**Sinorhizobium meliloti** low molecular mass phosphotyrosine phosphatase SMc02309 modifies activity of the UDP-glucose pyrophosphorylase ExoN involved in succinoglycan biosynthesis

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In Gram-negative bacteria, tyrosine phosphorylation has been shown to play a role in the control of exopolysaccharide (EPS) production. This study demonstrated that the chromosomal ORF SMc02309 from *Sinorhizobium meliloti* 2011 encodes a protein with significant sequence similarity to low molecular mass protein-tyrosine phosphatases (LMW-PTPs), such as the *Escherichia coli* Wzb. Unlike other well-characterized EPS biosynthesis gene clusters, which contain neighbouring LMW-PTPs and kinase, the *S. meliloti* succinoglycan (EPS I) gene cluster located on megaplasmid pSymB does not encode a phosphatase. Biochemical assays revealed that the SMc02309 protein hydrolyses *p*-nitrophenyl phosphate (*p*-NPP) with kinetic parameters similar to other bacterial LMW-PTPs. Furthermore, we show evidence that SMc02309 is not the LMW-PTP of the bacterial tyrosine-kinase (BY-kinase) ExoP. Nevertheless, ExoN, a UDP-glucose pyrophosphorylase involved in the first stages of EPS I biosynthesis, is phosphorylated at tyrosine residues and constitutes an endogenous substrate of the SMc02309 protein. Additionally, we show that the UDP-glucose pyrophosphorylase activity is modulated by SMc02309-mediated tyrosine dephosphorylation. Moreover, a mutation in the SMc02309 gene decreases EPS I production and delays nodulation on *Medicago sativa* roots.

**INTRODUCTION**

Tyrosine phosphorylation is a reversible and dynamic process, governed by the activities of protein-tyrosine kinases (PTKs), and by two main classes of protein-tyrosine phosphatases (PTP): the conventional eukaryotic-like phosphatases and the acidic phosphatases of low molecular mass (LMW-PTPs) (Zhang, 2001).

In bacteria, the genes encoding the LMW-PTP and PTK pairs are generally included in large operons involved in the production or regulation of exopolysaccharides and capsular polysaccharides (CPS). Several studies have reported a direct relationship between the reversible tyrosine phosphorylation and the production of these polymers (Whitfield, 2006; Yother, 2011), which have been identified as significant virulence determinants in many plant and animal pathogens and are also required for symbiotic interactions in plant-associated bacteria (Skorupska *et al.*, 2006).

**Abbreviations:** ALP, commercial alkaline phosphatase; Ap, ampicillin; CPS, capsular polysaccharide; EPS, exopolysaccharide; Km, kanamycin; LMW-PTP, low molecular mass protein-tyrosine phosphatase; NaPP, sodium pyrophosphate; Nm, neomycin; Nx, nalidixic acid; PDB, Protein Data Bank; *p*-NPP, *p*-nitrophenyl phosphate; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase.
Additionally, many LMW-PTPs and PTKs are also involved in biofilm formation and community development (Whitmore & Lamont, 2012).

In *Escherichia coli*, Wzc and Etk are PTKs essential for the assembly of the group 1 and 4 CPS, respectively (Nadler et al., 2012). In addition to being able to autophosphorylate, PTKs can also phosphorylate endogenous proteins such as sugar-dehydrogenases or -transferases involved in the initial stages of polysaccharide biosynthesis. Indeed, one of the first identified substrates of PTKs was UDP-glucose dehydrogenase (Ugd) of *E. coli* (Grangeasse et al., 2003; Minic et al., 2007).

Wzc and Etk are dephosphorylated by the LMW-PTPs Wzb and Etp, respectively. These proteins have been identified in a number of bacterial species (Ferreira et al., 2007; Kennelly, 2001, 2002; Preneta et al., 2002; Shi et al., 1998; Tan et al., 2013). In addition to Wzc, Wzb dephosphorylates tyrosine residues from Ugd, thus influencing the CPS production (Lacour et al., 2008).

*Sinorhizobium meliloti* is a Gram-negative soil bacterium belonging to the *α*-proteobacteria group, which includes other bacteria interacting with eukaryotic hosts, such as *Agrobacterium tumefaciens* and *Brucella* spp. (Moreno et al., 1990). *S. meliloti* is able to establish a symbiosis with alfalfa plants, and fixes molecular nitrogen in root nodules enhancing plant growth and agricultural productivity. In the *S. meliloti–Medicago sativa* symbiosis, succinoglycan production is essential during the early stages of bacterial infection and root invasion (Battisti et al., 1992; González et al., 1996; Leigh & Walker, 1994; Leigh & Lee, 1988; Niehaus & Becker, 1998).

The biosynthetic pathways of *S. meliloti* exopolysaccharide (EPS) I, capsules of *Klebsiella pneumoniae* and group 1-like EPS of *E. coli* (colanic acid) and *Erwinia amylovora* (amylovoran) (Paiment et al., 2002) have similarities. Biosynthesis of EPS I is directed by the exoO and exo genes, which are grouped on megaplasmid pSymB (Becker et al., 1993, 1995). According to the assembly and translocation systems described for polysaccharides, and the genomic localization of the *exoPQT* genes, a Wzy-dependent pathway was proposed for *S. meliloti* EPS I biosynthesis (Ferreira et al., 2007; Reuber & Walker, 1993; Skorupska et al., 2006; Whitfield 2006). In several bacteria – such as *Acinetobacter sp.*, *E. amylovora*, *K. pneumoniae*, *Bacillus subtilis* and *Staphylococcus aureus* – a number of PTKs and their respective LMW-PTPs have been characterized (Bugert & Geider, 1997; Grangeasse et al., 1998; Gruszczyk et al., 2011; Mijakovic et al., 2005b; Preneta et al., 2002). In *S. meliloti* 2011, the PTKs ExoP and ExoP2 have been described (Jofré & Becker, 2009; Niemeyer & Becker, 2001). Moreover, the *S. meliloti* genome contains two ORFs, probably encoding LMW-PTPs (Niemeyer & Becker, 2001). The functional properties of these two ORFs, however, have not yet been reported. In the present work, we examined whether one of these *S. meliloti* ORFs – namely SMc02309, hereafter referred to as SMc02309 – is a LMW-PTP. We also determined the kinetic parameters as well as the PTP activity on the tyrosine kinase ExoP and on ExoN, a protein involved in the initial stages of succinoglycan biosynthesis. Finally, we demonstrated the influence of a mutation in the SMc02309 gene on EPS production and symbiotic performance of *S. meliloti* with *M. sativa* plants.

## METHODS

### Bacterial strains and growth conditions.

Table 1 lists the *S. meliloti* strains, primers and plasmids used in this work. The *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) broth (Sambrook et al., 1989).

*S. meliloti* 2011 and its derivatives were grown at 30 °C in TY or glutamate-α-mannitol-salts (GMS) medium (Beringer, 1974; Zevenhuizen & van Neerven, 1983). When required, antibiotics were added at the following concentrations (µg ml⁻¹): 10 nalidixic acid (Nx), 10 tetracycline (Tc), 40 gentamicin (Gm), 120 neomycin (Nm) for *S. meliloti*, and 100 ampicillin (Ap), 10 tetracycline and 50 kanamycin (Kn) for *E. coli*.

### Plasmids.

Standard molecular cloning techniques were used throughout this study (Sambrook et al., 1989). PCR was performed using standard conditions (Ausubel et al., 1995) with Pfx platinum polymerase (Invitrogen). All plasmid constructions generated in this study were verified by sequencing.

The SMc02309 gene – lacking the TGA codon – was amplified by PCR with the primers 2309f and 2309r and the *S. meliloti* strain Rm 2011 DNA as template. The resultant 477 bp fragment was digested with *Nde*I/*Sap*I, followed by ligation into *Nde*I/*Sap*I-digested pTYB1 (New England Biolabs). In the pTYB-02309, the C terminus of the target SMc02309 protein was fused to the self-cleavage intein tag containing a chitin-binding domain.

In a similar manner, the 906 bp amplicon corresponding to *exoN* from *S. meliloti* strain Rm2011 (generated by PCR with the primers *exoNf* and *exoNr*) was digested with *Nde*I and *Bam*HI, followed by ligation into *Nde*I/*Bam*HI-digested pET28a+(+) (Novagen). In the pET-*exoN*, the N terminus of the target protein was fused to the 6 × His tag.

### Construction of SMc02309 mutant strain.

The nonpolar mutant Rm42 was generated by the integration of plasmid pk18-02309 into the SMc02309 coding region of WT strain Rm 2011 following *E. coli* S17-1 mediated conjugal plasmid transfer and homologous recombination. The transconjugants were selected for resistance to Nac and Nm. Plasmid pk18-02309 carries an internal 187 bp fragment of the SMc02309 gene that was generated by PCR amplification (with the primers pk2309f and pk2309r) and subsequent insertion into the *EcoRI* and HindIII restriction sites of plasmid pk18mob2.

Plasmid pFA1-02309 was constructed by ligation of the XbaI/KpnI-digested product resulting from PCR amplification of the SMc02309 gene (with primers pFA1-2309f and pFA1-2309r and *S. meliloti* strain Rm 2011 DNA as template) to the XbaI/KpnI-digested plasmid pFA1708. Plasmid pFA1-02309 was mobilized by mating from *E. coli* S17-1 to *S. meliloti* mutant strain Rm42.

### Production and purification of the SMc02309-intein fusion protein.

For large-scale purification, 300 ml LB supplemented with 100 µg/ml tetracycline (Tc) and 40 µg/ml gentamicin (Gm) was inoculated with an overnight culture (1%, v/v) of *E. coli* BL21(DE3) containing plasmid pET-02309 and then cultured at 37 °C with shaking to an OD₆₀₀ of 0.6. The induction was initiated by adding 0.5 mM IPTG (final concentration) with the incubation being continued overnight with shaking at 22 °C. The cells were harvested and lysed by sonication in 5 ml column buffer (20 mM HEPES-Na, 50 mM NaCl, 10 mM Imidazole, pH 8.0).

Production and purification of the SMc02309-intein fusion protein. For large-scale purification, 300 ml LB supplemented with Ap was inoculated with an overnight culture (1%, v/v) of *E. coli* BL21(DE3) containing plasmid pTYB-02309 and then incubated at 37 °C with shaking to an OD₆₀₀ of 0.6. The induction was initiated by adding 0.5 mM IPTG (final concentration) with the incubation being continued overnight with shaking at 22 °C. The cells were harvested and lysed by sonication in 5 ml column buffer (20 mM HEPES-Na, 50 mM NaCl, 10 mM Imidazole, pH 8.0).
**Table 1. Bacterial strains, plasmids and primers used in this study**

<table>
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<tr>
<th>Strain, plasmid or primer</th>
<th>Relevant characteristics*</th>
<th>Reference or source</th>
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<tr>
<td><strong>S. meliloti strains</strong></td>
<td></td>
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<tr>
<td>Rm 2011</td>
<td>WT; Nod + Fix + Inf+ EPS+, Nxr Smr</td>
<td>(J. Dénarie, France)</td>
</tr>
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<td>Rm42</td>
<td>Nonpolar Smc02309 mutant in Rm2011; Nm + Nxr</td>
<td>This work</td>
</tr>
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<td>Rm43</td>
<td>Rm42 carrying pFAJ-02309; Nm' Tc' Nxr</td>
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</tr>
<tr>
<td>Rm148</td>
<td>Rm2011 carrying pFAJ1708; Tc' Nxr</td>
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<td>RmA9R0907</td>
<td>Rm2011; exoY-lacZ/uacC1, Gm' Nxr</td>
<td>Keller et al. (1995)</td>
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<td>Expression vector generating 6× His fusion proteins; Km'</td>
<td>Novagen</td>
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<tr>
<td>pGEX 5x-1</td>
<td>Expression vector generating glutathione S-transferase (GST) fusion proteins; Ap'</td>
<td>Amersham Biosciences</td>
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<td>pK18mob2</td>
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<td>Daniels et al. (2006)</td>
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<td>2309f</td>
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<td>exoNf</td>
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<tr>
<td>exoNr</td>
<td>CGAATTCCTATGGCGCGCGCGGGATGCC</td>
<td>This work</td>
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</table>

*Nxr, Smr, Nm', Ap', Tc' and Km': resistant to nalidixic acid, streptomycin, neomycin, gentamicin, ampicillin, tetracycline and kanamycin, respectively.

†Restriction sites are underlined.

pH 8.0, 500 mM NaCl, 1 mM Na-EDTA) containing 1 mg RNaseA ml⁻¹ and 1 mM PMSE. The resulting cell suspension was centrifuged (100 000 g for 1 h) and the supernatant loaded onto a chitin column (New England Biolabs). After several washings with column buffer, the proteins were eluted with the same buffer plus 50 mM DTT and incubated overnight to induce the autocleavage reaction. The resulting fractions were analysed by SDS-PAGE, and the purified proteins were eluted with the same buffer plus 50 mM DTT and 50 mM imidazole. The fractions were analysed by SDS-PAGE, and the purified ExoN protein was dialysed and concentrated by centrifugation. Finally, the purified ExoN protein was analysed by SDS-PAGE before storage at −20 °C until use.

**Production and purification of the ExoPc-GST fusion protein.** Production and purification of the ExoPc-GST fusion protein were performed as described by Niemeyer & Becker (2001). The purified protein was then analysed by SDS-PAGE before storage at −20 °C until use.

**Production and purification of 6× His-ExoN.** Briefly, an overnight culture of *E. coli* BL21 (DE3) cells containing plasmid pET28-exoN was used to inoculate 100 ml LB broth supplemented with Km. The cells were next induced with IPTG as described above, then harvested and lysed by sonication in 3 ml wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8) containing 1 mg RNaseA ml⁻¹ and 1 mM PMSE. The resulting suspension was centrifuged (100 000 g for 1 h) and the supernatant loaded onto an Ni-NTA agarose column (Qiagen). After several washings of the column, the proteins were eluted with the elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole). The fractions were analysed by SDS-PAGE, and the purified ExoN protein was dialysed and concentrated by centrifugation. Finally, the purified ExoN protein was analysed by SDS-PAGE before storage at −20 °C until use.

**MALDI-TOF-TOF spectrometry.** The overproduced proteins were separated by SDS-PAGE and the corresponding bands containing the purified proteins submitted to the Center for Chemical and Biological Studies Maldi Tof Spectrometry (CEQUIBIEM, University of Buenos Aires, Argentina) for spectrometric analysis in a MALDI-TOF-TOF spectrometer (Ultraflex II, Bruker).

**Assays for phosphatase activity.** The standard *in vitro* assay to measure acid-phosphatase activity (Preneta *et al.*, 2002) was performed at 37 °C in a reaction mixture containing, in a total volume of 0.1 ml, 20 mM sodium citrate buffer, pH 6.0, 0.1 mM ZnSO₄ and 10 mM p-nitrophenyl phosphate (p-NPP). The reactions were
incubated for 60 min and then stopped by the addition of sodium hydroxide to 1 M.

The effect of ions on the kinetic reactions was determined either with or without 0.1 mM of each of the following cations: Ca\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\), Fe\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), Ni\(^{2+}\) and Zn\(^{2+}\). Also, the PTP inhibitors sodium vanadate and sodium pyrophosphate (NaPP) were added to the reaction at final concentrations of 1 and 2.5 mM.

To determine the optimal pH for phosphatase activity, sodium acetate (pH 4.2) or sodium citrate buffer (with pH varying from 5.5 to 7.5) was used. In parallel, the optimal temperature was determined in the range from 30 to 85 °C.

Saturation curves with the addition of Zn\(^{2+}\) were determined in the presence of a variable concentration of p-NPP ranging from 0 to 60 mM. The times of the reactions, amounts of enzyme and concentrations of substrates were optimized to obtain linear kinetics. The kinetic parameters $K_{m}$ and $V_{max}$ were estimated by nonlinear fitting of the Michaelis–Menten curve with Origin software. The p-nitrophenol levels during a reaction were monitored by the increase in absorbance at 405 nm in a Tecan Infinite M200 reader. For calculations, the molar extinction coefficient 18 000 M\(^{-1}\) cm\(^{-1}\) was used (Ciri et al., 1993). One unit of acid phosphatase was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from p-NPP per min at 37 °C. The protein concentration was determined according to the method of Bradford (1976) with BSA as standard.

**Dephosphorylation assays.** Dephosphorylation of GST-ExoPc and 6 × His-ExoN was detected by immunoblot analysis. For that, 5 μg of GST-ExoPc or 6 × His-ExoN was incubated at 37 °C for 2 h with 5 μg of SMc02309 protein in 25 μl of a buffer containing 20 mM sodium citrate (pH 6) and 0.1 mM zinc sulfate. As a positive control for dephosphorylation, GST-ExoPc and 6 × His-ExoN were incubated with 1 U of commercial alkaline phosphatase (ALP; Fermentas). The reaction was stopped by the addition of an equal volume of 2 × SDS-PAGE sample buffer. The mixture was heated at 100 °C for 5 min and subsequently analysed by SDS-PAGE with immunodetection by means of a monoclonal anti-phosphotyrosine biotinylated antibody (clone PT-66, Sigma).

**Immunoblotting analysis.** Purified GST-ExoPc and 6 × His-ExoN proteins were separated and analysed by SDS-PAGE with either subsequent visualization by Coomassie blue R-250 staining or transfer onto PVDF membranes for immunoblotting in a semidry electrophoretic transfer cell, as described by Towbin et al. (1979). The GST-ExoPc protein was detected with an ExoP-specific peptide antibody (rabbit) (Eurogentec) raised with the peptide EWGRTPSRLVR. The anti-ExoP antibody was diluted in Tris-buffered saline supplemented with 0.1 % (v/v) Tween 20 and 0.3 % (w/v) nonfat dry milk. The binding of the secondary antibody, a biotinylated anti-rabbit immunoglobulin G (GE Healthcare), was detected with streptavidin biotinylated horseradish peroxidase complex (GE Healthcare). Immunoblots for the 6 × His-ExoN protein were probed with a monoclonal anti-His antibody from mouse (GE Healthcare). The binding of the secondary antibody, an mouse immunoglobulin G peroxidase conjugate (Sigma), was detected by ECL chemiluminescence reagents (Thermo Scientific). Phosphorylation on tyrosine residues was detected with the monoclonal anti-phosphotyrosine biotinylated antibody (clone PT-66 Sigma).

**Assays for UDP-glucose pyrophosphorylase activity.** 6 × His-ExoN protein was tested for UDP-glucose pyrophosphorylase activity by using a spectrophotometric assay as described elsewhere (Bergmeyer et al., 1983). Briefly, the 100 μl reaction mixture contained 100 mM MOPS buffer (pH 7.6), 1.5 mM MgCl\(_2\), 0.38 mM NADP, 20 μM glucose 1,6-diphosphate, 0.9 U glucose-6-phosphate dehydrogenase, 6.6 U phosphoglomucotase, 1 μmol UDP-glucose and 0.9 μg 6 × His-ExoN. The reaction was started by adding 1.5 μmol of pyrophosphate. The absorbance at 340 nm was measured for 15 min by using an Epoch Microplate Spectrophotometer (BioTek). The endogenous NADPH-oxidation rates were subtracted from the 6 × His-ExoN-induced NADPH values.

UDP-glucose pyrophosphorylase activity was also determined after dephosphorylation of 6 × His-ExoN. For this purpose, 0.9 μg 6 × His-ExoN was dephosphorylated either with 2 μg SMc02309 or with 1 U ALP by incubating 6 × His-ExoN for 10 min in the reaction mixture prior the determination of UDP-glucose pyrophosphorylase activity.

**Analysis of the EPS I content and distribution.** EPS I was obtained from dialysed supernatants (molecular-mass cut-off of 2000 Da) from 6-day-old cultures grown in GMS medium supplemented with 240 mM NaCl (Jofré & Becker, 2009). Total carbohydrates were determined by the anthrone method (Dische, 1962).

**Nodulation assays.** S. meliloti strains were assayed for their symbiotic phenotypes on M. sativa cv. Monarca (obtained from the Instituto Nacional de Tecnología Agropecuaria, Argentina). The seeds were surface sterilized and germinated as described by Müller et al. (1988). Inoculation of seedlings was carried out with exponential-phase cultures. The plantlets were grown on nitrogen-free medium as described by Rolle et al. (1980). Nodule formation was assayed over 4 weeks.

**Bioinformatics tools.** DNA and protein data were analysed through the use of the ORF-finder tool located at the National Center for Biotechnology Information (NCBI). The algorithm BLAST (Altschul et al., 1990) was used to compare the deduced amino acid sequences with those available in the NCBI database. Structure predictions and general protein characteristics were obtained from the Expasy server (http://www.expasy.org). Conserved orthologue neighbourhood regions were obtained from the Integrated Microbial Genomes (IMG) system (Markowitz et al., 2006) available at http://img.jgi.doe.gov.

Pairwise alignments between the SMc02309 protein and Protein Data Bank (PDB) template structures (1jl3_A, 1zgg_A, 2cwd_A, 2fek_A, 2ipa_B, 2wja_A, 2wnmy_A, 3rh0_A, 3rof_A and 3t38_A) were calculated with HHpred (Söding et al., 2005) and subsequently formatted as input for the software Modeller 9v10 (Sali et al., 1995). The resulting top model was predicted by ModEval (Eramian et al., 2008) to have a root-mean-square-deviation value of 4.552. In order to check the potential binding sites of this model, the ligands present in PDB entry 2WJA were merged to give the resulting final model.

**Statistical analyses.** All experiments were performed at least in triplicate. Data are presented as means ± SD of the indicated number of experiments. Statistical analyses were carried out using Student’s t-test and one-way ANOVA, and the means were compared using the Tukey test.

**RESULTS**

In S. meliloti the genes encoding the ExoP tyrosine-kinase and the putative phosphotyrosine phosphatase SMc02309 are not genetically linked

In S. meliloti, the PTP that dephosphorylates the C-terminal domain of the PTK ExoP has not yet been identified (Grangeasse et al., 2007). A gene neighbourhood analysis clearly indicated the absence of PTP homologues located upstream from exoP (Fig. S1a, available in the online Supplementary Material). Therefore, S. meliloti does not
possess the same genetic arrangement with regard to the BY-kinase and PTP loci that has been observed in other bacterial species (Arakawa et al., 1995; Bugert & Geider, 1995; Ferreira et al., 2007; Grangeasse et al., 1998; Huang & Schell, 1995; Vincent et al., 1999). This result prompted us to initiate a genome search to identify putative candidates encoding PTPs in S. meliloti. We detected a S. meliloti chromosomal ORF (locus tag SM2011_c02309, in this work referred to as SMc02309) containing pfam01451, a highly conserved domain in the well-characterized tyrosine-phosphatase Wzb from E. coli. The predicted SMc02309 protein was originally annotated as a putative arsenate reductase – as inferred by automated annotation – although SMc02309 is not included within the ars operon. BLASTP sequence analysis revealed that SMc02309 is significantly similar to other biochemically characterized LMW-PTPs and a synteny analysis of the SMc02309 gene showed conserved neighbouring orthologous genes when compared with other rhizobia and Brucella strains (Fig. S1b). The SMc02309 protein shares 1) the conserved motif CX5R(S/T) in the active site, including the arginine residue crucial for the binding of the phosphate substrate (Kennelly & Potts, 1999) and a conserved cysteine residue required for the enzymic activity (Zhang et al., 1994), and 2) an invariant aspartate in the DP(Y/T) motif (positions 116 in Wzb and 120 in Smc02309) (Fig. S1c). Furthermore, the SMc02309 protein is included in the tyrosine-phosphatase group and appears to be close to the LMW-PTP encoded by the locus tag Q57FA1/BruAb1_0278 of Brucella abortus (Fig. S2).

Taken together, these predictions suggest that the SMc02309 protein might be an LMW-PTP in S. meliloti.

**The molecular structure of SMc02309 resembles the LMW-PTP Wzb from E. coli**

Analysis by homology detection and structure prediction (HHPred; Söding et al., 2005) of the amino acid sequence of SMc02309 predicted three β-sheets and three α-helices. Scans within the PDB revealed that this protein is significantly similar to other arsenate reductase enzymes and amino acid phosphatases, but with rather low sequence identity (20–30%). Nevertheless, whereas SMc02309 does not conserve all the essential catalytic cysteine residues present among the former enzymes, the protein does contain a CX5R(S/T) motif, which forms the phosphate binding loop in the active site (known as the P-loop) present among the LMW-PTPs (cf. above). Therefore, on the basis of sequence conservation, it seems more likely that this protein might
be an LMW-PTP. Among the phosphatases with experimentally determined three-dimensional structures found in our searches, the most similar protein was Wzb (2WJA PDB entry). The superimposition of Wzb upon the model of SMc02309 was consistent with the hypothesis of SMc02309 being a phosphatase (data not shown).

**SMc02309 from *S. meliloti* is an acid phosphatase: optimal conditions for *in vitro* activity**

*In vitro* enzymic assays with purified SMc02309 protein were performed to test for the predicted LMW-PTP activity. The SMc02309 gene from *S. meliloti* 2011 lacking the stop codon was amplified and cloned into the expression vector pTYB1. SDS-PAGE analysis of proteins from *E. coli* cultures induced with IPTG exhibited an over-production of a 70 kDa protein consistent with the expected mass for the SMc02309 intein-tagged fusion protein. After affinity purification and on-column cleavage with DTT, a 17 kDa protein corresponding to the expected mass for the SMc02309 intein-tagged fusion protein. After affinity purification and on-column cleavage with DTT, a 17 kDa protein corresponding to the expected mass for the SMc02309 intein-tagged fusion protein.

**SMc02309 is not the phosphotyrosine phosphatase of the tyrosine kinase ExoP**

In order to assess whether SMc02309 exhibited phosphotyrosine phosphatase activity on ExoP, we proceeded to overexpress the *exoP* sequence encoding the C-terminal domain of ExoP as a glutathione-S-transferase (GST) fusion protein. Even when the protein was treated with the phosphatase inhibitors, we did not observe any change in activity (data not shown). The optimal conditions for SMc02309 phosphatase activity are summarized in Table 2. We also tested arsenate reductase activity by measuring NADPH oxidation (Anderson & Cook, 2004) and after three independent experiments concluded that SMc02309 does not catalyse that reaction (data not shown).
fusion protein (GST-ExoPc). For dephosphorylation assays, GST-ExoPc was incubated in the presence or absence of SMc02309 for 2 h. We were then able to show by immunoblotting that the GST-ExoPc fusion protein was indeed phosphorylated on tyrosine residues, as revealed by a monoclonal anti-phosphotyrosine antibody (Fig. 3a). Furthermore, we found that SMc02309 did not dephosphorylate GST-ExoPc. In contrast, ALP produced a complete dephosphorylation of the GST-ExoPc fusion protein under the same conditions. The GST-ExoPc was detected by immunoblotting with the anti-ExoP polyclonal antibody.

The SMc02309 acid phosphatase dephosphorylates tyrosine residues of the ExoN protein

ExoN is a UDP-glucose pyrophosphorylase that catalyses the conversion of glucose 1-phosphate into UDP-glucose – the latter being a precursor in EPS I biosynthesis (Becker et al., 1993). In addition, the ExoN amino acid sequence contains seven tyrosine residues that are potential targets for phosphorylation. Therefore, we determined whether ExoN was phosphorylated on tyrosine residues as well as whether ExoN could be an endogenous substrate for SMc02309. To demonstrate the predicted activity of SMc02309 as a phosphotyrosine phosphatase of ExoN, the exoN gene was overexpressed to produce a 6×His-ExoN recombinant protein that was subsequently purified. The 33 kDa protein obtained was identified – by MALDI-TOF-TOF mass spectrometry – as being consistent with ExoN from S. meliloti. Although 6×His-ExoN usually resulted in a double band in SDS-PAGE analysis, both bands were identified as ExoN.

We performed immunoblotting of 6×His-ExoN either with or without a previous incubation with the SMc02309 protein (Fig. 3b) and did likewise with ExoPc. The ExoN protein was phosphorylated at tyrosine residues as detected by using the monoclonal anti-phosphotyrosine antibody (Fig. 3b). Furthermore, the degree of ExoN tyrosine phosphorylation decreased when the protein was incubated in the presence of the acid phosphatase SMc02309, whereas a complete dephosphorylation of ExoN was observed in the presence of ALP. Since both ExoN bands were detected as tyrosine-phosphorylated and they were dephosphorylated by both phosphatases, we speculate that the slight difference in mass could be the result of differences in the degree of tyrosine phosphorylation.

In all these incubations, the 6×His-ExoN was detected by immunoblotting with an anti-His monoclonal antibody.

In vitro UDP-glucose pyrophosphorylase activity of ExoN was considerably reduced when the protein was previously incubated with either SMc02309 or ALP phosphatases (Table 3). Taken together, these results evidenced that 1) the ExoN protein was phosphorylated on tyrosine residues, 2) SMc02309 indeed exhibited tyrosine-phosphatase activity on ExoN and 3) ExoN dephosphorylation by SMc02309 could be physiologically relevant because it negatively affected the enzymic activity of ExoN in vitro.

A nonpolar mutation for the SMc02309 gene affects EPS I production

To determine the potential role of the SMc02309 gene in EPS I production and symbiosis with M. sativa, we

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific activity (μmol min⁻¹ mg⁻¹) ± SD</th>
</tr>
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<tbody>
<tr>
<td>ExoN</td>
<td>0.38 ± 0.0108</td>
</tr>
<tr>
<td>ExoN + SMc02309</td>
<td>0.23 ± 0.0043</td>
</tr>
<tr>
<td>ExoN + ALP</td>
<td>0.14 ± 0.0103</td>
</tr>
</tbody>
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Fig. 3. Dephosphorylation assays of (a) ExoPc and (b) ExoN by SMc02309 phosphatase. Purified ExoPc and ExoN proteins were incubated with SMc02309 protein and tested for tyrosine dephosphorylation by immunoblotting using anti-ExoPc (anti-ExoP) or anti-His, and anti-phosphotyrosine (anti-P-Tyr) antibodies, respectively. A decreased pattern of phosphorylation was observed when ExoN was incubated with SMc02309. When both ExoPc and ExoN proteins were incubated with ALP, complete tyrosine dephosphorylation was observed.
generated the mutant strain Rm42 by site-directed plasmid integration in which the lac promoter of plasmid pK18mob2 was driving transcription of the gene downstream of SMc02309. At 28 °C, no differences in growth rate were observed in the Rm42 mutant in comparison with the parental strain Rm 2011 (data not shown). EPS I production was measured in 6-day-old culture supernatants from GMS medium supplemented with 240 mM NaCl (an optimal condition for EPS I production). Rm42 accumulated significantly less EPS I compared with the Rm 2011 WT. As expected, complementation of the Rm42 mutant strain with the WT allele (Rm43) restored EPS production.

Extended incubation (9 days old) was also attempted but the results were ambiguous, possibly due to changes in the medium that can affect EPS I production at the transcriptional level (Geddes et al., 2014). To exclude the possibility that plasmid pFAJ1708 influences the EPS I production, we included S. meliloti strain Rm148 harbouring empty pFAJ1708 as a control. In addition, to confirm that the hexose equivalents measured were derived from EPS I, the S. meliloti exoY mutant (strain RmAR9007), deficient in production of EPS I (Keller et al., 1995), was included as another control (Fig. 4).

**Fig. 4.** Production of EPS I by S. meliloti strains Rm 2011 (WT), Rm42 (SMc02309 mutant), Rm43 (SMc02309 mutant complemented with pFAJ-02309), Rm148 (WT carrying empty vector pFAJ1708) and RmAR9007 (exoY mutant deficient in EPS I production). EPS I was obtained from supernatants of 6-day-old cultures grown in GMS medium supplemented with 240 mM NaCl. Bars represent the means of three biological replicates with two technical replicates each ± SD; **P<0.05 relative to Rm 2011 (WT).

Nodulation of *M. sativa* roots is delayed in the nonpolar mutant for the SMc02309 gene

EPS I in *S. meliloti* plays an essential role in nodule invasion. Mutants affected in EPS I biosynthesis give symbiotic phenotypes that range from non-invasive (e.g. exoY mutation) to not detectable (e.g. exoX mutation), the latter being capable of inducing the formation of nitrogen-fixing nodules on the host plant alfalfa (Reed et al., 1991). More recently it has been shown that increased EPS synthesis can be beneficial for symbiosis (Jones, 2012). Since exoN mutants have been shown to be symbiotically proficient and SMc02309 appears to affect the amount of EPS I being synthesized, we hypothesized that a mutation might lead to a delay in nodulation. To test this hypothesis, the symbiotic proficiency of mutant Rm42 was compared with that of WT Rm 2011. Although both strains established an effective symbiosis, as evidenced by the presence of pink and elongated nodules, the mutant strain Rm42 exhibited a significant delay in nodulation of *M. sativa* roots (Fig. 5).

**DISCUSSION**

In proteobacteria, genes encoding LMW-PTPs are often located immediately upstream from the genes encoding the BY-kinases (Grangeasse et al., 1998; Whitfield 2006). In *S. meliloti*, the chromosomal gene SMc02309 encoding a potential phosphatase is not genetically linked to exoP located on megaplasmid pSymB, suggesting that SMc02309 may not be specific for ExoP. The deduced amino acid sequence of SMc02309 shares 30 % identity and 43 % similarity with the *E. coli* wzb-encoded enzyme (Niemeyer & Becker, 2001).

Furthermore, biochemical analyses demonstrated that SMc02309 is able to hydrolyse the artificial substrate p-NPP, a commonly used substrate in phosphatase assays *in vitro* (Bennett et al., 2001; Mori et al., 2012; Standish & Morona, 2014). The estimated *Kₘ* and *Vₘₙₐₓ* values

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**Fig. 5.** Nodulation pattern of *S. meliloti* strains Rm 2011 and Rm42. Seeds were surface sterilized, germinated and inoculated as described in Methods. Nodule formation was monitored during 4 weeks. All nodules were pink. Bars indicate SD; **P<0.05; n=13.
obtained for Smc02309 were similar to those described for the LMW-PTP BceD of Burkholderia cepacia (Ferreira et al., 2007), the Wzb of Acinetobacter iwoffii (Nakar & Gün- nICK, 2003), the Yor5 of Klebsiella pneumoniae (Preneta et al., 2002), the Wzb of E. coli (Vincent et al., 1999) and the Ptp of Acinetobacter johnsonii (Grangeasse et al., 1998). In addition, the optimal pH and temperature for catalytic activity resemble those for the above-mentioned bacterial Cys-based protein phosphatases. The presence of Fe³⁺ or Cu²⁺ ions also significantly increased Smc02309 activity; while the presence of Zn²⁺ ions, a phosphatase cofactor, increased the activity by almost threefold. Na₂VO₄ was an efficient inhibitor of Smc02309. The pattern described of sensitivity to inhibitors is in line with the reported properties of other PTPs, such as the B. subtilis protein tyrosine phosphatase YwqE (Mijakovic et al., 2005a).

Protein phosphorylation plays a role during pathogenic or symbiotic processes (Grangeasse et al., 2007). A major breakthrough has been made by demonstrating a biological link between the activities of certain protein-tyrosine kinases and phosphatases and the production and/or transport of surface polysaccharides (Cozzozone 2005; Standish & Morona, 2014). Therefore, we explored a putative functional link between the activity of Smc02309 and the protein-tyrosine kinase ExoP from S. meliloti. It was reported that the molecular-mass distribution of EPS I is altered by amino acid substitutions in the tyrosine residues of the C-terminal domain of ExoP (Niemeyer & Becker 2001). Nevertheless, the Smc02309 phosphatase was not able to dephosphorylate the C-terminal domain of ExoP, suggesting that the Smc02309 phosphatase is not the cognate LMW-PTP of the BY-kinase ExoP. This observation is further supported by the fact that Smc02309 and exoP genes are not genetically linked.

In order to assess the implication of Smc02309 on polysaccharide biosynthesis we looked for possible substrates in this process. In several bacteria, UDP-glucose dehydrogenases (Ugd) catalyse the formation of glucuronic acid, whose product serves as a building block for polysaccharides biosynthesis. The transphosphorylation of Ugd of E. coli by the tyrosine kinase Wzc is highly relevant because the phosphorylated state of Ugd (Ugd-P) increases its enzymic activity, thus enhancing synthesis of UDP-glucuronic acid, a substrate in the production of colanic acid. Ugd-P also serves as a substrate for the LMW-PTP Wzb (Grangeasse et al., 2003; Lacour et al., 2008). In S. mellioti, ExoN functions as a UDP-glycosyl pyrophosphorylase involved in the synthesis of UDP-glucose from glucose 1-phosphate (Glucksmann et al., 1993). Here, we demonstrated that ExoN is phosphorylated on tyrosine residues and Smc02309 phosphatase dephosphorylates ExoN. In vitro assays also demonstrated that the enzymic activity of ExoN is negatively modulated by tyrosine dephosphorylation. In concordance with our results, the UDP-glucose dehydrogenase activity of E. coli Ugd was severely reduced by dephosphorylation mediated by Wzb (Grangeasse et al., 2003).

Our results suggest that in S. mellioti the enzymic activity of ExoN – required for activation of the precursors of EPS I – is modulated by phosphorylation–dephosphorylation cycles, with the latter step being mediated by the Smc02309 phosphotyrosine phosphatase.

Standish & Morona (2014) reviewed that both deletion and overexpression of phosphatases alter exopolysaccharide biosynthesis in a broad range of bacteria. For example, in addition to changes in Wzb levels affecting EPS biosynthesis in E. coli (Vincent et al., 1999, 2000), in Streptococcus thermophilus a lack of the EpsB phosphatase resulting in a slight reduction in the amount of EPS was reported (Minic et al., 2007). In contrast, PhpA, a tyrosine phosphatase of Myxococcus xanthus, was recently reported to be a putative negative regulator of EPS production (Mori et al., 2012).

The lower levels of EPS I synthesis, observed in the mutant strain affected in Smc02309, may affect early nodulation since a decrease in UDP-glucose subunits should lead to decreased production of EPS I as well as a delay in nodulation. Jones (2012) demonstrated that enhanced production of EPS I improves symbiosis of S. mellioti with Medicago truncatula. More recently, Geddes et al. (2014) suggested that an increased accumulation of EPS I can have a positive effect on nodulation and competition for nodule occupancy.

Since the S. mellioti genome contains another gene, ExoN2, encoding a probable UDP-glycosyl pyrophosphorylase, the possibility that ExoN2 is also a substrate for Smc02309 cannot be excluded and should be investigated. Moreover, the weak change of EPS production in the Smc02309 mutant could be the result of compensation effects caused by the enzymic activity of ExoN2.

Recently, strong evidence for a critical role of PTPs in Wzy-dependent capsule production was reported in several bacteria. Moreover, the discovery of small molecules inhibitory to the activity of PTPs – in both Gram-positive and Gram-negative pathogens – suggests that these PTPs constitute suitable targets for the development of antivirulence drugs (Standish et al., 2012). The evidence implying that Smc02309 acts as a phosphatase mediating cell signalling events in S. mellioti, though preliminary, is promising. In summary, this report described for the first time to our knowledge phosphatase characteristics of Smc02309, a protein annotated as a putative arsenate reductase of S. mellioti and suggests that ExoN is one of the endogenous substrates. Further studies will be required to unravel whether or not Smc02309 affects signalling pathways regulating interactions with the eukaryotic host and EPS biosynthesis.

ACKNOWLEDGEMENTS

This work was funded by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, and the Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto, Argentina. D. B. M., M. R. R., E. J. and S. E. F. are members of the
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