MucR and MucS Activate *exp* Genes Transcription and Galactoglucan Production in *Sinorhizobium meliloti* EFB1

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When grown under standard conditions, *Sinorhizobium meliloti* EFB1 simultaneously produces two acidic exopolysaccharides, succinoglycan and galactoglucan, yielding very mucoid colonies. In this strain, MucR is essential for galactoglucan synthesis. A mutation in the *mucS* gene resulted in less mucoid colonies than in the wild-type EFB1. This *mucS*[−] strain was complemented to the wild-type phenotype by the cloned *mucS* gene, indicating that *mucS* is necessary for a wild-type level of galactoglucan production. Reverse transcription–polymerase chain reaction analysis of *exp* genes, which encode the pathway for galactoglucan production, in EFB1 and in the mutants affected in *mucS*, *mucR*, and both genes simultaneously, showed that MucS is a transcriptional activator of the *exp* genes but does not affect its own transcription. Furthermore, MucR is necessary for *mucS* transcriptional activation. As introduction of a cloned *mucS* gene in a *mucR*[−] strain yielded colonies less mucoid than the wild type, MucR could also activate *exp* genes transcription through other pathways. Deletion analysis of the *expE* promoter showed a region important for transcription and MucS activation. This region, containing a palindrome, is present in the putative *expA*, *expC*, *expD*, and *expE* promoters but not in the *mucS* promoter, suggesting that it is the target for MucS. A *mucR*[−]*mucS*[−] mutant, which does not produce galactoglucan, was impaired in competitive nodulation of alfalfa in soil microcosms, indicating another possible role for this exopolysaccharide in symbiosis.

Sinorhizobium meliloti can produce two types of acidic exopolysaccharides, succinoglycan and galactoglucan. Succinoglycan production has been shown to be a necessary trait for nodule invasion and, therefore, for successful nitrogen-fixing symbiosis (Battisti et al. 1992; Gonzalez et al. 1996), although at least in symbiosis with alfalfa, it can be replaced by galactoglucan or capsular polysaccharide production (Glazebrook and Walker 1989; Leigh and Walker 1994). Galactoglucan production requires the expression of the *exp* genes that encode

the enzymes required for production of this exopolysaccharide and are clustered in the second megaplasmid (pSymb) (Astete and Leigh 1996). Regulation of galactoglucan production is complex and influenced by environmental factors such as cell density and salt and phosphate concentrations (Astete and Leigh 1996; Lloret et al. 1998; Rüberg et al. 1999). There is also a balance between galactoglucan and succinoglycan produced under each phosphate concentration (Mendrygal and Gonzalez 2000), indicating a coordinated regulation of the production of both exopolysaccharides.

Three loci implicated in regulating galactoglucan production have been identified, *expR*, *mucR*, and *mucS* (also called *expG*), although their precise roles have not been established (Glazebrook and Walker 1989; Keller et al. 1995; Martin et al. 2000). MucR is a zinc-finger protein encoded by a chromosomal gene (Keller et al. 1995) that, in strains such as Rm2011 that normally do not produce galactoglucan, acts as a repressor for the synthesis of the exopolysaccharide (Bertram-Drogatz et al. 1998; Keller et al. 1995). In strain Rm2011, the *mucR* mutant does produce galactoglucan, although only the high-molecular-weight-fraction (Gonzalez et al. 1996). However, in strain EFB1, which produces high amounts of galactoglucan showing a very mucoid phenotype, MucR is necessary for galactoglucan production (Martin et al. 2000). MucS is a helix-turn-helix protein encoded by a gene within the *exp* regulon. In Rm2011, MucS is required for the activation of the expression of at least one gene of the *expE* complementation group under low phosphate concentrations (Astete and Leigh 1996). In strain EFB1, a *mucS* mutant showed a 95% reduction in the expression of the *expE* promoter (Martin et al. 2000). However, as two different mutations within the gene coding region showed different phenotypes in the Rm2011 background (Becker et al. 1997), its role in galactoglucan production and *exp* genes activation has not been established. Furthermore, Rüberg and associates (1999) found that the deletion of the *mucS* gene had no influence on the level of *exp* genes expression in a Rm2011 *mucR* mutant.

Because *S. meliloti* EFB1 synthesizes galactoglucan under standard growth conditions, it is an adequate strain to study galactoglucan production (Lloret et al. 1998; Martin et al. 2000). This study analyzes the role played by the regulatory genes *mucR* and *mucS* in the production of galactoglucan by *S. meliloti* EFB1 and the possible role of this exopolysaccharide in symbiosis. We have studied the *exp* genes transcription level in different EFB1 backgrounds, wild type, a *mucS* mutant, a *mucR* mutant, and a double *mucRmucS* mutant. We have also analyzed the *expE* promoter by nested deletions to find regions implicated in its regulation.

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RESULTS AND DISCUSSION

mucS is essential for wild-type levels of galactoglucan production in EFB1.

We have previously shown that *mucR* is essential for galactoglucan production in *S. meliloti* EFB1 (Martín et al. 2000), as a *mucR* mutant is completely nonmucoid and produces colonies indistinguishable from the colonies produced by a mutant carrying a 3.8-kb deletion affecting *expD*, *mucS*, and *expC*. In order to investigate the role played by *mucS* in *exp* genes regulation and galactoglucan production, an EFB1 *mucS* deletion mutant (EFB207) was constructed by eliminating an internal *XhoI* fragment from a pLAFR3-cloned *mucS* gene (Fig. 1) followed by homologous recombination in an EFB1 wild type. Polymerase chain reaction (PCR) and Southern blotting (data not shown) confirmed the absence of a complete *mucS* gene in EFB207. This deletion eliminates most of the MucS open reading frame (ORF), including the helix-turn-helix motif. EFB207 produces a lower amount of galactoglucan than does EFB1 as judged by the less-mucoid colony morphology on tryptone-

yeast extract (TY) plates. These results indicate that *mucS* is necessary for the full production of galactoglucan, although it is not essential for the production of this exopolysaccharide. The EFB207 mutant was complemented to the wild-type mucoid phenotype by the cosmid pBG1000 (data not shown), which contains *mucS* and other *exp* genes. Furthermore, the plasmid pBG1140, which contains only the *mucS* gene, also complemented the mutation (Fig. 2C), while a similar construct, pBG1141, containing the *mucS* gene with the internal *XhoI* fragment deleted, did not (Fig. 2D). These results demonstrate the essentiality of a functional *mucS* gene for wild-type levels of galactoglucan production in *S. meliloti* EFB1.

MucS is a transcriptional activator of the *exp* promoters and is not autoregulated.

To investigate the transcriptional activation role that MucS and MucR play, we have analyzed the transcription level of all the *exp* promoters in the wild-type and mutant backgrounds. Transcription was analyzed by reverse-transcription PCR (RT-PCR) with total RNA extracted from strains EFB1, EFB207

Fig. 1. Physical and genetic map of part of the *exp* regulon in *Sinorhizobium meliloti* EFB1. Arrows indicate the primers used for reverse-transcription-polymerase chain reaction experiments and are numbered according to Table 1. The *mucS*-containing plasmids are indicated, with the vector used in parentheses. Only relevant restriction sites are shown. E = *EcoRI*, H = *HindIII*, S = *SphI*, and X = *XhoI*.

Fig. 2. Complementation analysis of the *mucS* mutant EFB207. **A**, EFB1 (wild type). **B**, EFB207 (*mucS*⁻). **C**, EFB207 pBG1140. **D**, EFB207 pBG1141.

(*mucS*⁻), EFB208 (*mucR*⁻), and EFB209 (*mucR*⁻*mucS*⁻), using primers designed from the sequence of an ORF in each *exp* operon (Table 1, Fig. 1). Double mutant EFB209 was constructed by cloning an internal fragment of the *mucR* gene (Martin et al. 2000) in suicide vector pK18mobsac followed by homologous recombination in the EFB207 background.

As shown in Figure 3, the transcript levels of *expA*, *expC*, *expD*, and *expE* were higher in EFB1 than in EFB207, indicating that MucS is activating the transcription of these *exp* genes. However, the *mucS* transcript was expressed at the same level in both EFB1 and EFB207, showing that the *mucS* promoter does not require MucS for its transcription, and therefore, it is not autoregulated. There was no transcript from *mucS* in *mucR*⁻ and *mucR*⁻*mucS*⁻ backgrounds, indicating that MucR is necessary for *mucS* transcription. Although galactoglucan production regulation is complex and influenced by different environmental factors, our results show that, in *S. meliloti* EFB1, MucR is regulating the activation of the *mucS* gene, which encodes a transcriptional activator required for the *exp* genes expression and for the production of galactoglucan observed under normal growth conditions. It is interesting to note that mutants affected in *mucR* homologues in *Agrobacterium tumefaciens* (Cooley et al. 1991) and *Rhizobium etli* (Bittinger and Handelsman 2000) also show a nonmucoid phenotype.

Fig. 3. Reverse-transcription–polymerase chain reaction (RT-PCR) analysis of the *exp* operons and *mucS* in EFB1 and mutant backgrounds; EFB1 (wild type), EFB207 (*mucS*⁻), EFB208 (*mucR*⁻), and EFB209 (*mucS*⁻*mucR*⁻). Total RNA was extracted, and 100 pg were subjected to RT-PCR. The *kdsB* gene was subjected to RT-PCR as a positive control. All the amplifications were performed with the same RNA samples and dilutions. Control reactions without reverse transcriptase showed no DNA contamination.

The transcript levels of the *expA*, *expC*, *expD*, and *expE* promoters were, if present, very low in the *mucR* and *mucRS* mutants, therefore the possibility that MucR is not only necessary for *mucS* transcription but also activates *exp* genes transcription through other pathways cannot be excluded. To test this hypothesis, a cloned *mucS* gene was introduced into the *mucR* mutant, either under the control of its own promoter (pBG1140) or under the control of the strong *npII* promoter (pBG1228). The introduction of pBG1140 had no effect on the colony morphology of the *mucR* mutant, which remained completely dry. However, when pBG1228 was introduced into the *mucR* mutant, colonies were mucoid, although at a lower level than in the wild type. Together with the RT-PCR results, these data indicate that *mucR* is a strict requirement for the transcription of *mucS* and that, in addition to a functional MucS, *mucR* is necessary for the full production of galactoglucan. If *mucR* has a role other than *mucS* transcriptional activation, it should influence *exp* genes transcription through another pathway.

A palindromic, conserved sequence is located at the distal part of *exp* promoters.

We have previously shown that the *expE* promoter is highly expressed in EFB1 and that there is a 95% reduction in expression in a *mucS* mutant strain (Martin et al. 2000). To identify putative regulatory regions within this promoter, we have analyzed the expression level from nested deletions of the promoter. To construct these deletions, nested forward primers and a common reverse primer were designed from the *expE* promoter sequence and were used to amplify genomic DNA from EFB1. The PCR-resulting fragments were cloned in the pCR-2.1 plasmid, sequenced, and directionally fused to a promoterless *lacZ* gene in plasmid pMP220, giving rise to the reporter constructs described in Table 2. β -galactosidase activity assays were done in EFB1, EFB207, EFB208, and EFB209 backgrounds. As shown in Figure 4, the expression of the *expE* promoter was very high in strain EFB1, and there was a drastic decrease in activity in the EFB207 background. Deletion of up to 138 nucleotides in the 5' region of the promoter (pRO1085 and pRO1086) did not have major effects on activity or regulation by MucS. However, a further deletion of only 30 bp (pRO1069) resulted in a drastic reduction of activity in the EFB1 background to levels found in EFB207, indicating the loss of *mucS* regulation. These results strongly indicate that this 30-bp region must be important for MucS as a transcriptional activator of *expE*. The lack of expression observed for any of the constructs tested in the EFB208 and EFB209 backgrounds is consistent with *mucR* playing an additional role in *expE* transcription.

The 30-bp *expE* putative regulatory region was compared with the *exp* intergenic sequences using the Bestfit and Gap

Table 1. Primers and annealing temperatures used for reverse transcription–polymerase chain reaction of the *exp* transcripts

<i>exp</i> gene	Primer no.	Primer sequence (5'–3')	Annealing temperature (°C)
<i>expA</i>	1	ATCGCCGACGAAGCGAAG	55
	2	TGCGGGCAGTCGATCC	
<i>expC</i>	3	GATGCCGCCATCCATCG	55
	4	CGCCGCCTCGGGCAG	
<i>mucS</i>	5	CCCCTAACGCGCGGC	53
	6	ATGGAGAGAGGGATGAAC	
<i>expD</i>	7	ACCGCTGATGCAACTGGAG	59
	8	ACGAAATAGGCGACATTGGAC	
<i>expE</i>	9	TGGCAGCGACTTCCTTCT	55
	10	CGACATTGCCTCCGACCTG	

programs (Genetic Computer Group, Madison, WI, U.S.A.). A region of 20 bp within the 30 bp was highly conserved in equivalent positions at the distal part of the putative *expA* and *expD* promoters in EFB1 (Fig. 5) and Rm2011 (data not shown) but not in the distal part of the putative *mucS* promoter of both strains. In the case of *expC*, the intergenic region located upstream of the gene is very short and does not contain putative regulatory sequences. However, the same conserved region is found 31 nucleotides upstream of the *mucS* start codon and in the sense of the transcription of *mucS* and *expC* (Fig. 1). It partially overlaps the conserved region in the putative *expD* promoter. As *mucS* is not autoregulated and this conserved region is located very close to the *mucS* ATG codon, it is likely that the *expC* promoter overlaps the *mucS* coding sequence, and therefore, the conserved region located just upstream of *mucS* corresponds to the *expC* promoter.

Strain or plasmid	Relevant characteristics	Source or reference
<i>Sinorhizobium meliloti</i>		
EFB1	Wild type, Sm ^r	Lloret et al. 1995
EFB207	EFB1 $\Delta mucS$	This study
EFB208	<i>mucR</i> ::pK18mobsacB	Martin et al. 2000
EFB209	EFB207 <i>mucR</i> ::pK18mobsacB	This study
<i>Escherichia coli</i>		
DH5 α	<i>recA1</i> $\Delta(lacZYA-argF)$	Gibco, Rockville, MD, U.S.A.
Plasmids		
pRK2013	Km ^r ColE1 replicon with PK2 <i>tra</i> genes	Figurski and Helinski 1979
pMP220	Tc ^r <i>mob</i> ⁺ <i>lacZ</i> IncP	Spaink et al. 1987
pCR 2.1-TOPO	Amp ^r Km ^r	Invitrogen, Carsbad, CA, U.S.A.
pBluescript	Amp ^r phagemid M13 derivative f1 origin of replication	Stratagene, La Jolla, CA, U.S.A.
pK18mobsacB	Km ^r pUC18 derivative <i>lacZ</i> <i>mob</i> site <i>sacB</i>	Schäfer et al. 1994
pLAFR3	Tc ^r host range P-group cloning vector, <i>mob</i> RK2 cosmid	Staskawicz et al. 1987
pML122	Km ^r RSF1010 derivative expression vector	Labes et al. 1990
pBG1000	pLAFR3 with 24 kb of the <i>exp</i> gene cluster of EFB1	Lloret et al. 1998
pBG1140	pLAFR3 with <i>mucS</i> gene	This study
pBG1141	pLAFR3 with $\Delta mucS$	This study
pBG1228	pML122 with <i>mucS</i> under the control of the vector Nm promoter	This study
pRO1052	pMP220 with 346bp <i>Sph</i> I fragment containing the <i>expE</i> promoter in sense orientation of EFB1	Lloret et al. 1998
pRO1069	pRO1052 with a 142-bp deletion at the distal part of <i>expE</i> promoter	This study
pRO1084	pRO1052 with a 187-bp deletion at the distal part of <i>expE</i> promoter	This study
pRO1985	pRO1052 with a 92b-p deletion at the distal part of <i>expE</i> promoter	This study
pRO1086	pRO1052 with a 112-bp deletion at the distal part of <i>expE</i> promoter	This study

The conserved regions contain a palindrome (Fig. 5, inverted arrows), although homology extends beyond it. Palindromes, such as these with an AT-rich axis, are typical binding sites for helix-turn-helix proteins such as MucS (Becker et al. 1997). The presence of this palindromic region in the putative *expA*, *expC*, *expD*, and *expE* promoters, and the fact that MucS is activating the transcription of the *exp* genes but not its own transcription, strongly indicates that it corresponds to the target site of the MucS protein. For this reason, we have termed the 20-bp region the *muc* box. These *muc* boxes do not correspond to the *pho* boxes described by Rüberg and associates (1999).

The biological significance of galactoglucan production is not well defined. Gonzalez and associates (1996) have shown

that galactoglucan can replace succinoglycan in noninfecting *exo* mutants that do not produce succinoglycan. Besides, the *expR* mutation, which results in overexpression of the *exp* genes, has been shown to suppress the symbiotic defect of succinoglycan-deficient strains (Glazebrook and Walker 1989). However, when galactoglucan is produced under phosphate-limiting conditions, it does not allow the invasion of alfalfa nodules (Mendrygal and Gonzalez 2000).

The mutant strain EFB209 does not show expression of any of the *exp* promoters and should not produce any galactoglucan. We have used this strain to investigate the possible role of this exopolysaccharide in *S. meliloti*–alfalfa symbiosis. Competition assays between EFB1 and EFB209 were performed in Leonard jars, using perlite as the solid substrate, and in sterilized and nonsterilized soil microcosms in Riviera pots. As shown in Table 3, no differences in competitiveness were observed in Leonard jar systems, with each strain forming approximately half of the analyzed nodules and with a small proportion of nodules formed by both strains simultaneously. However, when the experiments were performed in soil microcosms, the mutant strain EFB209 was clearly outcompeted by wild-type EFB1 and by the indigenous rhizobial population. It is interesting to note that the mutation of the *mucR* homologue *rosR* in *R. etli* results in a noncompetitive mutant (Bittinger et al. 1997). No differences in the lack of EFB209 competitiveness were observed between sterilized and nonsterilized soil microcosms, suggesting that galactoglucan might play a role in adaptation to abiotic stress conditions. In this sense, Mendrygal and Gonzalez (2000) have suggested that exopolysaccharides may play a role in the transition between the soil and the plant

environments. Because galactoglucan production is higher at low phosphate levels and the nodule is a high-phosphate environment, they assume that this exopolysaccharide is produced primarily while *S. meliloti* is in the soil and not inside the nodule.

Our results indicate that, in the *S. meliloti* strain EFB1, *exp* genes expression and, therefore, galactoglucan production is primarily regulated by MucR, which is necessary for the transcription of the *mucS* gene and possibly implicated in the transcription of the other *exp* promoters. This is in contrast to its role in strain Rm1021, in which MucR confers negative regulation of *exp* genes transcription. It should be noted that the *R. etli* MucR homologue (RosR) regulates more than 50 genes (Bittinger and Handelsman 2000). The fact that no MucR-binding sites (Keller et al. 1995) have been found in the *exp* promoters (including *mucS*) indicates that this regulation should be indirect. MucS, in turn, is a transcriptional activator of the *expA*, *expC*, *expD*, and *expE* operons and probably binds to the conserved *muc* boxes present within the *exp* promoters.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 2. *S. meliloti* was grown at 28°C in TY medium (Beringer 1974). *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) medium. Antibiotics were added at the following concentrations when required: 200 µg of streptomycin per ml, 10 µg of tetracycline per ml, 20 µg of kanamycin per ml, and 100 µg of ampicillin per ml.

DNA techniques.

Standard techniques for subcloning procedures, plasmid preparations, and agarose gel electrophoresis were used as described by Sambrook and associates (1989). Southern blot hybridizations were performed with a nonradioactive detection kit, and a chemiluminescence method was used to detect hybridization bands according to the manufacturer's instructions (Roche Diagnostics, Barcelona, Spain). DNA sequencing was done by the chain termination method using the DyeDeoxy terminator cycle sequencing kit protocol as described by the manufacturer (Applied Biosystems, Madrid, Spain). Homology search and sequence analysis were performed using software from the Genetics Computer Group (Madison, WI, U.S.A.).

Table 3. Percentage of nodules formed by each strain in competition experiments

Strain	Substrate		
	Perlite ^a	Sterilized soil ^b	Nonsterilized soil ^c
EFB1	49	91.4	33.3
EFB209	44	8.6	0
Other rhizobia	0	0	66.7
Co-occupancy ^d	7	0	0

^a *n* = 100 nodules.

^b *n* = 35 nodules.

^c *n* = 30 nodules.

^d EFB1 and EFB209.

Fig. 5. Alignment of the conserved region found in the distal part of the putative *expA*, *expC*, *expD*, and *expE* promoters of *Sinorhizobium meliloti* EFB1. Residues identical in the four promoters are boxed. Inverted arrows indicate a palindrome found within the conserved region. The distance in nucleotides to the ATG of the first open reading frame in each operon is indicated. The accession number for the sequence of the *S. meliloti* EFB1 *exp* region is Y08703.

RT-PCR assays.

Total RNA from strains EFB1, EFB207, EFB208, and EFB209 was extracted following the SV RNA isolation system from Promega (Madison, WI, U.S.A.). For subsequent RT-PCR, forward- and reverse-strand primers were designed from *expA*, *expC*, *expD*, *expE*, and *mucS* genes and are described in Table 1. The reactions were done following the RT-PCR system instructions from Promega. RT reaction was done at 48°C for 45 min, followed by a denaturation step and 40 PCR cycles. The appropriate band length was checked by agarose gel electrophoresis. Negative controls were done without RT reaction to detect putative DNA contamination of the RNA samples.

Deletions of the *expE* promoter and β -galactosidase assay.

PCR deletions were done using a common, reverse-strand primer, which contains an *SphI* site, 5' GCATGCGTCATCGG-CAC 3'. The forward-strand primer used to amplify the complete promoter was 5' CCCGCGGAAGTGCTGAT 3'. The nested forward primers used to amplify the different primer deletions were 5' GCATCAATTGGCAATTTACTT 3', 5' TTAAATTTTGAACCGGTTTGC 3', and 5' TCCGCAAGATAACTCCTAAAA 3'. The PCR products were cloned in the pCR-2.1 vector (Invitrogen, Carlsbad, CA, U.S.A.) and were digested with *EcoRI* and *SphI* to be directionally cloned in pMP220 in the sense of the transcription of the *lacZ* gene (Spaink et al. 1987). The reporter constructs (Table 2) were introduced in the rhizobial strains by triparental mating using the helper plasmid pRK2013. Reporter strains were grown on TY, and β -galactosidase activity was assayed after 18 h of growth. β -galactosidase units were calculated as described by Miller (1972).

Competition studies.

Competition experiments were performed in Leonard jar systems and soil microcosms. Alfalfa seeds were surface-sterilized with bleach and 70% ethanol, rinsed with sterile water, and germinated on agar plates. Seedlings were placed in Leonard jars with perlite and Riviera pots with either autoclaved or nonautoclaved sieved soil and inoculated with approximately 10^8 cells from exponentially growing TY cultures of each strain. Inoculated plants were grown for 4 weeks in a growth cabinet with a photoperiod of 16 h of light at 25°C and 8 h of dark at 18°C. Nodules were excised, surface-sterilized with 70% ethanol, and crushed in liquid TY medium. Crushed nodules were inoculated on TY plates containing the appropriate antibiotics to differentiate the strain forming the nodule. Nodule occupancy was also assessed by the morphology of the colonies.

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