RESEARCH LETTER

Analysis of the *mucR* gene regulating biosynthesis of exopolysaccharides: implications for biofilm formation in *Sinorhizobium meliloti* Rm1021

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Abstract

Bacterial surface polysaccharides are crucial for establishment of successful rhizobia–legume symbiosis, and in most bacteria, are also critical for biofilm formation and surface colonization. In *Sinorhizobium meliloti*, the regulatory protein MucR controls exopolysaccharide production. To clarify the relationship between exopolysaccharide synthesis and biofilm formation, we studied *mucR* expression under growth conditions that influence attachment to polyvinylchloride, developed a microtiter plate assay to quantify biofilm formation in *S. meliloti* strain Rm1021 and mutants defective in succinoglycan (EPS I) and/or galactoglucan (EPS II) production, and analyzed expression of EPS I and EPS II genes by quantitative reverse transcriptase-PCR. Consistent with previous studies of planktonic bacteria, we found that disruption of the *mucR* gene in Rm1021 biofilms increased EPS II, but reduced EPS I gene expression. *mucR* expression was not affected by environmental conditions that influence biofilm formation on polyvinylchloride, and biofilm formation by Rm1021 was independent of exopolysaccharide synthesis. Other factors on the Rm1021 cell surface, and growth conditions, presumably regulate attachment and/or growth as a biofilm on polyvinylchloride.

Introduction

Rhizobia are soil bacteria with the capability to establish a symbiotic relationship with legume plants when soil nitrogen is limited. Rhizobial surface polysaccharides play important roles in symbiosis and formation of active nodules. Mutants defective in the production of exopolysaccharides, lipopolysaccharides, and capsular polysaccharides usually show reduced induction of effective nodules, and are particularly affected in the process of infection through infection threads (Hirsch, 1999).

One of the best-studied exopolysaccharides produced by *Sinorhizobium meliloti* is succinoglycan (EPS I) (Reinhold *et al.*, 1994), which consists of repeated units of an octasaccharide containing one galactose and seven glucoses, and has characteristic succinyl, acetyl, and pyruvyl modifications. A 25-kb region located in the second symbiotic megaplasmid (pSymB) in *S. meliloti* clusters the *exo–exs* genes necessary for the production of EPS I. The roles of most of these genes have already been defined (Reuber & Walker, 1993). *Sinorhizobium meliloti* is also capable of producing a second exopolysaccharide known as galactoglucan (EPS II) (Her *et al.*, 1990; Zevenhuizen, 1997), which is synthesized under conditions of phosphate limitation (as often found in soils) (Zhan *et al.*, 1991; Mendrygal & González, 2000), in the presence of a mutation in the regulatory gene *mucR* (Zhan *et al.*, 1989; Keller *et al.*, 1995) or an intact copy of the transcriptional regulator *expR* (Glazebrook & Walker, 1989; Pellock *et al.*, 2002). EPS II is a polymer of disaccharide repeating units consisting of an acetylated glucose and a pyruvylated galactose (Her *et al.*, 1990). A 32-kb cluster of genes (the *exp* genes) also located in pSymB is responsible for the production of EPS II (Glazebrook & Walker, 1989).

EPS I and EPS II are synthesized in two different fractions: high molecular weight (HMW) and low molecular weight (LMW). External addition of the LMW fractions of EPS I (trimers of the octasaccharide), and oligomers (15–20 units of the disaccharide) of EPS II, can restore defective infection phenotypes in exopolysaccharide mutants, indicating that

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the establishment of symbiosis requires the presence of at least one of the LMW forms of either EPS I or EPS II (Battisti *et al.*, 1992; González *et al.*, 1996).

Bacterial surface components, such as exopolysaccharides, flagella, and lipopolysaccharides, are important not only in rhizobia–legume symbiosis but also in biofilm formation. Biofilms are defined as microbial communities surrounded by a self-produced polymeric matrix and attached to a surface (Costerton *et al.*, 1995). The major components of biofilms are water (up to 97% of the total volume) and bacterial cells. The minor components are exopolysaccharides (Sutherland, 2004), proteins, DNA, and bacterial lysis products (Branda *et al.*, 2005). Exopolysaccharides play important roles in surface attachment and development of mature biofilms (Watnick & Kolter, 1999; Sutherland, 2001). The biofilm matrix provides bacteria with a physical barrier against antibiotics and defense compounds from the host (Gilbert *et al.*, 1997), and against various environmental stresses including UV radiation, pH changes, osmotic shock, and desiccation (Flemming, 1993).

In *S. meliloti*, the regulatory protein MucR plays a key role in the control of EPS I and EPS II production by binding to promoter regions in both exopolysaccharide biosynthesis gene clusters (Keller *et al.*, 1995; Bahlawane *et al.*, 2008). A mutation in *mucR* results in the production of high levels of the HMW fraction of EPS II, and the reduction of EPS I to trace levels (Zhan *et al.*, 1991; González *et al.*, 1996). MucR also causes feedback inhibition of its own transcription by binding to a short transcribed region located upstream of the coding region of *mucR* (Bertram-Drogatz *et al.*, 1997).

Rhizobia face a diversity of natural environments ranging from a rhizosphere rich in nutrients and root exudates, to soils deficient in nitrogen, phosphorus, water, and/or other nutrients. The behaviors of biofilms on abiotic and biotic surfaces provide the basis for several survival strategies in bacteria, particularly nonspore formers such as rhizobia. Previous studies by our group suggest that biofilm formation in *S. meliloti* is altered by changes in environmental conditions and the nutritional status of the medium (Rinaudi *et al.*, 2006). Adhesion of bacteria to different surfaces, and their self-aggregation, may be modulated by regulation of exopolysaccharide synthesis. The present study is focused on the roles of transcriptional regulator *mucR*, and exopolysaccharide synthesis, in biofilm formation by S. *meliloti* Rm1021.

Materials and methods

Bacterial strains

The strains used in this study are listed in Table 1. Mutations carried in Rm3131 (Keller *et al.*, 1995), Rm9020 (Glazebrook & Walker, 1991), and Rm10002 (Glazebrook & Walker, 1989) were transferred between *S. meliloti* strains by phage Φ M12 general transduction as described previously by Finan *et al.* (1984). Antibiotics were added at the following concentrations: streptomycin, 500 μg mL^{-1}; neomycin, 200 μg mL^{-1}; tetracycline, 10 μg mL^{-1}; oxytetracycline, 0.75 μg mL^{-1}; and chloramphenicol, 20 μg mL^{-1}.

Culture media and growth conditions

Sinorhizobium meliloti was grown in minimal *Rhizobium* defined medium (RDM) [5 g sucrose, 100 mL RDM A stock (6 g KNO_3; 1 g $CaCl_2 \cdot 2H_2O$; 2.5 g $MgSO_4 \cdot 7H_2O$; 1000 mL H_2O), 100 mL RDM B stock (10 g K_2HPO_4; 10 g KH_2PO_4; 0.1 g $FeCl_3 \cdot 6H_2O$; 1000 mL H_2O), 4 mL biotin stock (0.25 mg mL^{-1}), 1 mL thiamine stock (10 mg mL^{-1}), H_2O q.s. to 1000 mL] (Vincent, 1970), Luria–Bertani (LB) broth (Sambrook *et al.*, 1989), or tryptone–yeast extract (TY) medium (Beringer, 1974) at 30 °C. RDM medium was supplemented when needed with 0.3 M sucrose, 0.15 M NaCl, 0.1–100 mM phosphate, or 7 mM $CaCl_2$.

Construction of the *mucR::lacZ* transcriptional fusion

A DNA region containing the *mucR* promoter was generated by PCR amplification of Rm1021 genomic DNA using the following primers (*mucR* sense, 5′-TTCGGGTCTGCTGC TCTTTG-3′; *mucR* antisense, 5′-ATGTGATCTGGTCGTC CTGC-3′) designed from the *S. meliloti* Rm2011 *mucR* sequence (Martín *et al.*, 2000). The PCR-amplified fragment was cloned upstream of a promoterless *lacZ* gene in the wide-host-range vector pMP220 (Spaink *et al.*, 1987). The *mucR::lacZ* fusion plasmid was introduced by triparental mating into *S. meliloti* Rm1021.

β-Galactosidase assay

Bacterial liquid cultures comprising 10–15% of the flask volume were grown in a rotary shaker (Model SI4-2 Shel Lab, 12-mm orbit, Sheldon Manufacturing Inc., OR) at 200 r.p.m. and at 30 °C for 72 h. Planktonic cells were removed from the flasks and biofilm rings growing on the glass in the interface between air and the culture medium were gently washed twice with a sterile physiological saline solution, collected in an Eppendorf tube, centrifuged, and resuspended in cold Z-buffer [100 mM sodium phosphate (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol] for β-galactosidase activity assays, performed as described by Miller (1972). β-Galactosidase activity in Miller units was calculated using the formula $(1000 \times OD_{420\,nm})/(OD_{600\,nm}\ \Delta T \times V)$, where ΔT is the reaction time (min) and V the initial volume of the culture used (mL).

Table 1. Strains and plasmids used in this study

Strain, plasmid or phage	Relevant properties	Source or reference
S. meliloti		
Rm1021	SU47 *str-21 expR102*::IS*Rm*2011-1	Leigh *et al*. (1985)
Rm1021 *mucR*	SU47 *str-21 mucR31*::Tn5	Present study
Rm1021 *exoY*	SU47 *str-21 exoY210*::Tn5-*132*	Present study
Rm1021 *expA*	SU47 *str-21 expA3*::Tn*5*	Glazebrook & Walker (1989)
Rm1021 *exoY expA*	SU47 *str-21 exoY210*::Tn5-*132 expA3*::Tn5	Present study
Rm1021 *mucR*::*lacZ*	SU47 *str-21* containing the pMP220mucR plasmid	Present study
E. coli		
MT616	*pro-82 thi-1 hsdR17 supE44 recA56* (pRK600), Cm^r	Finan *et al*. (1986)
Plasmids		
pMP220	Plasmid to detect promoter activity, transcriptional fusion to *lacZ* Tc^r Tra^-	Spaink *et al*. (1987)
pMP220mucR	pMP220 with the *mucR* promoter cloned upstream *lacZ*	Present study
Phage		
ΦM12	Generalized transducing phage for *S. meliloti*	Finan *et al*. (1984)

Biofilm formation assay

The biofilm formation assay, based on the method of O'Toole & Kolter (1998), relies on the ability of cells to adhere to the wells of 96-well microtiter dishes made of polyvinylchloride. To each well, 150 μL of a 1 : 100 dilution of an overnight culture ($OD_{600\,nm}$ 0.2) was added; the plates were covered with plastic to prevent evaporation and incubated without agitation at 30 °C for 48 h. Planktonic cells were gently homogenized manually by repeated pipetting and bacterial growth was quantified by measuring OD at 600 nm. Cultures were aspirated using an automatic hand pipette, and wells were washed three times with 180 μL of sterile physiological saline solution and stained for 15 min with 150 μL of 0.1% crystal violet (CV). Each CV-stained well was then rinsed thoroughly and repeatedly with water, and scored for biofilm formation by addition of 150 μL 95% ethanol. The $OD_{560\,nm}$ of solubilized CV was determined using a MicroELISA Auto Reader (Series 700 Microplate Reader, Cambridge Technology).

RNA purification

Biofilm rings from 3-day-old *S. meliloti* cultures growing in RDM medium or RDM supplemented with either 0.3 M sucrose or 25 mM phosphate were gently washed twice with a sterile physiological saline solution, collected in an Eppendorf tube, centrifuged (10 000 ***g*** for 5 min at 4 °C), and immediately used for RNA isolation. Total RNA was purified using the TRI ReagentLS kit (Cat # TS 120) following the manufacturer's protocol. Samples were DNAse treated and RNA was finally solubilized in RNAse-free water. RNA concentrations were determined using a spectrophotometer at $OD_{260\,nm}$.

Real-time reverse transcriptase (RT)-PCR analysis

First-strand cDNA from two independent biological samples was generated using an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Cat #600559) with 1 μg total RNA per reaction. An aliquot (1 μL) of the cDNA reaction was used as a template for real-time PCR. The primer sequences used for real-time PCR were described by Glenn *et al*. (2007). The probes and oligonucleotide sequences used were:

SMc00128 probe, 5′-[HEX]TCAGCATGAACGACCAGA CAGCCGTCA[DBH2]-3′;

expE2 probe, 5′-[DFAM]CAACCCGTCCCGCTCGT CAGCAC[DBH1]-3′;

SMc00128 sense, 5′-GCAGTTCGACGAGCTGGATC-3′;

SMc00128 antisense, 5′-TTGCGATCTTCGACAGCGG-3′;

expE2 sense, 5′-GCCAAACACACGCTCGTCAT-3′;

expE2 antisense, 5′-GCCACTCTCCGCAAGAGAAA-3′;

exoY sense, 5′-AATCATAGAGTTCGAGTTCGTCTTC-3′;

exoY antisense, 5′-TCCTGCGTAAGCTCAGCCT-3′

(DFAM, 6-carboxyfluorescein; HEX, hexachlorofluorescein; DBH1, Black Hole Quencher 1; DBH2, Black Hole Quencher 2). For real-time PCR analysis using SMc00128 or *expE2* probes, Brilliant II QPCR master mix (Stratagene, Cat #600804) was used. For the SYBR Green protocol, the Brilliant SYBR® Green QPCR Master Mix (Stratagene, Cat #600548) was used. The experiment was performed using the Mx3005P™ Real-Time PCR System (Stratagene), programmed as follows: stage 1, 95 °C for 120 s; stage 2, 95 °C for 15 s and 60 °C for 30 s (two-temperature cycle repeated 40 times). The expression of SMc00128 was used as an internal control for normalization as described previously (Krol & Becker, 2004). A difference of one threshold cycle (C_T) value equals a twofold change in gene expression. The fold change was calculated as $2^{\Delta C_T}$, where C_T is the level of gene expression in the specified strain.

Statistical analysis

β-Galactosidase and biofilm formation assays were performed in triplicate and repeated at least three times. Values were averaged, and the SDs were calculated. Data were subjected to one-way ANOVA, followed by comparison of

multiple treatment levels with the control, using *post hoc* Fisher's LSD test. All statistical analyses were performed using INFOSTAT software version 1.0.

Results and discussion

Effects of environmental conditions on *mucR* expression

MucR is a transcriptional repressor of the *exp* genes involved in the biosynthesis of EPS II, and an activator of EPS I biosynthesis in *S. meliloti* (Keller *et al.*, 1995). Our previous studies showed that the ability of *S. meliloti* strain Rm1021 to attach and develop a biofilm on polyvinylchloride is strongly affected by environmental conditions (Rinaudi *et al.*, 2006). To determine the role of MucR in this process, we studied the effect of various environmental conditions on *mucR* expression, using a *mucR* promoter fusion to the *lacZ* gene that encodes β-galactosidase. Growth of *S. meliloti* in a nutritionally limiting environment is known to promote the transition from a planktonic to a sessile mode of life. For example, Rm1021 forms a larger biofilm biomass when it is grown in a nutrient-poor RDM medium, as compared with enriched media such as LB or TY (Fujishige *et al.*, 2005). On the other hand, similar levels of *mucR* expression were observed in cells resuspended from 3-day-old biofilms grown in any of these three media (data not shown).

We also studied *mucR* expression in biofilm cells grown in RDM medium supplemented with 0.3 M sucrose, 0.15 M NaCl, 25 mM phosphate, or 7 mM calcium chloride, conditions that influence attachment to the polyvinylchloride surface (Rinaudi *et al.*, 2006). Although biofilm formation was enhanced when any of these three components was added to the medium, *mucR* expression was only slightly increased by addition of phosphate, and unaffected by addition of sucrose or calcium (Fig. 1). Addition of 0.15 M sodium chloride, which reduces biofilm formation, had no effect on reporter expression from the *mucR* promoter (Fig. 1). These observations suggest that the ability of *S. meliloti* Rm1021 to sense nutritional and environmental conditions, with the consequent transition from a planktonic to a sessile mode, and formation of biofilms (Rinaudi *et al.*, 2006), is not mediated by changes in *mucR* expression.

Because expression of the *mucR* promoter was slightly increased in the presence of 25 mM phosphate as compared with the regular RDM medium (12.5 mM phosphate) (Fig. 1), we evaluated *mucR* expression in biofilms from the Rm1021 *mucR::lacZ* strain under a range of phosphate concentrations (0.1–100 mM). The increase in phosphate availability was correlated with increased β-galactosidase activity (Fig. 2). The presence of *mucR* is necessary for EPS I production (Zhan *et al.*, 1991; Keller *et al.*, 1995; Bertram-Drogatz *et al.*, 1998). EPS I production is dramatically enhanced at high phosphate concentrations (Mendrygal & González, 2000). Our results suggest that this enhancement is mediated by increased *mucR* expression.

β-Galactosidase assays showed that *mucR* expression is maximal during the exponential phase of planktonic growth ($OD_{600\,nm}$ 0.8). Intermediate values of β-galactosidase activity were observed in the lag phase ($OD_{600\,nm}$ 0.2) and the stationary phase of growth ($OD_{600\,nm}$ 1.2). The expression of *mucR* was lower in a 3-day-old biofilm than at any stage of growth (Fig. 3), consistent with the results described above.

Effect of *mucR*, *exoY*, and *expA* mutations on attachment of strain Rm1021 to polyvinylchloride

To further elucidate the role of MucR in biofilm development, attachment of a *mucR* mutant to polyvinylchloride wells was evaluated by CV staining. Biomass of 2-day-old biofilms of the mutant grown in RDM medium was not different from that of wild-type Rm1021 (data not shown). Similar observations for these two strains were obtained in MGM medium with high (10 mM) and low (0.1 mM) phosphate (Rinaudi & González, 2009). The *mucR* mutant produces the HMW fraction of EPS II (González *et al.*, 1996), suggesting that the nonsymbiotically active fraction of EPS II of *S. meliloti* is not involved in attachment to polyvinylchloride under these conditions.

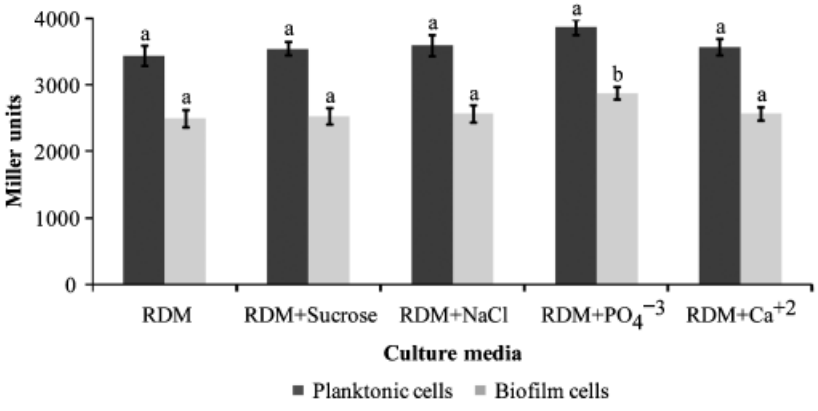

Fig. 1. Effect of RDM supplementation with various compounds on *mucR* expression. β-Galactosidase activity (expressed as Miller units) in planktonic and biofilm cells from Rm1021 *mucR::lacZ* grown for 3 days on RDM medium supplemented with 0.3 M sucrose, 0.15 M NaCl, 25 mM phosphate, and 7 mM calcium chloride. Assays were performed in triplicate, and the mean values and SDs are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

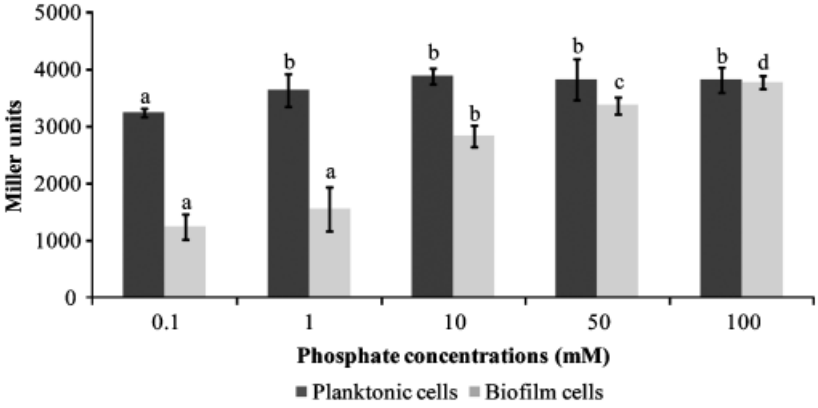

Fig. 2. Effect of phosphate on *mucR* expression. β-Galactosidase activity (expressed as Miller units) in planktonic and biofilm cells from Rm1021 *mucR::lacZ* grown for 3 days on RDM medium supplemented with phosphate at the indicated concentrations. Assays were performed in triplicate, and the mean values and SDs are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

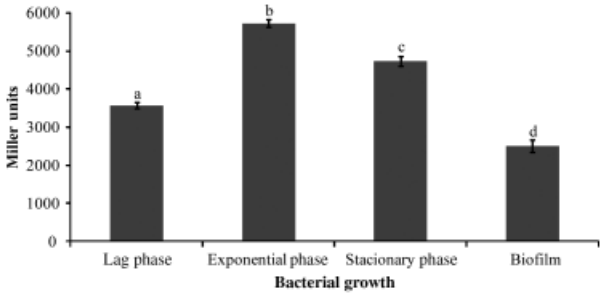

Fig. 3. *mucR* expression in planktonic and biofilm cells. β-Galactosidase activity (expressed as Miller units) of planktonic cells from various stages in the growth curve, and of biofilm cells from Rm1021 *mucR::lacZ* grown for 3 days on RDM medium. Assays were performed in triplicate, and the mean values and SDs are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

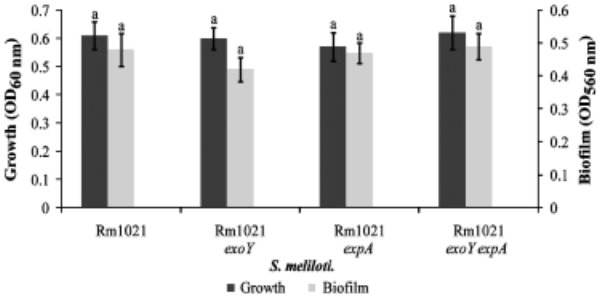

Fig. 4. Bacterial growth and biofilm formation of static cultures of *Sinorhizobium meliloti* Rm1021 and mutants in EPS I and/or EPS II. Biofilm was allowed to develop in RDM medium for 48 h, and then quantified. Assays were performed in triplicate, and the mean values and SDs are shown. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

To assess the contribution of EPS II and EPS I to biofilm formation of Rm1021 in RDM medium, we analyzed the polyvinylchloride attachment ability of *exoY* and *expA* mutants, which are defective in the biosynthesis of EPS I and EPS II, respectively. Biofilm biomass of both the mutants in RDM medium was similar to that of Rm1021, indicating that these polysaccharides are not crucial for polyvinylchloride attachment under our conditions (Fig. 4). An additional mutation in *expA* on the *exoY* mutant background did not result in a further decrease in biofilm formation (Fig. 4), which rules out the possibility of a compensation effect on exopolysaccharide production.

Expression of genes involved in the synthesis of exopolysaccharides in biofilms

Quantitative RT-PCR was used to evaluate the expression of genes involved in the production of EPS I and EPS II in biofilms of Rm1021 and its *mucR* mutant. Expression of *expE2* or *exoY* gene in biofilms grown in RDM medium (0.015 M sucrose, 12.5 mM phosphate), and RDM supplemented with 0.3 M sucrose or 25 mM phosphate was analyzed as described in M&M. The *expE2* gene encodes a glycosyltransferase involved in the synthesis of EPS II. The *exoY* gene encodes a galactosyltransferase responsible for incorporation of the first galactose into the intermediate lipid in EPS I biosynthesis.

Introduction of a mutation in the MucR regulator in Rm1021 led to increased transcription of the *expE2* gene, relative to the wild type. Transcription of *expE2* in biofilms formed by the *mucR* mutant was enhanced by addition of 0.3 M sucrose to culture medium, but was reduced by a high phosphate concentration (Fig. 5). This is consistent with the findings that increased phosphate availability in planktonic bacteria blocks EPS II synthesis (Zhan *et al.*, 1991; Mendrygal & González, 2000), and thereby reduces expression of the genes responsible for EPS II production. Because *expE2* is actively transcribed in biofilms of Rm1021 *mucR* (a strain that produces HMW EPS II) grown in RDM medium, and biofilm formation in Rm1021 *mucR* is similar to that in the wild type (present study, and Rinaudi & González, 2009), the above finding confirms that the HMW fraction of EPS II produced by the *mucR* mutant is not involved in biofilm formation.

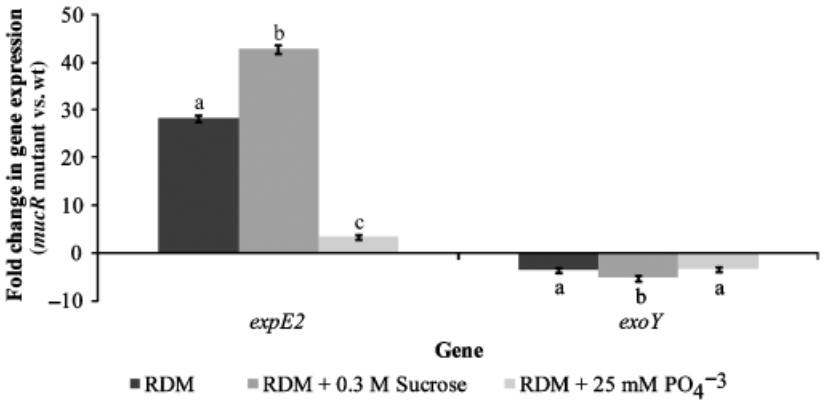

Fig. 5. Expression of the *expE2* and *exoY* genes in *Sinorhizobium meliloti* biofilms. Negative change values indicate downregulation in the mutant strain (Rm1021 *mucR*) compared with expression in wild-type Rm1021. Results are means from two independent experiments. Values having different letters are significantly different from each other according to Fisher's LSD test ($P < 0.05$).

exoY expression in biofilms of the *mucR* mutant was less than that in Rm1021 (Fig. 5). This result is consistent with previous observations that MucR promotes EPS I synthesis in planktonic bacteria (Bertram-Drogatz *et al.*, 1998). On the other hand, *exoY* expression was not activated by 25 mM phosphate (Fig. 5), suggesting that higher concentrations of phosphate are needed for induction of EPS I production. Mendrygal & González (2000) reported that *S. meliloti* achieves the maximal production of EPS I at phosphate concentrations higher than those used in the present study.

In conclusion, our findings suggest that *in vitro* polyvinylchloride attachment by Rm1021 does not depend on exopolysaccharide synthesis under our experimental conditions. In contrast, Fujishige *et al.* (2006) found that succinoglycan (EPS I) is involved in biofilm development. This apparent discrepancy may be explained by the fact that sucrose concentration in RDM medium for *S. meliloti* growth was 2% in the Fujishige study, but only 0.5% in the present study. High levels of sucrose in culture medium have been reported to cause increased exopolysaccharide synthesis in other microorganisms (van Geel-Schutten *et al.*, 1998; Lee *et al.*, 2003; Gross & Rudolph, 2008), probably as a result of facilitated carbon uptake.

Under our assay conditions, *mucR* gene expression and regulation of exopolysaccharide biosynthesis do not appear to be crucial for biofilm formation in *S. meliloti* strain Rm1021. This finding is important for understanding the contribution of rhizobial exopolysaccharides to legume colonization, a key step in the nodulation pathway.

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