Expression and Regulation of Phosphate Stress Inducible Genes in *Sinorhizobium meliloti*

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Sinorhizobium meliloti 104A14 was mutated with transposon Tn5B22, which creates *lacZ* transcriptional fusions when inserted in the correct orientation relative to the promoter. This promoter reporter allowed us to identify six phosphate stress inducible (*psi*) genes in *S. meliloti* that are up-regulated in response to inorganic phosphate (P_i) starvation. The transposon and flanking DNA were cloned from each *psi*::Tn5B22 reporter mutant and the junction DNA sequenced. High identity/similarity of the inferred peptides with those in major data bases allowed identification of the following genes: *dnaK*, *expC*, *pssB*, *ackA*, *vipC*, and *prkA*. The *prkA* homolog was also found to be up-regulated in response to carbon starvation and when nitrate replaced ammonium as the nitrogen source. Through allele replacement techniques, $PhoB^-$ mutants were generated for the *expC*, *ackA*, *vipC*, and *pssB* reporter strains. Loss of a functional *PhoB* resulted in the absence of P_i -sensitive induction in all four genes. These experiments suggest the *Pho* regulon in *S. meliloti* includes genes that presumably are not directly linked to P_i acquisition or assimilation. The *psi* strains were tested for their symbiotic properties under growth conditions that were P_i -limiting or P_i -nonlimiting for the host plant. All were *Nod*⁺ and *Fix*⁺ except the reporter strain of *dnaK* transcription, which was less effective than the wild-type strain under both *P* treatments, indicating *DnaK* is required for optimum symbiotic function.

Additional keywords: acetyl-phosphate, heat shock protein, polysaccharide.

In soil and aquatic environments, bacteria often experience conditions in which the bioavailability of carbon sources, electron donors/acceptors, and nutrients can fluctuate significantly. Energy inputs into these systems can be sporadic, with periods of weeks or perhaps months when substrates are in extremely low concentration and significantly depart from optimum growth conditions. Spore formation is a strategy used by some bacteria to survive these periods; however, a majority of known bacteria, including *Sinorhizobium meliloti*, do not have this survival mechanism. Such organisms must somehow

adapt to maintain cellular integrity and an energy state that will allow them to respond competitively when growth conditions are again favorable.

One of the best-studied bacterial responses to nutrient limitation is that of inorganic phosphate (P_i) stress (Wanner 1996). The phosphate (*Pho*) regulon in *Escherichia coli* has been examined in some detail and is estimated to be composed of at least 31 genes arranged in 8 different transcriptional units (Wanner 1996). These genes code for proteins involved in P_i acquisition and assimilation, and include alkaline phosphatase, proteins involved in organophosphate uptake and degradation, proteins required for phosphonate metabolism, and a high affinity P_i transport system (Wanner 1996). Studies of random insertion of *MudI* into the *E. coli* genome have also identified P_i stress inducible (*psi*) genes coding for enzymes presumably not directly involved in P_i acquisition and assimilation. These include *gltB* and *gltD* (code for the large and small subunits of glutamate synthase) and *glpB* (encodes anaerobically regulated glycerol-3-phosphate dehydrogenase) (Metcalfe et al. 1990). These observations were one of the first clues that the effects of P_i stress may extend beyond cell responses that involve attempts to maintain a steady supply of *P* when environmental P_i levels become depleted. A recent and more exhaustive estimate of P_i stress regulatory effects in *E. coli* showed that the impact of P_i starvation is global in nature, with the expression of approximately 400 proteins influenced by P_i availability (VanBogelen et al. 1996). Approximately half of these proteins are up-regulated in response to P_i stress, while the others are down-regulated. It is not known how many of the P_i -affected genes in *E. coli* are under the control of the *Pho* regulatory circuitry (reviewed by Wanner 1996). There are examples of P_i stress inducible genes in *E. coli* that are not under the control of known *Pho* regulatory components (Wanner and McSharry 1982). There are also P_i -affected genes in *E. coli* (Wanner and McSharry 1982) and a marine *Vibrio* sp. (Nystrom et al. 1992) that are inducible by additional environmental and nutrient stress situations.

We have been studying the *Pho* regulatory response in *S. meliloti* and *Rhizobium tropici* by assessing P_i concentrations that induce the P_i stress response in these bacteria (Al-Niemi et al. 1997a, 1997b), expression of *pho* genes during symbiosis (Al-Niemi et al. 1997a), and the regulatory genes involved (Al-Niemi et al. 1997b). In this paper, we report the use of a transposon-delivered promoter reporter to identify *S. meliloti* genes that are up-regulated in response to P_i stress, demonstrate that some of these genes are controlled by *Pho* regula-

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tory circuitry, and examine the symbiotic properties of the resulting mutants.

RESULTS

Isolation and identification of *psi* genes.

Screening of transconjugants for P_i -sensitive β -galactosidase expression resulted in the isolation of 6 *psi* strains in which the transposon-interrupted gene was identified. Following removal of P_i from the medium of broth cultures, increases in reporter enzyme activity in cell suspensions ranged from approximately twofold (*psi21*) to approximately 38-fold (*psi52*) after 16 h of incubation (Table 1). Apparent constitutive transcription of some *psi* genes under high P_i culture conditions varied considerably, i.e., from 50 Miller units for *psi25* to roughly 1,500 Miller units for *psi21*.

With Tn5B22 as a probe, Southern blot analysis verified the presence of a single transposon insertion in each *psi* strain and established that the entire Tn5B22, and suitable amounts of flanking chromosomal DNA, could be subcloned as an *Xma*I or *Sal*I fragment from DNA of each *psi* strain (results not shown). Single-strand sequence analysis of junction DNA ranging from 195 to 542 nucleotides allowed identification based on homology of the inferred peptide to those in major data bases (Table 1). *psi52* shared homology with *vipC* from *Salmonella typhi* and a smaller segment of the sequenced region also shared significant homology (66% similarity, 43% identity over 48 inferred amino acids) with *sus* from *Mycobacterium leprae*. *psi53* is a homolog to *pssB* found in *R. leguminosarum* bv. *viceae* (Ivashina et al. 1994), also referred to as *pss1* (Borthakur et al. 1988), and *psi10* showed nearly complete identity with *S. meliloti* ExpC (Becker et al. 1997). The insertion in *psi21* was found to be 84 bp upstream of the *S. meliloti* *dnaK* gene (Falah and Gupta 1994), which encodes the heat shock protein 70. At the nucleotide level, sequence for 318 bp downstream of the *psi21* insertion was 96% identical to the *dnaK* sequence previously determined for *S. meliloti* (Falah and Gupta 1994); the inferred peptide was 100% identical. Additional evidence for *dnaK* identification was obtained from the MOTIFS program (Devereux et al. 1984), which identified a perfect match between the inferred DnaK

peptide and the N-terminal signature pattern IDLGTNS that is unique to the heat shock protein 70 family. Primer extension with a primer designed from within the *dnaK* coding region resulted in a product extending beyond the site of transposon insertion (results not shown), indicating the transposon inserted between the promoter and the *dnaK* open reading frame and that *psi21* is a bona fide reporter of transcription for this gene.

In addition to P_i starvation, nutrient stresses such as starvation for carbon, oxygen, or nitrogen were examined to determine if the *psi* genes were influenced by factors other than P_i starvation. None of the *psi* reporter genes were influenced by these other factors except *psi57*, which showed significant induction in response to carbon starvation (Fig. 1A). Within 6 h of being resuspended in minimal media where the carbon source was omitted, reporter enzyme levels increased fivefold. Other aspects of nutrient availability such as nitrogen source were also studied. Again, only *psi57* was found to be influenced and only when nitrate replaced ammonium as the nitrogen source (Fig. 1B). No effect was observed when ammonium was replaced by glutamine (results not shown). Finally, reporter assays for *psi21* in rapid temperature upshift experiments showed no appreciable increase in β -galactosidase activity above the high constitutive levels observed at normal growth temperatures of $\leq 30^\circ\text{C}$ (results not shown).

Regulatory control of *psi* gene responses.

Introduction of a *PhoB*⁻ mutation would provide a means of determining whether these *psi* genes were controlled by *Pho* regulatory circuitry. A *phoB::Ω* allele was successfully introduced into the *psi10*, *psi25*, *psi52*, and *psi53* reporter strains. All recombinations were verified by Southern blot as was the absence of alkaline phosphatase induction following P_i starvation (results not shown). P_i stress induction of each of these *psi* genes was abolished by the *phoB* mutation (Fig. 2). Induction was also studied in P_i depletion experiments to determine the P_i concentration required to induce these *psi* genes and to compare their induction with that of *phoA*, the structural gene for alkaline phosphatase and a known *PhoB*-regulated enzyme (Al-Niemi et al. 1997b). In such experiments where P_i supply was not abruptly eliminated, differences in induction between the *psi* genes and *phoA* were readily apparent. Relative to

Table 1. Levels of reporter enzyme induction and identification of the *psi* genes

<i>psi</i> gene	Miller units ^v		Fold induction ^w	Inferred peptide (% similarity/% identity; no. inferred amino acids) ^x	Gene product or function	Organism	Reference
	+ P_i	- P_i					
<i>psi10</i>	55 ± 8	344 ± 36	6	ExpC (95/93:87)	EPS II synthesis	<i>Sinorhizobium meliloti</i>	Becker et al. 1997
<i>psi21</i>	1,506 ± 167	2,595 ± 128	2	DnaK (100/100:101)	Heat shock	<i>Sinorhizobium meliloti</i>	Falah and Gupta 1994
<i>psi25</i>	50 ± 7	1,350 ± 197	27	AckA (61/35:97)	Acetate kinase	<i>Clostridium acetobutylicum</i>	Boynton et al. 1996
<i>psi52</i>	41 ± 0	1,544 ± 17	38	VipC (50/28:84)	Polysaccharide synthesis ^y	<i>Salmonella typhi</i>	Hashimoto et al. 1993
<i>psi53</i>	52 ± 7	1,628 ± 184	31	PssB (75/68:38)	Polysaccharide synthesis ^y	<i>Rhizobium leguminosarum</i> bv. <i>viceae</i>	Ivashina et al. 1994
<i>psi57</i>	158 ± 5	500 ± 49	3	PrkA (57/37:105)	Protein kinase ^z	<i>Bacillus subtilis</i>	Fischer et al. 1996

^v Miller units are shown as the mean ± standard error of at least 3 experiments; all inductions were statistically significant ($\text{LSD}_{\alpha=0.05}$).

^w Induction data represents Miller units of the - P_i cells divided by Miller units of the + P_i cells after 16 h of incubation in minimal mannitol ammonium chloride (MMN) medium containing no P_i and MMN containing 5 mM P_i , respectively.

^x Inferred peptides were derived from nucleotide sequences with BLASTX 2.0 searches (Altschul et al. 1997) on the NCBI nonredundant data base posted on-line 2 June 1998.

^y Exact function of encoded protein is unknown.

^z Substrate identity in kinase reaction is unknown.

phoA, up-regulation of *psi10*, *psi52*, and *psi53* was consistently delayed 2 h. Results shown in Figure 3 are for *psi10* but are typical for *psi52* and *psi53*. Typically, up-regulated levels of alkaline phosphatase activity were first detected between 2 and 3 h when P_i levels had decreased to roughly 5 μ M (averaged across all strains). However, increases in β -galactosidase activity in these strains were not detectable until

after 4 h, well after medium P_i levels were below detection limits. Induction of *psi25* was more similar to that of *phoA*; delays were observed in some experiments, but never to the extent recorded for the other *psi* strains (results not shown).

Symbiotic phenotype of the *psi* reporter mutants.

The *psi* strains were tested for their symbiotic properties with alfalfa plants grown with limiting and nonlimiting levels

Fig. 1. Induction of *psi57* occurs in response to carbon starvation and when nitrate replaces ammonium as the nitrogen source. Culture optical density and β -galactosidase activity (shown as Miller units) were measured at 2-h intervals. Each data point is the average of two means (three observations per mean) obtained from two independent experiments; error bars (where visible) represent the range of these two means. *psi21* did not show a response to either treatment, but is not shown due to relatively large differences in constitutive reporter enzyme levels that would distort presentation of the other *psi* strains. **A**, Mid-exponential phase cells of each *psi* strain grown in minimal mannitol ammonium chloride (MMN) medium were washed in MMN lacking mannitol and resuspended in MMN lacking mannitol. There was no change in culture optical density after cells were resuspended in the zero carbon medium. **B**, Cells cultured as in **A**; then washed and resuspended in MMN in which ammonium was replaced by nitrate. All cultures resumed growth after resuspension in the nitrate medium (results not shown). Strain symbol designations are the same as in **A**.

Fig. 2. Involvement of PhoB in regulation of *psi* genes following inorganic phosphate (P_i) limitation. Reporter enzyme levels are shown as Miller units and were derived from cells that had been washed, resuspended, and incubated in + P_i medium or - P_i medium for 16 h. Results are from at least three separate cultures; where visible, error bars represent one standard error. Open bars, + P_i cells; filled bars, - P_i cells. For each set of bars for each strain, presence or absence of a functional PhoB is shown as + or -, respectively.

Fig. 3. Induction of *psi10* is delayed relative to alkaline phosphatase. Results for *psi52* and *psi53* were similar with respect to delayed induction; results are not shown to simplify documentation of this observation. Mid-exponential phase cells were washed and resuspended in minimal mannitol ammonium chloride medium containing 50 μ M inorganic phosphate (P_i) as described in Materials and Methods. At 1-h intervals, cell samples were withdrawn and measured for optical density, alkaline phosphatase, and β -galactosidase activity. P_i concentration in the medium was also measured at each time point. Measurement symbols are shown in axis labels. Each data point is the mean of three independent cultures; where visible, error bars represent one standard error.

of P_i . Across all symbiotically grown plants except those inoculated with *psi21*, average plant dry matter production was $10.3 \pm 0.1 \text{ mg} \cdot \text{plant}^{-1}$ and $14.2 \pm 0.2 \text{ mg} \cdot \text{plant}^{-1}$ for the P_i -limiting and P_i -sufficient treatments, respectively. Differences in plant dry matter between P_i treatments were statistically significant for all inoculant strains ($\text{LSD}_{\alpha} = 0.05$). Except for *psi21*, the *psi* strains were not different from the wild type with respect to symbiotic phenotype, therefore only results comparing the wild-type strain with *psi21* will be described in detail (Table 2). Under P_i -limiting and P_i -sufficient conditions, dry matter production by plants inoculated with *psi21* was 68 and 75%, respectively, of plants inoculated with the wild-type strain. While this difference was subtle, it was statistically significant ($\text{LSD}_{\alpha} = 0.05$) in each of two independent experiments. Comparing plants within each strain treatment, nodule number per plant was not affected by P treatment, whereas nodule fresh weight increased significantly at high P and is consistent with previous results (Al-Niemi et al. 1997b; Deng et al. 1998). Comparing strains within P_i treatments, plants inoculated with *psi21* tended to have more nodules than plants inoculated with 104A14, but these differences were not statistically significant. However, *psi21* formed significantly more nodules under high P conditions than the wild-type strain under low P conditions (Table 2). Acetylene reduction rates were not significantly different between strains nor between P_i treatments. This is again consistent with results of our previous work with alfalfa (Al-Niemi et al. 1997b; Deng et al. 1998).

Reporter enzyme levels were measured in bacteroids to assess whether any of these genes appeared up-regulated during symbiosis, and if bacteroid expression of any of these P_i -sensitive genes would differ when the host plant was subjected to P_i -limiting conditions (Table 3). Low levels of background reporter enzyme activity were observed in bacteroids of the wild-type strain and this is consistent with other studies (Uhde et al. 1997). P_i limitation of the host plant did not affect expression of these *psi* genes, as reporter enzyme levels in bacteroids of P_i -limited treatments were the same or slightly lower than in P_i -sufficient plants. Reporter enzyme levels in *psi21* bacteroids were very high, and higher than that observed when *psi21* free-living cells were grown under either high or low P_i culture conditions (Table 1). Bacteroid expression of the other *psi* genes was much lower and similar to levels observed for free-living cells grown in media containing high levels of P_i (Table 1).

To verify that nodules were formed by the appropriate *psi* strain and not by revertants, nodule occupants were tested for

antibiotic resistance and for transposon stability. All tested nodule isolates from each treatment were gentamicin resistant and Southern blot analysis demonstrated that nodule isolates were identical to the original mutant, showing no evidence of transposon relocation in the genome (data not shown).

DISCUSSION

We have previously reported that *S. meliloti* up-regulates the P_i stress marker enzyme alkaline phosphatase when medium P concentrations drop below $10 \mu\text{M}$, and that this response is controlled by the regulatory protein PhoB (Al-Niemi et al. 1997b). Similar results were obtained in the current study (Fig. 3). This type of response is not novel, as it has been demonstrated in other bacteria (Wanner 1996), but illustrates that it is likely that the Pho regulon in *S. meliloti* is induced under many/most normal soil situations, in which soluble P_i concentrations in the soil solution are often found to be 1 to $5 \mu\text{M}$ (Barber 1984).

Studies with *E. coli* (Metcalf et al. 1990; VanBogelen et al. 1990, 1996) and a marine *Vibrio* sp. (Nystrom et al. 1992) clearly show that the P_i stress response is global, that it involves proteins that do not necessarily participate in phosphorus acquisition or metabolism, and that some P_i -sensitive genes also up-regulate in response to other environmental cues such as carbon and nitrogen starvation. With *S. meliloti*, we have identified additional *psi* genes that suggest a variety of

Table 3. Reporter enzyme levels in bacteroids of *Sinorhizobium meliloti* 104A14 and *psi* reporter mutants taken from nodules of alfalfa plants grown under inorganic phosphorus (P_i)-limiting and P_i -sufficient growth conditions^y

Strain	Bacteroid β -galactosidase activity (Miller units) ^z	
	Low P_i plants	High P_i plants
Wild type	11 ± 3	5 ± 3
<i>psi21</i>	$3,956 \pm 726$	$4,242 \pm 605$
<i>psi25</i>	22 ± 12	35 ± 3
<i>psi52</i>	71 ± 20	61 ± 8
<i>psi53</i>	110 ± 36	126 ± 46
<i>psi57</i>	125 ± 2	213 ± 49

^y Data are presented as the mean $\pm$ standard error. Results are from a single experiment except for *psi21*, for which the data are summarized from two independent growth chamber experiments.

^z Nodules from each strain- P_i treatment combination were combined and then three subsamples were taken and processed for measurement of β -galactosidase activity as described in Materials and Methods. Bacteroid β -galactosidase activity was not determined for *psi10*.

Table 2. Symbiotic performance of *Sinorhizobium meliloti psi21* compared with the wild-type strain 104A14 under conditions of high and low phosphorus for the host plant^y

Plant phosphorus treatment	Strain	Shoot dry wt. (mg/plant)	Nodules per plant	Nodule fresh wt. (mg/plant)	Acetylene reduction activity ($\mu\text{mol C}_2\text{H}_2/\text{h/g-nodule}$)
Low	Uninoculated	4.5 a^z			
	<i>psi21</i>	7.1 b	7.0 ab	5.9 ab	6.0 a
	104A14	10.5 c	5.6 a	4.8 a	9.7 a
High	Uninoculated	5.3 a			
	<i>psi21</i>	10.4 c	7.9 b	7.8 c	7.8 a
	104A14	13.8 d	6.3 ab	6.8 bc	8.1 a

^y Except for acetylene reduction data that are derived from a single experiment, results are summarized from two independent growth chamber experiments, with 5 replicates per mean for each experiment.

^z Data were subjected to analysis of variance; means followed by the same letter in the same column are not significantly different as determined by $\text{LSD}_{\alpha} = 0.05$.

cellular functions are affected by P_i limitation (Table 1). Included are genes otherwise involved in the heat shock response (*dnaK*), polysaccharide synthesis (*expC*, *pssB*, and *vipC*), and acetyl-phosphate metabolism (*ackA*), and a gene coding for a protein kinase (*prkA*); the *prkA* homolog was also significantly induced by carbon starvation and to a lesser extent when the nitrogen source was changed from ammonium to nitrate (Fig. 1).

S. meliloti produces an acidic exopolysaccharide referred to as EPS II, which is coded by a gene cluster made up of five complementation groups (Glazebrook and Walker 1989; Becker et al. 1997) including *expC*, which constitutes complementation group C (Becker et al. 1997). Induction of *expC*::Tn5B22 by P_i starvation (Table 1, Fig. 2) is consistent with previous work that demonstrated that EPS II synthesis is significantly increased under low phosphate conditions (Zhan et al. 1991). Loss of P_i -starvation-dependent induction of *psi10* in a *phoB* mutant (Fig. 2) provides important evidence that a positive regulator of the *exp* gene cluster is PhoB. Positive regulation of *exp* genes by PhoB would be in addition to MucS (referred to as ExpG by Becker et al. 1997), which was found by Astete and Leigh (1996) to positively regulate *mucE* (otherwise referred to as *expE*, Becker et al. 1997). It is perhaps important to note, however, that induction of *expC* in response to P_i starvation was significantly greater than that observed for *mucE* (Astete and Leigh 1996) and was clearly evident after only a few hours (Fig. 3), as opposed to the several days required for *mucE* (Astete and Leigh 1996). Also, it should be noted that the difference in peptide identity inferred from *expC* between strain 104A14 used in this study and strain Rm2011 used to determine the sequence of the *exp* operon (Becker et al. 1997) is similar to the differences in *expD* and *expE* reported by Lloret et al. (1998). Two other *psi* genes, *vipC* and *pssB*, were identified as potentially being involved in polysaccharide synthesis, suggesting the possibility that, in addition to EPS II, *S. meliloti* has other polysaccharides that are expressed under low P_i conditions.

Acetyl-phosphate has been implicated as a nonspecific phosphodonor, apparently capable of short-circuiting normal sensor kinase-regulated pathways as well as leading to P_i -independent control of the Pho regulon (Wanner 1992; Wanner and Wilmes-Riesenburg 1992; McCleary and Stock 1994). Further investigation of the *ackA* locus revealed an adjacent gene coding for phosphotransacetylase (Summers and McDermott 1998). It is perhaps important that genes coding for enzymes involved in acetyl-phosphate metabolism, and therefore acetyl-phosphate pool size, are induced by P_i starvation. This could provide one possible explanation to connect P_i starvation with the global induction of large numbers of proteins.

While the extent to which acetyl-phosphate plays a regulatory role in *S. meliloti* is not yet known, the requirement of PhoB for normal P_i stress induction of *phoA* and increased levels of P_i transport has been demonstrated (Al-Niemi et al. 1997b). PhoB was also shown to be required for P_i -sensitive induction of four of the *psi* genes examined in the current study (Fig. 2). However, induction of *psi10*, *psi52*, and *psi53* lagged significantly behind that of *phoA* (Fig. 3). In *E. coli*, a consensus DNA sequence referred to as a Pho box has been found preceding *pho* genes and identified as the binding site for phosphorylated PhoB (Makino et al. 1986). A putative Pho box has been identified in the promoter region of *S. meliloti*

genes involved in phosphorus metabolism (*S. meliloti phnG*, GenBank accession no. M96263) and P_i transport (Bardin et al. 1996), and we have also identified a putative Pho box in the DNA sequence preceding the *pta-ackA* operon in *S. meliloti* (Summers and McDermott 1998). Degenerate changes in a Pho box sequence could result in variable affinity for phosphorylated PhoB (PhoB-P), thus resulting in variable induction kinetics. However, once *psi10*, *psi52*, and *psi53* were induced, the accumulation of β -galactosidase in all three reporter strains roughly paralleled that of alkaline phosphatase, suggesting transcription of these genes proceeded at rates comparable to that of *phoA*. This at least suggests the possibility that in the P_i stress response, an additional regulatory protein may participate as an intermediary between PhoB and P_i -sensitive genes that perhaps are not preceded by a Pho box. A limited search for Pho box sequences in regions of the *S. meliloti exp* operon (Becker et al. 1997) showed no likely Pho boxes upstream of the proposed *expC* coding region; however, a 17-bp sequence with some similarity to a Pho box was identified 39 bp upstream of *mucS*. Therefore, it is possible that parts of the P_i stress response in *S. meliloti* are cascades, with one possible example being PhoB-P acting as a positive regulator of *mucS*, with MucS in turn directing transcription of some or all of the *exp* operon. A similar cascade scenario has recently been demonstrated for P_i -sensitive control of alginate synthesis in *Pseudomonas aeruginosa* (Kim et al. 1998).

DnaK modulation of gene expression in response to nutritional stress such as phosphate starvation (Spence et al. 1990) is consistent with the observed increased expression of *psi21* after prolonged P_i starvation (Table 1). In addition to playing an important role in refolding thermally damaged proteins (Schroder et al. 1993; Ziemienowicz et al. 1993), DnaK also facilitates normal protein folding (Gragerov et al. 1992; Nelson et al. 1992; Mayhew and Hartl 1996), protein export (Wild et al. 1992), and general proteolysis (Keller and Simon 1988). These functions are required for normal cell growth (Bukau and Walker 1989, 1990), and consistent with the apparent extremely high constitutive expression of *psi21* in *S. meliloti*. This is also similar to DnaK expression in *E. coli*, which together with GroEL constitutes approximately 3% of cellular protein at normal growth temperature (Neidhardt et al. 1984).

As *S. meliloti* bacteroid cell morphology differs from that of free-living cells, optical density estimates of biomass in reporter enzyme assays do not allow for direct comparisons between free-living cells and bacteroids. However, relative comparisons between bacteroids of the different *psi* strains are valid, and when compared against differences between free-living cells of the same strains, some assessments of *psi* gene expression in bacteroids are possible. With the exception of *psi53* bacteroids, which showed slightly increased reporter enzyme levels relative to P_i -nonlimited free-living cells, reporter enzyme levels in bacteroids of the *psi* strains were never greater than that found in free-living cells and usually less (Tables 1 and 3). Furthermore, P_i limitation of the host plant did not appear to positively influence *psi* expression (Table 3). Reporter enzyme activity in strain *psi21* bacteroids was much higher than in bacteroids of the other *psi* strains and appeared higher than in *psi21* free-living cells. This is consistent with the symbiosis phenotype of *psi21* and suggests the

possibility that DnaK plays some role in nodule function. The phenotype may be a result of reduced efficiency in normal protein processing or trafficking as discussed above, or may possibly be due to secondary, compensatory mutations that have been documented for other *dnaK* mutants (Bukau and Walker 1990) but have thus far gone undetected in *psi21*.

MATERIALS AND METHODS

S. meliloti wild-type strain 104A14 (Somerville and Kahn 1983) was the parent strain from which all transconjugants were derived. The basic medium used for culturing all strains was a minimal mannitol ammonium chloride (MMN) medium (Somerville and Kahn 1983) that was modified to contain no added P_i salts in the basal medium, but was buffered by 10 mM morpholinepropanesulfonic acid (MOPS; pH 7.0) and amended with variable amounts of P_i as indicated. In some experiments *S. meliloti* was cultured on YMB agar (Somerville and Kahn 1983). *E. coli* was cultured on LB medium (Sambrook et al. 1989). Ampicillin ($100 \mu\text{g} \cdot \text{ml}^{-1}$), gentamicin ($25 \mu\text{g} \cdot \text{ml}^{-1}$ for agar media; $15 \mu\text{g} \cdot \text{ml}^{-1}$ in broth cultures), streptomycin/spectinomycin (each $200 \mu\text{g} \cdot \text{ml}^{-1}$), or 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal, $40 \mu\text{g} \cdot \text{liter}^{-1}$ agar media) were included as required.

Transposon mutagenesis and reporter gene assays.

Transposon Tn5B22 contains a promoterless *lacZ* (Simon et al. 1989) and was used as a promoter probe to identify genes up-regulated in response to P_i stress conditions. Tn5B22 contained in the plasmid pSUP1021 (Simon et al. 1989) was transferred from *E. coli* strain S17-1 ($\text{Pro}^- \text{Mob}^+$, Simon et al. 1983) to *S. meliloti* by conjugation. Transconjugants were spread on MMN agar containing $60 \mu\text{M}$ P_i amended with gentamicin and X-Gal. Blue colonies were replica plated on MMN agar containing 5 mM P_i and MMN agar containing $60 \mu\text{M}$ P_i , with both media containing gentamicin and X-Gal. Transconjugants appearing darker blue on the low P_i medium were saved and the reporter gene activity (β -galactosidase) quantified in broth-cultured cells of each strain by the protocol described by Miller (1972), but as modified by Mulligan and Long (1985). In *E. coli*, genes affected by phosphate availability have been designated as *psi* (phosphate stress inducible; Wanner and McSharry 1982). In this study where we describe *S. meliloti* phosphate stress inducible genes, we adhere to this gene symbol and strains carrying the transposon Tn5B22 in a *psi* gene are referred to as *psi* strains.

Transposon cloning and *phoB* allele replacement.

The protocols of Sambrook et al. (1989) were used for all manipulations of plasmid and chromosomal DNA. For each *psi* strain, the transposon and flanking DNA were cloned to facilitate sequencing of the transposon insertion site for identification of the *psi* gene. Total chromosomal DNA was harvested, digested with *Xma*I or *Sal*I, and then ligated into pBluescript KS⁺ (Stratagene, La Jolla, CA). The ligate was transformed into *E. coli* strain DH5 α (Sambrook et al. 1989), and plasmids from transformants resistant to ampicillin and gentamicin were analyzed by restriction analysis to verify that each contained a single cloned fragment. Southern blots were used to verify the cloned fragment was identical to that in the genome from the respective *psi* strains.

The chromosomal DNA flanking the transposon was sequenced by the dideoxy nucleotide method with a kit purchased from United States Biochemical (Cleveland, OH). Primers were 5'-AACGACGGGATCCATAAT-3' and 5'-CCATGTTAGGAGGTCACATGGAAGTCAG-3' to initiate sequencing from the *lacZ* and transposase termini, respectively (Simon et al. 1989). Gene identification was based on sequence from a single strand, with homology searches conducted with the BLAST 2.0 network service (Altschul et al. 1997). Sequence alignments were done with the GAP program (Devereux et al. 1984). Sequences for *psi10* through *psi57* have been assigned accession numbers AF074450 through AF074455, respectively.

The *S. meliloti phoB* gene from strain 104A14 was previously cloned on a 2.3-kb *Hind*III fragment (Al-Niemi et al. 1997b) and for experiments in this study was subcloned to pUC19 (Yanish-Peron et al. 1985) and inactivated by insertion of an Ω cassette (Elhai and Wolk 1988) into a unique *Eco*RV site located within the *phoB* coding region. This entire construct was linearized with *Xba*I and subcloned into pJQ200SK⁺ (Quandt and Hynes 1993) to create pMLS90. pMLS90 was transformed into *E. coli* S17-1 to facilitate conjugal transfer to the *S. meliloti psi* strains. Isolation of *phoB::\Omega* allelic replacements was on MMN agar containing 5 mM P_i amended with gentamicin, streptomycin, spectinomycin, and 10% (wt/vol) sucrose. Gene replacement was verified with Southern blots with the wild-type *phoB* as probe (Al-Niemi et al. 1997b) and by loss of inducible alkaline phosphatase activity under P_i starvation conditions (Al-Niemi et al. 1997b).

Nutrient stress induction experiments.

For P_i starvation experiments, cells were grown to mid exponential phase in MMN broth containing 5 mM P_i and gentamicin, washed twice with MMN containing 0 P_i (25°C), resuspended in MMN containing 0 P_i to an optical density (OD) of 0.2 at A_{595} , and then divided into two cultures. No P_i was added to one of the cultures ($-P_i$ cells), while the other culture was amended to 5 mM P_i ($+P_i$ cells). $+P_i$ and $-P_i$ cultures were incubated in a reciprocal shaking water bath at 30°C. After the desired incubation period, a culture sample was taken for measurement of β -galactosidase activity.

P_i depletion experiments were conducted to determine the P_i concentration at which P_i -sensitive induction occurred, and to monitor induction of the *psi* genes relative to alkaline phosphatase. The *psi* strains were grown to mid log phase in MMN containing 10 mM P_i , washed twice in MMN containing 50 μM P_i (centrifuged at $5,000 \times g$, 8 min at 25°C), and then resuspended in MMN containing 50 μM P_i to an OD of 0.2 at A_{595} . At 60-min intervals, cell samples were measured for reporter enzyme and alkaline phosphatase activities as previously reported (Al-Niemi et al. 1997b). At each sampling time, the P_i concentration in the cleared spent medium was measured as described by Penney (1976).

MMN medium was also the base medium in experiments conducted to determine if the *psi* genes were sensitive to other nutrient limitations or to changes in growth conditions. For carbon or nitrogen starvation experiments, the cells were grown to mid exponential phase in MMN, washed in MMN or MMN that lacked the test nutrient, and then resuspended in the respective wash medium to an OD of 0.2 at A_{595} . For oxy-

gen limitation experiments, mid exponential phase MMN cells were diluted to an OD of 0.2 at A_{595} with fresh MMN, transferred to sterile serum-stoppered Vacutainer tubes (Becton-Dickinson, Rutherford, NJ), and bubbled with filter-sterilized N_2 gas to purge the tubes of oxygen. To assess nitrogen source as a possible factor in *psi* gene induction, exponential phase MMN cultures were washed and subsequently incubated in MMN in which either glutamine or nitrate was substituted for ammonium. All cultures were incubated as described above for P_i starvation experiments and control cultures were always included. In temperature up-shift experiments, cultures were grown in waterbath shakers to an OD of 0.18 to 0.30 at A_{595} in MMN at temperatures ranging from 21 to 30°C. Cultures were then incubated at 37 and 42°C for 5 min, 10 min, or continuously, with reporter enzyme activity monitored periodically over several hours.

Plant growth and inoculation.

Axenic alfalfa plants were cultured under growth conditions that are limiting or nonlimiting with respect to P_i availability as previously described (Al-Niemi et al. 1997b). Briefly, seeds of alfalfa (*Medicago sativa* L.) cv. Ladak were surface sterilized by soaking in 5.25% sodium hypochlorite for 5 min, rinsed six times in sterile distilled water, and germinated on YMB agar (Somerville and Kahn 1983). Seedlings showing no evidence of contamination were transferred to sterile growth box units (four seedlings per box, five replicate boxes per treatment) and inoculated immediately with approximately 1.0×10^6 washed *S. meliloti* cells per box. Plants were cultured in growth box units, described previously (McDermott and Kahn 1992), by the sand-alumina plant culture technique (Gourley et al. 1993) to control P_i supply at levels that were P_i limiting and P_i nonlimiting for the plants (Al-Niemi et al. 1997b). Plants were cultured in a growth chamber at 25°C with a light intensity of approximately $400 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ and a photoperiod of 16 h (Al-Niemi et al. 1997b).

Nodulation, nitrogen fixation, and bacteroid reporter gene activity.

Nitrogen fixation was estimated by acetylene reduction, by methods previously described (McDermott and Kahn 1992; Al-Niemi et al. 1997b). Nodules were removed, counted, and weighed, and plant shoots were dried at 65°C and weighed.

Reporter enzyme activity was also assayed in bacteroids isolated from these nodules. Nodules from each treatment were combined and then three subsamples (10 to 15 nodules per subsample) were washed by resuspending in 1.0 ml of Z buffer lacking β -mercaptoethanol (Miller 1972) in a 1.8-ml microcentrifuge tube and vortexing, and then crushed in the same tube with 1.0 ml of fresh complete Z buffer. The nodule homogenate was centrifuged at $1,000 \times g$ for 5 s to pellet crude debris and then 750 μl of the supernatant was centrifuged at $10,000 \times g$ for 5 min to pellet the bacteroids. The bacteroid pellet was then resuspended in 1.0 ml of Z buffer (Miller 1972) and β -galactosidase measured as for free-living cells.

For each *psi* strain, transposon stability during symbiosis was assessed. Nodules were surface sterilized as described previously (Al-Niemi et al. 1997b), and then crushed in 0.85% (wt/vol) saline solution and serially diluted, and aliquots spread onto YMB agar. Sixty isolated colonies of each mutant

were subcultured onto MMN and MMN-gentamicin to determine retention of antibiotic resistance. Tn5B22 was used as probe in Southern blot analysis of chromosomal DNA from five gentamicin-resistant nodule isolates for each *psi* strain to determine if the location of the transposon had changed.

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