

## HPrK Regulates Succinate-Mediated Catabolite Repression in the Gram-Negative Symbiont *Sinorhizobium meliloti*<sup>∇</sup>

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The HPrK kinase/phosphatase is a common component of the phosphotransferase system (PTS) of gram-positive bacteria and regulates catabolite repression through phosphorylation/dephosphorylation of its substrate, the PTS protein HPr, at a conserved serine residue. Phosphorylation of HPr by HPrK also affects additional phosphorylation of HPr by the PTS enzyme EI at a conserved histidine residue. *Sinorhizobium meliloti* can live as symbionts inside legume root nodules or as free-living organisms and is one of the relatively rare gram-negative bacteria known to have a gene encoding HPrK. We have constructed *S. meliloti* mutants that lack HPrK or that lack key amino acids in HPr that are likely phosphorylated by HPrK and EI. Deletion of *hprK* in *S. meliloti* enhanced catabolite repression caused by succinate, as did an S53A substitution in HPr. Introduction of an H22A substitution into HPr alleviated the strong catabolite repression phenotypes of strains carrying  $\Delta hprK$  or *hpr*(S53A) mutations, demonstrating that HPr-His22-P is needed for strong catabolite repression. Furthermore, strains with a *hpr*(H22A) allele exhibited relaxed catabolite repression. These results suggest that HPrK phosphorylates HPr at the serine-53 residue, that HPr-Ser53-P inhibits phosphorylation at the histidine-22 residue, and that HPr-His22-P enhances catabolite repression in the presence of succinate. Additional experiments show that  $\Delta hprK$  mutants overproduce exopolysaccharides and form nodules that do not fix nitrogen.

*Sinorhizobium meliloti* is a member of the alphaproteobacteria and can grow as free-living organisms, or as intracellular, nitrogen-fixing symbionts of alfalfa and other legumes (5, 13, 35, 54).

*S. meliloti* is able to utilize a large variety of compounds for growth, but succinate and other C<sub>4</sub>-dicarboxylic acids play an especially important role in metabolism during both free-living and symbiotic states. C<sub>4</sub>-dicarboxylic acids are used to fuel and provide reducing equivalents for nitrogen fixation by bacteroids (20, 49). Free-living *S. meliloti* also utilize succinate and do so in preference to many sugars and other substrates (6, 28, 31, 45, 57).

It has been shown that succinate represses genes needed for utilization of secondary carbon sources such as lactose (31, 57) and that it can prevent the intracellular accumulation of secondary carbon sources, lactose and raffinose, through inducer exclusion (6). This is called succinate-mediated catabolite repression (SMCR) and, though well documented in *S. meliloti*, the molecular mechanisms of its operation remain obscure. In many other model bacteria where catabolite repression in response to sugars is well understood, sensing of primary carbon sources takes place during their transport through the phosphotransferase system (PTS) (14, 50, 53). The regulatory and physiological responses that constitute catabolite repression are often controlled by the phosphorylation state of the of the PTS proteins HPr and EIIA, which in turn depends on whether or not the PTS is transporting a favored sugar (22, 24, 58, 59).

There are key differences between how some gram-negative bacteria, exemplified by *Escherichia coli*, and some gram-positive bacteria, exemplified by *Bacillus subtilis*, regulate catabolite repression via the PTS (Fig. 1). In these organisms the PTS delivers a phosphate group to a preferred sugar such as glucose. The phosphate originates from PEP, is transferred to the histidine-15 residue of HPr by enzyme EI, and is then transferred from HPr to EIIA.

In *E. coli* and other enteric bacteria, EIIA is the main player in catabolite repression. In the presence of glucose, EIIA is mainly unphosphorylated because the phosphate group it obtains from HPr eventually phosphorylates the incoming sugar. Unphosphorylated EIIA blocks transporters of secondary carbon sources, resulting in inducer exclusion (30, 32, 34). In the absence of glucose, EIIA is mainly in its phosphorylated form, which activates cyclic AMP synthesis by adenylate cyclase. Cyclic AMP binds to the regulator Crp and activates genes necessary for catabolism of secondary carbon sources (7, 23, 51, 56). In contrast, gram-positive bacteria such as *B. subtilis* rely mainly on HPr for regulation of catabolite repression (48, 58). HPr can be phosphorylated at the serine-46 residue by the HPr kinase/phosphatase HPrK under energy-replete conditions (33, 38, 42, 47). HPr-Ser46-P, after binding to the transcriptional regulator CcpA, can repress genes associated with secondary carbon source utilization (1, 25, 47, 58). Inducer exclusion is also used by some gram-positive organisms for catabolite repression. In these cases the permease blocking is effected by HPr-Ser46-P (41, 58).

*S. meliloti* possesses genes encoding some of the PTS proteins. Genes for an EI-type protein (SMc02437), HPr (SMc02754), EIIA-type proteins ManX (SMc02753) and EIIA<sup>Ntr</sup> (SMc01141), and HPrK (SMc02752) are present in the chromosome. Transport-related PTS proteins EIIB and

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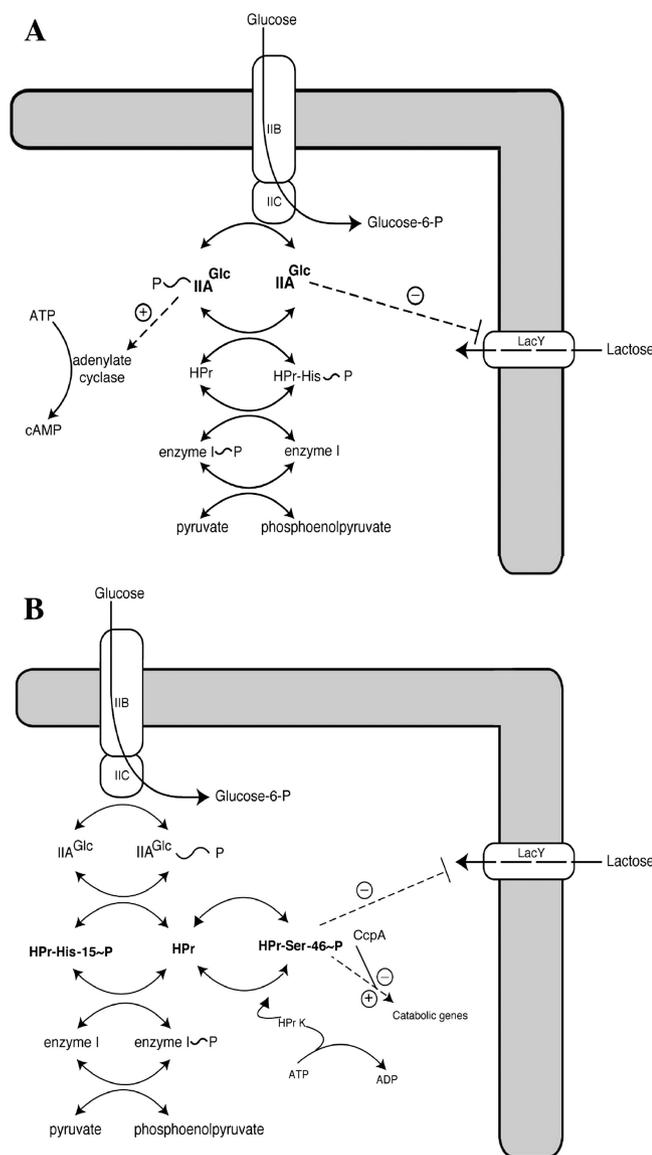


FIG. 1. Mechanisms of catabolite repression in *E. coli* (A) and *B. subtilis* (B).

EIIC are absent, indicating that PTS proteins that have been retained in *S. meliloti* probably provide critical regulatory functions even though they likely do not catalyze transport (4, 29). In support of this hypothesis, recent work in *S. meliloti* has shown that the HPr and ManX proteins are involved in regulation of many aspects of cellular physiology, including carbon metabolism, catabolite repression, exopolysaccharide synthesis, and survival in stationary phase (44).

While HPrK is a common regulator in gram-positive bacteria, it is lacking in most gram-negative bacteria, including *E. coli* and other enteric bacteria. However, it is encoded in the genomes many of alphaproteobacteria (Fig. 2A) (2, 29). In *S. meliloti* and many other alphaproteobacteria, the gene for *hprK* is found upstream of the *hpr* and *manX* genes (4, 29) (Fig. 2A). The putative *S. meliloti* HPrK is shorter than HPrK in model gram-positive organisms (see Fig. 2B). In fact, all alphapro-

teobacteria known to contain a HPrK homolog contain a short version, similar to the one in *S. meliloti*, rather than the longer version typical of gram-positive organisms (29, 55). In addition, *S. meliloti* appears to lack the regulator CcpA. Interestingly, an artificially truncated HPrK from *Lactobacillus casei*, which contained the same regions as the shorter *S. meliloti* HPrK, exhibited wild-type kinase and phosphatase activities toward HPr (9, 18). This suggests that the *S. meliloti* HPrK may also have kinase and phosphatase activities toward HPr. Given its unusual presence in *S. meliloti* and related bacteria and its association with the *hpr* and *manX* genes, we generated and characterized *hprK* mutants as part of an ongoing project to understand the role of the PTS system in regulating SMCR, carbon metabolism, and other aspects of physiology in *S. meliloti*.

MATERIALS AND METHODS

**Media, plasmids, and strains.** *S. meliloti* strain Rm1021 was used as the parent for the construction of all mutants. *S. meliloti* strains were grown in TY or in M9 mineral salts medium (52) supplemented with 5 ng/ml of cobalt chloride and various carbon sources at the concentrations noted in the text. *E. coli* XL1B MRF' (Stratagene) was the host for construction of all plasmids and was grown in LB medium (52) supplemented with antibiotics, IPTG (isopropyl-β-D-thiogalactopyranoside) at 48 μg/ml, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 40 μg/ml as needed. All other strains and plasmids used are listed in Table 1. Antibiotics were added at the following concentrations as needed: streptomycin at 500 μg/ml, ampicillin at 100 μg/ml, gentamicin at 30 μg/ml (*S. meliloti*) or 10 μg/ml (*E. coli*), neomycin at 100 or 200 μg/ml, and streptomycin at 200 μg/ml.

**Construction of in-frame deletions of *hprK*.** In-frame deletion of *hprK* was carried out following the gene replacement approach of Quandt and Hynes (46). For construction of the deletion, the N-terminal coding end and upstream region of *hprK* were amplified from *S. meliloti* Rm1021 genomic DNA with the primers 229 and 238. The C-terminal coding end and downstream flanking sequence of *hprK* were amplified by using primers 242 and 243. The primers are listed in Table 2. The two fragments were separately cloned into pGEM-T Easy, excised with the restriction enzymes SacI-NsiI and SpeI-NsiI, respectively. Fragments were ligated together, eliminating a 243-bp fragment of the gene, and cloned into the *sacB*-containing suicide vector pJQ200SK, creating plasmid pCAP31. pCAP31 construction was confirmed by PCR and sequencing. The plasmid was transformed into *S. meliloti* by electroporation, integrating into the chromosome by homologous recombination, yielding strain CAP13. A second recombination resulted in loss of the integrated plasmid and the wild-type copy of *hprK* and in retention of the *hprK* in-frame deletion. Isolates that had lost the plasmid were selected on TY-sucrose plates, and the presence of the  $\Delta hprK$  allele was screened for by PCR. The  $\Delta hprK$  strain obtained, CAP14, was used in the present study. The same plasmid (pCAP31) was delivered into strain RB111 ( $\Delta hpr$ ), yielding strain CAP78( $\Delta hpr/\Delta hprK$ ) which carries in-frame, unmarked deletions of *hpr* and *hprK*.

**Site-directed mutagenesis of *hpr*.** Site-directed mutants of HPr were constructed by the overlap extension method (52), using two internal primers that were complementary to each other and carried the desired mutation (amino acid substitution), and two external primers downstream and upstream of the gene (Table 2). Briefly, one internal primer and one external primer constituted a primer set used to amplify each one of two segments of the gene. Plasmid pDG127, which contains *hpr* under its native promoter region, was used as a template. The amplified segments shared an overlapping sequence (carrying the desired mutation), which mixed, denatured, and annealed served as templates for a second round of amplification using only the external primers. The product of this second amplification was the complete gene carrying the desired mutation. The different site-directed mutant genes constructed were *hpr*(H22A), *hpr*(S53A), and *hpr*(H22A/S53A). All were verified by sequencing.

The DNA fragments carrying the mutations were cloned into pCAP77, a suicide vector that contains an internal fragment of the first gene of the rhamnose utilization operon in *S. meliloti* Rm1021, SMC02324 (36). Suicide plasmids with the mutant alleles were then delivered into the chromosome of strains RB111( $\Delta hpr$ ) and CAP78( $\Delta hpr/\Delta hprK$ ). Strain CAP49, a complemented RB111( $\Delta hpr$ ) strain that carries a wild-type copy of *hpr* in SMC02324, was used

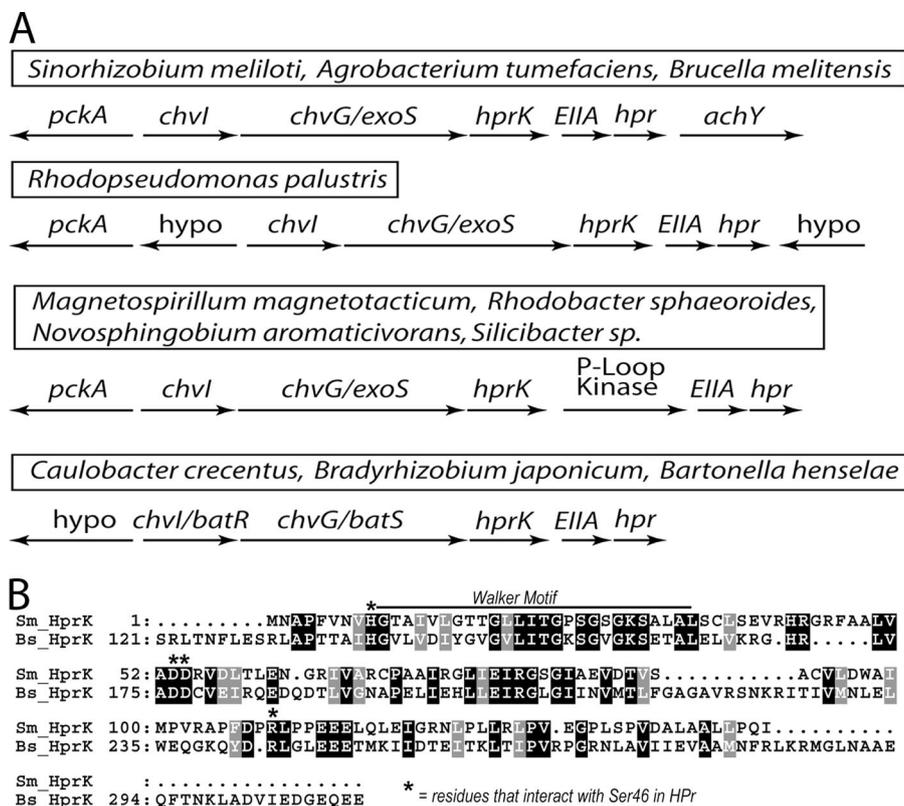


FIG. 2. Genes encoding HPrK-like proteins in *S. meliloti* and other alphaproteobacteria. (A) Maps of chromosomal regions containing PTS-like genes from various sequenced alphaproteobacteria. (B) CLUSTALX alignment of HprK from *B. subtilis* and *S. meliloti*. Important motifs and residues are indicated. Black boxes indicate identical amino acids and gray boxes conserved amino acids. Note that the *S. meliloti* protein lacks the N-terminal domain present in the *B. subtilis* protein.

as a control strain for experiments with site-directed mutant alleles of *hpr* integrated in SMc02324. Strain CAP60, an RB111-derived strain that carries an empty vector (pCAP77) in SMc02324, was used as the  $\Delta hpr$  control strain in these experiments. These strains are listed in Table 1.

**Complementation of  $\Delta hprK$ .** A copy of the *hprK* gene was amplified from Rm1021 genomic DNA and was cloned as a 447-bp fragment into plasmid pCAP84, resulting in pCAP83. Plasmid pCAP84 is a derivative of pCAP77 that contains a weak promoter upstream of the multiple cloning site. The promoter is a 490-bp sequence upstream from the operon comprised of genes *sma0113* and *sma0114*. This region exhibited very weak promoter activity when fused to the reporter gene *gfp* (P. P. Garcia, D. J. Gage, and C. Arango, unpublished data). Strain CAP14( $\Delta hprK$ ) was complemented *in trans* by recombination of pCAP83 in the chromosome at SMc02324. The resulting strain was called CAP83. A control strain, that exhibited wild-type phenotypes, was constructed by recombination of pCAP77 at SMc02324 in Rm1021.

**Growth curves and growth rates.** Growth curves and growth rates were obtained as explained previously (44). Concentration of single carbon sources was 0.4%, while concentrations for dual carbon sources were 0.05% for succinate and 0.1% for the secondary carbon source. Yield measurements at different succinate concentrations confirmed that at 0.05% the cultures were limited for carbon when they reached stationary phase. Growth curve cultures were tested for the number of fast-growing suppressors upon reaching stationary phase. Experiments with a fraction of suppressors higher than 10% were not included in the results. Growth experiments for evaluation of gene expression were conducted with strains transformed with plasmid pCAP11, and fluorescence was measured as described previously (44).

**$\beta$ -Galactosidase assays.** Strains were streaked, or spread, onto M9 minimal plates with succinate (0.4%), lactose (0.4%), or succinate plus lactose (0.1 and 0.05%, respectively) as carbon sources. Cells were scraped off the plates, rinsed, resuspended in 15% glycerol, and frozen at  $-80^{\circ}\text{C}$  until the time of the assay. Enzyme assays were conducted as described previously (39). For qualitative evaluation of  $\beta$ -galactosidase activity, cells were pregrown on M9 minimal medium with glycerol as carbon source, rinsed, resuspended in no-carbon M9, and

plated on M9 minimal plates with succinate (0.1%) plus lactose (0.05%) as carbon sources and X-Gal (40  $\mu\text{g/ml}$ ) as an indicator of  $\beta$ -galactosidase activity (SLX plates).

**Nodulation assays.** Alfalfa seeds were sterilized and sprouted as described previously (19), with the following modifications. Single seedlings were placed on Nod3 (BNM) agar slants in 18-mm glass tubes and inoculated with 100  $\mu\text{l}$  of a suspension of *S. meliloti*. Suspensions were made by scraping colonies from M9 plates with glycerol as a carbon source, rinsing, and resuspending in Nod3 medium to a final optical density (OD) of 0.1 at 595 nm. Plant growth tubes were loosely capped and incubated in a growth chamber with 16/8-h light/dark cycle at  $26^{\circ}\text{C}$ . Nodules were counted weekly. At 36 days after inoculation, plant shoots were cut above the cotyledons, dried, and weighed.

**Nodule structure microscopy.** Nodules were excised from alfalfa plants, fixed in phosphate-buffered saline with 4% paraformaldehyde, dehydrated in ethanol, and embedded in JB4 embedding resin as described previously (17, 40). Embedded nodules were sliced to give 2- $\mu\text{m}$ -thick sections, dyed with a mixture of DAPI (4',6'-diamidino-2-phenylindole) and acridine orange, and photographed under epifluorescence illumination using a filter set with a 330- to 380-nm excitation filter and a long-pass emission filter with a 435-nm cutoff.

## RESULTS

**Deletion of *hprK* results in greatly reduced growth rates and reduced catabolic gene expression.** Although the main objective of deleting *hprK* was to investigate whether the protein had a role in catabolite repression, it became evident that the deletion of *hprK* strongly affected growth. Colonies of strain CAP14( $\Delta hprK$ ) grown on M9 plates with a variety of carbon sources were significantly smaller than those of strain Rm1021 (wild type) (Fig. 3). Growth rates were measured in minimal

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Relevant genotype or marker <sup>a</sup>	Source or reference
<b>Strains</b>			
Rm1021	<i>S. meliloti</i> , wild type	<i>hpr hprK</i> (wild type)	37
RB111	Rm1021, $\Delta hpr$	$\Delta hpr$	44
CAP14	Rm1021, $\Delta hprK$	$\Delta hprK$	This study
CAP43	Rm1021, SMc02324::pCAP77	<i>hpr hprK</i> (wild type); Nm <sup>r</sup>	This study
CAP49	RB111, SMc02324::pCAP81	<i>hpr hprK</i> (wild type); Nm <sup>r</sup>	44
CAP60	RB111, SMc02324::pCAP77	$\Delta hpr$ ; Nm <sup>r</sup>	This study
CAP62	CAP14, SMc02324::pCAP83	$\Delta hprK/P_{sma0113}::hprK$ ; Nm <sup>r</sup>	This study
CAP78	Rm1021, $\Delta hpr/\Delta hprK$	$\Delta hpr \Delta hprK$	This study
CAP92	CAP78, SMc02324::pCAP81	$\Delta hprK$	This study
CAP93	RB111, SMc02324::pCAP113	<i>hprH22A</i> ; Nm <sup>r</sup>	This study
CAP94	CAP78, SMc02324::pCAP113	$\Delta hprK/hpr(H22A)$ ; Nm <sup>r</sup>	This study
CAP95	RB111, SMc02324::pCAP114	<i>hpr(S53A)</i> ; Nm <sup>r</sup>	This study
CAP97	RB111, SMc02324::pCAP118	<i>hpr(H22A/S53A)</i> ; Nm <sup>r</sup>	This study
<b>Plasmids</b>			
pGEM-T Easy	Cloning vector	Amp	Promega
pJQ200 SK	Suicide plasmid with <i>sacB</i>	Gm	46
pMB439	Suicide plasmid	Nm	3
pCAP11	pMB393, $P_{melA}::gfp$	Sp	44
pDG127	pGEM-T Easy, <i>hpr</i>	Amp	44
pCAP31	pJQ200SK carrying $\Delta hprK$ allele	Gm	This study
pCAP77	pMB439 with an internal fragment of SMc02324	Nm	44
pCAP81	pCAP77 with <i>hpr</i>	Nm	44
pCAP83	pCAP77 with $P_{sma0113}::hprK$	Nm	This study
PCAP84	pCAP77 with $P_{sma0113}$	Nm	This study
pCAP113	pCAP77 with <i>hpr(H22A)</i>	Nm	This study
pCAP114	pCAP77 with <i>hpr(S53A)</i>	Nm	This study
pCAP118	pCAP77 with <i>hpr(H22A/S53A)</i>	Nm	This study

<sup>a</sup> Resistance markers; Nm, neomycin; Sp, spectinomycin; Amp, ampicillin; Gm, gentamicin.

medium with succinate, fructose, arabinose, maltose, glycerol, glucose, raffinose, and lactose as the sole carbon sources. The strain lacking *hprK* exhibited growth rates that were reduced 70 to 80% relative to wild-type growth rates (Table 3). Growth on TY was reduced as well, but not as drastically as in minimal medium. Suppressors readily arose in strain CAP14( $\Delta hprK$ ), which resulted in faster-growing strains that gave rise to colonies much larger than those of the parental  $\Delta hprK$  strain (Fig. 3).

Attempts to alleviate the growth defect by addition of growth factors were unsuccessful (data not shown). The addi-

tion of tryptophan or vitamins to M9 plates with lactose as the carbon source did not result in larger colonies for either the wild-type strain or the CAP14( $\Delta hprK$ ) strain compared to nonamended plates. The addition of Casamino Acids and yeast extract slightly stimulated the growth of wild-type strain Rm1021 but did not fix the growth phenotype of strain CAP14( $\Delta hprK$ ). These results, together with the slow growth on all types of carbon sources tested, suggest that the altered growth phenotype of strain CAP14( $\Delta hprK$ ) may be a result of alteration in central metabolic pathways rather than a defect in the biosynthesis of amino acids or cofactors.

TABLE 2. Primers used in this study

Primer	Sequence (5'-3')	Description	Notes
229	GGACTAGTCGCATCGTCCGGC	Amplification of the N-terminal coding end of <i>hprK</i>	
238	TGATCATGCATGAAAGCGGAGC GCCGACTTGCC	Amplification of the N-terminal coding end of <i>hprK</i>	
242	CGAGCTCATGGCCAGATATCGCAA TGCAG	Amplification of the C-terminal coding end of <i>hprK</i>	
243	CATGCATGATCAGAAATCTGCCGC TGTTGC	Amplification of the C-terminal coding end of <i>hprK</i>	
278	ATGGCGGGCAGCGTGGTC	External primer for site-directed mutagenesis of <i>hpr</i>	
279	CGCCGGAGGAGTTGCAG	External primer for site-directed mutagenesis of <i>hpr</i>	
462-463	GTCGGCGGGACCGCCATCATGGG GCTGATGA	Internal primers for <i>hpr(S53A)</i> mutagenesis	Primer 463 is the reverse complement of 462
464-465	ACAAACGCGGCTGGCCGCGCGT GCCTCTG	Internal primer for <i>hpr(H53A)</i> mutagenesis	Primer 465 is the reverse complement of 464

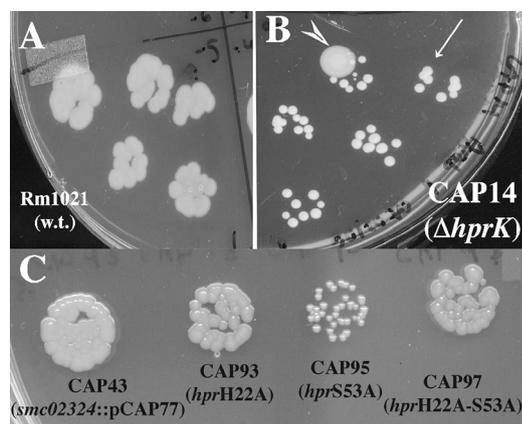


FIG. 3. Colony morphology of *S. meliloti* containing altered HprK and HPr proteins. (A) Colonies of wild-type strain Rm1021. (B) Colonies of strain CAP14( $\Delta hprK$ ) (arrows) and  $\Delta hprK$  suppressor strains (arrowheads). (C) Colonies of control strain CAP43(SM02324::pCAP77) and substitution mutants CAP93[*hpr*(H22A)], CAP95[*hpr*(S53A)], and CAP97[*hpr*(H22A/S53A)] on M9 plates with 0.4% glycerol. Plates were incubated for 2 weeks (A and B) or for 1 week (C) before being photographed.

Low growth rates on certain carbon sources could be caused by either low expression of the catabolic or transport genes or by low activity of the corresponding proteins. Expression of the *mela-agp* operon, necessary for utilization of  $\alpha$ -galactosides such as raffinose and melibiose, was evaluated using the reporter plasmid pCAP11, which carries a  $P_{mela}::gfp$  fusion inducible by  $\alpha$ -galactosides. Specific fluorescence of  $\Delta hprK$  cells at mid-exponential phase grown on raffinose as the sole carbon source was reduced sixfold compared to the wild-type cells [ $10,700 \pm 1,500$  fluorescence units/OD units for CAP14( $\Delta hprK$ ) versus  $66,900 \pm 2,200$  for the wild type,  $n = 2$ ]. Even when grown in M9 with succinate, a condition that represses the *mela-agp* operon, the measured levels were significantly lower for strain CAP14( $\Delta hprK$ ) [ $900 \pm 90$  fluorescence units/OD units for CAP14( $\Delta hprK$ ) versus  $4,000 \pm 670$  for the wild type]. The  $\beta$ -galactosidase activity was also low in strain CAP14( $\Delta hprK$ ) when measured in cells grown on plates with lactose as the sole carbon source. The enzyme activity in strain Rm1021 was  $20.7 \pm 0.7$  U (average  $\pm$  the standard error, three independent experiments), while in strain CAP14( $\Delta hprK$ ) it

TABLE 3. Growth rate constants of strains Rm1021 (wild type) and CAP14( $\Delta hprK$ ) in different media ( $n = 2$  to 4)

Carbon source <sup>a</sup>	Avg growth rate constant $k$ (hr <sup>-1</sup> ) $\pm$ SE	
	Rm1021	CAP14( $\Delta hprK$ )
Succinate	$0.17 \pm 0.015$	$0.05 \pm 0.004$
Glucose	$0.14 \pm 0.002$	$0.04 \pm 0.011$
Raffinose	$0.11 \pm 0.013$	$0.02 \pm 0.003$
Lactose	$0.09 \pm 0.002$	$0.02 \pm 0.001$
Fructose	$0.19 \pm 0.017$	$0.06 \pm 0.011$
Arabinose	$0.10 \pm 0.002$	$0.03 \pm 0.001$
Maltose	$0.11 \pm 0.012$	$0.02 \pm 0.0003$
Glycerol	$0.21 \pm 0.015$	$0.04 \pm 0.002$
TY (rich medium)	$0.27 \pm 0.009$	$0.15 \pm 0.0002$

<sup>a</sup> Single carbon source added to M9 minimal medium. All cultures were grown at 30°C with shaking.

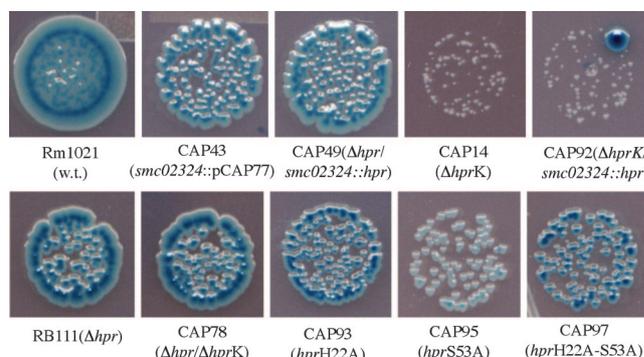


FIG. 4. Colony phenotype of *S. meliloti* containing altered HPrK and HPr proteins on SLX plates. Colonies of the indicated strains were grown for 7 days on succinate (0.1%) plus lactose (0.05%) M9 plates with indicator X-Gal before being photographed. Blue coloration indicates the activity of endogenous  $\beta$ -galactosidase.

was  $4.3 \pm 0.12$  U, a fivefold reduction. These results suggest that HPrK is involved, directly or indirectly, in regulation of carbon metabolism, through the regulation of genes or enzymes needed for transport and catabolism.

**Loss of HPrK results in severe SMCR.** HPrK regulates catabolite repression in gram-positive organisms via phosphorylation of HPr on a conserved serine residue. Since *S. meliloti* HPr protein retains this phosphorylatable residue, it seemed likely that HPrK could regulate SMCR in this organism. In a preliminary assessment of SMCR phenotypes, strains Rm1021 (wild type) and CAP14( $\Delta hprK$ ) were grown on M9 minimal plates with succinate (0.1%) plus lactose (0.05%). The plates also contained X-Gal, which turns blue when cleaved by endogenous  $\beta$ -galactosidase. Colonies from strain Rm1021 were initially white, indicating growth on succinate and repression of  $\beta$ -galactosidase production, but turned blue after succinate was depleted and lactose utilization started. In contrast, strain CAP14( $\Delta hprK$ ) never turned blue, even after colonies reached the maximum size (Fig. 4). This suggested that strain CAP14( $\Delta hprK$ ) had a severe alteration of SMCR, leading to very low levels of lactose utilization. If HPrK were generally involved in SMCR, its absence would affect the catabolite repression of other secondary carbon sources, and the effects should be observed in growth curves of the mutant strain on dual carbon sources. The growth of strains Rm1021 and CAP14( $\Delta hprK$ ) was monitored in M9 medium with succinate plus either raffinose (an  $\alpha$ -galactoside) or lactose (a  $\beta$ -galactoside). When grown on succinate plus lactose, the wild-type strain Rm1021 exhibited a short lag of 2 to 3 h after succinate was exhausted, before growth resumed using lactose as a carbon source. This lag was 20 or more hours for the strain CAP14( $\Delta hprK$ ) (Fig. 5A). An extended diauxic lag was also exhibited by strain CAP14( $\Delta hprK$ ) in succinate plus raffinose medium (Fig. 5B). The diauxic lag was extended from 5 h in the wild-type strain to approximately 30 h for strain CAP14( $\Delta hprK$ ). These results indicated that SMCR was stronger in strain CAP14( $\Delta hprK$ ) than in the wild-type strain, suggesting that HPrK has a role in the process of repression or derepression and that its role is not specific to a single type of sugar.

**Epistatic relation between  $\Delta hpr$  and  $\Delta hprK$ .** To explore the epistatic relation between the *hpr* deletion and the *hprK*

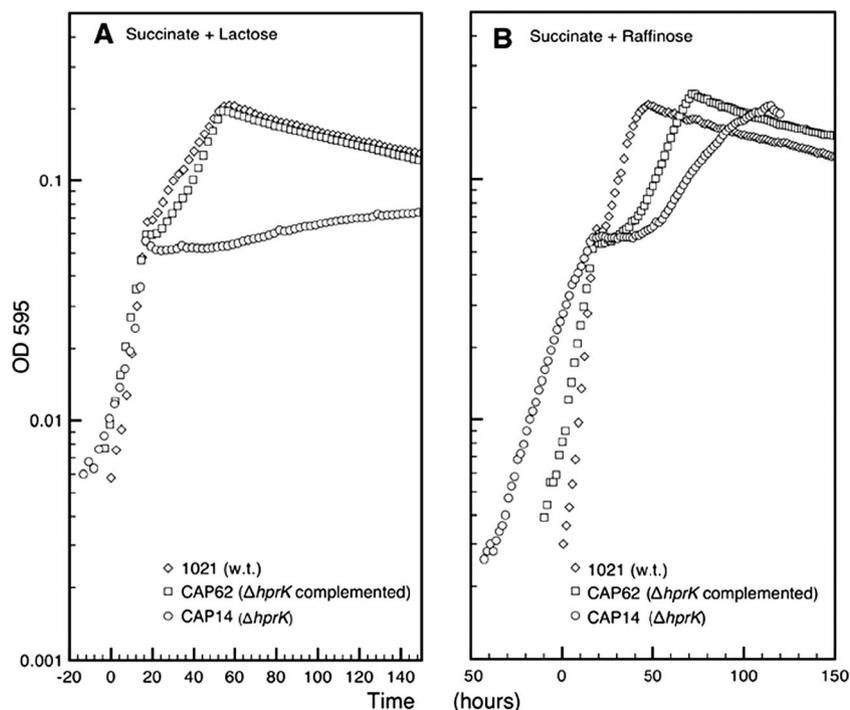


FIG. 5. Growth of  $\Delta hprK$  mutants on dual carbon sources. The diauxic growth of wild-type strain Rm1021, strain CAP14( $\Delta hprK$ ), and strain CAP62 (which was complemented with *hprK* in *trans*) was examined. Cells were grown in M9 media with 0.1% succinate plus 0.05% lactose (A) or with 0.1% succinate plus 0.05% raffinose (B). Curves were time shifted to align the point of succinate exhaustion.

deletion, a double  $\Delta hpr/\Delta hprK$  mutant strain (CAP78) was constructed by introducing a  $\Delta hprK$  allele into the  $\Delta hpr$  mutant strain RB111. Growth and SMCR phenotypes of strain CAP78( $\Delta hpr/\Delta hprK$ ) were similar to those of strain RB111( $\Delta hpr$ ), i.e., relaxed SMCR compared to the wild-type strain, indicating that the phenotypes caused by the deletion of *hprK* require HPr in one or more of its forms (data not shown, Fig. 4, and Fig. 6A).

Due to the high frequency of secondary mutations that suppress the phenotypes of  $\Delta hprK$  mutants, it was possible that the strain isolated as ( $\Delta hpr/\Delta hprK$ ) was one of these suppressor mutants. To verify that this was not the case, plasmid pCAP81 (carrying a copy of *hpr*) was introduced into the chromosome of CAP78( $\Delta hpr/\Delta hprK$ ). The resulting strain (CAP92) exhibited phenotypes typical of strain CAP14( $\Delta hprK$ ): small colonies on M9 minimal plates with glycerol (data not shown) and white coloration on M9 minimal plates with succinate plus lactose plus X-Gal (SLX plates), confirming that CAP78( $\Delta hpr/\Delta hprK$ ) had no *hprK* suppressor mutations (Fig. 4).

***hprK* complementation reverses the observed phenotypes of the  $\Delta hprK$  mutant.** To confirm that the observed phenotypes were a direct result of deleting *hprK*, a copy of the gene was supplied in *trans* by inserting it into the SMC02324 gene in the chromosome of strain CAP14( $\Delta hprK$ ). The gene was supplied in single copy, with its expression controlled from the promoter region of genes sma0113 and sma0114 (P. P. Garcia and D. J. Gage, unpublished results). The resulting strain, CAP62, was evaluated for growth and SMCR on various carbon sources. Introduction of the *hprK* gene reestablished wild-type phenotypes, fully or partially, in all cases tested (Fig. 5 and data not shown).

To verify that strain CAP62( $\Delta hprK$ , SMC02324::*hprK*) was truly a complemented  $\Delta hprK$  mutant and did not contain a spontaneous suppressor of  $\Delta hprK$ , the integrated plasmid containing *hprK* was exchanged for another suicide plasmid without *hprK*. Transductants plated on M9 minimal medium with glycerol as the sole carbon source exhibited limited growth (small colonies) compared to the parental strain CAP62( $\Delta hprK$ , SMC02324::*hprK*), confirming that the phenotypes of strain CAP62( $\Delta hprK$ , SMC02324::*hprK*) were due to complementation of  $\Delta hprK$  in *trans* and not due to a suppressor mutation (data not shown).

**An *hpr(S53A)* mutant behaves like a mutant devoid of HPrK.** Certain HPr proteins can be phosphorylated on two different residues. In *B. subtilis* EI phosphorylates HPr at the histidine-15 residue, while HPrK phosphorylates (and dephosphorylates) HPr at the serine-46 (47, 48). The latter form of HPr acts as a corepressor of catabolic genes in response to the presence of favored carbon sources (1, 25, 47, 58). Alignment of *S. meliloti* HPr to HPr proteins of *B. subtilis* and other bacteria revealed well-conserved regions around Ser-53 and His-22 in the *S. meliloti* protein that are similar to regions around phosphorylatable serine and histidine residues in other HPr proteins (4). These results suggested that residues His-22 and Ser-53 may be phosphorylated in *S. meliloti* HPr. It has been suggested that in *S. meliloti* HPr-His22-P is involved, directly or indirectly, in repression of secondary carbon source utilization in the presence of succinate (44). This hypothesis does not exclude the possibility that HPr-Ser53-P is also involved in SMCR or in regulating phosphorylation of HPr at the histidine-22 residue. Slow growth on single carbon sources and extended diauxic lags on dual carbon sources in mutants lack-

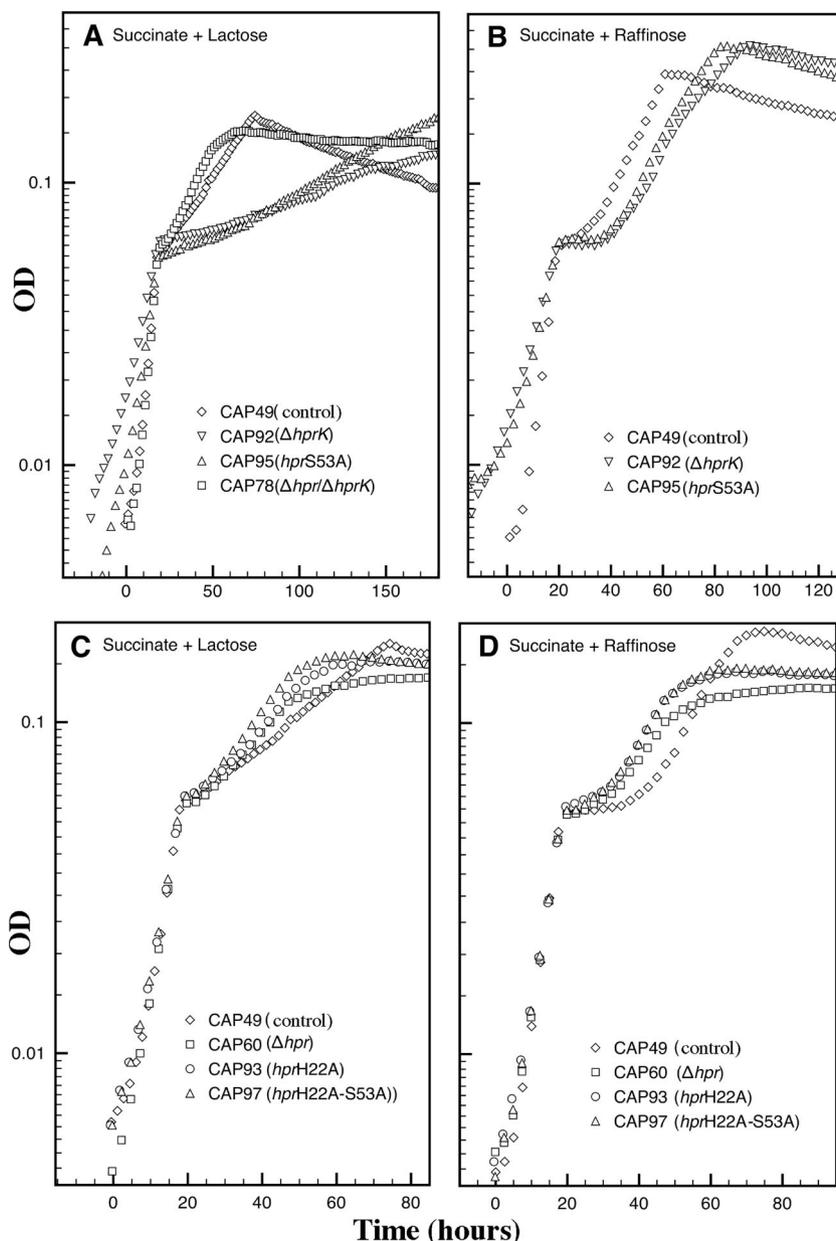


FIG. 6. Growth of  $\Delta hprK$  and HPr amino acid-substitution mutants on dual carbon sources. (A and B) Diauxic growth of the control strain CAP49, strain CAP92( $\Delta hprK$ ), strain CAP78( $\Delta hprK/\Delta hprK$ ) and strain CAP95[*hpr*(S53A)]. (C and D) Diauxic growth of the control strain CAP49, strain CAP60( $\Delta hpr$ ), strain CAP93[*hpr*(H22A)] and strain CAP97[*hpr*(H22A/S53A)]. Cells were grown in M9 media with 0.1% succinate plus 0.05% lactose (A and C) or with 0.1% succinate plus 0.05% raffinose (B and D). All strains are isogenic and came from the same strain, RB111( $\Delta hpr$ ), into which wild-type *hpr* or site-directed mutants of *hpr*, or an empty vector, were introduced at the SMc02324 site. Curves were time shifted to align the point of succinate exhaustion.

ing the *hprK* gene (strains CAP14 and CAP92) suggested that HPr-Ser53-P is important for activation of catabolic genes. To explore the importance of phosphorylation at the two different critical residues, genes coding for HPr-H22A, HPr-S53A, and HPr-H22A/S53A were inserted in single copy in the SMc02324 gene of strain RB111( $\Delta hpr$ ), yielding strains CAP93, CAP95, and CAP97, respectively.

Colonies of CAP95[*hpr*(S53A)] were smaller than colonies of control strain CAP43 when grown on M9 plates with glycerol as a carbon source (0.4%) (Fig. 3C). Slow growth was also

observed in M9 minimal medium with succinate, lactose, or raffinose as carbon sources (data not shown). When grown on SLX plates, colonies of strain CAP95[*hpr*(S53A)] were white for a longer period of time than the control strains CAP43 and CAP49. The final color of strain CAP95[*hpr*(S53A)] was pale blue, in contrast to the dark blue colonies of control strains CAP43 and CAP49 (Fig. 4). The levels of  $\beta$ -galactosidase observed for strain CAP95[*hpr*(S53A)] on SLX plates resembled those of strain CAP14( $\Delta hprK$ ), although they were slightly less severe (Fig. 4). This suggested that lack of phosphorylation at

the serine-53 residue on HPr contributes to the altered phenotypes in both  $\Delta hprK$  and  $hpr(S53A)$  mutants. Enhanced catabolite repression was observed in CAP95[ $hpr(S53A)$ ] during growth in M9 medium with succinate plus lactose or raffinose (Fig. 6A and B). Once succinate was exhausted, the strain carrying the  $hpr(S53A)$  allele exhibited longer diauxic lags than strain CAP49 which carried a wild-type  $hpr$ . In summary, the growth and catabolite repression phenotypes observed for strain CAP95[ $hpr(S53A)$ ] are similar to those observed in strain CAP14( $\Delta hprK$ ), suggesting that HPrK phosphorylates HPr at the serine-53 residue and that phosphorylation at this residue by HPrK plays a significant role in regulation of carbon utilization.

**Phenotypes caused by the lack of phosphorylation of HPr-Ser53 are reversed by an H22A substitution in HPr.** To investigate whether enhanced SMCR in strains unable to phosphorylate serine-53 of HPr was due to the absence of HPr-Ser53-P or was due to an excess of HPr-His22-P, a strain in which both the serine-53 and the histidine-22 residues of HPr were unphosphorylatable was constructed. The resulting strain, CAP97[ $hpr(H22A/S53A)$ ], was characterized during growth on single and mixed carbon sources. Normal growth on M9 plates with glycerol was restored (Fig. 3C), as was growth in liquid M9 medium with succinate as carbon source (data not shown). Normal catabolite repression phenotype was restored as well in strain CAP97[ $hpr(H22A/S53A)$ ]. Diauxic lags were shortened to less than the wild-type length in M9 medium with succinate plus lactose or raffinose (Fig. 6C and D). The  $\beta$ -galactosidase levels in strain CAP97[ $hpr(H22A/S53A)$ ] reverted to wild-type levels, as shown by their darker blue coloration on SLX plates, compared to the pale blue colonies of strain CAP95[ $hpr(S53A)$ ] (Fig. 4). Similar results were seen in strain CAP94, which has the  $hpr(H22A)$  allele in a  $\Delta hprK$  background (data not shown).

These results are consistent with the idea that inability to phosphorylate the histidine-22 residue of HPr relieves the enhanced catabolite repression imposed by an unphosphorylatable serine-53 residue, suggesting that the accumulation of HPr-His22-P in the  $\Delta hprK$  and  $hpr(S53A)$  mutants slows growth in single carbon sources and enhances repression of lactose and raffinose utilization in the presence of succinate.

**Mutants with unphosphorylatable HPr-His22 show partial relief from catabolite repression.** If HPr-His22-P is an effector of SMCR, it would be expected that a strain with unphosphorylatable histidine-22 residue should exhibit a relaxed catabolite repression phenotype. To test this hypothesis, the  $hpr(H22A)$  allele was introduced into strain RB111( $\Delta hpr$ ). The resulting strain, CAP93[ $\Delta hpr/hpr(H22A)$ ], exhibited normal growth on M9 glycerol plates and in liquid M9 with succinate (Fig. 3C and data not shown). Growth rates on lactose and raffinose were reduced compared to control strains CAP43 and CAP49 (data not shown).

The  $\beta$ -galactosidase levels in strain CAP93[ $hpr(H22A)$ ] were slightly higher than the wild-type control strains CAP43 and CAP49, as judged from its darker blue color on SLX plates (Fig. 4). Strain CAP93[ $hpr(H22A)$ ] exhibited a slightly relaxed SMCR phenotype compared to the isogenic control strain CAP49 during diauxic growth in M9 with succinate plus lactose (Fig. 6C) and succinate plus raffinose (Fig. 6D). These results

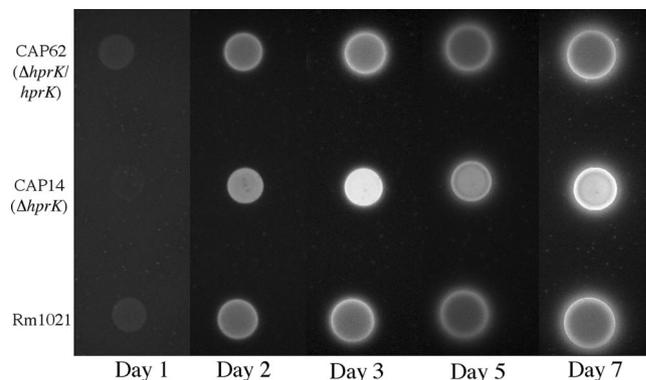


FIG. 7. Succinoglycan production in  $\Delta hprK$  mutants. Strains were pregrown in M9-glycerol medium, rinsed, resuspended, and spotted onto TY plates with 0.02% calcofluor (0.02%). Plates were photographed under UV light to document succinoglycan fluorescence. The exposure time was 0.05 s for all images, except for the day 1 images, which were exposed for 0.1 s.

confirm that HPr-His22-P, directly or indirectly, causes repression in the presence of succinate, since both CAP93[ $hpr(H22A)$ ] and the control strain CAP49 have the ability to produce HPr-Ser53-P but only CAP93 cannot produce HPr-His22-P.

**Deletion of  $hprK$  alters succinoglycan production and symbiotic properties.**  $hprK$  is located immediately downstream of  $chvG$  (also known as  $exoS$ ) in the chromosome of *S. meliloti* and is likely cotranscribed with  $chvG$ , since the two genes overlap. Reports that  $chvG$  mutants of *S. meliloti* exhibit alterations in succinoglycan production and in symbiotic phenotypes (10–12) prompted us to investigate these phenotypes in strain CAP14( $\Delta hprK$ ). When the  $\Delta hprK$  strain was plated on TY plates with calcofluor, it exhibited a very bright phenotype starting on the second day of incubation, which lasted for several days. In contrast, strain Rm1021 (wild type) initially presented a bright phenotype that dimmed gradually as a halo developed around the colony (Fig. 7). The bright calcofluor phenotype of CAP14( $\Delta hprK$ ) indicated that this strain had upregulated production of long-chain succinoglycan. Normal development of a halo suggested that breakdown of long-chain succinoglycan or synthesis of short-chain succinoglycan had occurred and had not been altered by the absence of HPrK.

In planta phenotypes of strain CAP14( $\Delta hprK$ ) were evaluated in alfalfa (*Medicago sativa*). Inoculation with the  $\Delta hprK$  strain induced nodule formation in the plants, and the number of nodules per plant was similar to the plants inoculated with wild-type strain Rm1021. However, the nodules formed were ineffective, as judged by the white coloration of many of them and by the reduced weight of the plant shoots compared to plants inoculated with strain Rm1021 (Fig. 8). Crushed nodules from plants inoculated with both  $\Delta hprK$  and wild-type strains yielded colonies when spread on TY plates. Colonies recovered from nodules elicited by strain CAP14( $\Delta hprK$ ) included the  $\Delta hprK$  mutants (small colonies), as well as larger colonies with suppressor mutations, showing that strain CAP14( $\Delta hprK$ ) was able to grow in infection threads and occupy the nodules but either failed to transition to the bacteroid state or to fix nitrogen once in that state (data not shown). Nodules occupied by strain Rm1021 show clear meristematic,

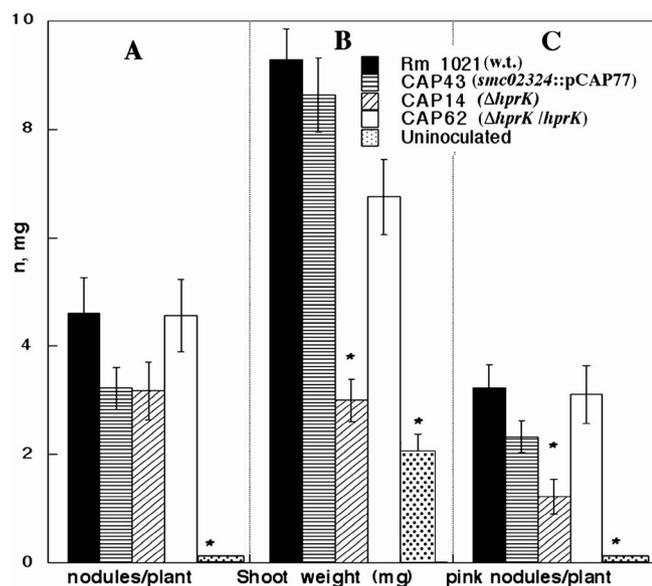


FIG. 8. Nodulation phenotype of  $\Delta hprK$  mutants. Average number of nodules per plant (A), shoot weight (B), and number of pink nodules per plant (C) on alfalfa plants inoculated with *S. meliloti* wild-type strain Rm1021, strain CAP14( $\Delta hprK$ ), and the  $\Delta hprK$  complemented strain CAP62. \*, Significantly different from the wild-type strain ( $P < 0.003$ ,  $n = 18$ ).

infection, and nitrogen fixation zones (Fig. 9A) and densely populated bacteroid-infected plant cells (Fig. 9C). Examination of the internal structure of CAP14( $\Delta hprK$ ) induced nodules revealed low bacteroid numbers and the presence of starch granules in bacteroid-infected plant cells in both white and pink nodules (Fig. 9B and D). There was also a poorly defined infection/nitrogen-fixation zone in white nodules (Fig. 9B), perhaps explaining the low nitrogen fixation efficiency of these nodules.

When strain CAP62( $\Delta hprK$ , SMC02324::hprK) was tested for succinoglycan and symbiotic phenotypes, both of the phenotypes were complemented by the introduction of *hprK* in trans (Fig. 7 and 8). This result indicated that the observed alterations in succinoglycan production and symbiosis in strain CAP14( $\Delta hprK$ ) were due to the deletion of *hprK*.

## DISCUSSION

Many bacteria do not have a complete PTS and are thought to have retained the partial systems for regulation (4, 8). Possession of a partial PTS is common in the alphaproteobacteria, and is also found in certain gamma-, delta-, and betaproteobacteria; in spirochetes (*Treponema denticola*, *T. pallidum*, and *Leptospira interrogans*); and in *Chlorobium tepidum* (2, 4, 55).

The PTS of *S. meliloti* includes the general proteins EI and

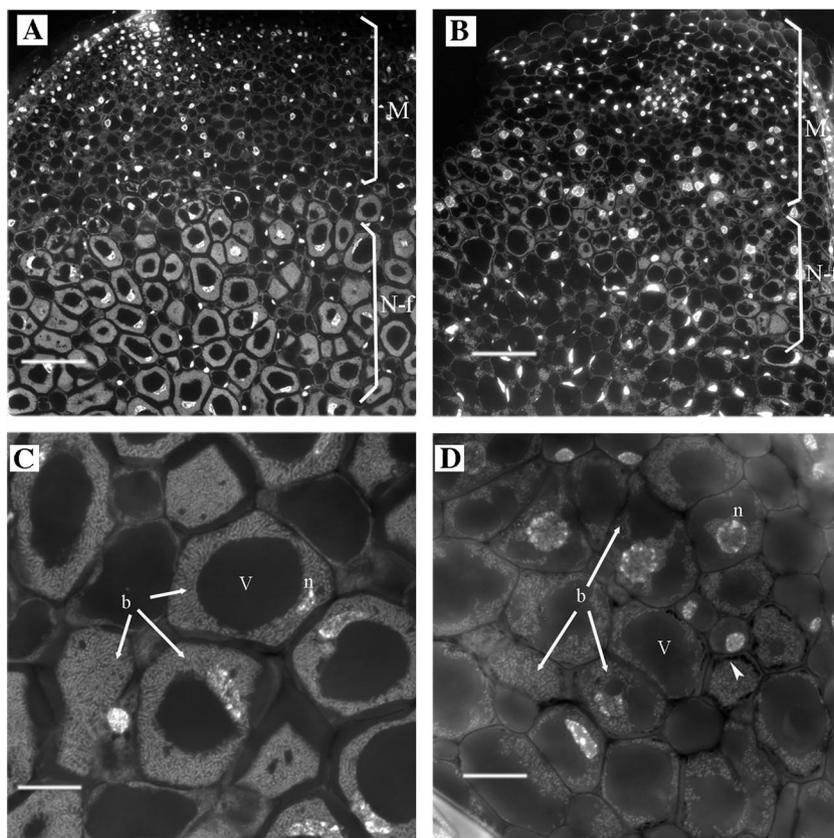


FIG. 9. Internal structure of nodules induced by wild-type strain and  $\Delta hprK$  mutant. Images of *S. meliloti* strain Rm1021 (A and C, gray nodules) or strain CAP14( $\Delta hprK$ ) (B and D, white nodules) are shown. Panels A and B show the meristematic (M) and nitrogen fixation (N-f) zones. Panels C and D show bacteroids in plant cells from the nitrogen fixation zone. b, bacteroids; n, nucleus; V, vacuole. Arrowheads indicate starch granules which can be seen around the perimeter of nodule cells in panel D. Scale bar, 98  $\mu\text{m}$  in panels A and B and 24  $\mu\text{m}$  in panels C and D.

HPr and the EIIA-type proteins ManX and EIIA<sup>Ntr</sup>. Since the permease components EIIB and EIIC are missing, it is unlikely that sugar transport and phosphorylation directs the regulation of catabolite repression in this organism. The EI protein in *S. meliloti* is similar to EI<sup>Ntr</sup> proteins in other bacteria. Although it has not been proved that HPr is phosphorylated by EI<sup>Ntr</sup>, it is likely that this is the case, given the lack of a classical EI and the similarity of HPr to NPr (an Ntr-type HPr) (data not shown). In addition, *S. meliloti* and many other alphaproteobacteria possess HPrK proteins that are shorter than their gram-positive counterparts (4, 55). These contain all of the critical residues involved in both the kinase and the phosphatase activities. This suggests that *S. meliloti* uses its incomplete PTS to regulate metabolism and may do so through phosphorylation of HPr by HPrK. It has been shown previously that HPr and ManX participate in SMCR in *S. meliloti* (44) and that research suggested that HPr-His22-P was, directly or indirectly, responsible for exerting repression in the presence of succinate.

**HPrK is involved in catabolite repression through phosphorylation of HPr-Ser53.** In *B. subtilis* and other firmicutes, HPrK phosphorylates HPr on a conserved serine-46 residue. We investigated the role of HPrK and its possible phosphorylation of HPr in SMCR in *S. meliloti* by construction of mutant strains lacking HPrK and other strains in which potential phosphorylation of HPr at the conserved histidine-22 and serine-53 residues was prevented. A strain devoid of the *hprK* gene exhibited strengthened SMCR, as evidenced by long diauxic lags. It also showed that downregulation of *mela* and *lac* genes was needed for utilization of raffinose and lactose even in the absence of succinate, indicating strong repression of those genes. These phenotypes were replicated by a strain with a mutant HPr that could not be phosphorylated on the conserved serine-53 residue. This indicated that HPrK most likely phosphorylates HPr at the serine-53 residue, the lack of which results in enhanced catabolite repression. We noted that some of the phenotypes observed in the strain with an unphosphorylatable serine-53 in HPr were less severe than those of a strain lacking HPrK. It has been reported that substitution of an alanine for the conserved serine residue can affect phosphorylation at the histidine residue by EI, because the serine residue is part of the HPr-EI interaction interface (15). Thus, the slight differences in phenotype between the  $\Delta hprK$  and the *hpr(S53A)* strains may be caused by a lessening of phosphorylation of the HPr-S53A at its histidine-22 site.

Two, not mutually exclusive, hypotheses could explain the enhanced SMCR seen in the  $\Delta hprK$  strain. Either HPrK is required for activation of catabolic genes or accumulation of HPr-His22-P in the cell results in enhanced SMCR. A mutant strain in which both the serine-53 and the histidine-22 residues of HPr were unphosphorylatable eliminated the strong phenotype caused by an unphosphorylatable serine-53. Also, the strong phenotype of the  $\Delta hprK$  strain was eliminated when HPr was replaced with HPr-H22A. This suggests that HPr-His22-P mediates the  $\Delta hprK$  phenotypes and that HPrK may act by slowing down phosphorylation at the histidine-22 residue when it phosphorylates the serine-53 residue. In *B. subtilis* and other gram-positive organisms, phosphorylation of HPr at the conserved serine residue significantly slows down phosphorylation by EI at the conserved histidine residue (26). In support of the

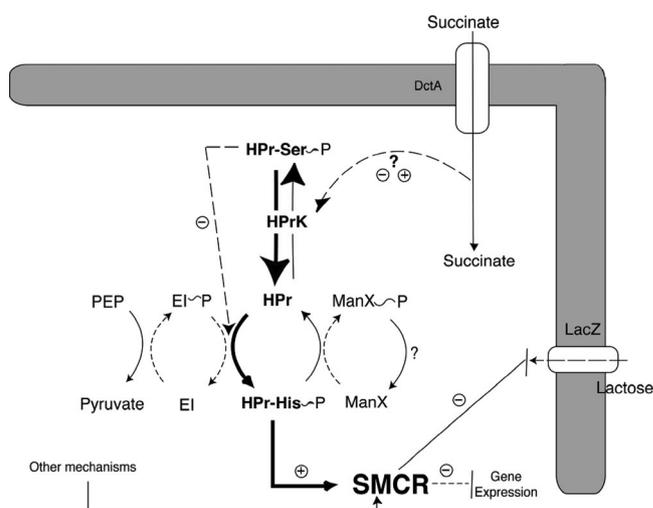


FIG. 10. Proposed model for the effects of HPrK on SMCR in *S. meliloti*. Phosphate groups enter the PTS by transfer from PEP to the histidine-22 residue of HPr. Phosphatase activity or low kinase activity of HPrK in the presence of succinate allows for accumulation of HPr-His22-P, which elicits SMCR. In the absence of succinate, phosphorylation of the serine-53 residue of HPr prevents HPr-His22-P formation, relieving catabolite repression. Dashed lines indicate phosphorylation events that are assumed to take place based on work in other model systems.

idea that HPr-His22-P is important for exerting SMCR, a mutant devoid of ManX, which likely accumulated HPr-His22-P, exhibited enhanced SMCR (44).

If HPr-His22-P were solely responsible for establishing SMCR, it would be expected that a strain containing only HPr-H22A would exhibit completely relieved SMCR. Strain CAP93[*hpr(H22A)*] did exhibit relaxed SMCR, but not a complete relief from catabolite repression. A likely explanation for this result is that there is more than one mechanism that operates to establish SMCR. An example of another protein that is involved in regulation of catabolite repression in *S. meliloti* is the sensor kinase Sma0113 (Garcia and Gage, unpublished).

We have provided evidence consistent with a model wherein HPrK phosphorylates HPr at the serine-53 residue, an event that is important in the regulation of SMCR in *S. meliloti*. Phosphorylation at serine-53 most likely affects SMCR control by slowing down or preventing phosphorylation by EI at the histidine-22 residue. Our results, along with previous results, indicate that HPr-His22-P, directly or indirectly, strengthens SMCR. A simplified model of phosphate transfer and SMCR regulation is shown in Fig. 10. During metabolism, HPr is phosphorylated at the histidine-22 residue by EI<sup>Ntr</sup> and transfers the phosphate group to ManX. In the presence of succinate or other preferred carbon sources HPr-Ser53-P levels are low through HPrK phosphatase activity or through a reduction in the synthesis of HPr-S53-P by HPrK. The reduction in HPr-Ser53-P levels allows HPr-His22-P to increase and elicit strengthened SMCR gene regulation and/or inducer exclusion.

It is interesting that phosphorylation of the conserved serine-53 residue weakens SMCR in *S. meliloti* because, in *B. subtilis* and other firmicutes, phosphorylation of the conserved serine strengthens catabolite repression (16, 27). Thus, the

regulation of catabolite repression in *S. meliloti* is mechanistically different from other organisms that possess HPr and HPrK. This difference extends not only to the role of HPr and HPrK but also to how the PTS receives information about carbon status, since succinate is neither imported into the cell by PTS-related proteins nor phosphorylated upon entry.

We have also shown that HPrK is involved in other cellular processes, such as carbon utilization, regulation of succinoglycan production, and symbiosis with alfalfa. It was reported that an *S. meliloti* strain lacking HPr was also upregulated for succinoglycan production (44). This suggests that the succinoglycan phenotype of the  $\Delta hprK$  strain is regulated through HPr, as are the SMCR phenotypes. This, however, has yet to be further investigated. Many strains with modified succinoglycan production also exhibit alterations in their symbiotic properties, in particular in their abilities to infect root hairs and construct proper infection threads (11, 21, 43, 60). In contrast, the  $\Delta hprK$  strain in the present study successfully invaded its host but exhibited a nitrogen fixation defect. The inability to fix nitrogen in strain CAP14( $\Delta hprK$ ) could be related to its slow-growth phenotype or to failure to differentiate into bacteroids, since the internal structure of alfalfa nodules populated by this strain exhibits a low density of bacteroids.

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