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EPS II-Dependent Autoaggregation of *Sinorhizobium meliloti* Planktonic Cells

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Abstract Planktonic cells of *Sinorhizobium meliloti*, a Gram-negative symbiotic bacterium, display autoaggregation under static conditions. ExpR is a LuxR-type regulator that controls many functions in *S. meliloti*, including synthesis of two exopolysaccharides, EPS I (succinoglycan) and EPS II (galactoglucan). Since exopolysaccharides are important for bacterial attachment, we studied the involvement of EPS I and II in autoaggregation of *S. meliloti*. Presence of an intact copy of the *expR* locus was shown to be necessary for autoaggregation. A mutant incapable of producing EPS I displayed autoaggregation percentage similar to that of parental strain, whereas autoaggregation was significantly lower for a mutant defective in biosynthesis of EPS II. Our findings clearly indicate that EPS II is the essential component involved in autoaggregation of planktonic *S. meliloti* cells, and that EPS I plays no role in such aggregation.

Introduction

Bacterial autoaggregation is the process whereby bacteria physically interact with each other and settle to the bottom in static liquid suspensions. This process is industrially significant since separation of biomass from culture medium is facilitated when bacteria remain aggregated [21]. Extracellularly secreted polysaccharides, i.e., cellulose and an arabinose-rich exopolysaccharide, were shown to be involved in autoaggregation of the soil-borne bacteria *Agrobacterium tumefaciens* and *Azospirillum brasilense*, respectively [2, 16].

Sinorhizobium meliloti, a symbiotic Gram-negative rhizobacterium, has the potential to produce two different exopolysaccharides: EPS I (succinoglycan) and EPS II (galactoglucan). EPS I consists of a repeated octasaccharide structure containing one galactose and seven glucose units, with succinyl, acetyl, and pyruvyl modifications [23]. The genetic determinants for EPS I biosynthesis are located in a 25-kb region containing the *exo-exs* genes [24]. EPS II consists of a repeated disaccharide containing an acetylated glucose and a pyruvylated galactose unit [11]. Biosynthesis of EPS II is controlled by a 32-kb cluster of *exp* genes [8, 29]. In two strains (Rm2011 and Rm1021) derived from *Sinorhizobium meliloti* SU47, EPS II synthesis is activated under phosphate limitation [18, 31], in the presence of either a mutated *mucR* locus [14, 30], or an intact, functional copy of the *expR* gene [8, 22]. The low molecular weight fractions of EPS I and EPS II (trimers of the octasaccharide and 15–20 units of the disaccharide, respectively) are involved in symbiosis, whereas the corresponding high molecular weight fractions (more than 2000 subunits of the octasaccharide unit in the case of EPS I; more than 25 disaccharide subunits in the case of EPS II) are not [3, 10].

The regulatory protein MucR is a transcriptional repressor of the *exp* genes involved in biosynthesis of EPS II, and an activator of EPS I biosynthesis in *S. meliloti* [14]. We found recently that disruption of *mucR* gene in strain Rm1021 biofilms increased EPS II, but reduced EPS I gene expression; however, biofilm formation in this strain was independent of EPS synthesis [27]. These results are

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consistent with those from previous studies of planktonic bacteria. In this study, we evaluated the possible involvement of EPS I and II in autoaggregation of planktonic cells of various *S. meliloti* strains.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

Various *S. meliloti* strains were grown at 30°C in TY medium [4] on a rotary shaker at 200 rpm. Antibiotics were used at the following final concentrations: streptomycin, 500 μg/ml; neomycin, 200 μg/ml; gentamycin, 50 μg/ml. Strains, plasmid, and phage used are listed in Table 1.

Phage Transductions

The mutant alleles *expA3*::Tn5-233, *exoY210*::Tn5, and *mucR31*::Tn5 were transferred from Rm1021 *expA*::Tn5-233, Rm7210, and Rm3131, respectively, to recipient strains Rm8530 or Rm1021, using generalized transduction with phage φM12, as described by [9]. Cotransduction of the resistance markers (neomycin or gentamycin), and specific relevant phenotypes, were verified in each transductant strain, i.e., reduction or absence of fluorescence in LB plates supplemented with 0.05% Calcofluor (for the *mucR* and *exoY* mutants), dry colony phenotype (for the *expA* mutant). Donor and recipient strains were included as controls.

Autoaggregation Assay

Each *S. meliloti* strain was grown for 48 h in 3 ml TY medium supplemented with appropriate antibiotic, diluted (1/1000), and grown in 100 ml TY for additional 48 h. Bacterial suspension (5 ml) was transferred to a glass tube (10 × 70 mm) and allowed to settle for 24 h at 4°C. A 0.2 ml aliquot of the upper portion of suspension was carefully transferred to a microtiter plate, and OD_{600} was measured (OD_{final}). A control tube was vortexed for 30 s, and OD_{600} was determined ($OD_{initial}$). Autoaggregation percentage was calculated as $100[1 - (OD_{final}/OD_{initial})]$. For both homologous and heterologous autoaggregation assays, cultures were centrifuged at 4200*g* for 20 min prior to the settling period. For homologous assay, the resulting bacterial pellet of a given strain was gently resuspended in cell-free supernatant from independent culture of the *same* strain. For heterologous assay, the pellet was resuspended in cell-free supernatant from culture of a *different* strain.

Statistical Analysis

Autoaggregation assays were performed in quadruplicate and experiments were repeated at least three times. Mean values and standard deviations were calculated. Data were subjected to one-way Analysis of Variance (ANOVA) test, followed by comparison of multiple treatment levels with control using *post hoc* Fisher's Least Significant Difference (LSD) test. Statistical analyses were performed with Infostat software version 1.0.

Results and Discussion

When a suspension of planktonic cells of strain Rm8530 (*expR*$^+$ derivative of Rm1021) was placed in a glass tube and left without shaking for 24 h at 4°C, most of the bacteria settled at the bottom, forming a type of aggregate, termed

Table 1 Strains and plasmids used in this study

Strain, plasmid, or phage	Relevant properties	Reference
S. meliloti		
Rm1021	SU47 *str21 expR102*::IS*Rm*2011-1	Meade et al. [17]
Rm8530	SU47 *str21 expR101* (*expR*$^+$)	Glazebrook and Walker [8]
Rm2011	Wild-type reference strain	Casse et al. [5]
102F34	Wild-type reference strain	Ditta el al. [6]
Rm7210	Rm1021 *exoY210*::Tn5	Leigh et al. [15]
Rm8530 *exoY*	*exoY210*::Tn5	Present study
Rm1021 *expA*	*expA3*::Tn5-233	Glazebrook and Walker [8]
Rm8530 *expA*	*expA3*::Tn5-233	Present study
Rm8530 *expA exoY*	*expA3*::Tn5-233 *exoY210*::Tn5	Present study
Rm3131	Rm2011 *mucR31*::Tn5	Keller et al. [14]
Rm1021 *mucR*	*mucR31*::Tn5	Present study
Phage		
ΦM12	Generalized transducing phage for *S. meliloti*	Finan et al. [7]

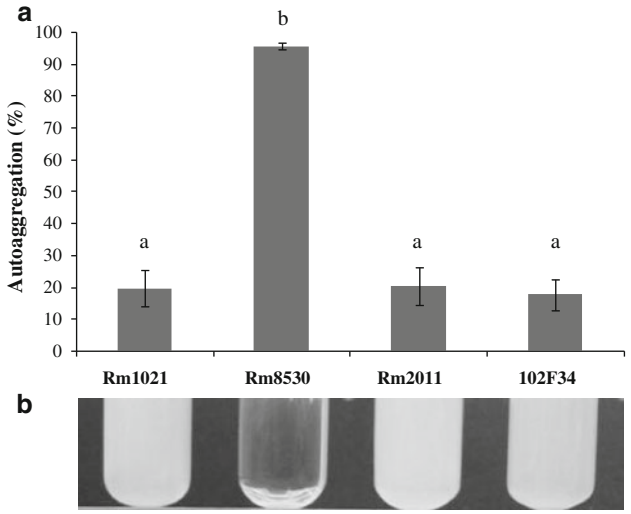

Fig. 1 Autoaggregation assay of wild-type reference strains of *S. meliloti*. **a** Quantitative assay, showing mean aggregation percentage for each strain. Each experiment was performed in four or more independent replicates, each in quadruplicate. Bars represent standard deviation, and different letters indicate significant differences ($P \leq 0.05$) between mean values according to Fisher's LSD test. **b** Macroscopic appearance of tubes containing bacterial suspensions, following a 24-h resting period at 4°C

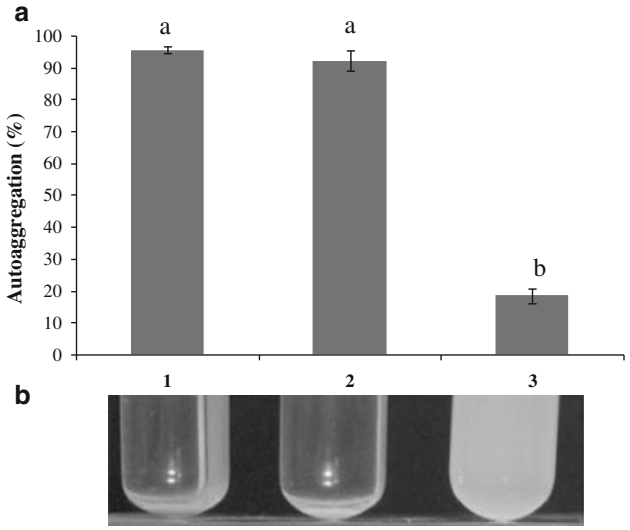

Fig. 2 Modified autoaggregation assays of strain Rm8530. **a** Quantitative assay. Statistical considerations as in Fig. 1. **b** Macroscopic appearance of tubes. 1. Autoaggregation of Rm8530 suspension (control). 2. Homologous autoaggregation assay: Rm8530 cells were resuspended in cell-free supernatant from independent culture of the same strain. 3. Autoaggregation of washed cells of Rm8530

"floc," bound together by extracellular mucopolysaccharides (Fig. 1a). Under standard shaking conditions, such visible autoaggregation of Rm8530 did not occur. This finding is in contrast to results from autoaggregation studies of *Azospirillum*, in which floc formation was evident under shaking conditions [20]. Much of the Rm8530 floc remained at the bottom of the tube following 2–3 gentle inversions, indicating a certain degree of integrity. However, the floc was easily dispersed by repeated pipetting or a vigorous shake. The autoaggregation of bacteria at the bottom of the tube was associated with decreased OD_{600} of the upper portion of the suspension, and autoaggregation percentage was quantified by spectrophotometry as described in "Materials and Methods". When suspensions of wild-type strains Rm1021, Rm2011, and 102F34 were left without shaking for 24 h at 4°C, most of the bacteria remained suspended, without visible autoaggregation (Fig. 1b). Autoaggregation percentage was similar (18-20%) for the three wild-type strains, but significantly higher (95%) for Rm8530 (Fig. 1a).

When Rm8530 cells, prior to the resting period, were washed and resuspended in fresh, sterile TY media, their autoaggregation was almost completely abolished (Fig. 2), suggesting that some extracellularly secreted and/or labile surface factor was responsible for the autoaggregation. When Rm8530 pellets were resuspended in cell-free supernatant from the same (or different) Rm8530 culture, the capacity for aggregation was not significantly decreased (Fig. 2a, b). This finding indicates that an extracellular component, rather than a centrifugation-labile surface factor, was the main determinant of autoaggregation.

In contrast to Rm8530, non-autoaggregating wild-type strains Rm1021, Rm2011, and 102F34 have a modified, non-functional *expR* locus [22]. This suggests a link between status of *expR* locus (i.e., intact versus altered sequence) and autoaggregation ability in these *S. meliloti* strains. Presence of a functional copy of the *expR* regulator gene is clearly necessary for autoaggregation. ExpR controls many aspects of *S. meliloti* physiology, including exopolysaccharide production [12, 22]. We performed quantitative autoaggregation assays of a set of *S. meliloti* mutants defective in biosynthesis of various exopolysaccharides to clarify the roles of these components in autoaggregation.

Rm8530 *exoY210*, a mutant incapable of producing succinoglycan, showed autoaggregation percentage (93%) similar to that of parental Rm8530 (Fig. 3). Autoaggregation percentage was much lower (18%) for Rm8530 *expA*, a mutant defective in biosynthesis of galactoglucan. Using phage φM12, we transduced the *exoY210::Tn5* mutant allele from Rm7210 to Rm8530 *expA*, and obtained double mutant Rm8530 *expA exoY*, as described in "Materials and Methods". Degree of autoaggregation was low (similar to that of Rm8530 *expA*) for Rm8530 *expA exoY* and Rm1021, which are also unable to produce galactoglucan under normal conditions (Fig. 3). These findings suggest that galactoglucan is the extracellular factor mainly responsible for autoaggregation of *S. meliloti*.

Galactoglucan is produced in two major fractions: one has low molecular weight and is symbiotically active; the

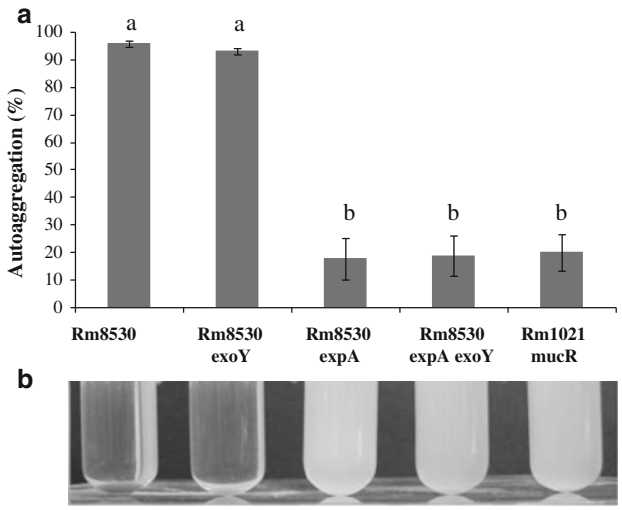

Fig. 3 Autoaggregation of mutant strains of *S. meliloti* defective in exopolysaccharide production. **a** Quantitative assay. Statistical considerations as in Fig. 1. **b** Macroscopic appearance of tubes

other has high molecular weight and no symbiotic function [10]. Although galactoglucan synthesis is activated in Rm1021 *mucR*, specific synthesis of the low molecular weight fraction does not occur. As an initial attempt to elucidate the roles of the two fractions in autoaggregation, we transduced the *mucR31*::Tn5 allele from Rm3131 to Rm1021, and performed quantitative autoaggregation assay of the resulting Rm1021 *mucR* strain, which is unable to synthesize low molecular weight EPS II fraction. Autoaggregation percentage for this mutant was much lower than that of Rm8530, and did not differ significantly from those of Rm1021 and Rm8530 *expA* (Fig. 3). This initial finding allowed us to conclude that high molecular weight EPS II, by itself, cannot mediate cellular interactions leading to autoaggregation.

To further substantiate our results, we performed extracellular complementation assays, in which cell pellets were resuspended in cell-free supernatant from early stationary phase bacterial cultures. Homologous and heterologous assays were conducted as described in "Materials and Methods". Under our experimental conditions, autoaggregation of the homologous and heterologous suspensions depended only on identity of the supernatant, as shown in Table 2. As expected, cell-free supernatants from cultures of both galactoglucan-producing strains, Rm8530 and Rm8530 *exoY*, enhanced autoaggregation of all the *S. meliloti* strains tested. In contrast, there was no significant aggregation-promoting effect by cell-free supernatants from the non-galactoglucan-producing strains (Rm1021, Rm8530 *expA*, Rm8530 *expA exoY*), or from Rm1021 *mucR* (which does not produce low molecular weight galactoglucan). These findings suggest that the low molecular weight fraction of EPS II is responsible for the autoaggregation phenotype. Further experiments, including direct testing of purified low molecular weight galactoglucan, will be necessary to confirm this hypothesis.

DNA microarray analysis has shown that *expR* regulates many other genes, in addition to EPS and motility genes, in *S. meliloti* [12]. Under our experimental conditions, and in the absence of EPS II, it appears that no additional *expR*-regulated factors play a role in autoaggregation, since autoaggregation percentages of Rm1021 and Rm8530 *expA* (or washed cells of Rm8530 and Rm8530 *exoY*) were similar.

Bacterial cell–cell interactions contribute to both autoaggregation and microcolony development, which are important early steps in formation of biofilms. Several factors, including EPS, are involved in these interactions [28]. Interestingly, biofilm formation and autoaggregation of planktonic cells seem to be related processes in *S. meliloti*, since EPS II (but not EPS I) was shown to be critical for development of highly structured biofilms. A high molecular weight EPS II producer, Rm1021 *mucR* strain, failed to form organized biofilms [26], consistent with the poorly autoaggregative phenotype of this mutant in our assays.

Table 2 Extracellular complementation of non-autoaggregating strains with cell-free supernatant from culture of autoaggregating strains

	Cell-free supernatants derived from					
	Rm8530	Rm8530 *exoY*	Rm8530 *expA*	Rm8530 *expA exoY*	Rm1021	Rm1021 *mucR*
Pellets						
Rm8530	92.52 ± 3.00 a	91.63 ± 1.65 a	18.14 ± 4.15 b	18.66 ± 5.45 b	16.88 ± 2.09 b	17.87 ± 2.59 b
Rm8530 *exoY*	92.5 ± 1.85 a	92.35 ± 1.98 a	16.74 ± 5.48 b	18.53 ± 4.31 b	18.01 ± 3.33 b	17.04 ± 3.35 b
Rm8530 *expA*	91.56 ± 1.84 a	91.58 ± 1.99 a	18.74 ± 5.98 b	17.48 ± 2.27 b	18.91 ± 2.15 b	16.76 ± 3.64 b
Rm8530 *expA exoY*	92.05 ± 1.56 a	92.15 ± 1.48 a	14.62 ± 3.39 b	15.37 ± 2.62 b	15.38 ± 3.48 b	18.65 ± 4.64 b
Rm1021	91.6 ± 2.00 a	92.44 ± 2.01 a	17.68 ± 4.01 b	16.07 ± 3.47 b	16.35 ± 5.72 b	15.42 ± 4.71 b
Rm1021 *mucR*	92.31 ± 1.41 a	91.5 ± 1.67 a	18.37 ± 1.56 b	15.40 ± 2.79 b	18.61 ± 2.61 b	16.13 ± 3.60 b

Values indicate mean autoaggregation percentage ± SD. Each experiment was performed in four or more independent replicates, each in quadruplicate. Different letters indicate significant differences ($P \leq 0.05$) between mean values according to Fisher's LSD test

We speculate that other molecular determinants, besides EPS, may be involved in both autoaggregation of planktonic cells and biofilm formation (at least, the early steps) in *S. meliloti*. Inoculation of plants with EPS-producing rhizobacteria, such as *Rhizobium* sp. YAS34 [1] and *Rhizobium* sp. KYGT207 [13], modifies the aggregation of root-adhering soil and leads to improvement of plant growth. Biofilm formation, although it may provide rhizobia with an advantageous microenvironment to persist in the soil, colonize root surfaces, and establish symbiosis, is not essential for legume invasion [25]. On the other hand, host plants, as well as other soil microorganisms, may benefit from the biofilm formation capability of plant growth-promoting rhizobacteria, since EPS within biofilms improve soil structure and help maintain soil moisture [19].

Results of this study strongly suggest that *expR*-regulated EPS II plays a key role in promoting bacterial self-aggregation. Low molecular weight EPS II, either alone or in combination with high molecular weight fraction, may function as the polymeric extracellular matrix that agglutinates bacterial cells. Future extracellular complementation experiments, using purified low molecular weight EPS II, will help clarify this point. We are currently in the process of identifying a bacterial surface component that interacts with EPS II, and expect that results of this study will provide a more complete picture of the *S. meliloti* autoaggregation process.

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