Research Note

MucR Is Necessary for Galactoglucan Production in *Sinorhizobium meliloti* EFB1

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Sinorhizobium meliloti can produce two types of acidic exopolysaccharides, succinoglycan and galactoglucan, that are interchangeable for infection of alfalfa nodules. Strain SU47 and derivatives produce only succinoglycan, unless it grows under phosphate limitation or carries a mutation in either of two regulatory loci, *mucR* or *expR*. It has been proposed that MucR acts as a transcriptional repressor that blocks the expression of the *exp* genes responsible for galactoglucan production. Strain EFB1 simultaneously produces both exopolysaccharides. Heterologous expression of *lacZ* transcriptional fusions of the *expE* promoters has shown that genetic background is more important than promoter sequence for *exp* gene expression, since *expE* promoters from both strains are expressed at high level in EFB1 and not in SU47. We have found that *mucR* is present in mucoid and nonmucoid strains, and in EFB1 differs from SU47 in only one conservative amino acid change. MucR proteins from both strains are interchangeable. An *mucR* mutant of EFB1 cannot produce galactoglucan and does not express *mucS*.

Exopolysaccharide (EPS) production in *Sinorhizobium meliloti* is necessary for the infection of plant nodules and it has been proposed that the low-molecular-weight forms of EPS act as a specific signal that promotes growth of the infection thread in the plant (Battisti et al. 1992; Gonzalez et al. 1996). *S. meliloti* can produce two types of acidic EPSs: succinoglycan (EPS I), needed for the symbiosis with its host plants, and galactoglucan (EPS II), which can replace succinoglycan at least in its symbiosis with alfalfa (Glazebrook and Walker 1989; Leigh and Walker 1994). Studies on EPS production have been carried out mainly in derivatives of strain SU47, which under normal culture conditions produces only EPS I. Strain SU47 also produces and secretes EPS II under conditions of phosphate limitation, yielding mucoid colonies (Zhan et al. 1991). The same phenotype is observed in mutants affected in a phosphate transport system named *phoCDET* (originally designated *ndvF*), probably due to an artificial phosphate limitation (Oresnik et al. 1994; Bardin et

al. 1996). However, *ndvF* mutants are impaired in nodule invasion (Charles et al. 1991).

Most of the genes responsible for the production and secretion of both EPSs in SU47 have been located unlinked in the second megaplasmid (Charles and Finan 1991). They are organized in two clusters, the *exo/exs* cluster for EPS I and the *exp* cluster for EPS II, both of which have been totally sequenced (Reed et al. 1991; Buendia et al. 1991; Becker et al. 1993a, 1993b, 1993c, 1995, 1997; Glucksmann et al. 1993a, 1993b; Müller et al. 1993). Within the *exp* cluster, a regulatory gene, *mucS* (*expG*) has been found (Astete and Leigh 1996; Becker et al. 1997). *mucS* is necessary for the expression of *exp* genes and for EPS II production under phosphate limitation conditions (Astete and Leigh 1996).

In addition, SU47 mutants affected in either of two regulatory chromosomal loci, *expR* or *mucR*, produce EPS II independently of the phosphate condition (Glazebrook and Walker 1989; Zhan et al. 1989; Keller et al. 1995). *mucR* has been cloned and sequenced in strain SU47 and codes for a protein that contains a zinc finger motif of the C₂H₂ type (Keller et al. 1995). MucR binds to a short transcribed region located upstream of the coding region of *mucR*, regulating its own transcription negatively (Bertram-Drogatz et al. 1997). Furthermore, MucR has been proposed to be a transcriptional repressor of the *exp* genes and to be an activator of EPS I biosynthesis, being essential for the production of high-molecular-weight EPS I (Zhan et al. 1989; Keller et al. 1995; Bertram-Drogatz et al. 1998). It has the capability of binding to DNA regions upstream of *exoH* and *exoY*, although only a weak influence on the transcription of these genes has been demonstrated (Bertram-Drogatz et al. 1998). Unexpectedly, there are not MucR DNA binding sequences near the *exp* genes in spite of the strong influence on the transcription of several of these genes (Keller et al. 1995; Becker et al. 1997).

EPS regulation is different in *S. meliloti* EFB1. This strain, which was isolated from nodules of a *Medicago* sp. plant growing in a salt marsh, simultaneously produces EPS I and EPS II, which results in the formation of very mucoid colonies. In this strain, EPS II production is severely diminished when growing in the presence of NaCl, and the colonies appear with a less mucoid phenotype. By using *lacZ* transcriptional fusions of the *expE* promoter, we have previously shown that in EFB1 the *expE* gene transcription is constitutive under normal laboratory growth conditions but growth phase dependent when the medium is supplemented with 0.3 M

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NaCl. During early exponential phase, *expE* expression is more than 10 times lower in the presence of salt, which can account for the different phenotype observed on NaCl-supplemented media (Lloret et al. 1998).

Different regulation of galactoglucan production in both strains can be caused either by differences in promoter sequences in the *exp* region or by differences in the genetic background. We have previously reported the sequence of a 3.7-kb DNA fragment from *S. meliloti* EFB1 containing part of the *expD* and *expE* operons (Lloret et al. 1998). Sequence was extended by cloning a 3.6-kb *HindIII* fragment situated upstream of the *expD2* gene (Fig. 1). In order to fill the gap between the two clones, two primers were designed to amplify the intervening DNA fragment. Figure 1 shows the genetic organization of this 8.2-kb region, which contains seven open reading frames (ORFs), two of them incomplete, and four intergenic spaces, three of which are interoperonic. The sequence that we present is representative of the *exp* regulon, as it contains ORFs corresponding to four out of the five *exp* operons described (Becker et al. 1997) and is likely to contain promoters for all of them except the *expA* operon. The genetic organization of this region is identical to that of SU47 (Becker et al. 1997) but DNA sequence homology is only 85%, indicating genetic divergence between the two strains. Homology is maximal within coding regions (Table 1) especially at the predicted amino acid sequence, which is indicative of conservative changes. Two types of intergenic regions can be differentiated as a function of sequence homology (Table 1). Two of the intergenic regions present a homology level comparable to that of the coding regions. One of them (IG2) should contain the promoters for the *mucS* (*expG*) and *expD* genes while the other (IG4), situated within the *expE* operon between *expE1* and *expE2*, contains an incomplete mosaic sequence, RIME2, that has only been found in *Sinorhizobium meliloti* (Østerås et al. 1995; Lloret et al. 1998). The other two intergenic regions show a significantly lower level of homology between the two strains. The first one (IG1) is likely to contain the *expC* promoter, although there are no obvious promoter sequences. The second region (IG3) contains an *expE* promoter, as has been shown by a transcriptional *lacZ* fusion (Lloret et al. 1998). Because of the presence of this promoter and the reduced homology with SU47, IG3 was selected for further analysis.

The *expD-expE* intergenic regions (IG3) of *S. meliloti* Rm2011, a streptomycin-resistant derivative of SU47, and EFB1 were cloned into pMP220 to create *lacZ* transcriptional fusions. The resulting plasmids, pRO1052 and pRO1091 (described in Table 2), were mobilized by conjugation to EFB1 and Rm2011, and β -galactosidase activity was determined in the presence and absence of NaCl. As shown in Fig. 2A, the *expE* promoters from both strains were expressed at a high level in the EFB1 background and expression was reduced in the presence of NaCl. The expression level was slightly higher with the EFB1 promoter than with the Rm2011 promoter, suggesting that, despite the promoter sequence divergences, there is very little promoter contribution to the differences in gene expression. On the contrary, no expression was observed in the Rm2011 background with any of the promoters in both conditions (Fig. 2D). Interestingly, these results strongly indicate that the different galactoglucan production in both *S. meliloti* strains does not depend on the observed differences in promoter sequence. The results also show that salt regulation of the Rm2011 *expE* promoter occurs in EFB1 background. Plasmids pRO1052 and pRO1091 were also mobilized into Rm3131, a Rm2011 derivative that contains a Tn5 insertion in *mucR* and is derepressed for *exp* gene expression. Both promoters were effectively expressed in this strain (Fig. 2B), although at a lower level than in EFB1. Salt regulation was not observed in Rm3131.

Table 1. Homology between *exp* regions of *Sinorhizobium meliloti* EFB1 and SU47

Region ^a	DNA identity (%)	Predicted amino acid identity (%)
<i>expC</i>	85.9	93.5
IG1	63.7	—
<i>mucS</i> (<i>expG</i>)	87.9	91.2
IG2	87.2	—
<i>expD1</i>	90.1	95.4
<i>expD2</i>	86.2	90.1
IG3	79.4	—
<i>expE1</i>	93.2	97.7
IG4	84.0	—
<i>expE2</i>	84.4	84.9
<i>expE3</i>	85.6	91.4

^a Open reading frames and intergenic regions as defined in Figure 1.

Fig. 1. Physical and genetic map of part of the *exp* region of *Sinorhizobium meliloti* EFB1 implicated in galactoglucan production. All DNA fragments were sequenced by the chain termination method with the DyeDeoxy terminator cycle sequencing kit protocol and an Applied Biosystems automatic sequencer. Homology search and sequence analysis were performed with software from the Genetics Computer Group (University Research Park, Madison, WI). Intergenic regions (IG 1–4) and relevant restriction sites are indicated. E: *EcoRI*, H: *HindIII*.

Despite the observed differences in the *expE* promoter sequence there are regions of very high conservation that might reflect common regulatory elements that interact with the promoter. One of these elements might be *mucS* (*expG*), which is highly conserved in both strains and encodes a protein that contains an helix-turn-helix motif and shows homology with proteins of the transcriptional regulator MarR family (Becker et al. 1997). In SU47 it has been shown that, under phosphate limitation, MucS is essential for *exp* gene expression (Astete and Leigh 1996). In order to test the role of *mucS* in EFB1, an *mucS* null mutant was generated by replacing the 3.8-kb *HindIII* fragment that contains *mucS* with a chloramphenicol-resistance cassette, thus creating EFB200. The reporter constructs were mobilized into EFB200 and β -galactosidase activity was determined under the same conditions as described above. As shown in Figure 2E, *expE* expression in the *mucS* mutant was 95% lower than in EFB1, indicating that MucS is a transcriptional activator of the *expE* promoter.

The MucR protein has been proposed to be a transcriptional repressor of *exp* gene expression in *S. meliloti* Rm2011 (Keller et al., 1995). In this strain, *mucR* mutants produce galactoglucan and allow the expression of *expE::lacZ* fusions that are not expressed in the wild-type strain (Becker et al. 1997). It was therefore possible that galactoglucan production and *expE* expression in EFB1 were due to a lack of a functional *mucR* gene in this strain. To test this hypothesis, primers designed from the amino and carboxi terminus of Rm2011 MucR protein were used to amplify this gene in EFB1. Polymerase chain reaction (PCR) yielded a single band with the appropriate size that was sequenced and showed 96% identity

with the Rm2011 *mucR* gene. Figure 3 shows a comparison between *mucR* in *S. meliloti* Rm2011 and EFB1. Twenty-one codons are different between them, indicating again a substantial genetic divergence between both strains. However, 20 of these changes are silent, as the same amino acids are encoded. The remaining codon difference produces a conservative change of an aspartic acid for a glutamic acid residue, suggesting that EFB1 MucR protein is functionally identical to the Rm2011 protein. This change is not located within the zinc finger motif and therefore it is not likely that it would affect binding of the protein to DNA. To examine whether *mucR* is correctly transcribed in EFB1, the same primers that amplified the *mucR* gene were used for a reverse transcription (RT)-PCR experiment and a band of the correct size was amplified from total RNA extracted from both Rm2011 and EFB1 strains (not shown). Furthermore, Northern (RNA) blot analysis showed that an *mucR* probe recognized a band of identical size in total RNA extracts from Rm2011 and EFB1 (Fig. 4), demonstrating that EFB1 produces an *mucR* transcript equivalent to the one produced by Rm2011. To demonstrate that MucR is translated and is functional in EFB1, an *mucR* mutant was constructed by plasmid insertion (Schäfer et al. 1994). An internal fragment of *mucR* (nucleotides 99 to 320) was amplified and cloned in pK18mobsac. This suicide vector was mobilized by conjugation into EFB1, a kanamycin-resistant transconjugant was selected, and homologous recombination was confirmed by Southern hybridization. The mutant (EFB208) showed a nonmucoid phenotype indistinguishable from that of Rm2011 and does not produce galactoglucan, as judged from H^+ -nuclear magnetic resonance

Table 2. Strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
<i>Sinorhizobium meliloti</i>		
EFB1	Wild type, Sm ^r	Lloret et al. 1995
EFB200	EFB1 Δ (<i>expC' mucS expD1 expD2'</i>)	This work
Rm2011	Wild-type Sm ^r derivative of SU47	Casse et al. 1979
Rm3131	Rm2011 <i>mucR31::Tn5</i>	Keller et al. 1995
EFB208	EFB1 <i>mucR</i> mutant	This work
<i>Escherichia coli</i>		
DH5α	<i>endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF) U169 φ80d lacZΔM15</i>	Gibco B.R.L., Rockville, MD
TOPO10	F [−] <i>mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galK rpsL endA1 nupG</i>	Invitrogen, Carsbad, CA
XL1-Blue	<i>recA1 lac[F' proAB lacI^q ZΔM15 Tn10(Tet^r)] thi</i>	Lab collection
JM109	<i>recA endA1 gyrA96 thi hsdR17 supE44 Δ(lac-proAB) relA1</i>	
Plasmids		
pRK2013	Km ^r ColE1 replicon with RK2 <i>tra</i> genes	Figurski and Helinski 1979
pMP220	Tc ^r <i>mob⁺ lacZ IncP</i>	Spaink et al. 1987
pCR 2.1-TOPO	Amp ^r Km ^r	Invitrogen, Carsbad, CA
pBluescript	Amp ^r phagemid M13 derivative f1 origin of replication	Stratagene, La Jolla, CA
pK18mobsacB	Km ^r pUC18 derivative <i>lacZα mob</i> site <i>sacB</i>	Schäfer et al. 1994)
pLAFR3	Tet ^r host range P-group cloning vector, mobilizable RK2 cosmids	Staskawicz et al. 1987
pBG1000	pLAFR3 cosmid clone with 24 kb of the <i>exp</i> gene cluster of <i>S. meliloti</i> EFB1	Lloret et al. 1998
pBG1090	pLAFR3 cosmid clone that contains <i>mucR</i> of EFB1	This work
pBS1035	pBluescript containing 9.4-kb <i>EcoRI</i> fragment of the <i>exp</i> gene cluster	This work
pBS1076	pBluescript containing 3.8-kb <i>HindIII</i> fragment of the <i>exp</i> gene cluster	This work
pBS1077	Amp ^r Cm ^r pBS1035::Cm ^r Δ <i>mucS</i>	This work
pBS1078	pCR 2.1-TOPO containing an amplified <i>mucR</i> of <i>S. meliloti</i> EFB1	This work
pBS1087	pCR 2.1-TOPO containing the amplified <i>expE</i> promoter of <i>S. meliloti</i> Rm2011	This work
pK1088	Km ^r Cm ^r pBS1077 <i>EcoRI</i> fragment cloned in pK18 <i>mobsacB</i>	This work
pRO1052	pMP220 with 346-bp <i>SphI</i> fragment containing the <i>expE</i> promoter in sense orientation of <i>S. meliloti</i> EFB1	Lloret et al. 1998
pRO1091	pMP220 with <i>EcoRI-SphI</i> fragment containing the <i>expE</i> promoter in sense orientation of <i>S. meliloti</i> Rm2011	This work
pRO1109	pMP220 with 239-bp fragment containing the <i>mucS</i> promoter in sense orientation of EFB1	This work

Fig. 2. Expression of *expE* (pRO1052) and *mucS* (pRO1109) promoters of EFB1 and *expE* (pRO1091) promoter of Rm2011 in different backgrounds. **A**, EFB1 wild type (wt). **B**, Rm3131 (Rm2011 *mucR* mutant). **C**, EFB208 (EFB1 *mucR* mutant). **D**, Rm2011 wt. **E**, EFB200 (EFB1 *mucS* mutant). Cultures were grown in TY (black bars) or TY + 300 mM NaCl (open bars) to mid exponential phase ($OD_{600} \approx 0.2$). Results are expressed as β -galactosidase Miller units. Background activity of pMP220 was less than 80 U in all strains. Results are means of two independent assays with a standard deviation of less than 10%.

(NMR) analysis of the purified EPS (data not shown). The wild-type mucoid phenotype was restored by a cosmid (pBG1090) from the EFB1 gene bank (Lloret et al. 1998) that hybridized with the PCR-amplified *mucR* probe. This cosmid also complemented the *mucR* mutation on Rm3131, resulting in nonmucoid colonies. This result demonstrates that a functional MucR protein is produced by EFB1 and that this protein is functionally identical to the Rm2011 counterpart. This result also shows that MucR is necessary for galactoglucan production in EFB1. This observation contrasts with the proposed role for MucR as a transcriptional repressor of galactoglucan production in strain SU47. To test the role of MucR in *exp* gene expression in SU47 and EFB1, the intergenic region between *expD* and *mucS* (IG2) was transcriptionally fused to *lacZ* in the sense of the transcription of *mucS* in plasmid

pMP220 to create pRO1109. This plasmid and pRO1052 were introduced into SU47 (Rm3131) and EFB1 (EFB208) *mucR* mutants and β -galactosidase activity was determined in the presence and absence of salt. As shown in Figure 2B and D, *mucS* and *expE* are only expressed in SU47 when *mucR* is mutated. Conversely, in EFB1, expression of both promoters requires the presence of a functional *mucR* (Fig. 2A and C). Therefore, MucR plays opposite roles in the transcriptional regulation of galactoglucan production in SU47 and EFB1: it acts as a repressor of *mucS* expression in SU47 and as an activator in EFB1. The lack of MucR binding sites in the promoters, the homology between *mucS* promoters in both strains, and the different effect exerted by functionally identical proteins strongly indicate that *exp* promoters are not directly subjected to transcriptional regulation by MucR. Therefore,

EFB1	1	ATGACAGAGACTTCGCTCGGTACGAGCAATGAACTCCTGGTTGAGCTTAC	50
Rm2011	1	ATGACAGAGACTTCGCTCGGTACGAGCAATGAACTCCTGGTTGAGCTTAC	50
EFB1	51	GGCGGAGATCGTTGCCGCTATGTAAGCAACCATGTGGTTCCGGTTGCCG	100
Rm2011	51	GGCGGAGATCGTTGCCGCTATGTAAGCAACCATGTGGTTCCGGTTGCCG	100
EFB1	101	AGCTGCCGACGCTCATAGCCGACGTGCATTCCGGCACTCAACAACACGACC	150
Rm2011	101	AGCTGCCGACGCTCATAGCCGACGTGCATTCCGGCTCTCAACAACACCACG	150
EFB1	151	GCTCCAGCGCCGGTGGTTCGTTCCGGTGGAGAAGCCGAAGCCCGCGGTTTC	200
Rm2011	151	GCTCCTGCGCCGGTGGTTCGTTCCCGGTGGAAAAGCCGAAGCCTGCGGTTTC	200
EFB1	201	GGTGCGAAAGTCGGTGCAGGACGACCAGATCACTTGTCTCGAATGTGGCG	250
Rm2011	201	GGTCCGAAAGTCGGTGCAGGACGACCAGATCACATGTCTCGAATGCGGCG	250
EFB1	251	GGACCTTCAAGTCGTTGAAGCGGCATCTGATGACCCACCATAATCTTTTCG	300
Rm2011	251	GCACCTTCAAGTCGTTGAAGCGGCATCTGATGACGCACCACAACCTTTTCG	300
EFB1	301	CCGGAAGACTACCGCGACAAGTGGGATTTGCCTGCCGACTATCCGATGGT	350
Rm2011	301	CCGGAAGAAATACCGCGACAAGTGGGATTTGCCTGCCGATTATCCGATGGT	350
EFB1	351	CGCGCCGCGCTATGCGGAAGCCCGCTCGCGGCTCGCCAAGGAAATGGGTC	400
Rm2011	351	CGCAACCGGCTACGCGGAAGCGCGCTCGCGGCTCGCCAAGGAAATGGGTC	400
EFB1	401	TCGGACAGCGTCGTAAGCGTCGCGGCAAGTG	431
Rm2011	401	TCGGACAGCGTCGGAAGCGTCGCGGCAAGTG	431

Fig. 3. Comparison of the nucleotide sequence of the *mucR* genes from *Sinorhizobium meliloti* EFB1 and Rm2011. The EFB1 *mucR* gene was obtained by polymerase chain reaction (PCR) amplification of EFB1 genomic DNA with primers designed from the already published *S. meliloti* Rm2011 *mucR* sequence (Keller et al. 1995). Primers used were named mucr-F (ATGACAGAGACTTCGCTCGGT) and mucr-R (TCACTTGCCGCGACGCTT). Amplifications were carried out with a *Tth* polymerase (Biotools, Madrid). Two independently amplified fragments (431 nucleotides) were sequenced. The amplified fragment was also used as a probe to isolate cosmid pBG1090 (see text). The 21 nucleotides that produce codon changes are indicated in bold. Only the nucleotide change at position 309 (boxed) results in an amino acid change (D/E) in the predicted protein sequence.

the effect that MucR exerts on *exp* gene expression should be indirect, through a regulatory pathway that is different in both strains. Such *mucR*-mediated cascade has been suggested in Rm2011 (Bertram-Drogatz et al. 1998). The different role of MucR also explains the different morphology showed by colonies from both strains: while SU47 and EFB 208 showed identical colony morphology, EFB1 is more mucoid than Rm3131 and this mucoidy can be correlated to the expression level of both promoters in the different backgrounds tested. *mucS* expression is only affected by salt in the EFB1 background, suggesting that the reduction in galactoglucan production and *expE* expression observed in this strain might be the result of a lower level of *mucS* expression under this condition. However, the lower level of expression of the *expE* promoter in a *mucS* mutant background in salt-containing medium (Fig. 2E) indicates that other factors are implicated in this phenotype.

Thirteen *S. meliloti* field isolates from nodules of *Medicago* spp. plants growing in different fields around Madrid, which included mucoid and nonmucoid strains, were tested by PCR for the presence of an *mucR* gene. A single band, identically sized, was amplified from all the strains (not shown), demonstrating that *mucR* is present in all the strains. An *mucR* gene was also amplified from other laboratory strains with different origins, such as GR4 and AK631. The presence of *mucR* in mucoid strains that presumably produce galactoglucan indicates that the function of this gene is not to repress *exp* gene expression. The ubiquity and conservation of *mucR* also indicate that it plays an important role in more than galactoglucan production regulation. It has been shown that in Rm2011 *mucR* is necessary for the production of high-molecular-weight succinoglycan (Bertram-Drogatz et al. 1998) and a homologue (*rosR*) in *Rhizobium etli* is necessary for rhizosphere competitiveness (Bittinger et al. 1997). In this strain, RosR modulates the expression of at least six loci (Bittinger and Handelsman 1998).

The biological significance of galactoglucan production is not clear. Gonzalez et al. (1996) have shown that low-

molecular-weight galactoglucan can act as a signal for nodule infection and can replace succinoglycan in noninfecting *exo* mutants. On the other hand, the environmental regulation of galactoglucan production might indicate that galactoglucan can play a role in adaptation to different environments and/or in symbiosis under certain environmental conditions.

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Fig. 4. Expression of *mucR* in (lane 1) EFB1 and (lane 2) Rm2011 determined by Northern (RNA) blot analysis. Overnight bacterial cultures (1 ml) were sonicated, and RNA isolation and separation on agarose-formaldehyde gels were done as described (Logemann et al. 1987). RNAs were transferred to Hybond-N membranes (Amersham, Buckinghamshire, UK) under standard conditions (Sambrook et al. 1989) and hybridization of the filters was carried out with a digoxigenin (DIG) DNA-labeled probe (Boehringer Mannheim, Mannheim, Germany). Approximate transcript size was 700 nucleotides.

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