can be signaled by a variety of means: intracellular concentration of tricarboxylic acid cycle intermediates, flux of reducing equivalents through the electron transport chain, or proteins that bind dicarboxylic acids and cause inducer exclusion or expulsion directly.

We have shown that succinate establishes catabolite repression of the *melA-agp* genes, at least in part, by preventing intracellular inducer accumulation. Currently, we are working on identifying other mutants that are altered in this process. We hope to find mutants that are altered in the systems that convey information about the succinate status of the cell and transmit that information to secondary carbon source transporters. Such mutants should provide information on how succinate-mediated catabolite repression operates in rhizobia and may eventually shed light on how this process works in other

species which use dicarboxylic acids as preferred carbon sources.

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