Biosynthesis of the exopolysaccharide galactoglucan in *Sinorhizobium meliloti* is subject to a complex control by the phosphate-dependent regulator PhoB and the proteins ExpG and MucR

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The soil bacterium *Sinorhizobium meliloti* (*Rhizobium meliloti*) has the ability to produce the alternative exopolysaccharide galactoglucan (EPS II) in addition to succinoglycan (EPS I). In the wild-type strain EPS II production is induced by phosphate-limiting conditions or by extra copies of the *exp* gene cluster. Based on similarities to transcriptional regulators of the MarR family, an additional putative regulatory gene, *expG*, was identified in the *exp* gene cluster. Using *exp-lacZ* transcriptional fusions, a stimulating effect of extra copies of this *expG* gene on the transcription of all *exp* complementation groups was determined. Phosphate limitation also resulted in increased expression of the *exp-lacZ* fusions. This increase was reduced in strains characterized by a deletion of *expG*. The previously reported high level of *exp* gene transcription in a *mucR* mutant was further elevated under phosphate-limiting conditions. The *expA*, *expD*, *expG* and *expE* promoters contain sequences with similarities to the PHO box known as the PhoB-binding site in phosphate-regulated promoters in *Escherichia coli*. The *S. meliloti* *phoB* gene was required for the activation of *exp* gene expression under phosphate limitation, but not for induction of *exp* expression by MucR or ExpG.

Keywords: phosphate regulation, PHO box, *Rhizobium meliloti*

INTRODUCTION

The soil bacterium *Sinorhizobium meliloti* (*Rhizobium meliloti*) has the ability to produce the two acidic exopolysaccharides EPS I (succinoglycan) and EPS II (galactoglucan). The EPS I produced under standard conditions is required for nodule invasion of *Medicago sativa* (Leigh *et al.*, 1985; Battisti *et al.*, 1992; Leigh & Walker, 1994). It is composed of octasaccharide repeating units containing one galactose and seven glucose residues and can be decorated by acetyl, succinyl and pyruvyl groups (Reinhold *et al.*, 1994). The 36 kb *exo/exs* gene cluster directing the biosynthesis of EPS I was extensively studied and functions of the gene products were proposed (Buendia *et al.*, 1991; Becker *et al.*, 1993a, b, c, 1995a, b; Glucksmann *et al.*, 1993a, b; Müller *et al.*, 1993; Reuber & Walker, 1993).

EPS II can functionally replace the structurally different EPS I in nodule invasion (Glazebrook & Walker, 1989; Zhan *et al.*, 1989; González *et al.*, 1996). EPS II is

composed of alternating glucose and galactose residues which are decorated by acetate and pyruvate (Glazebrook & Walker, 1989). Recently, we reported on the 32 kb *exp* gene cluster directing the biosynthesis of EPS II and on the inferred properties of the encoded gene products (Becker *et al.*, 1997).

Under normal culture conditions, *S. meliloti* wild-type cells produce large amounts of EPS I and synthesize almost no EPS II. Zhan *et al.* (1991) showed that under phosphate-limiting conditions the wild-type strain is able to produce the alternative exopolysaccharide EPS II. Moreover, extra copies of the *exp* gene cluster resulted in the biosynthesis of EPS II (Glazebrook & Walker, 1989; Zhan *et al.*, 1989). The production of EPS II was also observed in the presence of a mutation in either of the regulatory genes *expR* (Glazebrook & Walker, 1989) or *mucR* (Keller *et al.*, 1995). In addition, it was shown that all *exp* complementation groups are transcribed at a substantially elevated level in a *mucR* mutant (Keller *et al.*, 1995; Becker *et al.*, 1997). While

the regulatory genes *expR* and *mucR* are located on the chromosome, an additional regulatory gene, *expG*, was identified on megaplasmid 2 in the *exp* gene cluster (Becker *et al.*, 1997). The proposed function of the *expG* gene product as a transcriptional regulator is based on weak homologies between ExpG and the PapX protein of *Escherichia coli*, a putative regulator of genes directing the biosynthesis of P pili (Marklund *et al.*, 1992). The strongest homologies were found to a conserved region of 35 amino acids which was described as the MarR family signature (Dehoux & Cossart, 1995). Transcriptional activators belonging to the MarR family bind DNA through a helix–turn–helix motif (Cohen *et al.*, 1993; Sulavik *et al.*, 1995). Recently, Astete & Leigh (1996) identified in *S. meliloti* the gene *mucS* which is identical to *expG*. *mucS* is required for EPS II induction by extra copies of the *exp* gene cluster. This gene is also required for the activation of the expression of one gene in the *expE* operon under phosphate limitation.

In this report, we investigate the complex regulation of *exp* gene expression by phosphate and the regulatory genes *expG* and *mucR*. Apart from an influence on the *exp* gene transcription by each factor alone, we focus on the interaction between the regulatory pathways involving phosphate availability and the regulators ExpG and MucR.

METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *E. coli* strains were grown in Penassay broth (Difco) or in LB medium (Sambrook *et al.*, 1989) at 37 °C. *S. meliloti* strains were grown in TY (Beringer, 1974) or LB (Sambrook *et al.*, 1989) medium at 30 °C. To study the effect of limiting phosphate on *exp* gene expression, the *S. meliloti* strains were grown in a MOPS-buffered medium described by Zhan *et al.* (1991). For the β -galactosidase assay, strains grown for 48 h in LB or TY liquid medium were washed once with MOPS medium containing either 2 mM phosphate (high phosphate) or 0.1 mM phosphate (low phosphate). A volume of washed cells corresponding to 75 μ l stationary culture was used to inoculate 10 ml of the appropriate MOPS medium.

Antibiotics were supplemented as required at the following concentrations (μ g ml⁻¹): streptomycin (Sm), 600; tetracycline (Tc), 10; gentamicin (Gm), 40; neomycin (Nm), 120 for *S. meliloti* and tetracycline, 10; gentamicin, 10; ampicillin (Ap), 100; and kanamycin (Km), 50 for *E. coli*.

DNA biochemistry. Preparation of plasmid DNA, DNA restriction, agarose gel electrophoresis, cloning procedures and transformations of *E. coli* cells were carried out according to established protocols (Sambrook *et al.*, 1989). Southern hybridizations were performed according to Kessler (1992). Total DNA from rhizobia was isolated according to Meade *et al.* (1982).

DNA sequencing. DNA sequencing to verify new plasmid constructs or mutations was carried out using the 'AutoRead Sequencing kit' (Pharmacia) according to a protocol devised by Zimmermann *et al.* (1990). Sequence data were obtained and processed using the ALF DNA Express Sequencer (Pharmacia) according to the manufacturer's instructions.

Construction of the *S. meliloti expG* deletion mutant. Plasmid pARII/1inv Δ *expG* characterized by a deletion comprising 490 nucleotides of the 3'-terminus of the *expG* coding region and 17 nucleotides downstream of *expG* was transferred by conjugation from the broad-host-range mobilizing strain *E. coli* S17-1 (Simon *et al.*, 1983) to *S. meliloti* 2011 as described by Simon (1984). Homogenization of the *expG* deletion was carried out as described by Masepohl *et al.* (1988). Selection by neomycin resistance conferred by plasmid pARII/1inv Δ *expG* resulted in the identification of a merodiploid strain carrying the plasmid integrated into the genome. This strain was cultured in liquid LB medium without antibiotic stress until stationary phase. Subsequently, homogenotes that lost neomycin resistance were tested for the absence of the vector and the presence of the *expG* deletion by Southern hybridization. The resulting strain was designated SmSRAG.

Interposon mutagenesis. Using appropriate restriction sites, a promoterless *lacZ*-Gm interposon (Becker *et al.*, 1995c) was inserted in the direction of transcription into fragments of the *exp* gene region, which were subcloned into the mobilizable suicide vector pK18mob (Schäfer *et al.*, 1994). Resulting hybrid plasmids were transferred from *E. coli* S17-1 (Simon *et al.*, 1983) to the *S. meliloti expG* deletion mutant SmSRAG as described by Simon (1984). Homogenization of the *lacZ*-Gm insertions was carried out as described by Masepohl *et al.* (1988). All homogenotes were verified by Southern hybridization. *S. meliloti exp-lacZ/mucR* double mutants (Becker *et al.*, 1997), *exp-lacZ/mucR/ Δ expG* triple mutants, *exp-lacZ/phoB* double mutants and *exp-lacZ/mucR/phoB* triple mutants were obtained via Φ M12-mediated transduction (Finan *et al.*, 1984).

β -Galactosidase assay of *S. meliloti* strains carrying *lacZ*-Gm insertions. *S. meliloti* strains were grown to an OD₆₀₀ of 0.6–0.8 in MOPS medium initially containing 0.1 mM phosphate or 2 mM phosphate. The assay for β -galactosidase activity was carried out according to the protocol devised by Miller (1972). Relative β -galactosidase units were calculated per cell number according to Miller (1972).

RESULTS

Phosphate limitation stimulates the expression of *exp-lacZ* transcriptional fusions

Previous complementation and operon analyses using *exp-lacZ* transcriptional fusions revealed the presence of five transcriptional units in the *exp* gene cluster (Glazebrook & Walker, 1989; Becker *et al.*, 1997). To determine whether the transcription of these five *exp* complementation groups is affected by phosphate limitation, the β -galactosidase activities of one representative *exp-lacZ* transcriptional fusion for each *exp* operon (Becker *et al.*, 1997) were determined in MOPS medium with 0.1 mM phosphate (low phosphate) or 2 mM phosphate (high phosphate) added. The transcription of the *exp-lacZ* fusions was studied in wild-type and *mucR* mutant backgrounds.

In the wild-type background *exp-lacZ* fusions showed only basal levels of β -galactosidase activity when the strains were cultured in MOPS medium containing 2 mM phosphate (Fig. 1). In MOPS medium containing 0.1 mM phosphate the expression of the *expA1-lacZ* and *expC-lacZ* fusions was stimulated about eightfold, whereas the expression of the *expE2-lacZ* and the

Table 1. Strains and plasmids

Strain/plasmid/phage	Relevant characteristics	Source/reference
<i>S. meliloti</i>		
Rm2011	Wild-type, Nx ^r Sm ^r	Casse <i>et al.</i> (1979)
Rm3131	Rm2011, <i>mucR31::Tn5</i>	Keller <i>et al.</i> (1995)
Rm101	Rm2011, Spc ^r cassette of pHP45Ω inserted into the <i>Pmacl</i> site of <i>mucR</i> (<i>mucR101-Spc</i>)	Becker <i>et al.</i> (1997)
RmAR1014	Rm2011, <i>expA1-lacZ</i> Gm ^r , insertion site: <i>KpnI</i>	Becker <i>et al.</i> (1997)
RmAR1016	Rm2011, <i>expC-lacZ</i> Gm ^r , insertion site: <i>XhoI</i>	Becker <i>et al.</i> (1997)
RmAR1018	Rm2011, <i>expG-lacZ</i> Gm ^r , insertions site: <i>StuI</i>	Becker <i>et al.</i> (1997)
RmAR1020	Rm2011, <i>expD1-lacZ</i> Gm ^r , insertion site: <i>PstI</i>	Becker <i>et al.</i> (1997)
RmAR1022	Rm2011, <i>expE2-lacZ</i> Gm ^r , insertion site: <i>EcoRI</i>	Becker <i>et al.</i> (1997)
RmAR1114	Rm3131, <i>expA1-lacZ</i> Gm ^r , insertion site: <i>KpnI</i>	Becker <i>et al.</i> (1997)
RmAR1116	Rm3131, <i>expC-lacZ</i> Gm ^r , insertion site: <i>XhoI</i>	Becker <i>et al.</i> (1997)
RmAR1118	Rm3131, <i>expG-lacZ</i> Gm ^r , insertion site: <i>StuI</i>	Becker <i>et al.</i> (1997)
RmAR1120	Rm3131, <i>expD1-lacZ</i> Gm ^r , insertion site: <i>PstI</i>	Becker <i>et al.</i> (1997)
RmAR1122	Rm3131, <i>expE2-lacZ</i> Gm ^r , insertion site: <i>EcoRI</i>	Becker <i>et al.</i> (1997)
Sm5RAG	Rm2011, deletion of the <i>expG</i> gene comprising 490 nucleotides of the 3'-terminus of the <i>expG</i> coding region and 17 nucleotides downstream of <i>expG</i>	This work
RmH615	Lac ⁻ , Ω <i>phoB3::TnV</i>	Bardin & Finan (1998)
RmH406	Ω <i>phoB3::Tn5-132</i>	T. M. Finan, McMaster University, Hamilton, Ontario, Canada
<i>E. coli</i>		
XL-1 Blue	<i>recA1 lac [F' proAB lacI^a ZΔM15 Tn10 (tet^r)] thi</i>	Bullock <i>et al.</i> (1987)
S17-1	<i>E. coli</i> 294, <i>thi</i> RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Simon <i>et al.</i> (1983)
Plasmid		
pARII/linvΔ <i>expG</i>	pK18mob containing a 2.9 kb <i>KpnI</i> - <i>BglII</i> fragment with a deletion comprising 490 nucleotides of the 3'-terminus of the <i>expG</i> coding region and 17 nucleotides downstream of <i>expG</i>	A. Roxlau
pPHU231	pRK290 derivative, broad-host-range vector	Hübner <i>et al.</i> (1991)
pSR1018	pPHU231 containing a 1 kb <i>PstI</i> fragment with the <i>expG</i> gene under control of its own promoter	This work
pSR1018-5	pPHU231 containing a 0.7 kb <i>RsaI</i> - <i>PstI</i> fragment with the <i>expG</i> gene under control of the <i>E. coli lac</i> promoter	This work
pK18mob	pUC18 derivative, <i>lacZα</i> Km ^r , <i>mob</i> site	Schäfer <i>et al.</i> (1994)
Phage		
ΦM12	<i>S. meliloti</i> transducing phage	Finan <i>et al.</i> (1984)

expG-lacZ fusions was enhanced about 12–18-fold. Expression of the *expD1-lacZ* fusion was only weakly influenced by phosphate starvation. Growth in MOPS medium with 0.1 mM phosphate resulted in a twofold increase of β-galactosidase activity.

In contrast to the β-galactosidase activities mediated by *exp-lacZ* fusions in the wild-type background, β-galactosidase activities of the *mucR* mutant carrying the *exp-lacZ* fusions were already detectable at a substantial level under high phosphate conditions (Fig. 1). Negative regulation of *exp* gene transcription by *mucR* has already been shown for *S. meliloti* strains grown in LB medium (Becker *et al.*, 1997). However, phosphate limitation of the *mucR* mutant resulted in an additional increase in the expression of the *exp-lacZ* fusions (Fig.

1). This indicates that MucR is dispensable for the activation of *exp* gene expression under phosphate-limiting conditions.

An *expG* mutation reduces the stimulating effect of phosphate limitation on the expression of the *expA* and *expE* operons

Recently it was reported that under phosphate limitation the regulatory gene *expG* is essential for the activation of the transcription of one gene in the *expE* operon (Astete & Leigh, 1996). To investigate the role of the *expG* gene in the phosphate-mediated regulation of additional *exp* operons, the β-galactosidase activities of the *expA1-lacZ*, *expD1-lacZ* and *expE2-lacZ* fusions

Fig. 1. Histogram showing the β -galactosidase activities expressed by *exp-lacZ* transcriptional fusions in the wild-type Rm2011, the *expG* mutant SmSRΔ*G*, the *mucR* mutant Rm3131 and the Δ*expG*-*mucR* double mutant dependent on phosphate concentration and copy number of the *expG* gene. Relative β -galactosidase activities mediated by the *expA1*(1014)-*lacZ* (a), *expD1*(1020)-*lacZ* (b), *expE2*(1022)-*lacZ* (c), *expC*(1016)-*lacZ* (d) and *expG*(1018)-*lacZ* (e) transcriptional fusions in the background of the wild-type (WT), the *expG* deletion mutant (Δ*expG*), the *mucR*3131::Tn5 mutant (*mucR*) and the Δ*expG*-*mucR*101-Spc mutant (Δ*expG*/*mucR*) are shown. The cells were grown in MOPS medium with 2 mM phosphate or 0.1 mM phosphate added in the presence or absence of extra copies of the *expG* gene on plasmid pSR1018-5 as indicated by the different shading of the bars (■, 2 mM phosphate; ▨, 0.1 mM phosphate; □, 2 mM phosphate + pSR1018-5; ▩, 0.1 mM phosphate + pSR1018-5). The β -galactosidase activity values are means of at least five independent assays. The background β -galactosidase activity of the wild-type strain Rm2011 was 4 ± 1 Miller units. Error bars denote 1 SD. ND, Not determined.

were determined in the *expG* deletion (Δ*expG*) mutant SmSRΔ*G*. The observed increase in the expression of the *expA1-lacZ* and *expE2-lacZ* transcriptional fusions

under phosphate limitation was reduced in the Δ*expG* mutant (Fig. 1). In the wild-type strain β -galactosidase activities mediated by the *expA1-lacZ* transcriptional fusion were enhanced sevenfold under phosphate limitation. However, phosphate limitation resulted in only a threefold increase in β -galactosidase activity in the Δ*expG* background (Fig. 1). The *expG* deletion had a stronger effect on the expression of the *expE2-lacZ* transcriptional fusion under phosphate limitation. Whereas β -galactosidase activity in the wild-type strain carrying the *expE2-lacZ* fusion was enhanced 12-fold under phosphate starvation, the activity mediated by this fusion in mutant SmSRΔ*G* was only enhanced twofold (Fig. 1). No effect of the *expG* deletion could be detected for the *expD1-lacZ* fusion. In the wild-type background as well as in the Δ*expG* background, transcription of the *expD1* gene was only increased at a very low level under phosphate limitation. Compared to the wild-type background, the expression of the *expD1-lacZ* fusion was not decreased.

Previously, we have demonstrated that a mutation in the regulatory gene *mucR* resulted in the enhanced expression of the five *exp* transcriptional units. To analyse whether *expG* influences the effect of the *mucR* mutation on *exp* gene transcription, the β -galactosidase activities of the *expA1-lacZ*, *expD1-lacZ* and *expE2-lacZ* fusions were measured in a Δ*expG*-*mucR* double mutant. In comparison to the results obtained with the *mucR* mutant, no significant difference was detected in the Δ*expG*-*mucR* double mutant under high or under low phosphate concentrations (Fig. 1). This indicates that the deletion of the *expG* gene had no influence on the level of *exp* gene expression in a *mucR* mutant.

Extra copies of the *expG* gene stimulate the transcription of the five *exp* operons

To study the effect of the *expG* gene dosage on *exp* gene regulation, the *expG* gene was subcloned under the control of its own promoter and the *E. coli lac* promoter in the low copy vector pPHU231 (Hübner *et al.*, 1991). The resulting plasmids pSR1018 (native promoter), pSR1018-5 (*lac* promoter) and as a control vector pPHU231 were transferred to the *S. meliloti* strains containing the *exp-lacZ* fusions. β -Galactosidase activities were determined in the wild-type background, and in the Δ*expG*, *mucR* and Δ*expG*-*mucR* mutants. No differences were observed when the strains lacking the plasmids were compared to strains carrying vector pPHU231. In the wild-type and Δ*expG* backgrounds, extra copies of the *expG* gene stimulated expression of all five *exp-lacZ* transcriptional fusions under high phosphate conditions (Fig. 1); the extent differed for each *exp* operon. While extra copies of *expG* resulted in 10–25-fold enhanced β -galactosidase activities for the *expA1-lacZ*, *expD1-lacZ* and *expE2-lacZ* fusions, the *expG-lacZ* and *expC-lacZ* fusions showed only a twofold increase. Phosphate limitation further increased

Table 2. Putative PHO boxes in the promoter regions preceding the *expG*, *expA*, *expD* and *expE* operons

The first row shows the *E. coli* consensus PHO box with two direct repeats separated by an AT-rich spacer of two to seven nucleotides (Wanner, 1993, 1996; VanBogelen *et al.*, 1996). The typical consensus sequence for the spacer of *E. coli* PHO boxes is shown. Weakly conserved nucleotides of the consensus sequence found in promoters shown to be regulated by a phosphorus stimulus (VanBogelen *et al.*, 1996) are printed in lower case. The second row shows the putative PHO boxes identified by Bardin *et al.* (1996) preceding the *phoC* gene. Below, sequences located upstream of the *expG*, *expA*, *expD* and *expE* operons are shown. Nucleotides in these regions which are identical to the consensus PHO box are printed in bold.

Gene	Direct repeat	AT-rich spacer (2–7 nt)	Direct repeat	No. of nucleotides to ATG*
	CTTTcat G	AT (A) AT (A)	CTTTCAC G A T	
<i>phoC</i>	CTGTTAC	AGAA	CCTACAC	84
	CTGACAC	TGCG	CTTTCAT	62
<i>expG</i>	GTGTTAT	GAAATTA	CTTCAAG	38
<i>expA1</i>	TTTGTCT	GAATATTG	CTTCAAT	170
	CTTCTGG	ATAAATCG	ATTTATT	25
<i>expD1</i>	CTTATCG	CAACAATA	CTGTGAT	64
<i>expE1</i>	CTCGTCG	CAAATTA	CTTTAAA	229
	GTTTAT	TTTATGCAT	CTGTGTT	91

* The position of the PHO-box-like sequences is given by the number of nucleotides to the ATG start codon of the relevant gene.

β -galactosidase levels for all fusions. Under high phosphate conditions it made no difference if extra copies of *expG* were provided by the plasmid pSR1018 or pSR1018-5. However, under low phosphate conditions, the presence of plasmid pSR1018 carrying the *expG* gene preceded by the native *expG* promoter region caused a slightly higher expression of the *expA1-lacZ* and *expE2-lacZ* fusions than the presence of plasmid pSR1018-5 containing the *expG* gene preceded by the *lac* promoter (data not shown).

The β -galactosidase activities detected in the *expG* deletion mutant SmSRAG when extra copies of *expG* were present showed no significant difference in comparison to the wild-type carrying extra copies of *expG*. In the *mucR* and $\Delta\text{expG-mucR}$ backgrounds, extra copies of the *expG* gene did not have any effect on the β -galactosidase activities mediated by the *exp-lacZ* fusions (Fig. 1).

PhoB is required for the activation of *exp* gene transcription under phosphate limitation

Sequence analysis of promoter regions preceding *E. coli* genes whose expression is influenced by phosphate resulted in the identification of a consensus sequence for PhoB binding, the so-called PHO box (Makino *et al.*, 1994; VanBogelen *et al.*, 1996). Putative PHO boxes were also identified in the intergenic regions preceding the *exp* complementation groups (Table 2). Whereas both in front of the *expG* and the *expD1* gene one PHO

box was found, we identified two putative PHO boxes in front of the *expA1* and *expE1* genes. In comparison to the PHO box preceding the *expG* gene the other PHO boxes are only weakly conserved. No PHO-box-like sequence was identified in the intergenic region preceding the *expC* gene.

The data showing *exp* operon induction under phosphate starvation (Fig. 1) and the identification of the sequences with similarities to the *E. coli* PHO box consensus in the *exp* promoter regions led to the hypothesis that the response regulator PhoB plays an important role in the phosphate-dependent regulation of *exp* gene transcription. To investigate this hypothesis, a *phoB::TnV* mutation (*phoB3*) (Bardin & Finan, 1998) was transferred to *S. meliloti* strains carrying the different *exp-lacZ* fusions. β -Galactosidase activities were determined in the *exp-lacZ/phoB::TnV* double mutants cultured in MOPS medium containing either 2 mM phosphate or 0.1 mM phosphate (Fig. 2). In the wild-type strain, phosphate limitation resulted in increased *exp* gene transcription; however, no effect of phosphate starvation could be detected in the *phoB* background. The level of β -galactosidase activities of all *exp-lacZ* fusions in the *phoB* mutant corresponded to the level of β -galactosidase activities detected in the wild-type background for the cells grown in MOPS medium with 2 mM phosphate added (Fig. 2).

To analyse if the stimulating effect of *mucR* on *exp* expression is dependent on *phoB*, expression of the

Fig. 2. Effect of a *phoB* mutation on *exp* gene expression. Relative β -galactosidase activities mediated by the *expA1*(1014)-*lacZ*, *expC*(1016)-*lacZ*, *expG*(1018)-*lacZ*, *expD1*(1020)-*lacZ* and *expE2*(1022)-*lacZ* transcriptional fusions in the wild-type (WT) and *phoB*::TnV mutant (*phoB*) strains are shown. The cells were grown in MOPS medium with 2 mM phosphate (■) or 0.1 mM phosphate (▨) added. The β -galactosidase activity values are means of at least five independent assays. The background β -galactosidase activity of the wild-type strain Rm2011 was 4 ± 1 Miller units. Error bars denote 1 SD.

exp-lacZ fusions was determined in a *mucR-phoB* mutant. In the presence of 2 mM phosphate, *exp-lacZ* expression was induced at a level comparable to the induction level in a *mucR* mutant (data not shown). This indicates that PhoB is not required for the induction of *exp* expression in a *mucR* mutant. To analyse if *phoB* is required for activation of *exp* transcription by ExpG, plasmid pSR1018-5 carrying *expG* preceded by the *lac* promoter was transferred to the *exp-lacZ/phoB* mutants. In a *phoB* mutant, extra copies of *expG* induced *exp-lacZ* expression under high and low phosphate conditions at the same level as additional copies of *expG* induced *exp-lacZ* expression under high phosphate conditions in the wild-type background (data not shown). This indicates that ExpG is able to activate *exp* transcription independent from PhoB.

DISCUSSION

Using *exp-lacZ* transcriptional fusions for each of the five *exp* operons, we detected increased *exp* gene expression in response to phosphate limitation. These results confirm the observation by Zhan *et al.* (1991), who reported that phosphate limitation induces the production of EPS II. Our data indicate that the phosphate effect on EPS II biosynthesis is at the gene transcription level of all known *exp* operons. Sequence comparisons of the intergenic regions of the *exp* operons to promoter regions preceding genes regulated by PhoB in *E. coli* led to the identification of sequence elements similar to the PHO boxes present in phosphate-regulated promoters of *E. coli*. Since expression of the *exp-lacZ* fusions was induced in response to phosphate limitation,

it is likely that these putative PHO boxes located upstream of the *expA1*, *expG*, *expD1* and *expE1* genes mediate the regulation by a PhoB-like protein in response to phosphate. The phosphate-dependent regulation of the phosphate (PHO) regulon in *E. coli* is controlled by the two-component regulatory system PhoR-PhoB (Wanner, 1993, 1996). Under phosphate-limiting conditions, the sensor PhoR activates the response regulator PhoB via phosphorylation. PhoB activates transcription by binding to promoters that are characterized by the PHO box. Recently, a high-affinity phosphate uptake system which is required for an effective symbiotic interaction between *S. meliloti* and alfalfa was identified in *S. meliloti* (Bardin *et al.*, 1996; Voegelé *et al.*, 1997). This transport system is encoded by the *phoCDET* genes, which are also induced in response to phosphate starvation. Interestingly, analysis of the *phoC* promoter led to the identification of two putative PHO boxes. The assumption that the PHO boxes in *S. meliloti* are controlled by a system like PhoR-PhoB was recently supported by the identification of the *S. meliloti phoB* gene (Al-Niemi *et al.*, 1997; Voegelé *et al.*, 1997; Bardin & Finan, 1998; P. A. McLean and others, GenBank accession no. M96261). Additionally, Bardin & Finan (1998) showed that *phoB* is required for *phoCDET* expression and that PhoB may also act as a repressor, since the expression of the alternative *orfA* *pit*-encoded low-affinity phosphate transport system is negatively regulated by the *phoB* gene product. As inferred by the presence of a PHO box preceding genes repressed or induced by phosphate limitation the regulator PhoB may also have activating or repressing properties in *E. coli* (VanBogelen *et al.*, 1996). In *S. meliloti*, a *phoB* mutation abolished induction of the *exp-lacZ* transcriptional fusions under phosphate-limiting conditions. The facts that (i) *exp* expression was strongly activated in response to phosphate limitation, (ii) PHO boxes were identified in the *exp* promoter regions and (iii) *phoB* was required for the activation of *exp* gene transcription under phosphate-limiting conditions strongly suggest that PhoB functions as an activator of *exp* gene transcription.

We previously reported that MucR acts as a transcriptional repressor of the *exp* genes (Becker *et al.*, 1997). The high levels of β -galactosidase activities detected in the *mucR* mutant under high phosphate concentrations (2 mM phosphate) were further increased under phosphate limitation (0.1 mM phosphate). This additional increase indicates that MucR may act independently from the phosphate-specific regulatory system, or it influences this system but it is not an essential part thereof. This hypothesis is supported by the finding that PhoB was dispensable for induction of *exp* expression in a *mucR* mutant.

We recently proposed that ExpG functions as a transcriptional regulator based on similarities to transcriptional regulators of the MarR family (Becker *et al.*, 1997). This is supported by Astete & Leigh (1996), who reported that ExpG is essential for the expression of one gene of the *expE* operon under phosphate limitation. In

the wild-type background additional copies of the *expG* gene resulted in increased *exp-lacZ* transcription, indicating that ExpG functions as an activator of *exp* gene transcription; however, the level of increase differed. Expression levels of *expG* and *expC* were at least five to seven times lower than those of the three other *exp* genes. Since the effects of extra copies of *expG* on the transcription of *expC* and *expG* were comparable and no PHO-box-like sequence was found upstream of the *expC* coding region, the phosphate- and *expG*-dependent regulation of *expC* might be mediated by the promoter and the PHO box preceding *expG*. The effect of extra copies of *expG* was further increased under phosphate-limiting conditions. This result might be explained by PhoB either acting directly to increase *exp* gene transcription under phosphate limitation or activating transcription indirectly by first increasing copies of ExpG.

We further showed that a deletion of the *expG* gene had no effect on the phosphate-dependent transcription of the *expD1-lacZ* fusion, but reduced the stimulating effect of phosphate limitation on the expression of the *expA1-lacZ* and *expE2-lacZ* fusions. The levels of reduction differed for both genes. In particular, the *expE2-lacZ* fusion appeared most influenced by the *expG* deletion. A possible explanation for these results is that under phosphate-limiting conditions the affinity of the response regulator PhoB for the PHO box in the *expG* promoter region is greater than for the less-conserved putative PHO boxes preceding the *expA*, *expD* and *expE* operons. Relative differences in PhoB affinity for the different PHO boxes may also explain the function of ExpG, which could act to balance overall *exp* expression. As the *expG* deletion did not completely block the stimulation of *exp* gene expression under phosphate-limiting conditions, we suggest that *expG* is not required for mediating the phosphate signal. Moreover, *phoB* was not required for induction of *exp* expression by ExpG if transcription of *expG* was directed by a constitutive promoter. This indicates that ExpG and PhoB are able to activate *exp* expression independently from each other.

Three different regulators (MucR, ExpG and PhoB) influencing *exp* gene expression in *S. meliloti* were identified. Previously, it was shown that MucR has DNA-binding activity. By *in vitro* experiments binding sites for MucR were identified upstream of the *mucR*, *exoY* and *exoH* genes, but not upstream of the five *exp* operons (Bertram-Drogatz *et al.*, 1997, 1998). This indicates that MucR does not bind directly to the *exp* promoter regions or that at least one additional factor is necessary to promote binding of MucR. Since PhoB and ExpG were not required for the induction of *exp* expression in a *mucR* mutant, it is unlikely that these proteins are necessary to promote binding of MucR. ExpG shares similarities with transcriptional regulators of the MarR family which bind DNA through a helix-turn-helix motif (Cohen *et al.*, 1993; Sulavik *et al.*, 1995), and thus ExpG might regulate *exp* gene transcription by direct binding to the *exp* promoter

regions. However, binding sites for ExpG remain to be identified.

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