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Sinorhizobium meliloti Mutants Lacking Phosphotransferase System Enzyme HPr or EIIA Are Altered in Diverse Processes, Including Carbon Metabolism, Cobalt Requirements, and Succinoglycan Production▽†

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***Sinorhizobium meliloti* is a member of the *Alphaproteobacteria* that fixes nitrogen when it is in a symbiotic relationship. Genes for an incomplete phosphotransferase system (PTS) have been found in the genome of *S. meliloti*. The genes present code for Hpr and ManX (an $EIIA^{Man}$-type enzyme). HPr and EIIA regulate carbon utilization in other bacteria. *hpr* and *manX* in-frame deletion mutants exhibited altered carbon metabolism and other phenotypes. Loss of HPr resulted in partial relief of succinate-mediated catabolite repression, extreme sensitivity to cobalt limitation, rapid die-off during stationary phase, and altered succinoglycan production. Loss of ManX decreased expression of *melA-agp* and *lac*, the operons needed for utilization of α- and β-galactosides, slowed growth on diverse carbon sources, and enhanced accumulation of high-molecular-weight succinoglycan. A strain with both *hpr* and *manX* deletions exhibited phenotypes similar to those of the strain with a single *hpr* deletion. Despite these strong phenotypes, deletion mutants exhibited wild-type nodulation and nitrogen fixation when they were inoculated onto *Medicago sativa*. The results show that HPr and ManX ($EIIA^{Man}$) are involved in more than carbon regulation in *S. meliloti* and suggest that the phenotypes observed occur due to activity of HPr or one of its phosphorylated forms.**

Bacteria belonging to the genera *Sinorhizobium*, *Rhizobium*, and *Bradyrhizobium* are members of the *Alphaproteobacteria*, a group of organisms that contains many intracellular symbionts and pathogens. *Sinorhizobium meliloti* can grow in soil as a free-living organism or as a nitrogen-fixing symbiont inside root nodules of alfalfa and several other plants belonging to the family Leguminosae (9, 19, 34, 46, 53, 66).

Free-living rhizobia are like many heterotrophic bacteria in that they can utilize a large number of compounds as sources of carbon, including sugars, amino acids, and tricarboxylic acid cycle intermediates. Although *S. meliloti* can utilize a variety of compounds, succinate and other C_4-dicarboxylic acids play an especially important role in metabolism during both the free-living and symbiotic states. For example, C_4-dicarboxylic acids are used to fuel and provide reducing equivalents for nitrogen fixation by bacteroids (7, 28, 30, 62). Mutants unable to transport C_4-dicarboxylic acids are able to nodulate plants, but the bacteroids do not fix nitrogen (26, 63). In addition to its role in symbiotic nitrogen fixation, succinate is a favored carbon and energy source for free-living rhizobia. It supports one of the highest rates of growth in laboratory medium and also exerts catabolite repression on the utilization of other carbon sources. In *S. meliloti*, succinate is used in preference to the secondary carbon sources glucose (36), fructose (36), galactose (36), lactose (39, 69), *myo*-inositol (57), and several pentoses and polyols (36). This preference for succinate can be manifested as diauxie when *S. meliloti* is grown on succinate plus a secondary carbon source (36). When *S. meliloti* is grown on succinate plus lactose, succinate downregulates transcription of the *lac* operon (39, 69). It has been shown that succinate also prevents the accumulation of lactose and raffinose by inducer exclusion or inducer expulsion (11).

Glucose-mediated catabolite repression in *Escherichia coli* is probably the best-studied system of global regulation of carbon utilization (Fig. 1). It consists of two mechanisms of control: inducer exclusion and gene regulation by cyclic AMP (cAMP) (12, 32, 38, 40, 43). The glucose phosphotransferase system (PTS) transports and phosphorylates glucose, which makes it ready for metabolism. $EIIA^{Glc}$ is phosphorylated by HPr and donates its phosphate to glucose molecules transported into the cell; thus, $EIIA^{Glc}$ exists mainly in the unphosphorylated state when glucose is being utilized (Fig. 1). Unphosphorylated $EIIA^{Glc}$ inhibits transporters required for the uptake of secondary carbon sources when these transporters are bound with ligand. When glucose is absent, $EIIA^{Glc}$-P predominates and activates adenylate cyclase, the enzyme responsible for cAMP synthesis. In conjunction with the Crp protein, cAMP activates transcription of genes needed to catabolize secondary carbon sources (65, 67). Thus, the $EIIA^{Glc}$ enzyme plays two important roles in carbon metabolism in *E. coli*: it prevents secondary carbon sources from entering the cell when a primary carbon source is present, and it participates in the induction of the genes responsible for catabolism of secondary carbon sources when the primary carbon source is absent, by activating adenylate cyclase.

Gram-positive bacteria regulate carbon utilization through the PTS enzyme HPr rather than through EIIA (12, 42, 60, 71)

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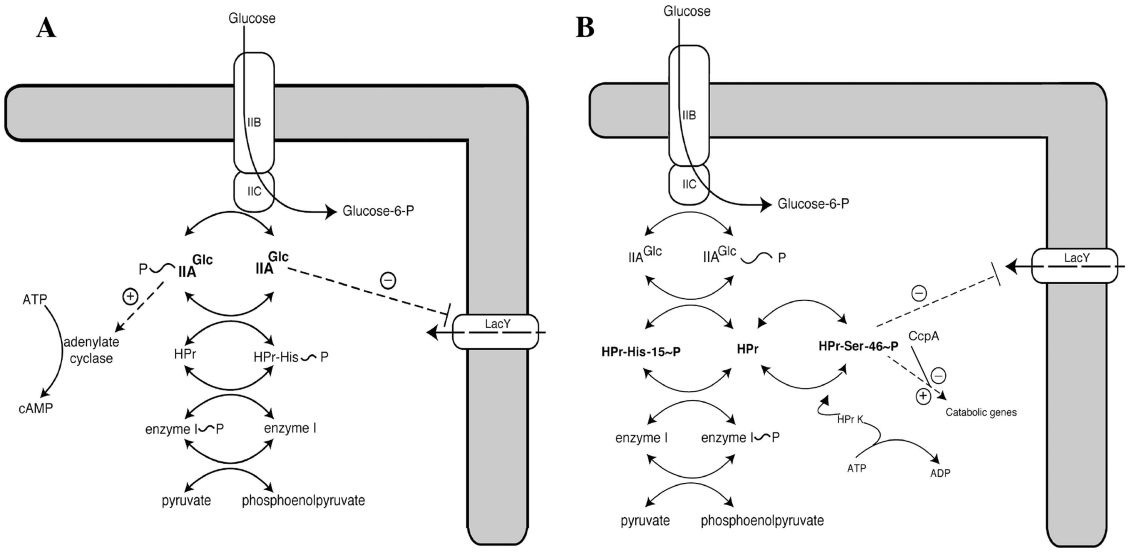

FIG. 1. Glucose-mediated regulation of secondary carbon source (lactose) metabolism in *E. coli* (A) and *B. subtilis* (B).

(Fig. 1). *Bacillus subtilis* and other low-G+C-content gram-positive bacteria possess a complete PTS for uptake and utilization of carbohydrates. As it does in gram-negative bacteria, phosphate flows from phosphoenolpyruvate to the transported carbohydrate. During this process HPr is phosphorylated at the histidine-15 residue by PTS enzyme I. Additionally, an HPr kinase/phosphatase (HPrK) can phosphorylate the serine-46 residue of HPr using either ATP or pyrophosphate (49, 54). HPr-Ser46-P is a poor substrate for additional phosphorylation at HPr-His15. High levels of HPr-Ser46-P accumulate under energy-replete conditions due to high levels of ATP and PP_i and high HPrK activity. HPr-Ser46-P can associate with the LacI-type transcriptional regulator CcpA, and the complex can activate or repress transcription of numerous genes (2, 60, 71). The commonly repressed genes are associated with the catabolism of secondary carbon sources. There is evidence that in some gram-positive bacteria HPr-Ser46-P interacts with sugar transport systems, resulting in inducer exclusion or inducer expulsion (51, 71). Thus, the ratio of HPr-Ser46-P to HPr-His15-P is important in regulating carbon metabolism and may also mediate inducer exclusion or expulsion (8).

The PTS not only regulates utilization of sugars but also regulates other bacterial processes, often in a manner linked to sugar utilization. For example, in *Listeria monocytogenes* several virulence genes are repressed in the presence of glucose by a CcpA-independent mechanism (21, 29). In contrast, in bacteria belonging to the group A streptococci, expression of the virulence regulator Mga is stimulated by CcpA. In addition to virulence genes, Mga regulates many metabolic genes, thereby linking metabolism to virulence (1, 35). The *Bacillus anthracis* virulence transcription factor AtxA is also controlled in a carbon-source-dependent manner. There is evidence that phosphorylation by HPr and EIIB modulates the activity of AtxA (68).

In *Pseudomonas putida* the PTS orthologs EI^{Ntr}, NPr, and $EIIA^{Ntr}$ are distinct from homologs involved in carbohydrate transport and are usually involved in nitrogen regulatory networks (20). In *P. putida* they are involved in the control of polyhydroxyalkanoate synthesis, and in *E. coli* $EIIA^{Ntr}$ regulates the potassium transporter TrkA and affects growth on organic nitrogen (44, 58, 70).

It appears that *S. meliloti* is missing genes required to assemble a complete PTS. The genome of *S. meliloti* contains smc02754 (*hpr*), a gene similar to *ptsH* (coding for HPr in *E. coli*) and smc02753 (*manX*) (coding a protein similar to the N-terminal half of *E. coli* ManX, which contains an $EIIA^{Man}$ PTS domain). However, the *S. meliloti* genome has no genes encoding proteins similar to the EIIB or EIIC components of PTS transport systems (4, 14, 25). The genes encoding *S. meliloti* EI^{Ntr} and $EIIA^{Ntr}$ (smc02437 and smc01141, respectively) are at a chromosomal site separate from *hpr* and *manX*.

The *manX* and *hpr* genes are adjacent to each other on the *S. meliloti* chromosome, are oriented the same direction, and are likely cotranscribed because the start codon of *hpr*, the downstream gene, overlaps the stop codon of the upstream *manX* gene. Importantly, *S. meliloti* HPr contains both the critical histidine that is phosphorylated in other bacteria during carbohydrate transport and the serine residue that is phosphorylated by HPrK in gram-positive bacteria during catabolite control (22, 72). In addition, the *S. meliloti* EIIA homolog, ManX, contains the histidine residue that is phosphorylated by HPr (24). Upstream of the *S. meliloti manX* gene is an open reading frame (smc02752) that encodes a protein similar to HPrK.

No genes encoding proteins similar to the gram-positive transcriptional regulator CcpA, which also contain known Hpr-binding motifs, are present in the *S. meliloti* genome (41; D. J. Gage, unpublished results). A search of the genome for genes encoding Crp-like proteins revealed genes encoding two potential proteins belonging to the Crp/FNR family of regulators. Members of this family are versatile transcription factors that are involved in processes such as nitrogen fixation, photosynthesis, sensing of oxidative stress, metabolism, and catabolite repression.

It is clear that succinate-mediated catabolite repression (SMCR) in *S. meliloti* is mechanistically different (at least

partially) from catabolite repression in the well-studied models described above. This is because preferred carbon sources in the model bacteria are transported via the PTS. In contrast, succinate enters *S. meliloti* via a very different system, the DctA permease. This paper describes results showing that some of the PTS genes needed for PTS-dependent catabolite repression in well-studied models affect SMCR and other aspects of carbon metabolism in *S. meliloti*. The results also show that in *S. meliloti* the PTS proteins are involved in regulation of other cellular processes as well.

MATERIALS AND METHODS

Media, strains, and plasmids. *S. meliloti* strain Rm1021 was used as the parent for construction of all mutants. *S. meliloti* strains were grown in TY medium or in M9 mineral salts medium supplemented with biotin (5 μg/ml), cobalt (5 ng/ml $CoCl_2$), and carbon sources at the concentrations indicated below. *E. coli* XL1B MRF′ (Stratagene) was the host used for construction of all plasmids and was grown in LB medium supplemented with ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) (48 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml). Media were supplemented with antibiotics at the following concentrations when necessary: streptomycin, 500 μg/ml; ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 10 μg/ml; and gentamicin, 30 or 10 μg/ml.

Construction of *hpr* and *manX* in-frame deletion mutants. For construction of an unmarked, in-frame deletion of the *hpr* gene (smc02754), the 5′ end of *hpr* and the upstream sequence were amplified from strain Rm1021 genomic DNA using primers 192 (5′-GAGCTCGACGGCGTCATCATCCTGA-3′) and 193 (5′-TCTAGATGCATGCGCGTCAGAGCCGTGTC-3′). In the same manner, a piece of DNA including the 3′ end of *hpr* and downstream sequences was amplified using primers 194 (5′-TCTAGATGCATGCGGTTCGCGACAGGTT CGG-3′) and 195 (5′-CTCGAGCATTTCCGTTTCGGCGA-3′). The primers were designed so that when the fragments were cut with NsiI and ligated together, they would remain in frame and a central 228-bp region of the *hpr* gene would be left out. After the fragments were ligated, the product was cloned into pJQ200SK, a suicide vector that carries *sacB* and a gene for gentamicin resistance, creating plasmid pRB88. After confirmation of the in-frame deletion by sequencing, plasmid pRB88 was moved into strain Rm1021 by triparental mating. The suicide plasmid was integrated by homologous recombination into the chromosomal *hpr* gene, resulting in strain RB108. This strain was then grown in TY broth without antibiotics to allow a second recombination that would eliminate the suicide vector and leave the Δ*hpr* allele in the chromosome. The culture was spread plated onto TY medium with 5% sucrose, and sucrose-resistant isolates were screened for gentamicin sensitivity to select for isolates that had lost the suicide vector. Isolates were screened by PCR for the presence of the Δ*hpr* allele. One strain, RB111 (Δ*hpr*) having an in-frame *hpr* deletion, was purified and used for further study. A *manX* (smc02753) in-frame deletion mutant was constructed in a similar manner. The primers used to construct the *manX* in-frame deletion mutant were primers 186 (5′-ATCTAGAGCGGGATGAGCAA AAGTGTTT-3′) and 187 (5′-TGATCATGCATGCCGAAACTCATCCGCC-3′) for amplification of the 5′ fragment and primers 188 (5′-TGATCATGCAT GCAGGCGTGCGCGG-3′) and 189 (5′-CTGCAGGCCGCGTTTGTTGACG-3′) for amplification of the 3′ fragment. The primers were designed so that when the fragments were cut with SphI and ligated together, they would remain in frame and a central 243-bp region of the *manX* gene would be left out. The final *S. meliloti* strain having the unmarked deletion in *manX* was designated RB105. An *hpr-manX* double mutant was constructed using primers 186 and 187 for amplification of the 5′ end of *manX* and primers 194 and 195 for amplification of the 3′ end of *hpr*. The strain with the deleted *manX-hpr* fragment was designated CAP32.

Complementation of deletion mutants. The unmarked deletion mutants were complemented by inserting a single copy of each gene into the chromosome under the control of its native promoter. The DNA fragment for the *hpr-manX* operon, including the intergenic region upstream of *manX*, was amplified by PCR and cloned into pGEM-T Easy (Promega), and using this construct plasmids were constructed by "outward PCR" (by amplifying the plasmid with primers directed outward from the boundaries of the fragment to be deleted and then ligating the PCR product). One resulting plasmid had an in-frame deletion of the *manX* gene and an intact *hpr* gene (pDG127). The other had an in-frame deletion of the *hpr* gene and an intact *manX* gene (pDG126). The promoter gene sequences from pDG127 and pDG126 were cloned into pCAP77 as EcoRI fragments, creating plasmids pCAP81 (*hpr*) and pCAP80 (*manX*), respectively. pCAP77 is a pMB439-based suicide plasmid (5) that contains a fragment of the *S. meliloti* smc02324 gene followed by a *trp* terminator upstream of a multiple cloning site. This plasmid recombines into smc02324 and renders cells unable to grow on rhamnose as the sole carbon source, and it inserts into the chromosome any gene that has been cloned into the multiple cloning site (C. Arango Pinedo and D. J. Gage, unpublished). Plasmids pCAP80 and pCAP81 were moved into the Δ*manX* and Δ*hpr* mutant strains, respectively. Insertion into the smc02324 gene was confirmed by the inability of the strains to grow on M9 medium plates containing rhamnose and by the results of PCR screens with appropriate primers.

Construction of a *melA* promoter reporter plasmid. To construct a reporter plasmid for the *melA* promoter, a 338-bp fragment containing the 5′ end of *agpT* (21 bp), the *melA* promoter region (195 bp), and the 5′ end of *melA* (122 bp) (10) was amplified from plasmid pRB27 with primers 180-RB27 (5′-CCGCTC GAGCCATCGATTGGCGGCCGCTCTAGAACTA-3′; XhoI restriction site underlined) and 181-*melA* (5′-ACGCGTCGACTCAGATCCGTCAACGC GAA-3′; SalI restriction site underlined). The product was cut with XhoI and SalI and cloned into the SalI site of pDG64, which is between the *trp* terminator and *gfp*(*mut1*), creating pCAP03. pDG64 is the same as pDG65 described by Rosado and Gage (64), but pDG64 contains *gfp*(*mut1*) instead of *gfp*(*mut3*) (16, 23). The terminator, promoter, and *gfp* were excised from pCAP03 with HindIII and KpnI and cloned into plasmid pMB393 to obtain pCAP11. pCAP11 was electroporated into strain Rm1021. Green fluorescent protein (GFP) expression patterns during growth on raffinose and melibiose (α-galactosides), on succinate, and on succinate plus α-galactosides confirmed that all P*melA* regulatory sequences were present and functional in the reporter plasmid.

Growth curves and growth rates. Growth curves were obtained by removing 100-μl samples from liquid cultures growing in flasks with constant shaking at 30°C and determining the optical density at 595 nm (OD_{595}) with a microwell plate reader (Microplate Reader 550; Bio-Rad). Alternatively, growth curves were obtained using 48-well plates (culture volume, 200 μl) and a Biotek Synergy HT-1 multidetection microplate reader at 30°C with automatic reading and shaking every 10 min. Growth rates were calculated from the data using a least-squares fit method and data points for the exponential phase of growth. Cultures used for growth curve analyses were inoculated to obtain an initial OD_{595} of 0.005 using starter cultures in M9 medium containing glycerol in the exponential phase of growth that had been rinsed in carbon-free M9 salts.

GFP fluorescence measurement. GFP fluorescence was measured while 200 μl cultures were growing in 48-well plates at 30°C with shaking (excitation wavelength, 480 nm; emission wavelength, 520 nm). Carbon sources were used at a concentration of 0.4% (succinate alone or raffinose alone) or (when succinate and raffinose were supplied together) at concentrations of 0.05% (succinate) and 0.1% (raffinose). Specific fluorescence was calculated by dividing the background-corrected fluorescence by the OD_{595} of the sample.

Determination of β-galactosidase activity. Strains were grown in tubes with succinate or lactose as the sole carbon source (0.4%) or with succinate plus lactose (0.2 and 0.1%, respectively). The optical density was monitored, and samples were retrieved during the early exponential to mid-exponential phase. Culture samples were pelleted and frozen. At the time of the assay, the samples were thawed and resuspended in Z-buffer. β-Galactosidase assays were conducted as previously described (50). OD_{415} values were used to calculate the slope of the linear portion of the curve by a least-squares fit method. The values were normalized by dividing by the cell mass of the sample (initial OD_{415}) to obtain arbitrary β-galactosidase units.

Evaluation of succinoglycan production. Succinoglycan production and low-molecular-weight succinoglycan production were evaluated as previously described (75). Briefly, strains were pregrown in M9 broth with glycerol as the carbon source, rinsed, and resuspended to an optical density of 0.03, and 5 μl of each cell suspension was placed on MGS plates that contained calcofluor (0.02%). Succinoglycan production was visualized as bright fluorescence of the bacterial mass under UV light. Production of low-molecular-weight succinoglycan was indicated by the presence of a halo of fluorescence around the bacterial mass under UV light.

Nodulation assays. Alfalfa (*Medicago sativa*) seeds were sterilized and sprouted as described previously (27). Briefly, seedlings were placed on Nod3 agar slants in 18-mm glass tubes and inoculated with 100 μl of a suspension of *S. meliloti*. Suspensions were prepared by washing 1 ml of a TY medium-grown culture with 1 ml of Nod3 medium and resuspending the pellet in 10 ml of Nod3 medium. Plant growth tubes were loosely capped and incubated in a growth chamber with a cycle consisting of 16 h of light and 8 h of darkness at 26°C. Thirty-six days after inoculation nodules were counted, and plant shoots were cut above the cotyledons, dried, and weighed.

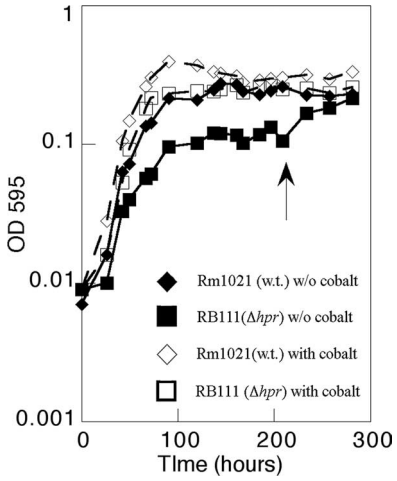

FIG. 2. Growth of wild-type strain Rm1021 and strain RB111 (Δ*hpr*) in M9 minimal medium with and without added cobalt (5 ng/ml) following two dilutions in cobalt-free medium. The arrow indicates when cobalt was added to the cobalt-deficient cultures. The data are representative of three independent experiments. w.t., wild type.

RESULTS

Construction of in-frame *hpr* and *manX* deletions. HPr and EIIA-type proteins have been shown to be involved in catabolite repression control in both high-G+C-content gram-positive and enteric gram-negative bacteria and could be expected to be involved in catabolite repression in *S. meliloti* as well. Therefore, we constructed in-frame, unmarked deletions to investigate the role of these two proteins in SMCR in *S. meliloti*.

Strain RB111 (Δ*hpr*) is especially sensitive to cobalt limitation. *S. meliloti* strain Rm1021 requires cobalt for proper growth (17, 73). Cobalt deficiency results in a decrease in the B_{12} coenzyme content in the cells and consequently a reduction in the growth rate (17). The effect of cobalt limitation is not commonly noted in the laboratory, even during growth in minimal medium lacking added cobalt, because cobalt impurities in medium components are enough to support growth (17). Strain RB111 (Δ*hpr*) frequently exhibited premature growth arrest in minimal medium. We investigated if adding cobalt could alleviate the inconsistent growth of the Δ*hpr* strain. Strains Rm1021 (wild type) and RB111 (Δ*hpr*) were grown to exponential phase in a no-cobalt M9 minimal medium with glycerol as the carbon source. Cultures were diluted into fresh minimal medium with and without added cobalt (5 ng/ml $CoCl_2$). After repeated dilution in medium without added cobalt, the final yield of strain Rm1021 (wild type) was reduced around 20%, but usually there was not a significant reduction in the growth rate compared to that of the cobalt-amended cultures. For the Δ*hpr* strain variability in growth was observed without cobalt, and often the growth rate was lower or there was growth arrest in early exponential phase resulting a 25 to 50% decrease in the growth yield. A typical growth curve demonstrating the lower yields of the Δ*hpr* strain in medium without cobalt is shown in Fig. 2. When cobalt was added early in stationary phase, growth resumed, and the culture yield was the same as the culture yield of the cobalt-amended culture, confirming that the effect was due to cobalt limitation (Fig. 2). Since the enzymes involved in methionine synthesis in *S. meliloti* require B_{12} cofactors and thus cobalt for proper function (6, 48, 73), we investigated the ability of methionine to rescue cobalt-limited cells. Cells exhibited the wild-type growth rate and yield when methionine was supplied (data not shown). Because of the dramatic effect of cobalt on the growth phenotype of strain RB111 (Δ*hpr*), cobalt was added to all minimal media used, unless otherwise noted.

Δ*hpr* mutation affects diauxic growth on succinate plus galactosides. Catabolite repression can be manifested as diauxic growth when primary and secondary carbon sources are provided to bacteria. To investigate if the lack of HPr affected SMCR phenotypes, strains Rm1021 (wild type) and RB111 (Δ*hpr*) were grown in liquid M9 medium with succinate (0.05%) plus either raffinose or lactose (0.1%) as carbon sources. When grown in medium containing succinate plus raffinose, the wild-type strain utilized succinate first, and growth lagged for about 30 h before growth on raffinose started (Fig. 3A). Strain RB111 (Δ*hpr*) exhibited a shorter lag phase (only 15 h) and resumed growth sooner than the wild type. When lactose (0.1%) was provided as the secondary carbon source, the wild type exhibited a very short diauxic lag (3 to 4 h), while strain RB111 (Δ*hpr*) showed almost no lag, and diauxie was evident only because of the abrupt change in the growth rate that resulted from switching from succinate utilization to lactose utilization (Fig. 3C). These results showed that catabolite repression was weaker in the Δ*hpr* strain and suggest that the HPr protein has a role in succinate-mediated catabolite repression.

The lower level of repression in the Δ*hpr* strain could have been due to constitutive or higher levels of expression of the *melA-agp* and *lac* operons, which code for proteins necessary for utilization of α- and β-galactosides, respectively.

To test for constitutive or elevated levels of transcription from the *melA-agp* operon, the expression from a P*melA*::*gfp* fusion plasmid (pCAP11) in the Δ*hpr* strain was measured and found to be comparable to the wild-type expression when the strains were grown in M9 medium containing raffinose alone, succinate alone, or succinate plus raffinose (Fig. 4A). These results showed that the Δ*hpr* mutation did not result in constitutive or elevated levels of *melA-agp* expression in these media.

Expression of the *lac* operon was investigated by measuring endogenous β-galactosidase levels in strains Rm1021 (wild type) and RB111 (Δ*hpr*) (Fig. 4B). The levels of activity of β-galactosidase in strain RB111 (Δ*hpr*) were equivalent to the levels in the wild-type strain during growth on lactose alone and on lactose plus succinate. However, the activity of β-galactosidase in cultures growing on succinate alone was slightly, but significantly, higher in the Δ*hpr* strain than in the wild-type strain. In all cases the activity levels were 5- to 20-fold lower than the fully induced levels, showing that SMCR was not relieved in strain RB111 (Δ*hpr*), as succinate still efficiently suppressed expression of the *lac* operon in the presence of lactose.

Thus, the Δ*hpr* mutation did not result in high-level expression of the *lac* or *melA-agp* operon in the presence of single carbon sources, nor did it result in relief of succinate-mediated repression in medium containing succinate plus lactose or raffinose.

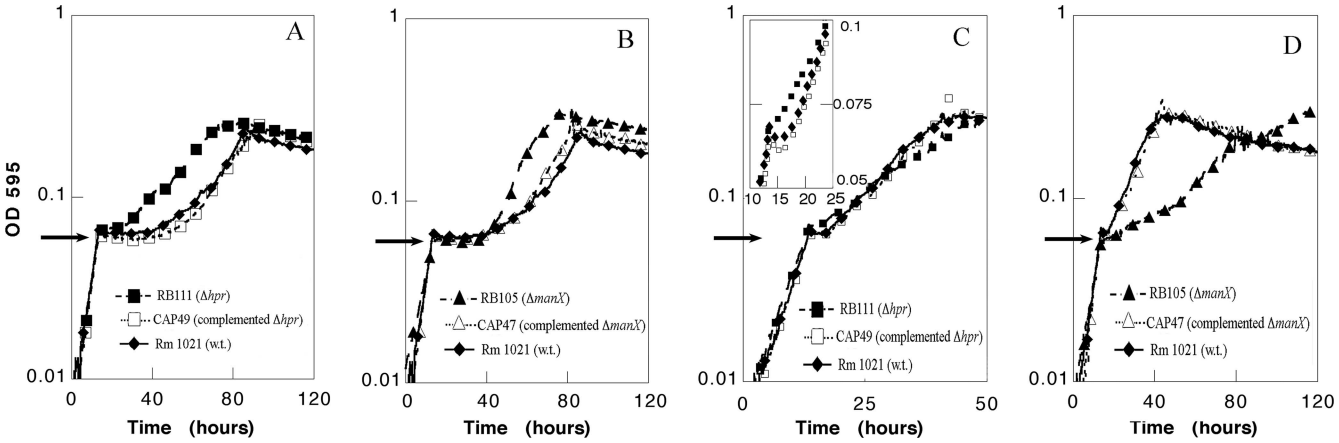

FIG. 3. Diauxie phenotypes of the Δ*hpr* strain (A and C) and the Δ*manX* strain (B and D). Data for the corresponding complemented strains are also shown. (Inset in panel C) Detail of the diauxic curve showing no diauxic lag in the Δ*hpr* strain. Strains were grown in M9 minimal medium with succinate (0.05%) plus raffinose (0.1%) (A and B) or with succinate plus lactose (0.1%) (C and D). The data are representative of three independent experiments. Curves were time shifted so that the points of succinate exhaustion in each panel were aligned. The arrows indicate the OD_{595} attained by strain Rm1021 when it was grown on 0.05% succinate (0.06 ± 0.003 [average ± standard error; $n = 4$]). w.t., wild type.

Δ*manX* mutation affects diauxic growth on succinate plus galactosides. The growth of strain RB105 (Δ*manX*) on succinate (0.05%) plus raffinose (0.1%) or on succinate (0.05%) plus lactose (0.1%) was compared to the growth of strain Rm1021 (wild type). During growth on succinate plus raffinose there was earlier release from succinate repression for the Δ*manX* strain, manifested by a slightly shorter diauxic lag (Fig. 3B). This phenomenon was not observed during growth on succinate plus lactose (Fig. 3D). In this case, strain RB105 (Δ*manX*) lagged longer than the wild type before it started to grow on lactose. The absence of ManX (EIIA^{Man}) may have altered regulation of raffinose and lactose utilization and not SMCR directly. This was tested by measuring *melA-agp* expression and β-galactosidase levels during growth (Fig. 4). The level of expression of the *melA-agp* operon during growth on raffinose, as measured by using the P*melA*::*gfp* reporter fusion (pCAP11), was twofold lower in the Δ*manX* strain than in strain Rm1021 (wild type) (Fig. 4A). The expression of the P*melA*::*gfp* reporter fusion during growth on succinate and during growth on succinate plus raffinose was not significantly different from the expression in strain Rm1021 (wild type) (Fig. 4A). These experiments showed that the Δ*manX* mutation resulted in lower levels of *melA-agp* expression in the presence of raffinose, conditions in which the genes should be fully induced, and it had no effect on *melA-agp* promoter activity under conditions in which full expression was not expected.

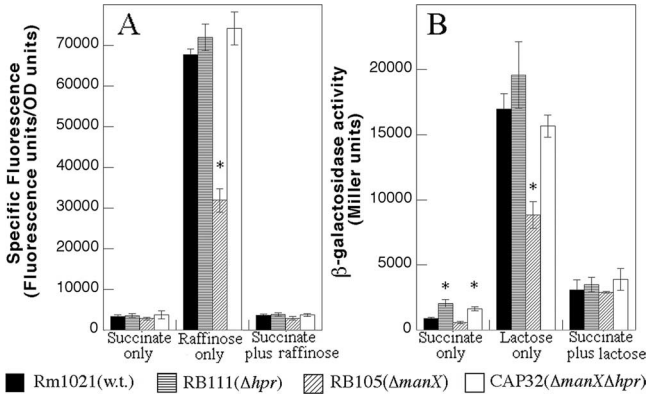

FIG. 4. (A) Expression of the *melA-agp* operon as monitored with the P*melA*::*gfp* fusion in plasmid pCAP11. Samples from mid-exponential-phase growth were used, and specific fluorescence was calculated. For dual-carbon-source growth, samples from early to mid-exponential phase during growth on succinate were used. (B) β-Galactosidase activity in PTS mutant strains. Samples from mid-exponential growth on the indicated carbon sources were assayed to determine the β-galactosidase activity. For dual-carbon-source growth, samples from the mid-exponential phase were used, when succinate was still being utilized. The bars indicate the averages of three to eight independent experiments. The error bars indicate standard errors. An asterisk indicates that a value is statistically different from the wild-type value ($P < 0.01$). w.t., wild type.

Levels of endogenous β-galactosidase were measured during growth on lactose, on succinate, and on succinate plus lactose, and the results paralleled the results for *melA-agp* expression (Fig. 4B). The enzyme levels were twofold lower in the Δ*manX* strain during growth on lactose but were not significantly different from the wild-type levels during growth on succinate or on succinate plus lactose. These results indicate that expression of both the *lac* and *melA-agp* genes was lower in the absence of ManX (EIIA^{Man}) under inducing conditions.

***hpr* deletion affects growth on specific carbon sources.** Strain RB111 (Δ*hpr*) showed alterations in diauxic growth. To determine if this strain also showed altered growth on single carbon sources, the growth rates of strains Rm1021 (wild type) and RB111 (Δ*hpr*) in M9 minimal medium with succinate, raffinose, lactose, maltose, glucose, and glycerol as carbon sources were determined (Table 1).

Strain RB111 (Δ*hpr*) exhibited wild-type growth rates in medium containing succinate, glucose, and glycerol, but the growth rates on raffinose and maltose were significantly lower. Growth of the Δ*hpr* strain on lactose was noticeably slower than growth of the wild type, but the difference was not statistically significant because of high experimental variation. Strains Rm1021 (wild type) and RB111 (Δ*hpr*) exhibited comparable growth rates on rich, complex TY medium (Table 1).

TABLE 1. Growth rate constants in M9 minimal media containing cobalt or in rich medium

Carbon source	Growth rate constant (1/h)[a]			
	Rm1021 (wild type)	RB111 (Δ*hpr*)	RB105 (Δ*manX*)	CAP32 (Δ*manX* Δ*hpr*)
Succinate	0.18 ± 0.003	0.16 ± 0.005	0.11 ± 0.009[b]	0.18 ± 0.004
Raffinose	0.12 ± 0.008	0.08 ± 0.007[b]	0.08 ± 0.007[b]	0.07 ± 0.015[b]
Lactose	0.09 ± 0.013	0.06 ± 0.007	0.07 ± 0.012[b]	0.06 ± 0.010
Maltose	0.13 ± 0.005	0.10 ± 0.006[b]	0.10 ± 0.005[b]	0.11 ± 0.007
Glucose	0.14 ± 0.010	0.13 ± 0.012	0.10 ± 0.010[b]	0.12 ± 0.015
Glycerol	0.08 ± 0.008	0.08 ± 0.009	0.06 ± 0.006[c]	0.08 ± 0.007
TY medium	0.27 ± 0.008	0.29 ± 0.003	0.23 ± 0.006[b]	0.29 ± 0.009

[a] The values are averages ± standard errors for two to five experiments.
[b] The value is statistically different from the value for the wild-type strain ($P < 0.01$).
[c] Growth started at the normal rate (data shown) and soon changed to very slow growth.

Thus, the Δ*hpr* strain had lower growth rates on carbon sources that are used as secondary sources after succinate or glucose.

***man*X deletion affects growth rates on single carbon sources.** Since the induced levels of β-galactosidase and *melA-agp* expression were lower in the Δ*manX* strain, we investigated the effect of these lower levels on growth rates in M9 minimal medium with various single carbon sources. With all carbon sources tested (succinate, glycerol, lactose, raffinose, maltose and glucose) the growth rates of strain RB105 (Δ*manX*) were lower than the growth rates of strain Rm1021 (wild type) (Table 1). The growth rate of the deletion mutant in a rich medium (TY medium) was also lower. The low growth rates with many carbon sources, along with the low levels of expression of catabolic and transport genes needed for utilization of at least some carbon sources, suggest that ManX ($EIIA^{Man}$) may have a general role in the regulation of carbon metabolism or in other important processes that impact the growth rate. The slow growth of the Δ*manX* mutant is not likely to be the cause of its low levels of *melA*::*gfp* and β-galactosidase gene expression because the Δ*hpr* mutant, which grew as slowly in raffinose and lactose (Table 1), did not show decreased expression of *melA*::*gfp* or β-galactosidase (Fig. 4). In fact, in the Δ*hpr* mutant the level of expression of these genes is slightly higher than that in strain Rm1021 (wild type). Therefore, slow growth, by itself, is not sufficient to explain the altered expression seen in the Δ*manX* mutant.

***hpr* deletion results in poor survival during stationary phase.** The stationary-phase survival of strains Rm1021 (wild type) and RB111 (Δ*hpr*) was evaluated in four different media: rich medium (TY medium), M9 medium containing succinate, M9 medium containing glycerol, and M9 medium containing glycerol plus cobalt. Die-off in TY medium cultures started as soon as the stationary phase was reached for both the wild type and Δ*hpr* strains. The number of viable wild-type cells in the TY medium culture decreased by 1 order of magnitude and then stabilized (Fig. 5A). In TY medium, the number of strain RB111 (Δ*hpr*) cells decreased by 3 orders of magnitude, and then recovery was observed, perhaps resulting from the growth of Δ*hpr* suppressor mutants in the culture (Fig. 5A).

In cobalt-deficient M9 media, strain Rm1021 (wild type) reached the stationary phase, and then the viable counts slowly decreased twofold during the experiment. Strain RB111 (Δ*hpr*) exhibited growth arrest in early exponential phase, which is typical of cobalt limitation for this strain, and die-off started at the onset of this arrest (Fig. 5B). In contrast to the results for strain Rm1021, the viable counts in the strain RB111 (Δ*hpr*) culture decreased 2 to 4 orders of magnitude. The effect of *hpr* deletion on stationary-phase survival was not as striking when cobalt was added to the minimal medium. The number of cells was reduced by 1 order of magnitude for strain RB111 (Δ*hpr*) and by 50% for Rm1021 (wild type) (Fig. 5C). In contrast to these findings, deletion of *manX* did not affect the die-off in TY or minimal medium (Fig. 5A and data not shown).

Effects of the Δ*hpr* and Δ*manX* mutations on succinoglycan production and root nodule formation. Expression of succinoglycan, the most abundant exopolysaccharide in *S. meliloti* strain Rm1021, can be detected by plating bacteria on MGS plates with calcofluor, which fluoresces when it binds to succinoglycan (45). Fluorescence in strain RB111 (Δ*hpr*) was apparent sooner than fluorescence in strain Rm1021 (wild type) (Fig. 6). The mutant strain also exhibited a fluorescent halo 1 day before the wild type exhibited a halo, which indicated that there was earlier production of low-molecular-weight succinoglycan (75).

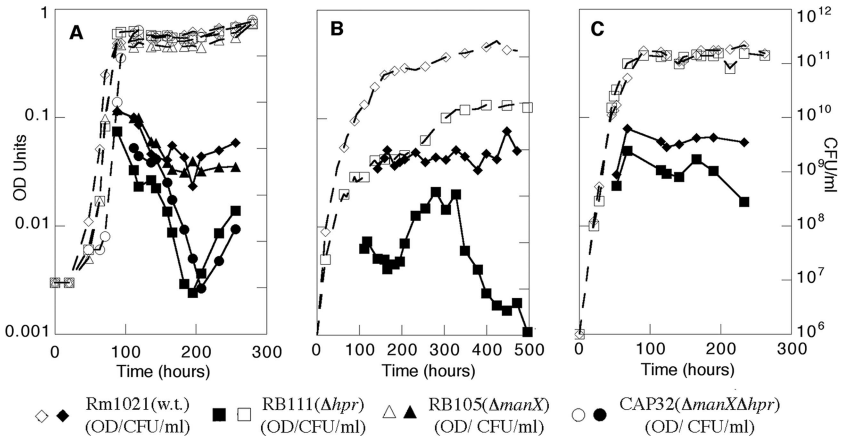

FIG. 5. Survival of strains during stationary phase. (A) TY medium. (B) M9 minimal medium plus glycerol (0.4%) without cobalt. (C) M9 minimal medium plus glycerol (0.4%) with cobalt (5 ng/ml). The data are representative of three independent experiments. w.t., wild type.

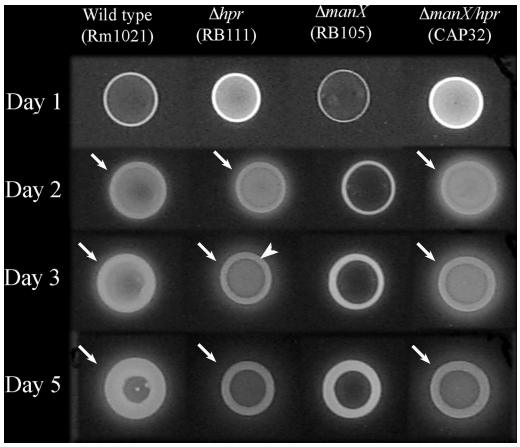

FIG. 6. Production of succinoglycan on MGS plates with calcofluor. Strains were grown in M9 broth with glycerol as the carbon source and rinsed before plating. All photographs were taken with an exposure time of 0.1 s and identical camera settings, and the images were not level or contrast adjusted. The arrows indicate the diffuse fluorescence in the low-molecular-weight succinoglycan halos. The nondiffuse rings around all of the colonies resulted from preferential cell growth at the edge of the spot (arrowhead) The fluorescence inside the spots and in the nondiffuse rings resulted from accumulated high-molecular-weight succinoglycan.

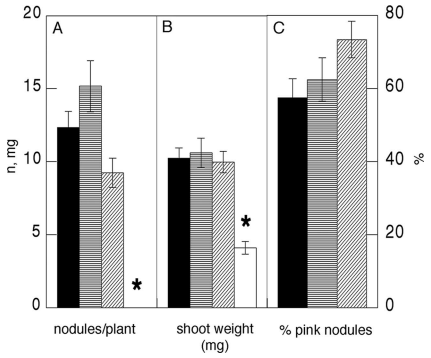

FIG. 7. Symbiotic capabilities of PTS mutant strains with *M. sativa* (alfalfa) plants: average number of nodules per plant (A), weight of plant shoots (B), and percentage of nodules that were pink (C). Solid bars, Wild-type strain Rm1021; bars with horizontal lines, Δ*hpr* strain RB111; bars with diagonal lines, Δ*manX* strain RB105; open bars, uninoculated control. The error bars indicate standard errors. An asterisk indicates that a value is significantly different from the wild-type value ($P < 0.00001$).

When strain RB105 (Δ*manX*) was plated on MGS plates with calcofluor, it exhibited a calcofluor-bright phenotype compared to strain Rm1021 (wild type) after day 1, indicating that there was enhanced accumulation of succinoglycan (Fig. 6). Production of the low-molecular-weight succinoglycan halo in the Δ*manX* strain was delayed, and the halo was smaller than that observed with strain Rm1021 (wild type).

Some *S. meliloti* mutants with altered succinoglycan synthesis are symbiotically defective, typically forming nodules that do not fix nitrogen (15, 55). Both strain RB111 (Δ*hpr*) and strain RB105 (Δ*manX*) elicited nodules on alfalfa grown in nitrogen-free medium, and the numbers were not different from the numbers observed with the wild type (Fig. 7). The nodules were functional, as judged from the presence of leghemoglobin, by the shoot weight of plants, and by the normal appearance of the internal structure of the nodules (Fig. 7 and data not shown).

Phenotypes of a Δ*manX* Δ*hpr* strain are the same as those of the Δ*hpr* mutant. An in-frame deletion that removed both *manX* and *hpr* was constructed, and the phenotypes were examined to investigate epistatic relationships between the mutations. Strain CAP32 (Δ*manX* Δ*hpr*) was evaluated for the SMCR phenotype on succinate plus raffinose and on succinate plus lactose, for growth rates on different carbon sources, for enzyme expression and activity levels, for sensitivity to cobalt limitation, for survival in stationary phase, for succinoglycan production, and for symbiotic capabilities. In all of these experiments the Δ*manX* Δ*hpr* strain exhibited phenotypes similar to those of the Δ*hpr* strain (Fig. 4 to 6 and Table 1; data not shown). This suggests that the phenotypes associated with the *manX* deletion are mediated by HPr.

Complementation of the *hpr* and *manX* deletions. To confirm that the observed phenotypes of the Δ*hpr* and Δ*manX* strains were a result of the gene deletions rather than secondary mutations, a single wild-type copy of each gene under the control of its native promoter was inserted into the rhamnose operon on the chromosome of strains RB111 (Δ*hpr*) and RB105 (Δ*manX*). The wild-type genes complemented all phenotypes tested (Fig. 3 and data not shown), confirming that the altered phenotypes were indeed due to deletion of the *hpr* and *manX* genes.

DISCUSSION

S. meliloti lacks the EII components EIIB and EIIC, as well as a classical PTS EI. It does have genes coding for HPr, ManX (EIIA^{Man}), EIIA^{Ntr}, and EI^{Ntr}. The last two proteins are part of an orthologous PTS system that is often involved in nitrogen metabolism regulation. In addition, this organism contains a gene encoding an HPrK, which is typically found in gram-positive bacteria, although it is also present in many other alphaproteobacteria (3, 37). HPrK phosphorylates HPr on a serine residue rather than at the histidine residue targeted by EI.

The *S. meliloti* Hpr protein is interesting because it displays characteristics of both the Hpr and Npr proteins. *hpr* is in a gene cluster with *manX* and *hprK*. This gene cluster is highly conserved in alphaproteobacteria, implying that the interaction of Hpr with HprK and ManX (a typical EIIA) is important and has been maintained by selection (37). This suggests that the protein has properties characteristic of classic Hpr proteins. In addition, the *S. meliloti* Hpr protein has sequence similarity to Npr proteins which interact with the EI^{Ntr} and EIIA^{Ntr} proteins. Also, it likely is phosphorylated at His15 by EI^{Ntr}, the only EI in *S. meliloti*.

In order to shed light on the role of HPr and ManX (EIIA^{Man}) in *S. meliloti*, we constructed mutants with unmarked, in-frame deletions of these genes and characterized the resulting phenotypes.

Carbon source utilization. Deletion of *manX* in *S. meliloti* resulted in strong phenotypes. The Δ*manX* strain grew slower than the wild-type strain on all carbon sources tested (succinate, glucose, glycerol, raffinose, lactose, and maltose). It ex-

hibited lower levels of expression of the P*melA*::*gfp* fusion during growth on raffinose, as well as lower β-galactosidase activity during growth on lactose. When cells were tested for SMCR on succinate plus raffinose or on succinate plus lactose, the length of the diauxic lag was altered in a secondary-carbon-source-dependent fashion. The lag was shorter than that of the wild type on succinate plus raffinose and longer than that of the wild type on succinate plus lactose.

In contrast, deletion of *hpr* did not result in altered expression of the *melA-agp* or *lac* operon as measured by P*melA*::*gfp* fusion and by endogenous β-galactosidase activity. Growth of the Δ*hpr* strain on succinate, glycerol, and glucose was normal. Growth on lactose, maltose, and raffinose, which are carbon sources subject to repression by succinate, was slower. The diauxic growth curves for succinate plus raffinose and for succinate plus lactose showed a shorter diauxic lag than the diauxic growth curves obtained with the wild-type strain, indicating that there was earlier derepression of genes needed for utilization of the secondary carbon sources. Deletion of *manX* and *hpr* together resulted in phenotypes identical to those of the Δ*hpr* strain.

One hypothesis is that the low levels of expression of raffinose and lactose catabolic genes in the Δ*manX* strain were a direct consequence of the absence of one of the forms of ManX ($EIIA^{Man}$), i.e., that ManX or ManX-P was required for normal expression of these catabolic genes. Normal P*melA*::*gfp* expression and enzyme levels in the Δ*hpr* strain, which should lack ManX-P, suggested that this form of ManX was not required. Additionally, the Δ*manX* Δ*hpr* strain, which lacked both ManX-P and ManX, also exhibited normal levels of enzyme activity and gene expression, ruling out the possibility that there was direct involvement of ManX ($EIIA^{Man}$) in the observed Δ*manX* phenotype.

Given the results described above, an alternative hypothesis is that one of the forms of HPr is responsible for the phenotypes observed for the Δ*manX* strain. Removal of HPr in a Δ*manX* background (Δ*manX* Δ*hpr* strain) restored normal levels of expression, providing strong evidence that supports this hypothesis. It is likely that the absence of ManX ($EIIA^{Man}$) results in accumulation of histidine-phosphorylated HPr (HPr-His-P), as ManX ($EIIA^{Man}$) dephosphorylates this form of HPr. Therefore, elevated levels of HPr-His-P in the Δ*manX* strain may have resulted in downregulation of the *melA-agp* and *lac* operons. As the levels of HPr-His-P increase, the levels of HPr-Ser-P should decrease because HPr-His-P is less amenable to phosphorylation by HprK at the serine residue (61). The same phenotype (downregulation of catabolic genes) should be observed for the Δ*manX* strain if HPr-Ser-P were required for relief from catabolite repression. The two strains lacking *hpr* (Δ*hpr* and Δ*manX* Δ*hpr* mutants) have no HPr-Ser-P, and they do not exhibit low levels of *melA-agp* or *lac* expression. These strains lack HPr-His-P as well, and this strongly suggests that HPr-His-P is directly or indirectly responsible for the low levels of expression of *melA-agp* and *lac* observed in the Δ*manX* strain. This does not rule out the possibility that it is the balance between HPr-His-P and HPr-Ser-P which regulates the levels of expression of *melA-agp* and *lac*. It has been suggested that in some gram-positive organisms a high proportion of HPr-His-P plus HPr relative HPr-Ser-P slows the metabolism of specific carbon sources (56). This may also be the case for *S. meliloti*.

The SMCR phenotypes of the Δ*hpr* and Δ*manX* Δ*hpr* strains also suggested that one of the forms of HPr is involved. Both of these mutants lack HPr and exhibited early release from the diauxic lag. However, the levels of expression of *melA-agp* and *lac* in these strains during the first phase of diauxic growth on succinate plus raffinose or on succinate plus lactose were similar to the levels of expression in the wild-type strain. The unaltered levels of expression were a surprise given the early release from the diauxic lag. This indicated that the time necessary for *melA-agp* or *lac* induction and the resulting increase in the enzyme level, after succinate was depleted, was shorter in the Δ*hpr* strains than in the wild-type strain. We do not currently know how this might occur.

When the SMCR phenotypes of the Δ*manX* mutant were considered, the diauxic lag on succinate plus raffinose was shorter than the diauxic lag for the wild-type strain, while its diauxic lag was longer than that of the wild type on succinate plus lactose. This was in spite of the fact that the expression of *melA-agp* and *lac* was not different from the expression in the wild-type strain during the first phase of growth in either condition. This suggests that differences in the molecular mechanisms of induction of *melA-agp* and *lac* may be important for how ManX influences gene activation. It is known that inducer exclusion is involved in SMCR in *S. meliloti* (11). It is possible that ManX ($EIIA^{man}$) or HPr mediates carbon metabolism at least partially through inducer exclusion. HPr and ManX ($EIIA^{man}$) could also interact with the nitrogen-PTS proteins encoded by genes in the *S. meliloti* genome and influence carbon metabolism in conjunction with them.

There is no evidence that there is a homologue of CcpA in *S. meliloti*, and a regulator with the role of Crp has not been found yet, although there are two genes that could encode Crp-like proteins. However, it is possible that there is a mechanism similar to one present in *Pseudomonas* spp. *P. putida* and *Pseudomonas aeruginosa* are also subject to SMCR; they possess the Crc (*c*atabolite *r*epression *c*ontrol) global regulator that acts at the translational level, preventing the synthesis of activators necessary for expression of genes for secondary carbon source utilization in the presence of succinate (33, 47, 52). Analysis of the *S. meliloti* genome yields at least three genes coding for probable exodeoxyribonucleases with similarity to Crc. Whether such proteins influence SMCR in *S. meliloti* is currently unknown.

Cobalt requirements and survival in stationary phase. Deletion of *hpr* from *S. meliloti* resulted in increased sensitivity to cobalt deficiency. In the absence of cobalt, growth of the mutant was frequently arrested, and the growth rates were often lower than those on minimal medium plus cobalt. The Δ*hpr* and Δ*manX* Δ*hpr* strains also exhibited rapid die-off during stationary phase, which was greater in rich medium or in cobalt-deficient minimal medium than in cobalt-amended minimal medium. In minimal medium the inability to survive in stationary phase seemed to be linked to the extreme sensitivity of the mutants to cobalt deficiency. However, in rich medium there was steep die-off during stationary phase when, presumably, cobalt was not limiting. The fact that the Δ*manX* mutant did not exhibit altered survival in stationary phase or sensitivity to cobalt deficiency suggests that the phenotype of the Δ*hpr*

mutant is due to the absence of one or more forms of HPr and not due to its inability to phosphorylate ManX (EIIA^{Man}).

Cobalt is the central atom in the tetrapyrrole ring structure of vitamin B_{12} cofactors. These cofactors are critical for methyltransferases, ribonucleotide reductases, and other enzymes (17, 48). *S. meliloti* possesses several B_{12} (cobalt)-dependent enzymes involved in DNA synthesis, methionine synthesis, and propionate metabolism (6, 17, 18). We did not investigate which of the cobalt-dependent enzymes mentioned above, if any, is involved in the response to cobalt limitation or how these enzymes could be related to the function of HPr.

Exopolysaccharide production and symbiotic characteristics. The Δ*hpr* strain and the Δ*manX* Δ*hpr* strain exhibited calcofluor-bright phenotypes and a fluorescent halo sooner than the wild type, indicating that deletion of HPr resulted in an alteration in the control of succinoglycan synthesis. In contrast, the Δ*manX* strain showed accumulation of high-molecular-weight succinoglycan, as judged from the calcofluor-bright phenotype and the very late appearance of a halo. Succinoglycan is critical for symbiosis; mutants unable to synthesize succinoglycan or low-molecular-weight succinoglycan cannot colonize their host or fix nitrogen, while some mutants that overproduce the polymer are also defective in colonization or nitrogen fixation (15, 31, 45, 59, 74). The three mutant strains (Δ*hpr*, Δ*manX*, and Δ*manX* Δ*hpr*) were symbiotically unimpaired. Clearly, the succinoglycan-related phenotypes exhibited by the mutants in this study were mild enough not to alter symbiosis. It is interesting that a recently isolated mutant defective in B_{12} cofactor synthesis was identified by a succinoglycan overexpression screen, establishing a link between regulation of succinoglycan synthesis and the cobalt-containing cofactor (13).

We show here that deletion of the genes encoding the PTS proteins HPr and ManX (EIIA^{Man}) resulted in profound physiological changes and affected carbon catabolism, succinoglycan production, and the ability to cope with nutrient stresses. We also present evidence that HPr-His-P is the factor most likely to be directly or indirectly responsible for the observed phenotypes. We speculate that HPr acts as a regulator for a variety of processes in the cell and that the phenotypes observed in the absence of ManX (EIIA^{Man}) are due to the accumulation of HPr-His-P.

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