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APPLIED AND ENVIRONMENTAL MICROBIOLOGY:

A positive correlation between bacterial autoaggregation and biofilm formation in native *Sinorhizobium meliloti* isolates from Argentina

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Running title: Cell-cell interactions in *S. meliloti* native strains

Abbreviations: CV, crystal violet; EPS, exopolysaccharide; HMW, high-molecular-weight; IS, insertion sequence; LMW, low-molecular-weight; ORF, open reading frame; PCR, polymerase chain reaction

ABSTRACT

Sinorhizobium meliloti is a symbiotic nitrogen-fixing bacterium that elicits nodule formation on roots of alfalfa plants. *S. meliloti* produces two exopolysaccharides, termed EPS I and EPS II, that are both able to promote the symbiosis. EPS I and EPS II are secreted in two major fractions that reflect differing degrees of subunit polymerization, designated as high- and low-molecular-weight fractions. We reported previously that EPSs are crucial for autoaggregation and biofilm formation in *S. meliloti* reference strains and isogenic mutants. However, the previous observations were obtained using "domesticated" laboratory strains, with mutations resulting from successive passages under unnatural conditions, as has been documented for reference strain Rm1021. In the present study, we analyzed the autoaggregation and biofilm formation abilities of native *S. meliloti* strains isolated from root nodules of alfalfa plants grown in four regions of Argentina. *16S rRNA* gene analysis of all the native isolates revealed a high degree of identity with reference *S. meliloti* strains. PCR analysis of the *expR* gene in all the isolates showed that, as in the case of reference strain Rm8530, this gene is not interrupted by an insertion sequence (IS) element. A positive correlation was found between autoaggregation and biofilm formation abilities in these rhizobia, indicating that both processes depend on the same physical adhesive forces. Extracellular complementation experiments using mutants of the native strains showed that autoaggregation and biofilm formation abilities, as well as the mucoid phenotype, were all dependent on EPS II production. Our results indicate that a functional EPS II synthetic pathway and its proper regulation are essential for cell-cell interactions and surface attachment of *S. meliloti*.

INTRODUCTION

Sinorhizobium meliloti is a Gram-negative alphaproteobacterium found in soil that, under nitrogen limitation conditions, is able to engage in a symbiotic association with the agriculturally important legume *Medicago sativa* (alfalfa). In nature, the bacterium plays an important role in the conversion of atmospheric nitrogen into forms that can be utilized by the plant. This process of nitrogen fixation is carried out in specialized structures called nodules that are formed in the legume roots. The interaction of the bacteria (termed rhizobia) and the plants shows a high degree of host specificity (8), and the successful infection of the roots is dependent upon a reciprocal molecular dialogue between the host plant and the rhizobia (11).

Biofilms are defined as bacterial communities surrounded by a self-produced polymeric matrix and reversibly attached to an inert or a biotic surface (7). Bacteria may develop on plant roots as isolated cells, microcolonies, bacterial aggregates, or biofilms (31). Bacterial surface components, particularly exopolysaccharides (EPSs), flagella, and lipopolysaccharides (LPSs), in combination with bacterial functional signals, are crucial for the formation of rhizobial biofilms in all species studied so far (39). Rhizobial surface polysaccharides play important roles in symbiosis and formation of active root nodules. Mutants defective in the production of EPSs, LPSs, and capsular polysaccharides usually show reduced induction of effective nodules, and are particularly affected in the process of infection through infection threads (18). *S. meliloti* produces two different EPSs, succinoglycan (also known as EPS I) and galactoglucan (EPS II) (22), that are both able to promote symbiosis. The perceptions of EPSs in the two basic types of nodule ontogeny (determinate vs. indeterminate) appear to display differing rhizobial EPS requirements. *E.g.*, EPS mutants of *R. loti* (in which LPSs are conserved) are fully effective with a determinate nodulating host but ineffective with an indeterminate nodulating host (20).

EPS I, the best-understood symbiotically important EPS, is required for invasion of alfalfa roots by *S. meliloti* strain Rm1021. EPS I is a polymer of repeating octasaccharide subunits

(seven glucose, one galactose), bearing succinyl, acetyl, and pyruvyl substituents (36). Mutations affecting EPS I biosynthesis result in a variety of developmental abnormalities during nodule formation, including delayed root hair curling, defective or aborted infection threads, and empty nodules with no bacteria or bacteroids. These findings suggest that EPS I has a signaling function (12, 26). EPS II is composed of alternating glucose and galactose residues that are respectively acetylated and pyruvylated (47). EPSs are produced in dual forms having high vs. low molecular weight, termed HMW and LMW. The LMW fraction is an active biological form of EPS that is essential for successful infection of leguminous plants that form indeterminate-type nodules (45). Under non-starvation conditions in the laboratory, wild-type *S. meliloti* Rm1021 produces detectable quantities of succinoglycan, but does not produce EPS II. Production of EPS II was observed under low-phosphate conditions (54), and in a *mucR* mutant (23). Strain Rm1021 carries an insertional mutation within the *expR* gene (35), that prevents EPS II production under standard culture conditions. The presence of a functional *expR* open reading frame (ORF) on a plasmid or in the genome is sufficient to promote production of symbiotically active EPS II, *e.g.*, in strain Rm8530, which has an intact *expR* and is termed *expR*$^+$ (17). EPS II-producing strain Rm8530, which has a mucoid phenotype, displays a highly structured architectural biofilm, in contrast to the unstructured one formed by non-EPS II-producing strain Rm1021. In experiments with *M. sativa* (alfalfa), strain Rm8530 *expR*$^+$ formed biofilms that covered the entire surface of the root, including root hairs, whereas strain Rm1021 formed clusters of cells that adhered mostly to the main root (40). Wild-type *S. meliloti* reference strains carrying non-functional *expR* loci (and therefore unable to synthesize EPS II) fail to autoaggregate, and develop a relatively small biomass attached to plastic surfaces.

Bacterial autoaggregation is a process whereby bacteria physically interact with each other and settle to the bottom in static liquid suspension (33, 46). Adhesion of bacteria to various surfaces, and their self-aggregation, may be modulated by regulation of EPS synthesis (38). The

presence of a functional copy of the *expR* regulator gene is necessary for autoaggregation. LMW EPS II, either alone or in combination with HMW fraction, may function as a polymeric extracellular matrix that agglutinates bacterial cells (46).

Laboratory strains of *S. meliloti*, such as Rm1021, apparently often carry mutations resulting from successive passages under unnatural conditions. Two known examples in this strain are mutations in regulatory genes that control the expression of several genes, such as *expR* (16), and the mutation in the *pstC* gene that causes an increase in the expression of eight genes related to phosphate deficiency stress (24).

For the purpose of characterizing indigenous, undomesticated *S. meliloti* strains, we isolated bacteria from root nodules of alfalfa plants growing in fields that had not previously undergone inoculation procedures. We then examined the correlation between biofilm formation and autoaggregation in these native strains. Results of our analysis showed that EPS II plays a crucial role in cell-cell interactions in both sessile and planktonic bacterial cells.

MATERIALS AND METHODS

Bacterial strains. Wild-type reference *S. meliloti* strains Rm1021 (30) and Rm8530 (17) were grown as described previously (46)**.** Native alfalfa microsymbionts were obtained from plants growing in agricultural fields with no previous known inoculation procedures. Root nodules were taken from ten randomly chosen plants in each of four geographically distinct sites in Argentina (El Cerrito, San Rafael, Mendoza [SR]; UNRC field, Río Cuarto, Córdoba [CU]; La Escondida field, Río Cuarto, Córdoba [LE]; Paso de los Indios, Chubut [PI]). The nodules were surface sterilized and crushed, and their contents were plated on Petri dishes with tryptone yeast extract (TY) medium (50). Pure cultures were used in further experiments, and were grown in

TY medium on a rotary shaker (200 rpm) at 30 °C. The final concentrations of antibiotics used were: streptomycin 500 µg/ml; neomycin 200 µg/ml; gentamicin 40 µg/ml. The strains and phage used are listed in Table 1.

Plant nodulation tests. The nodulation phenotype was tested by inoculation with native strains. Seeds of alfalfa (*M. sativa*) "Pampeana" cultivar from INTA (Instituto Nacional de Tecnología Agropecuaria, Argentina) were surface sterilized, germinated, and grown in a chamber at 28 °C under a 16/8 h light/dark regime, supplied with nitrogen-free Hoagland solution as needed (28). Thirty days after planting, inoculated and uninoculated (control) plants were harvested. Nodules were separated from the roots, and the external morphology of the nodules was examined.

DNA extraction. Colonies were suspended in 500 µl sterile physiological saline solution and centrifuged at 10,000 rpm for 10 min. The supernatant was removed, and the pellet was suspended in 500 µl InstaGene Matrix (Bio-Rad, Hercules, CA, USA) (6). The suspension was incubated for 30 min at 56 °C, then heated for 10 min at 100 °C. The supernatant was used as a bacterial DNA template for PCR analysis.

Identification of isolated bacterial strains by partial *16S rRNA* gene sequencing. Direct PCR was performed utilizing 1 µl DNA template in 20 µl PCR mixture containing the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (25), numbering is based on the *Escherichia coli 16S rRNA* gene (3). Amplification was conducted for 35 cycles, at 94 °C for 45 sec, 55 °C for 60 sec, and 72 °C for 60 sec. Purified PCR products of approximately 1,400 bp were sequenced with an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) by Macrogen Inc. Laboratories (Korea). The *16S rRNA* gene sequences were subjected to

BLAST search program (National Center for Biotechnology Information) (1) to find identities between sequences.

Nucleotide sequence accession numbers. The nucleotide sequences of the *16S rRNA* gene from alfalfa-nodulating strains PI1, PI2, CU4, CU5, CU9, CU10, LE7, LE16, LE17, SR1, SR2, SR3, SR4, SR6, SR7, SR8, SR9, SR10, SR11 and SR15 determined in this study have been deposited in the GenBank nucleotide sequence database under accession numbers JQ666174, JQ666175, JQ666176, JQ666177, JQ666178, JQ666179, JQ666180, JQ666181, JQ666182, JQ666183, JQ666184, JQ666185, JQ666186, JQ666187, JQ666188, JQ666189, JQ666190, JQ666191, JQ666192 and JQ666193 respectively (Table 1).

Phylogenetic tree construction. Phylogenetic analyses were conducted using *MEGA* version 4 in order to produce a phylogenetic tree reflecting the evolutionary relationship between alfalfa-nodulating strains and reference strains by the neighbor-joining method (42), using the Kimura 2-parameter model.

Diagnostic PCR analysis of *expR* gene. This analysis was conducted using the procedure of Pellock *et al.* (2002) (35), with minor modifications. The two primers used to amplify the *expR* region were: Rm*ndvA*5′out (5′-GCGAGGAGATCCTGCCCGAG-3′) and Rm*pyc*5′out (5′AGAGTGGCGTGAACATTCGG-3′). We used 1 µl DNA template in 20 µl PCR mixture containing 2.5 U Taq polymerase (Invitrogen), under the manufacturer′s recommended buffer conditions. Primers and deoxynucleoside triphosphates were used at concentrations of 1 µM and 200 µM, respectively. The PCR program used was: 1) 95 °C for 5 min, 2) 94 °C for 30 s, 3) 65 °C for 30 s, 4) 72 °C for 5 min, 5) hold at 4 °C. Steps 2 to 4 were repeated 29 times. The reaction

147 was performed at final volume 25 µl. The PCR product was analyzed by electrophoresis in 0.8%
148 w/v agarose gel, with ethidium bromide (1 mg/ml), at 90 V for 45 min.
149
150 **Phage transduction.** The mutant alleles *expA*::Tn5 were transferred from
151 Rm1021*expA*::Tn5 to recipient strains SR4, SR6, and SR9 using the generalized transduction
152 method described by Finan *et al.* (1984) (10), with some modifications. Co-transduction of the
153 resistance markers (neomycin) and dry colony phenotype were verified in each transductant
154 strain. Donor and recipient strains were include as controls.
155
156 **Autoaggregation assay.** The bacteria were grown in 2 mL TY medium supplemented with
157 appropriate antibiotic, incubated for 24 h at 30 ºC, diluted 1/100 in TY or MGM low phosphate
158 medium, and incubated for 48 h under the same conditions. The bacterial suspensions (5 mL)
159 were then transferred to a glass tube (10 x 70 mm) and allowed to settle for 24 h at 4 °C. A 0.2
160 mL aliquot of the upper portion of the suspension was transferred to a microtiter plate, and OD_{600}
161 was measured (OD_{final}). A control tube was vortexed for 30 s, and OD_{600} was determined
162 ($OD_{initial}$). The autoaggregation percentage was calculated as $100[1-(OD_{final}/OD_{initial})]$. For both
163 homologous and heterologous autoaggregation assays, cultures were centrifuged at 4200 *xg* for
164 20 min prior to the settling period. For homologous assay, the pellet of a given strain was
165 resuspended in cell-free supernatant from an independent culture of the *same* strain. For
166 heterologous assay, the pellet was resuspended in cell-free supernatant from a culture of a
167 *different* strain.
168
169 **Biofilm formation assay.** Biofilm formation was determined macroscopically by a
170 quantitative assay in 96-well microtiter dishes, whereby biofilms were stained with crystal violet
171 (CV) based on the method of O'Toole and Kolter (34) with modifications (14). The bacteria

were grown in 2 ml TY medium supplemented with appropriate antibiotic, and incubated with agitation for 48 h at 30 ºC. The cultures were diluted with fresh medium to give OD_{600}= 0.1. One hundred µl of the suspension was added to each well and incubated with agitation for 24 h at 30 ºC. Bacterial growth was quantified by measuring OD_{600}. Planktonic cells were gently removed, 180 µl CV aqueous solution (0.1% w/v) was added, and staining proceeded for 15 min. Each CV-stained well was rinsed thoroughly and repeatedly with water, then scored for biofilm formation by addition of 150 µL 95% ethanol. The OD_{560} of solubilized CV was measured in a MicroELISA Auto Reader (Series 700 Microplate Reader, Cambridge Technology). Parallel, sterile control cultures were made in TY medium.

Quantification of rhizobia adsorption to roots. For this quantification procedure, we followed the protocol of Caetano-Anollés and Favelukes (4), except that our experimental unit consisted of a group of 15 alfalfa plants in which the total number of adsorbed microcolonies was counted. For each experimental condition, at least 4 independent experiments were performed.

Statistical analysis. The autoaggregation assays were performed in quintuplicate. For the biofilm assays, each strain was plated in at least 8 wells of each microtiter dish. The data were subjected to one-way ANOVA, followed by comparison of multiple treatment levels with the control, using *post hoc* Fisher´s least significant difference (LSD) test. All statistical analyses were performed using Infostat version 1.0.

RESULTS

Isolation and phylogenetic analysis of alfalfa-nodulating strains. Native alfalfa microsymbionts were able to develop highly mucoid colonies after a 24 h incubation period in YEMA (yeast extract mannitol) or TY medium. Acidification and lack of adsorption of congo red were observed when strains were grown in YEMA medium supplemented with bromothymol blue and congo red, respectively. In order to confirm the symbiotic nature of the isolates, the nodulation phenotype was tested by inoculating the bacteria on sterile alfalfa seeds. After 30 days, all isolates elicited characteristic root nodules in the host plant.

Phylogenetic analysis of *16S rRNA* gene sequences grouped all the isolates with *S. meliloti* reference strains (Fig. 1). High identity percentages were obtained when comparing each isolate with the sequenced strain *S. meliloti* Rm1021. The genetic relationships between different strains can be determined by comparative analysis of the 16S rRNA encoded gene sequence. This method is useful for taxonomic analysis of bacteria because there are few variations in the evolutionary level, and the gene product is universally essential and functionally conserved. When closely related strains are compared, the differences in gene sequence are minimal. Using the criteria of Stackebrandt and Goebel (1994) (48), the majority of the strains were identified to the species level, as their sequences showed >97% identity with the *16S rRNA* gene sequences of *S. meliloti* available in the EMBL database.

Determination of biofilm formation and autoaggregation. The ability to attach and develop sessile biomass on a plastic surface was assessed for the native rhizobial strains by growing them on polystyrene microtiter dishes and using the CV method to indirectly quantify the sessile biomass. The observed biofilm formation abilities ranged from strains with low attachment ability to strains that showed high attachment ability and developed a biofilm biomass on the plastic surfaces (Fig. 2).

Planktonic autoaggregative behavior was quantified as described in Materials and Methods. Similarly to the range of biofilm formation abilities, we observed a wide heterogeneity in the autoaggregative phenotypes; some strains displayed strong autoaggregation while others were much weaker (Fig. 3).

We hypothesized that cell-cell interactions for both biofilm populations and planktonic aggregates depend, to some extent, on the same physical adhesive forces. To test this hypothesis, we conducted a correlation analysis to determine whether the planktonic autoaggregation and biofilm formation abilities of the strains in our collection were quantitatively related phenotypes. A scatter plot was generated (Fig. 4) and the Pearson correlation coefficient (r) was calculated. A statistically significant correlation was observed between the two phenotypes ($r = 0.78$; $p \leq 0.05$).

Analysis of *expR* gene in native and reference strains. Expression of a functional *expR* gene regulator is important for the production of the symbiotically important EPS II (35) and therefore for colony phenotype, biofilm formation, and planktonic autoaggregation (40). We tested for the possible presence of a functional *expR* locus in our collection of native rhizobia using primers designed to PCR-amplify the complete ORF of this gene (35).

The presence of a non-functional gene was demonstrated previously by PCR analysis in wild-type reference strains. The size of the PCR product from strain Rm8530 (Rm1021 *expR*$^+$ [formerly *expR101*]) is 0.9 kb, while that of the PCR product from wild-type strain Rm1021 is 2.2 kb. Sequence analysis of the 2.2 kb PCR product indicated that the *expR* ORF was disrupted in Rm1021 by a copy of IS*Rm*2011-1, a previously described 1,319-bp insertion sequence (IS) element (35, 44). Amplification products were detected in all strains and, as expected, size-fragment analysis revealed the presence of 0.9 kb amplicons similar to that obtained from an Rm8530 template (see Figure S1 in the supplemental material). This finding indicates that there is no IS element similar to IS*Rm*2011-1 interrupting the *expR* gene. However, this finding does

not provide direct evidence that the sequence of the gene is intact. For example, a previous study showed that although the *expR* region of *S. meliloti* strain 102F34 does not contain an IS element, the 102F34 *expR* ORF has an 11-bp deletion in its coding sequence (35). This deletion is consistent with the dry colony morphology of strain 102F34, which is distinct from the typical mucoid phenotype of the native strains isolated in the present study. The combination of phenotypic and genotypic results as above supports the presence of a functional *expR* gene in all the native strains assayed in the present study.

The role of EPSs in cell-cell interactions. Autoaggregation, mucoid phenotype, and biofilm formation are three traits that were shown previously to depend on EPS II production (40, 46). In order to determine whether the adhesive and mucoid phenotypes in our collection of indigenous strains also depend on EPS II production, we utilized a genetic approach involving the transduction of the *expA*::Tn5 mutant allele to the indigenous isolates SR4, SR6, and SR9 (which displayed high autoaggregative and biofilm formation abilities) followed by a phenotypic evaluation of the transductant strains. In contrast to the parental strains, the three transductant daughter strains SR4 *expA*, SR6 *expA*, and SR9 *expA* displayed dry colony phenotypes (Fig. 5A), drastic reductions in autoaggregation percentage (Fig. 5B), and low biofilm formation on plastic surface (Fig. 5C). These findings indicate that the adhesive phenotypes of these native rhizobia, similarly to those of the reference strains, are closely related to EPS II production.

Extracellular complementation assays. Since the *expA* mutants showed low autoaggregative behavior, we speculated that the deficiency in biofilm formation and autoaggregation observed in some of the native rhizobial strains could be explained in terms of EPS II production and/or abnormal EPS II- bacterial surface interaction. Extracellular complementation experiments were performed in order to distinguish between these two

possibilities. Rhizobial pellets from the native strains were resuspended in bacteria-free culture supernatant from Rm8530 *exoY* (containing EPS II), and the resulting suspensions were subjected to quantitative autoaggregation assay (Fig. 6). The supernatant containing EPS II induced significantly higher autoaggregative behavior in all the tested strains (Fig. 6), whereas the supernatants from native *expA* mutant cells did not promote autoaggregation of Rm8530 *exoY*. These findings provide strong evidence for the role of EPS II in bacterial cell-cell interactions.

The *S. meliloti* mutant Rm1021 *mucR* has a mucoid phenotype and synthesizes EPS II, but only in the form having a high degree of polymerization (HMW fraction). This mutant does not develop architecturally complex biofilms and does not display efficient autoaggregation, indicating that the LMW fraction is the form of EPS II essential for both these processes (40, 46). Surprisingly, several native rhizobial strains showing low autoaggregative and biofilm formation abilities (CU5, CU10, PI1, LE17) were highly mucoid and therefore likely to produce EPS II. In order to elucidate the reason for this apparent discrepancy, we transduced the *expA*::Tn5 allele into these four native strains. As expected, the *expA* mutation induced a strong reduction in mucoid phenotype, and our daughter mutant strains displayed a dry appearance when grown on TY solid medium, similar to the colony phenotype of other *expA* mutants. Our *expA* mutants also did not display efficient autoaggregation (data not shown). These findings strongly support the existence of an EPS II-associated mucoid phenotype in the native isolates.

Heterologous autoaggregation assays were also conducted in order to explain the low autoaggregative ability of native strains CU5, CU10, PI1, and LE17. As mentioned above, *expA* mutation in these strains induced a strong reduction in mucoid property, indicating an EPS II-associated phenotype. Treatment with culture supernatant of Rm8530 *exoY* increased the autoaggregation of these four strains, whereas culture supernatants of the four strains did not induce autoaggregation of Rm8530 *exoY* (Fig. 7). The surfaces of CU5, CU10, LE17, and PI1

296 cells were presumably able to interact normally with the EPS II present in the Rm8530 *exoY*
297 supernatant. Taken together, these observations suggest that the native rhizobial strains showing
298 low autoaggregative and biofilm formation abilities do not produce the extracellular factors
299 required for strong autoaggregation, presumably because of a difference in the HMW: LMW
300 ratio of EPS II in the extracellular media.

301 To evaluate the role of *S. meliloti* EPSs in early interactions with alfalfa roots, adsorption
302 assays were performed using *S. meliloti* mutants with specific defects in EPS synthesis (Table 2).
303 The results suggest that EPS II partially inhibits rhizobial adhesion to roots, presumably through a
304 "shielding effect". The *mucR* mutant (which secretes only the HMW fraction of EPS II) attached
305 to roots in higher numbers than did Rm8530 (which synthesizes both EPS II fractions), suggesting
306 that the LMW fraction of EPS II may partially block rhizobial attachment to roots. Because
307 planktonic rhizobia were incubated with alfalfa plants for 4 h (4), these findings reflect the role
308 that EPSs may play during the initial access to the root; this test should therefore not be
309 interpreted as a biofilm assay. Additional experiments are needed to better clarify the associations
310 between biofilm formation and other adhesion phenotypes.

311

312 **DISCUSSION**

313 Inoculation of legume crop plants with selected, highly efficient rhizobia is an important
314 method for improvement of symbiotic nitrogen fixation in agricultural ecosystems, and
315 constitutes a major strategy for the sustainable input of nitrogen into agricultural soils (27).
316 However, the native rhizobial populations present in soils often display a superior competitive
317 ability over inoculated strains on the basis of their large population size, positional advantage,
318 and/or superior adaptation to local environmental conditions (2, 49). The selection of efficient
319 rhizobial strains based on their adaptation to local ecological conditions can therefore lead to
320 increased grain production of crops (27, 32).

We used several approaches to evaluate the rhizobial strains present in root nodules of alfalfa plants growing in fields in Argentina that had no previous history of inoculation procedures. The isolated strains showed a mucoid phenotype when grown on Petri dishes. Such phenotype was indicative of EPS II synthesis in previously characterized reference strains.

16S rRNA gene analysis of all the isolates revealed a high degree of identity (approximately 98%) with reference *S. meliloti* strains, corresponding to a value of sequence divergence less than the 3.0% required for differentiation between species (48). PCR analysis of the chromosomal *expR* gene in these isolates revealed that this gene is not interrupted by an IS element, as is also the case in the reference strain Rm8530 (35). ExpR is a LuxR-homologue, whose functions include activation of EPS II production in the presence of N-acyl-homoserine lactone (AHL), which is produced by the *sinR/sinI* system in *S. meliloti*. Strain Rm1021 displays a dry (as opposed to mucoid) phenotype because its *expR* gene is interrupted by an IS element and it therefore cannot produce EPS II. EPS II-producing strain Rm8530 displays a highly mucoid phenotype. Rm8530 and the native strains isolated in this study harbor an intact (not interrupted) copy of the *expR* gene giving a 0.9 kb PCR product. Rm1021 yields a larger amplicon (2.2 kb PCR product) because the *expR* ORF is disrupted by a copy of IS*Rm*2011-1, a 1,319-bp IS element.

We have shown previously that rhizobial cell surface components such as EPSs, in combination with bacterial functional signals, are essential for the processes of autoaggregation (46) and biofilm formation (39). Both processes play important ecological roles for the survival of rhizobia in their natural soil environment, and probably for the nitrogen-fixing symbiosis that occurs within root nodules, in which EPSs are essential for early stages of infection (12). The findings of the present study illustrate a great variability in both autoaggregation and biofilm formation abilities among native soil isolates. This phenotypic diversity may result from differential selective pressures in the soil microenvironment or in the root nodules. Interestingly,

346 correlation analysis of autoaggregation and biofilm formation abilities gave a Pearson correlation
347 coefficient of 0.78, indicating a positive correlation between these two variables. These findings
348 suggest that the two processes are related and that cell-cell interaction in the context of both
349 biofilm populations and planktonic aggregates depends, at least under the conditions of our
350 assays, on the same physical adhesive forces. A similar positive correlation between the
351 autoaggregation and biofilm formation abilities was showed in *Myroides odoratus*, a Gram-
352 negative bacillus (21).

353 The results of transduction of the *expA*::Tn5 mutant allele to native strains displaying
354 strong autoaggregation and biofilm formation abilities showed that these processes, and the
355 expression of mucoid phenotypic characteristics, depend mainly on EPS II synthesis. This
356 *expA*::Tn5 mutation also abolished the expression of mucoid colonies in four native strains (Cu5,
357 Cu10, PI1, LE17) that showed weak autoaggregation and biofilm formation abilities, indicating
358 that the mucoid phenotype depends on EPS II production even in these strains. Autoaggregation
359 in these four strains and their *expA* mutants could be complemented by exogenous addition of
360 culture supernatants from Rm8530 *exoY*, indicating that the cell surfaces of these strains can
361 interact normally with EPS II. These findings, taken together, suggest that the low
362 autoaggregation and biofilm formation abilities of some of the isolates that showed a mucoid
363 phenotype were due to a low LMW: HMW ratio of EPS II. Further experiments, including direct
364 testing of purified EPS II fractions, will be necessary to test this hypothesis.

365 Bacterial surface components, particularly EPSs, are crucial for biofilm formation in
366 rhizobia (39). *S. meliloti* has been the subject of studies on the effects of nutritional and
367 environmental conditions (37), EPSs and flagella (13), ExoR with an ExoS–ChvI two-
368 component system (51), *nod* genes (15), and regulation of EPS biosynthesis (40). However, in
369 other rhizobial species a connection between EPS production and biofilm formation ability is not
370 clear. EPS production in *Rhizobium* sp. YAS34 is not essential for biofilm formation on inert

supports or on roots of *Arabidopsis thaliana* or *Brassica napus* (43). *Rhizobium leguminosarum* mutants defective in the synthesis of acid EPSs in were also deficient in biofilm formation (41) and showed alteration of the pattern of adherence to pea roots (52). *R. leguminosarum* mutants defective in the synthesis of glucomannan, another EPS, attached and formed normal biofilms *in vitro*, but did not display normal attachment or biofilm formation on root hairs (52).

It is likely that different polymer types mediate attachment depending on differing substrate chemistries and medium compositions. For example, polymers with nonpolar sites, such as LPSs, may dominate in binding to hydrophobic surfaces, whereas polymers capable of hydrogen bonding or electrostatic interactions, such as polysaccharides, may dominate in binding to hydrophilic surfaces. Different polymer types may act cooperatively in binding to a surface to stabilize the adhesive interaction. *E.g.*, a *Pseudomonas fluorescens* mutant that lacks the O antigen of the LPS, with consequent increased exposure of the lipid moiety of the LPS, displays increased adhesion to hydrophobic substrates (53). In *S. meliloti*, the *lpsB* mutant lacks glycosyltransferase I, which is responsible for the biosynthesis of the LPS core (5), while the *bacA* mutant is defective in the distribution of fatty acids on the lipid-A component of LPS (9). The *lpsB* mutation resulted in a slight reduction of biofilm formation compared with wild-type, whereas the *bacA* mutation resulted in a roughly 50% reduction of biofilm formation (19). In view of these observations, it would be very interesting to evaluate the contributions of LPS (by themselves or in combination with EPSs) in the adhesion properties of the native strains used in the present study. In the case of our subgroup of mucoid isolates that displayed weakly autoaggregative and poor biofilm formation phenotypes, a complete complementation of autoaggregation was observed when the isolates were resuspended in cell-free EPS II-containing supernatants from Rm8530 *exoY*. This finding indicates that, under our experimental conditions, all bacterial surfaces are equally effective for EPS II autoaggregative interactions and that

395 possible LPS structural heterogeneity among the strains has no impact on planktonic
396 autoaggregation.

397 Increased knowledge of the genotypic and phenotypic characteristics of rhizobial
398 populations will help improve agricultural legume production worldwide, through application of
399 inoculation strategies and other sustainable management practices (29). In view of the economic
400 importance of alfalfa production in Argentina and its status as the most extensively cultivated
401 forage legume worldwide, it is essential to better understand the factors that affect the growth of
402 this crop, including its associated nodulating rhizobial populations. Further detailed studies on
403 genotypic and phenotypic composition, seasonal shifts in populations, and effects of rhizobia on
404 different varieties of alfalfa, in combination with biogeographic analysis, will clarify the
405 behavior of local rhizobial populations, and have direct application for improved agricultural
406 production.

407

408

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566 **Table 1.** Bacterial strains and phage used in this study.

Strain	Origin	Source or reference
Reference S. meliloti strains		
Rm1021	SU47 *str21 expR102*::IS*Rm*2011-1	(30)
Rm8530	SU47 *str21 expR101* (*expR* $^{+}$)	(17)
Rm8530 *exoY*	*exoY210*::Tn5	(46)
Rm1021 *expA*	*expA3*::Tn5-233	(17)
Rm8530 *expA*	*expA3*::Tn5-233	(46)
Rm8530 *expA exoY*	*expA3*::Tn5-233 *exoY210*::Tn5	(46)
Rm1021 *mucR*	*mucR31*::Tn5	(46)
Phage		
ΦM12	Generalized transducing phage for *S. meliloti*	(10)
Indigenous S. meliloti strains		
PI1 (JQ666174)	Paso del Indio	Present study
PI2 (JQ666175)	Paso del Indio	Present study
CU4 (JQ666176)	Campus UNRC	Present study
CU5 (JQ666177)	Campus UNRC	Present study
CU9 (JQ666178)	Campus UNRC	Present study
CU10 (JQ666179)	Campus UNRC	Present study
LE7 (JQ666180)	La Escondida	Present study
LE16 (JQ666181)	La Escondida	Present study
LE17 (JQ666182)	La Escondida	Present study
SR1 (JQ666183)	San Rafael	Present study
SR2 (JQ666184)	San Rafael	Present study
SR3 (JQ666185)	San Rafael	Present study
SR4 (JQ666186)	San Rafael	Present study
SR6 (JQ666187)	San Rafael	Present study
SR7, (JQ666188)	San Rafael	Present study
SR8 (JQ666189)	San Rafael	Present study
SR9 (JQ666190)	San Rafael	Present study
SR10 (JQ666191)	San Rafael	Present study
SR11 (JQ666192)	San Rafael	Present study
SR15 JQ666193	San Rafael	Present study

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571 **Table 2.** *S. meliloti* adsorption to alfalfa roots.

	Rm8530	Rm8530 *exoY*	Rm8530 *expA*	Rm8530 *expA exoY*	Rm1021 *mucR*	Rm1021
Adsorption (‰)	0.4 ± 0.1^{a}	0.5 ± 0.1^{a}	2.8 ± 0.1^{b}	2.7 ± 0.1^{b}	2.6 ± 0.1^{b}	2.7 ± 0.1^{b}

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573 Adsorption (permillage, ‰) of *S. meliloti* mutant and wild-type strains to alfalfa roots (groups of
574 15 plants). The data shown are mean of adsorption permillage ± standard error.
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578 **FIGURE LEGENDS**

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580 FIG. 1. Phylogenetic tree based on *16S rRNA* gene sequences, showing the evolutionary
581 relationships between alfalfa-nodulating native and reference strains. The tree was constructed
582 using the neighbor-joining method. Alfalfa-nodulating native strains are indicated by boldface.
583 Sequence accession numbers are listed in parentheses.

584 FIG. 2. Quantitative comparison of biofilm formation in isolated native strains of *Sinorhizobium*
585 *meliloti* based on CV assay. Bars represent standard deviation of the mean based on four or more
586 independent experiments with seven replicates each.

587 FIG. 3. Quantitative autoaggregation assay of isolated native strains of *S. meliloti*. Bars represent
588 standard deviation from four or more independent experiments with four replicates each.

589 FIG. 4. Scatter plot of two variables: autoaggregation (%) and relative biofilm formation ability
590 (OD_{560}/ OD_{600}). The diamonds are ordered pairs that represent different isolates.

591 FIG. 5. Colony phenotype, autoaggregation, and biofilm formation in native strains and their
592 mutants. A: Appearance of native and mutant strains deficient in EPS II production (*expA*),
593 following 48 h incubation in TY medium. 1) Rm8530; 2) SR4; 3) SR6; 4) SR9; 5) Rm8530
594 *expA*; 6) SR4 *expA*; 7) SR6 *expA*; 8) SR9 *expA*. B: Quantitative autoaggregation of native strains
595 SR4, SR6, and SR9 and their respective *expA* mutants (non-EPS II producers). C: Relative
596 biofilm formation ability of native strains and their respective *expA* mutants using the CV assay.
597 Bars represent standard deviation of three or more independent experiments performed in
598 triplicate. Different letters indicate significant differences ($p \leq 0.05$) according to Fisher's LSD
599 test.

FIG. 6. Extracellular complementation of autoaggregation assay in *expA* mutants of native strains. First 3 bars: pellets from culture of autoaggregative strain Rm8530 *exoY* were resuspended in cell-free supernatant from *expA* mutant cultures. Last 3 bars: pellets from *expA* mutant cultures were resuspended in cell-free supernatant from Rm8530 *exoY*. Bars represent standard deviation of two or more independent assays with four replicates each. Different letters indicate significant differences ($p \leq 0.05$) according to Fisher's LSD test.

FIG. 7. Extracellular complementation of autoaggregation assays in native strain with low autoaggregation ability. Last 3 bars: pellets of native strains were resuspended in cell-free supernatant from autoaggregative strain Rm8530 *exoY*. First 3 bars: pellets of Rm8530 *exoY* were resuspended in cell-free supernatant of native strains. Bars represent standard deviation of two or more independent assays with four replicates each. Different letters indicate significant differences ($p \leq 0.05$) according to Fisher's LSD test.

Supplemental Material

Supplemental Figure S1. Agarose gel electrophoresis (0.8% w/v) showing the PCR products derived from amplification of the *expR* gene from native strains and reference strains Rm1021 (2.2 Kb) and Rm8530 (0.9 Kb). M= marker lane containing a 1 Kb ladder (Promega).

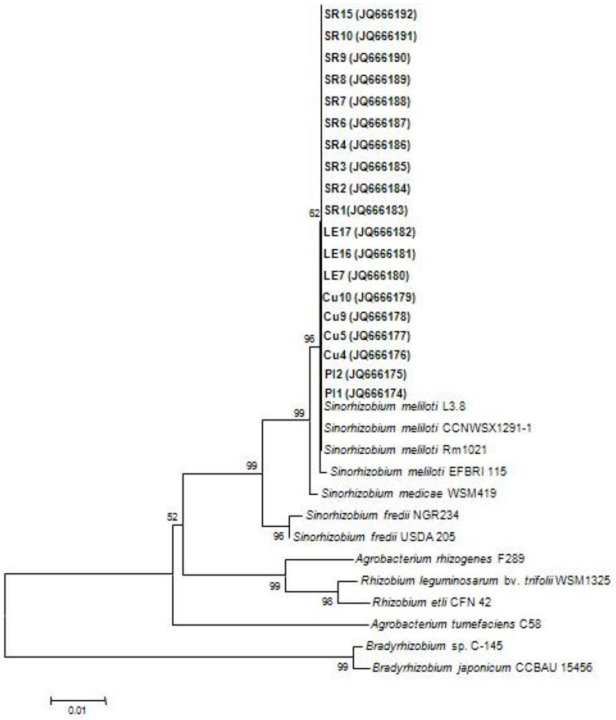

Fig. 1
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Fig. 2
Sorroche *et. al*

Fig. 3
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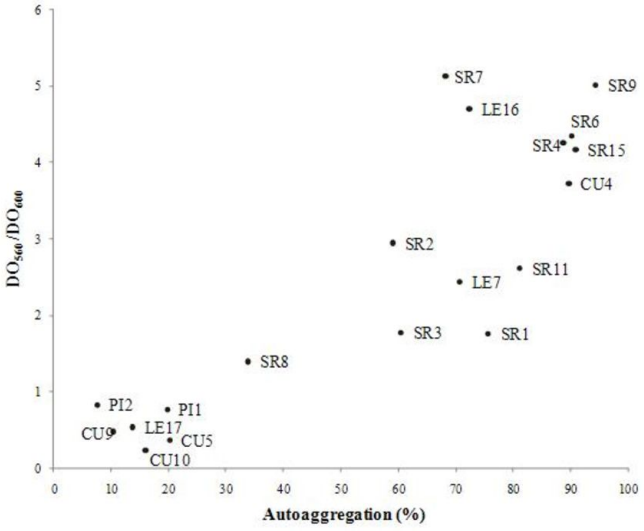

Fig. 4
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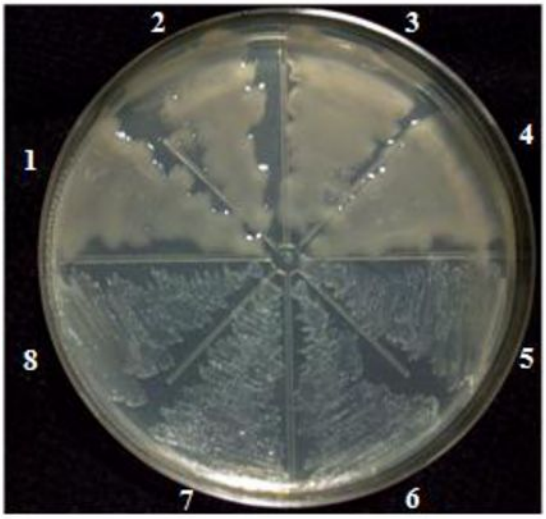

Fig. 5A
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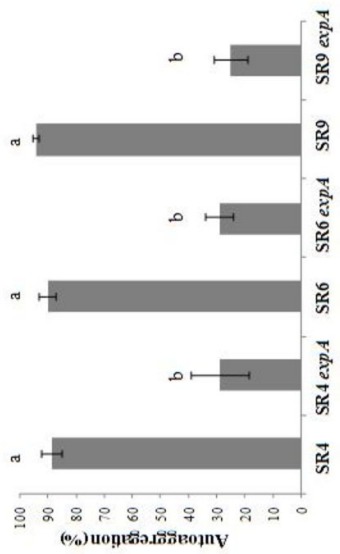

Fig. 5B
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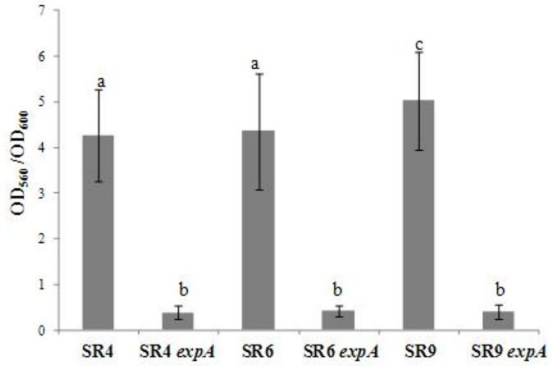

Fig. 5C
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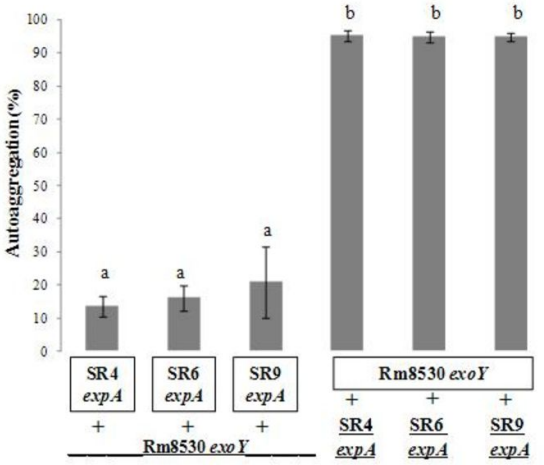

Fig. 6
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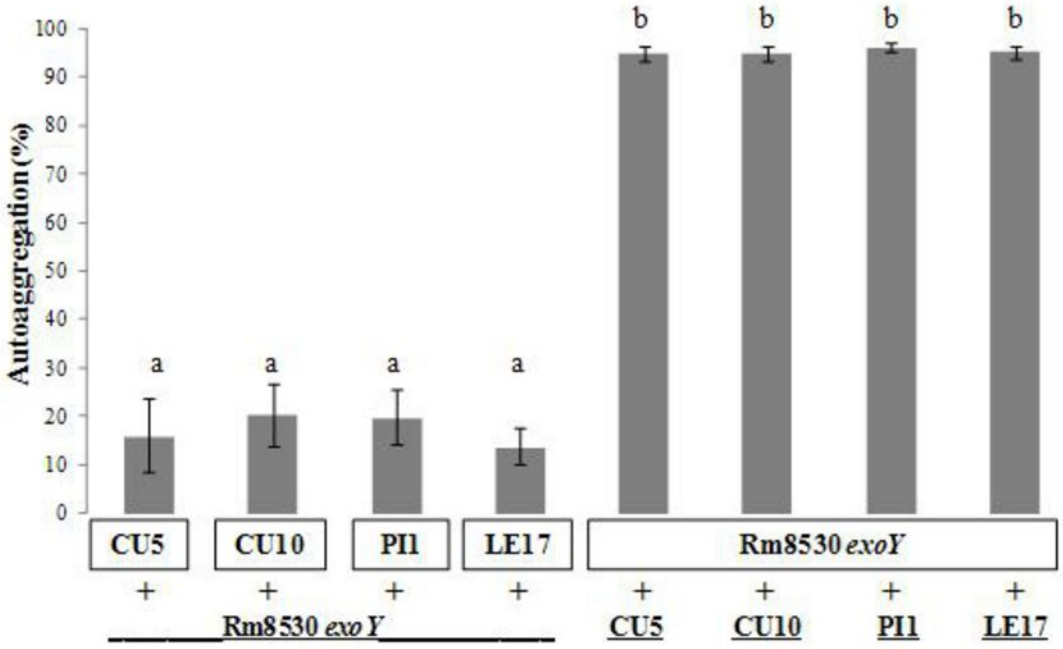

Fig. 7
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