JOURNAL OF BACTERIOLOGY, June 2006, p. 4474–4486 Vol. 188, No. 12
0021-9193/06/$08.00+0 doi:10.1128/JB.00246-06


# Proteins Exported via the PrsD-PrsE Type I Secretion System and the Acidic Exopolysaccharide Are Involved in Biofilm Formation by *Rhizobium leguminosarum*

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Received 16 February 2006/Accepted 27 March 2006

**The type I protein secretion system of *Rhizobium leguminosarum* bv. viciae encoded by the *prsD* and *prsE* genes is responsible for secretion of the exopolysaccharide (EPS)-glycanases PlyA and PlyB. The formation of a ring of biofilm on the surface of the glass in shaken cultures by both the *prsD* and *prsE* secretion mutants was greatly affected. Confocal laser scanning microscopy analysis of green-fluorescent-protein-labeled bacteria showed that during growth in minimal medium, *R. leguminosarum* wild type developed microcolonies, which progress to a characteristic three-dimensional biofilm structure. However, the *prsD* and *prsE* secretion mutants were able to form only an immature biofilm structure. A mutant disrupted in the EPS-glycanase *plyB* gene showed altered timing of biofilm formation, and its structure was atypical. A mutation in an essential gene for EPS synthesis (*pssA*) or deletion of several other *pss* genes involved in EPS synthesis completely abolished the ability of *R. leguminosarum* to develop a biofilm. Extracellular complementation studies of mixed bacterial cultures confirmed the role of the EPS and the modulation of the biofilm structure by the PrsD-PrsE secreted proteins. Protein analysis identified several additional proteins secreted by the PrsD-PrsE secretion system, and N-terminal sequencing revealed peptides homologous to the N termini of proteins from the Rap family (*R*hizobium *a*dhering *p*roteins), which could have roles in cellular adhesion in *R. leguminosarum*. We propose a model for *R. leguminosarum* in which synthesis of the EPS leads the formation of a biofilm and several PrsD-PrsE secreted proteins are involved in different aspects of biofilm maturation, such as modulation of the EPS length or mediating attachment between bacteria.**

Biofilms are bacterial populations in which individual cells adhere to each other and/or to surfaces or interfaces. It is widely accepted that most bacteria live principally in biofilms rather than in a planktonic life style in both natural and artificial environments (11, 55). Like many natural environments, soil is nutrient poor. Soil bacteria must persist within this environment, probably attached to surfaces, while scavenging a wide diversity of carbon sources simply to survive or at best to grow slowly. Some of these bacteria enter the relatively nutrient-rich environment around plant roots and have to compete with many other bacteria in order to remain in this niche. This behavior results in a complex consortium of interacting and competing bacteria probably living in a biofilm community, and communities of plant-associated bacteria have been observed to aggregate in the depressions of epidermal cells or to form biofilms on leaves and root surfaces (36). Genetic characterization of biofilms produced by organisms like *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Escherichia coli* helped to identify distinct steps in biofilm formation: (i) initial attachment to a surface, (ii) formation of microcolonies, (iii) maturation, and (iv) dispersion and cell death (11, 54, 55). Lipopolysaccharide, exopolysaccharide (EPS), outer membrane proteins, flagella, and pili have all been shown to play roles in biofilm formation in different bacteria (11).

Rhizobia are soil bacteria that clonally infect legume roots, leading to the formation of nitrogen-fixing nodules, and in so doing greatly increase their numbers. Such a lifestyle requires the ability to survive and compete both in the bulk soil and in the rhizosphere. One of the early stages in the formation of symbiotic root nodules on leguminous plants is the attachment of *Rhizobium* bacteria to the root hair tip surface. A *Rhizobium* $Ca^{2+}$-binding protein (rhicadhesin) was shown to participate in nonspecific attachment to the roots (49), while bacterial lectins were proposed to be involved in specific attachment (22, 57). It was proposed that at a later stage, bacterial aggregation and cellulose fibrils anchor the rhizobia to the root surface (49). In culture, the major exopolysaccharide produced by *Rhizobium leguminosarum* is an acidic EPS formed by the polymerization of a repetitive unit composed of five glucose residues, two glucuronic acid residues, and a galactose (42). This acidic EPS is essential for nodule infection (6), probably during the progression of the infection process through the infection threads, as was seen with *Sinorhizobium meliloti* (10, 37). Cellulose fibrils formed by *Rhizobium leguminosarum* RBL5523 have also been implicated in root hair infection, although they are not essential for nodulation (3, 28). Surface polysaccharides are likely to play an important role in both rhizosphere

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| Strains | | |
| 8401 | *R. leguminosarum* strain lacking a symbiotic plasmid; $Str^r$ | 27 |
| A34 | *R. leguminosarum* 8401 derivative carrying pSym plasmid pRL1JI | 13 |
| A412 | A34 *prsD1*::Tn*5* | 17 |
| A550 | A34 Δ*plyA-prs-pssC-V*::*npt* II | 60 |
| A600 | A34 *plyB1*::Tn*5* | 18 |
| A638 | A34 *plyA3*::$Spc^r$ | 18 |
| A640 | A34 *plyA3*::$Spc^r$ *plyB1*::Tn*5* | 18 |
| A755 | A34 *prsE1*::$Spc^r$ | This work |
| A1077 | A34 *pssA1*::Tn*5* | This work |
| Z10 | A34 *rapA1*::$Spc^r$ | This work |
| Plasmids | | |
| pRL1JI | *R. leguminosarum* bv. viciae *sym* plasmid | 24 |
| pLAFR1 | P1 group cloning vector; $Tet^r$ | 20 |
| pHP45Ω | 2-kb fragment containing the $Spc^r(\Omega)$ cassette | 16 |
| pIJ1427 | 30 kb of *R. leguminosarum* bv. *phaseoli* DNA, including *pssA*, in pLAFR1 | 6 |
| pIJ1891 | pLAFR3 with the pUC118 polylinker | 17 |
| pIJ7298 | pLAFR1 cosmid carrying *prsD*, *prsE*, and *plyA* | 17 |
| pIJ7331 | 9.6-kb EcoRI fragment carrying *prsD*, *prsE* region from pIJ7298 in pBluescript | This work |
| pIJ7349 | 9.6-kb EcoRI fragment carrying *prsD*, *prsE* region in pIJ1891 | 17 |
| pIJ7652 | Derivative of pIJ7331 with a 3.2-kb BamHI deletion carrying *prsE* | This work |
| pIJ7709 | 2-kb EcoRI-BamHI fragment carrying *plyB* in pIJ1891 | 18 |
| pIJ9109 | Derivative of pIJ7652 with *prsE1*::$Spc^r(\Omega)$ | This work |
| pIJ9121 | 6-kb SmaI fragment from pIJ9109 carrying *prsE1*::$Spc^r(\Omega)$ cloned in *sacB* suicide vector pJQ200(KS) | This work |
| pFC13 | pGEM T-easy (Promega) derivative carrying a 0.7-kb PCR product of *rapA1* homologue from A34 strain | This work |
| pFC18 | Derivative of pFC13 with *rapA1*::$Spc^r(\Omega)$ | This work |
| pFC21 | 2.7-kb NotI fragment from pFC18 carrying *rapA1*::$Spc^r(\Omega)$ cloned in pJQ200(KS) suicide vector | This work |
| pJQ200(KS) | *sacB* suicide vector | 41 |
| pMP4518 | pBBR1MCS-5 derivative carrying EYFP | 49a |
| pRU1319 | pOT1 derivative carrying GFPuv | 1 |

colonization and attachment to surfaces in the bulk soil, but biofilms under either condition have not been fully characterized.

The *R. leguminosarum* acidic EPS is cleaved by two closely related glycanases (PlyA and PlyB) secreted via the PrsD-PrsE type I secretion system (17, 18); the EPS produced by the *prsD* secretion mutant was considerably longer than normal (17), and the *plyB* mutant and *plyA plyB* double mutant showed increasingly greater lengths than that seen in the wild type (WT) (18). Both PlyB and PlyA can cleave mature acidic EPS but probably require nascent EPS synthesis for their activation; such regulation of their activity is thought to limit the cleavage of the acidic EPS to the region adjacent to the bacterial surface (60). During the course of our work on the secretion of PlyA and PlyB, we observed that *prsD* and *prsE* secretion mutants of *R. leguminosarum* bv. viciae consistently produced greatly reduced rings of biofilm compared with the wild type at the liquid-air interface of shaken flask cultures, suggesting a role for PrsD-PrsE secreted proteins in biofilm formation. In this work, we have analyzed the type of biofilm formed by *R. leguminosarum* in axenic culture and the effects on biofilm growth and maturation of mutations which block acidic exopolysaccharide formation and protein secretion. We propose that proteins secreted via the PrsD-PrsE type I secretion system are associated with different aspects of EPS processing and biofilm formation and show that the acidic EPS is essential for biofilm formation.

## MATERIALS AND METHODS

**Microbiological techniques.** Bacterial strains and plasmids are described in Table 1. *R. leguminosarum* strains were grown at 28°C in TY medium (4) or in Y minimal medium (46) containing mannitol (0.2%, wt/vol) as the carbon source. *Escherichia coli* was grown at 37°C in L medium (43). Bacterial growth was monitored at 600 nm using an MSE Spectroplus spectrophotometer. Plasmids were mobilized into *Rhizobium* by triparental mating using a helper plasmid.

For analysis of biofilm growth, bacteria were grown in TY medium (containing appropriate antibiotics) for 2 days (optical density at 600 nm [$OD_{600}$], about 1.5), and then the culture was used as an inoculum at a 1:1,000 dilution in Y medium. Rings of biofilm at the air-liquid interface were qualitatively scored after 3 to 5 days of growth in 100 ml of Y medium in a 250-ml conical flask shaken at 300 rpm in an orbital shaker. Biofilm growth on glass was monitored in static cultures by confocal microscopy (see below). In some experiments, bacterial attachment to the sides and the bottoms of the glass tubes and the wells of polystyrene plates was assayed by first growing the bacteria in shake flasks with Y mannitol medium to an $OD_{600}$ of 0.8 to 1.0 and then pipetting 5 ml of this culture into 10-ml glass tubes or 2 ml into the wells of polystyrene 24-well flat-bottom tissue culture plates (Corning Incorporated, Corning, NY), which were then allowed to stand at 28°C for 48 h. Unbound bacteria were removed by gently washing the tubes or the wells three times with fresh growth medium, and attached bacteria were quantified by staining them with 0.01% (wt/vol) crystal violet (Acros Organics, Geel, Belgium), as described previously (38).

To assay β-1,4-glycanase activity, carboxymethylcellulose (CMC) was incorporated into the Y 0.2% mannitol agar plates at 0.1%. Colonies were grown for 2 days at 28°C and washed off with water. The CMC was stained as previously described (60). Briefly, the plates were flooded with 0.1% (wt/vol) Congo red in water for 15 min, washed for 10 min with 1 M NaCl, and then washed for 5 min with 5% acetic acid. Degradation of CMC was observed as clearings (reduction of staining).

**Generation of mutant strains.** Standard DNA manipulations were carried out as described previously (43). To generate a mutant of A34 carrying a mutation in

*prsE*, a *prsE1*::Spc$^r$ allele was constructed. The 2-kb fragment containing the Spc$^r$(Ω) cassette from pHP45Ω (16) was ligated as an SmaI fragment into pIJ7652 digested with XmnI within the *prsE* gene to make pIJ9109. The resulting *prsE1*::Spc$^r$ allele was cloned from pIJ9109 as a 6-kb SmaI fragment into the *sacB* suicide vector pJQ200(KS) (41) digested with SmaI to make pIJ9121, which was transferred by triparental mating to A34; gentamicin-streptomycin-resistant colonies were selected, and double recombinants were selected on TY medium containing 5% sucrose and spectinomycin. The insertion in the mutant (A755) was confirmed by DNA hybridization. To generate a mutant in a *rapA1* homologue of A34, a *rapA1*:: Spc$^r$ allele was constructed. The *rapA1* homologue gene was PCR amplified from genomic DNA from strain A34 using sense and antisense oligonucleotide primers, ATGGCTGTTCACGCAACC and GGCTATT GACGATCAGAC, respectively. A PCR product of 700 bp was cloned in the pGEM T-easy vector (Promega) to make pFC13. The cloned product was sequenced and identified as an A34 *rapA1* homologue gene (90% identity with *Rhizobium leguminosarum* bv. trifolii autoaggregation *rapA1* gene). The 2-kb fragment containing the Spc$^r$(Ω) cassette from pHP45Ω was ligated as a BglII fragment into pFC13 digested with BamHI within the *rapA1* gene to make pFC18. The resulting *rapA1*::Spc$^r$ allele was cloned from pFC18 as a 2.7-kb NotI fragment into the *sacB* suicide vector pJQ200(KS) digested with NotI to make pFC21, which was transferred by triparental mating to A34; double recombinants were selected on sucrose and spectinomycin. The insertion in the mutant (Z10) was confirmed by sequencing the PCR product generated with the internal primers CCTTGATGTTACCCGAGAG (sense) and CTAGCGAGGGCTTT ACTA (antisense). To make a strain carrying the mutation in *pssA* (A1077), RL38 phage was propagated on strain A168 carrying pIJ1427 (44), and the phage was used to transduce A34 to kanamycin resistance as previously described (8).

**Analysis of extracellular proteins.** For analysis of secreted proteins, rhizobia were grown for 24 h at 28°C in TY medium to an optical density of 0.6. Culture supernatant proteins were concentrated by precipitation with 10% trichloroacetic acid as described previously (14), except that after precipitation, the trichloroacetic acid was extracted by washing the precipitate with acetone. Proteins from an equivalent of 5 to 10 ml of culture supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide and visualized by staining them with Coomassie brilliant blue R-250. Proteins were also stained with silver using the Bio-Rad (Richmond, California) Silver Stain kit following the manufacturer's protocol. For protein sequencing from gels, SDS-PAGE and sample purification were as previously described (56). Samples were separated on 12% (wt/vol) SDS/PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon P) in 10 mM 3-(cyclohexylamino) propane-1-sulfonic acid buffer (pH 11.0) containing 10% (vol/vol) methanol and rinsed thoroughly in deionized water. The blot was stained with 0.1% (wt/vol) Coomassie blue G250 in 50% (vol/vol) methanol and destained with 50% (vol/vol) methanol. Bands were excised and N-terminally sequenced by Edman degradation using an Applied Biosystems model 494 Procise protein sequencer in the pulsed-liquid mode.

**Confocal laser scanning microscopy (CLSM).** A confocal laser scanning microscope (Carl Zeiss LSM510-Axiovert 100 M) was used to visualize the different events of biofilm formation in a 4-day time course experiment using chambered cover glass slides containing a borosilicate glass base 1 μm thick (Lab-Tek Nunc; no. 155411). Confocal images were acquired from bacterial cultures carrying the plasmid pRU1319, which expresses the green fluorescent protein (GFPuv) (1) or the plasmid pMP4518 expressing the enhanced yellow fluorescent protein (EYFP) (49a). GFP- or EYFP-labeled bacterial cultures diluted 1:1,000 were grown in the chambers for up to at least 10 days at 28°C. Such static cultures typically reached an $OD_{600}$ of about 1.7, as determined by resuspending the biofilm bacteria and measuring their optical density. To prevent desiccation, the chambers were incubated in a humid sterile petri dish. A typical mature biofilm was developed by the A34 wild-type strain in static cultures in Y minimal medium containing mannitol after 4 days at 28°C, when the $OD_{600}$ was about 1.1. Three-dimensional images were reconstructed using the Zeiss LSM Image Browser version 3.2.0. Dual-color confocal images were acquired from mixed cultures carrying the GFP-expressing plasmid (pRU1319) or the EYFP-expressing plasmid (pMP4518) (49a). GFP-expressing bacteria appeared green, and EYFP-expressing bacteria appeared pseudocolored red. The detection of the emitted light was performed as described previously (49a). Dual-color images were acquired by sequentially scanning with settings optimal for GFP (488-nm excitation with argon laser line and 505-nm long-pass emission) or EYFP (488-nm excitation with argon laser line and detection of emitted light between 530 and 600 nm). Rates of biofilm formation by bacteria expressing both constructs were similar, and no difference in growth or biofilm formation could be detected using (nonfluorescence) microscopy of biofilms formed by bacteria containing or lacking the GFP or EYFP construct.

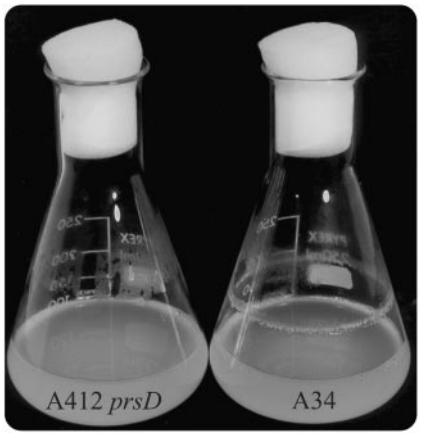


FIG. 1. Formation of a ring of biofilm in shaken cultures by the *prsD* secretion mutant is affected. *R. leguminosarum* strains A34 (WT) and A412 (*prsD*) were grown for 10 days in Y mannitol minimal medium. The *prsE* mutant (A755) produced a weak or greatly reduced ring of biofilm at the air-liquid interface similar to that seen with the *prsD* mutant (not shown).

## RESULTS

**Mutations in *prsD* or *prsE* reduced biofilm formation.** Different and distinguishable biofilms are formed by bacterial species, depending on the medium conditions used and the nature of the surface (15, 51, 38). Some such biofilms occur at the air-medium interface, which is often seen in standing bacterial cultures or in the form of a ring in shaken cultures (21, 45, 58). We observed that *R. leguminosarum* A34 forms a biofilm ring at the air-liquid interface on the sides of shaken flask cultures after 3 to 5 days of growth in Y minimal medium (Fig. 1). Such a biofilm ring was not observed when A34 was grown in TY medium. A34 also formed a thin but stable ring of biofilm in Y medium on a polypropylene surface after 9 days of growth in shaken cultures. These rings of biofilm remained firmly attached to the surface (glass or polypropylene) even after they were exposed to strong shear forces. A loopful of biofilm of GFP-expressing bacteria was removed from the glass surface of a shaken culture, and using CLSM, we confirmed that it consisted of highly compacted aggregates of bacteria (data not shown).

We consistently observed that the ring of biofilm was greatly reduced in strain A412 carrying a mutation (*prsD*::Tn5) blocking secretion via a type I secretion system (Fig. 1). This suggested that one or several of the proteins secreted by the PrsD-PrsE system (17, 18) play a role in the formation of the biofilm ring. Both PlyB and PlyA are acidic-EPS glycanases secreted by this system, and like the *prsD* mutant, both *plyB* and *plyA plyB* mutants produce longer chains of acidic EPS than the wild type (17, 18). The *plyB* and *plyA plyB* mutants (but not the *plyA* mutant) showed reduced rings of biofilm compared with the wild type (data not shown). This suggests that the ring of biofilm is not developed normally if the EPS is not cleaved into shorter lengths by PlyB. To analyze further the role of the acidic EPS in the formation of biofilm rings, a *pssA* mutant unable to produce the acidic EPS was grown under the same conditions. The *pssA* mutant (A1077) lacks an isoprenylphosphate glycosyl transferase responsible for the transfer

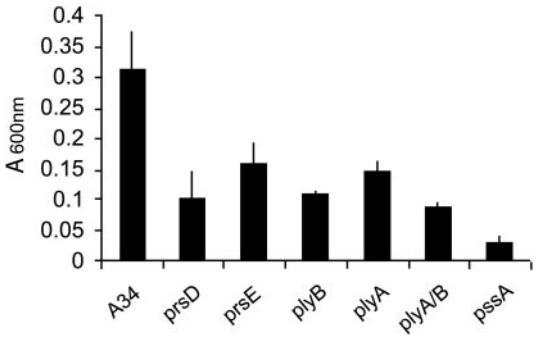


FIG. 2. Attachment to polystyrene is reduced by mutations affecting secreted proteins and the acidic EPS. Bacteria attached to the surfaces of wells in microtiter plates were stained with crystal violet, and the absorbance at 600 nm was measured. The strains used were A34 (WT), A412 (*prsD*), A755 (*prsE*), A600 (*plyB*), A638 (*plyA*), A640 (*plyA*/*B*), and A1077 (*pssA*). Data are presented as the mean absorbance values for four replicate wells plus standard errors from a representative experiment.

of the glucose-1-phosphate from the UDP-glucose to the polyisoprenylphosphate lipid intermediate, a key step specifically affecting acidic-EPS biosynthesis (6, 23, 39, 53). The *pssA* mutation does not affect other surface polysaccharides, such as lipopolysaccharide or the gel-forming polysaccharide (39, 53). Mutation of *pssA* abolished the formation of the ring of biofilm, although a layer of bacteria that was easily detached and dispersed was occasionally observed (data not shown). Taken together, these observations suggest that (i) formation of the biofilm ring requires the EPS and (ii) at least one of the PrsD-PrsE substrates, the PlyB glycanase, participates in the formation of the ring, suggesting that increasing the size of the EPS affects the formation of the biofilm.

A different type of attachment (more labile and dispersed) was observed when grown cultures of *R. leguminosarum* were incubated in glass tubes. Bacteria adhering to the sides and the bottoms of the tubes were quantified by crystal violet staining by measuring the absorbance at 600 nm Although mutation in *pssA* reduced staining ($A_{600}$, 0.06 $\pm$ 0.03) compared with A34 ($A_{600}$, 0.13 $\pm$ 0.03), the *prsD*, *plyA*, *plyB*, and *plyA plyB* mutants showed no significant difference from the wild type (A34) (data not shown). These observations suggest that under these conditions, the PrsD-PrsE secretion system is not critical or the crystal violet assay does not detect differences in the adhesion properties between the wild type and the mutants.

The type of substratum may influence bacterial attachment (40). To investigate the ability of *R. leguminosarum* to attach to other types of surfaces, bacterial adhesion to polystyrene 24-well plates was also analyzed by crystal violet staining. Interestingly, in the wells of the plate, the *prsD* secretion mutant showed significantly less crystal violet staining than the wild-type A34 (Fig. 2). Furthermore, the *plyB*, *plyA*, and *plyA plyB* glycanase mutants produced less biofilm on the plates as measured by crystal violet staining (Fig. 2). These results suggest that the secretion system is required for biofilm formation on polystyrene and that this is in part due to the EPS-glycanases secreted by PrsD-PrsE. The *pssA* EPS mutant showed very low crystal violet staining, supporting the idea that the EPS plays a critical role in surface attachment and/or biofilm growth.

To confirm these observations on the role of the PrsD-PrsE secretion system in attachment, we constructed a *prsE* mutant (A755) containing the Ω spectinomycin resistance cassette within *prsE*. The *prsE* gene codes for the membrane fusion component of the PrsD-PrsE type I system. The *prsE* mutant had a phenotype similar to that of the *prsD* mutant in that (i) it could not secrete the NodO protein (data not shown), (ii) A755 (*prsE*) was defective for cleavage of CMC (Fig. 3A) and exopolysaccharide (not shown), and (iii) A755 was impaired in the secretion of at least four additional extracellular proteins that are also absent in the *prsD* mutant (Fig. 3B). However, in contrast to the *prsD* mutant, the *prsE* mutant had residual glycanase activity (Fig. 3A). The low but detectable activity of CMC degradation observed with the *prsE* mutant suggests that another membrane fusion component may be able to partially compensate for the lack of PrsE; the partially leaky phenotype may explain why this mutant induced nitrogen-fixing nodules on peas (data not shown) whereas the *prsD* mutant formed non-nitrogen-fixing nodules (17). Although secreted proteins could not be detected by Coomassie blue staining of the *prsE* mutant, the assay of glycanase is probably a more sensitive indicator of protein export. Nevertheless, the *prsE* mutant A775 behaved similarly to the *prsD* mutant under all assay conditions described above (not shown) and on the polystyrene plates (Fig. 2), confirming the observations made with the *prsD* mutant.

***R. leguminosarum* differentiates from microcolonies to a three-dimensional structure.** We used CLSM to analyze the characteristics of an *R. leguminosarum* biofilm formed in vitro over a 4-day time course experiment in chambered cover slides. Inverted microscopy with a GFP-labeled strain of *R. leguminosarum* (A34/pRU1319) allowed observation of the different stages of biofilm growth on the bottom of the chamber. A flat lawn of bacteria without any apparent microcolony and organized structure formation was observed in static or shaken cultures over the course of the experiment in TY medium (data not shown). However, in Y minimal medium containing mannitol, clear stages of bacterial biofilm development were observed. During initial attachment, bacteria often contacted the glass surface via one cell pole (Fig. 4, day 1; see inset *z*-axis projection) and rotated anticlockwise (as observed from below the *x-y* axis) around that pole in a plane close to (or oblique to) that of the glass surface. Some of these attached cells vibrated rather than rotated. After initial attachment, some cell clusters (microcolonies) were formed, and progressive aggregations established more complex structures that consisted of multiple layers of cells in contact with each other and the surface (Fig. 4, day 2 and day 3). Bacteria were predominantly attached to each other through lateral interactions, forming a row of cells identically oriented (Fig. 4 inset, day 3), although pole-to-pole interactions were also observed. The 4-day architecture consisted of interconnected complex cell clusters (honeycomb-like) that were interlaced with water channels with an average thickness of 23.8 $\pm$ 5.6 μm (Fig. 4, day 4). For clarity, we call this architecture a typical mature *R. leguminosarum* biofilm. Bacteria were observed to swim freely within these channels and tumbled end-over-end on the surface when one of the cell poles approached the cell clusters. After 10 days, bacterial dispersion from the cell clusters was often observed (not shown). Similar biofilm stages were observed when A34 cham-

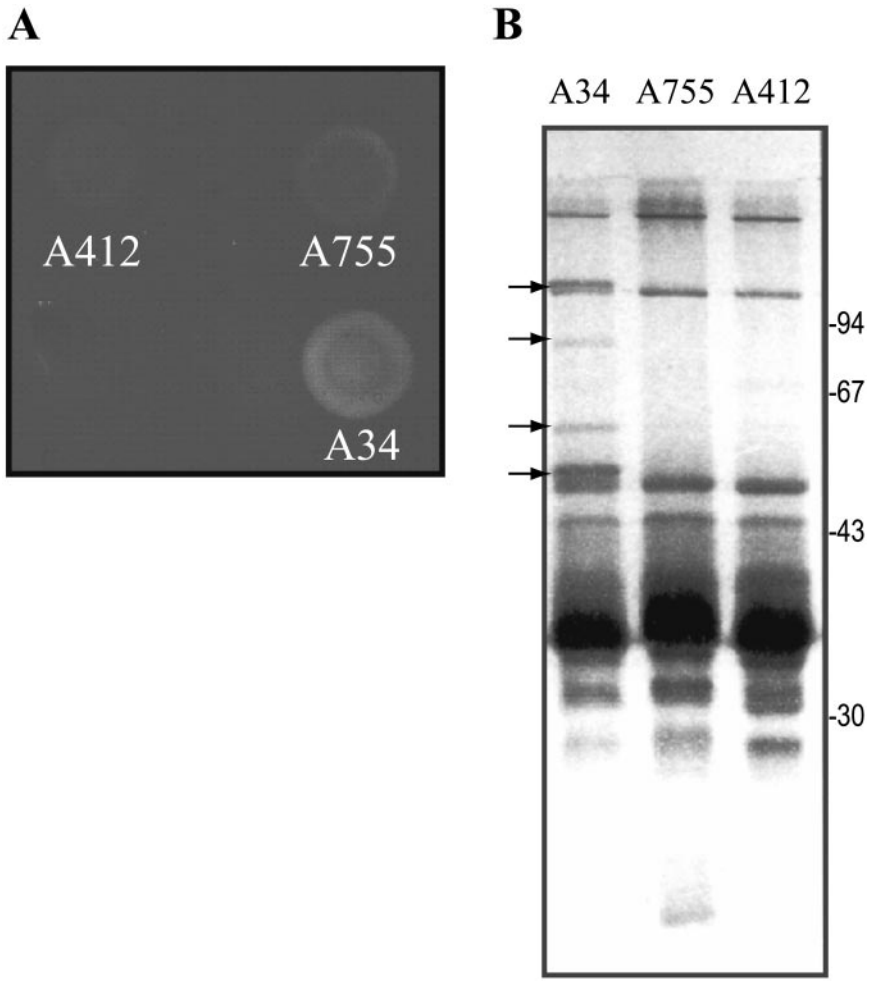


FIG. 3. CMC degradation and protein secretion by the *prsE* secretion mutant. (A) CMC degradation was estimated by the lack of Congo red staining (seen as clear regions) in the agar directly below the colonies. The strains used were A34 (WT), A412 (*prsD*), and A755 (*prsE*) on CMC agar. (B) Culture supernatant proteins were precipitated from A34 (WT), A755 (*prsE*), and A412 (*prsD*); separated by SDS-PAGE; and stained with Coomassie blue. The migration positions of molecular mass markers (94, 67, 43, and 30 kDa) are shown, and the arrows indicate the positions of the proteins absent from the supernatants of *prsE* and *prsD* mutants.

ber cultures were shaken gently, although more time (about 24 h) was required to reach each of the cell-clustering stages.

It has been proposed that aggregation during biofilm formation could be promoted by cellular migration, cell division, or both. We aimed to distinguish between these possibilities by using mixed cultures of the A34 wild-type strain labeled with different fluorescent proteins, GFPuv and EYFP (49a) (which is pseudocolored red), over a 4-day experiment (Fig. 4, A34g/A34r). From the first day, interactions between single-color and mixed-color bacteria were observed, indicating that cells grouped together not just by cell division, but also by cell migration. Moreover, after 2 days of culture, single and mixed microcolonies were formed (Fig. 4, A34g/A34r, days 2 and 3). These observations suggest that in *R. leguminosarum*, both cellular migration and division occur during cell clustering and biofilm formation.

**The *prsD* and *prsE* secretion mutants develop an immature biofilm.** To study the precise role of the PrsD-PrsE secretion system in biofilm development, the *prsD* mutant A412 labeled with GFP was grown in static culture in the chambered cover glass slide as described above (Fig. 4). Although no apparent alteration in cell attachment to the glass surface was seen in A412 (*prsD*) during the first and second days, the sizes of the microcolonies and aggregates observed during the third day were clearly reduced. After 4 days, the secretion mutant formed an immature biofilm composed of more but smaller clusters of bacteria with expanded water channels (Fig. 4), albeit with an average thickness rather similar to that of the wild type ($21.2 \pm 6.7$ μm). This arrangement of cells did not progress to a mature structure even after longer periods of observation and was reproducibly observed in all seven independent experiments. A similar immature biofilm was developed by the A755 (*prsE*::Spec) mutant labeled with GFP (Fig. 5B). The type of biofilm formed on the base of the chambers by the *prsD* and *prsE* mutants was probably similar to that formed on the surfaces of glass tubes, where no significant differences between the secretion mutants and the wild type were observed following staining with crystal violet. The *prsD-prsE* genes cloned in pIJ7349 restored the formation of a typical biofilm in A412 (Fig. 4) and A755 (not shown) grown in chambered cover glass slides.

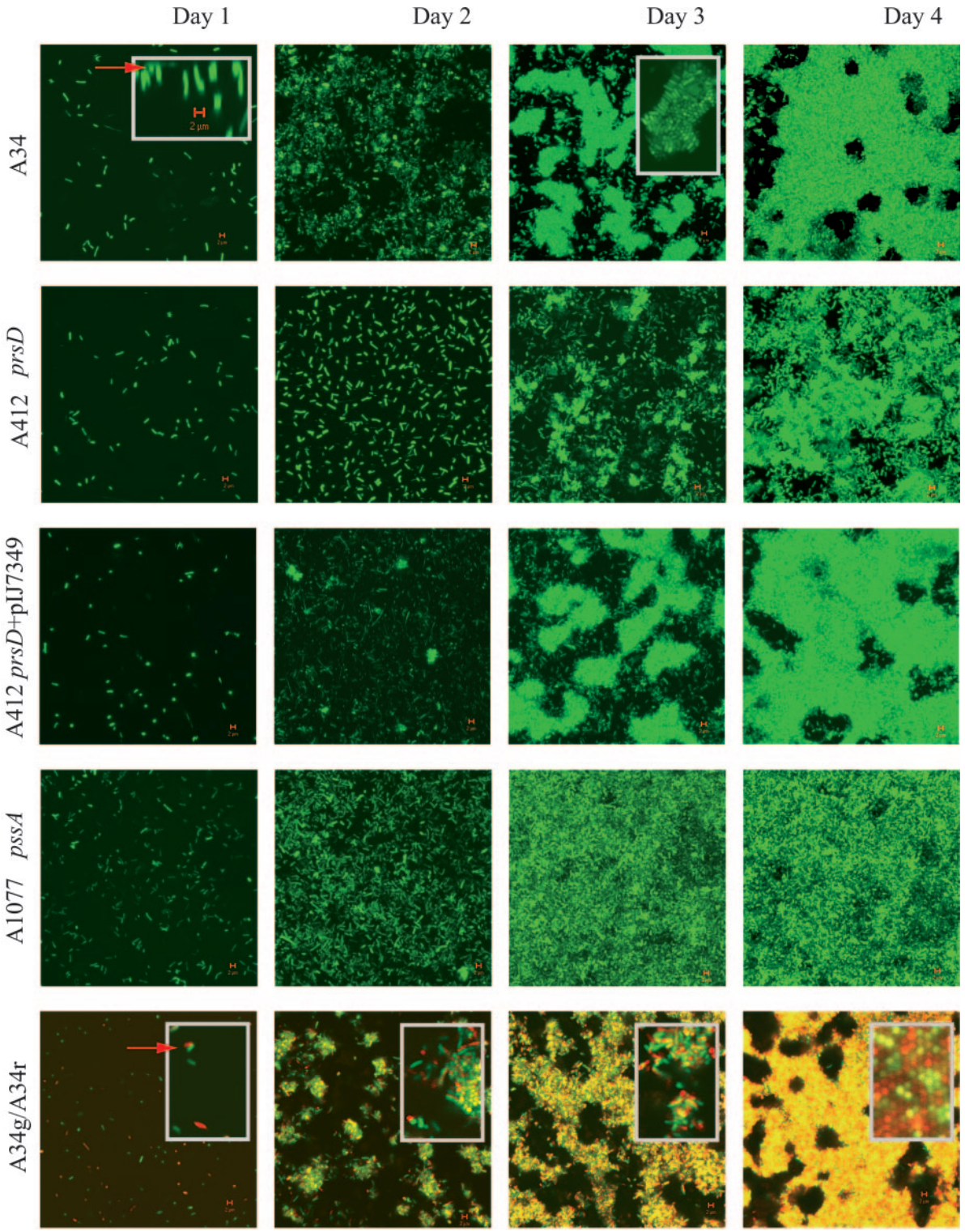


FIG. 4. Developing stages of biofilm formation in *R. leguminosarum* bv. viciae. A34 (WT), A412 (*prsD*), and A1077 (*pssA*) containing the GFP-expressing plasmid pRU1319 were grown for 4 days in chambered cover slides and observed daily by CLSM. The panels (left to right) show horizontal projected images (*x-y* axis) from each day. (A34, day 1) The arrow indicates cell attachment to the glass surface by one pole in vertical projected images (*z* axis). (A34, day 3) The inset shows a detail of a typical wild-type microcolony. The *prsD* mutant, A412, developed an immature biofilm after 4 days and was restored to wild-type phenotype with the *prsD-prsE* genes cloned in pIJ7349 (A412 *prsD* + pIJ7349). The *pssA* mutant, A1077, did not form a typical biofilm after 4 days. CLSM images were also acquired from mixed (1:1) cultures of A34/pRU1319 GFPuv-labeled (green) and A34/pMP4518 EYFP-labeled (red) (A34g/A34r). At early stages of biofilm development (A34g/A34r, days 1 and 2), dual-color A34 cultures showed mixed-color bacteria interacting (arrow) and developing microcolonies; at later stages, the typical microcolonies progressed to a honeycomb structure (A34g/A34r, day 4). Magnifications, ×1,000; zooms (insets), ×3,000. The size bars in panels and in the inset of the first panel indicate 2 μm.

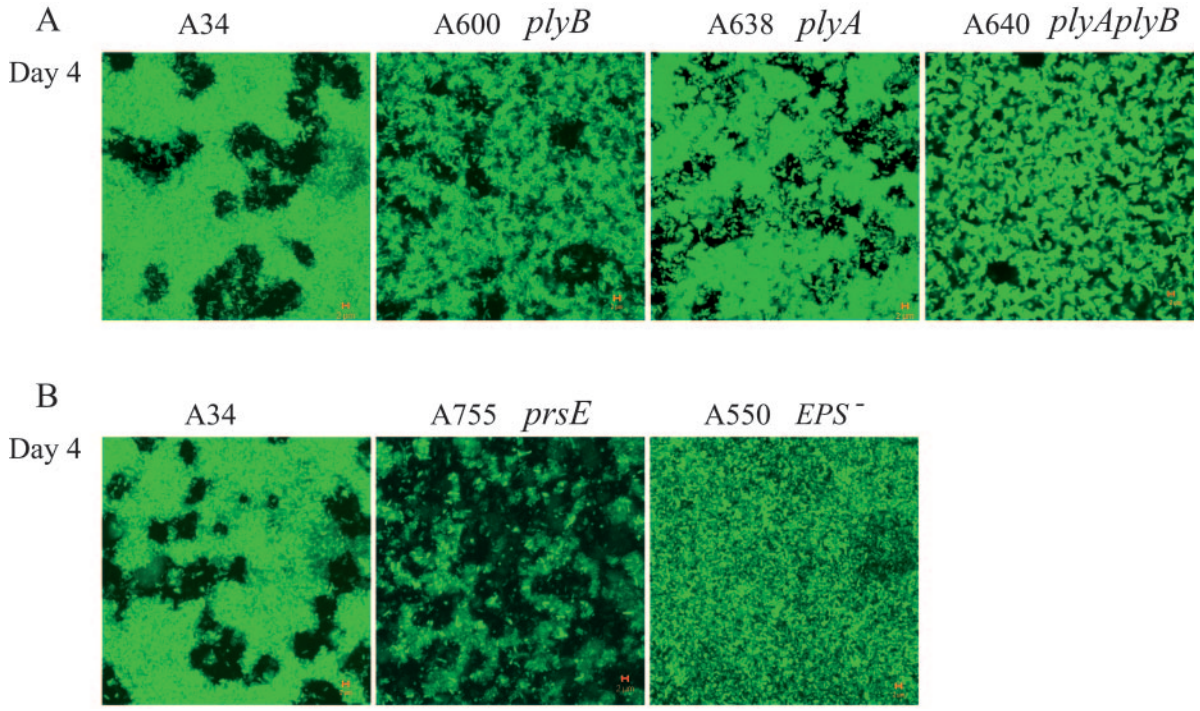


FIG. 5. Effects of different mutations on biofilm formation. (A) Altered biofilm phenotypes of the *plyB* (A600) and *plyA plyB* (A640) mutants observed by CLSM. The panels show horizontal projected images (*x-y* axis) from 4-day static cultures in chambered cover slides of A34/pRU1319, A600 *plyB*/pRU1319, A638 *plyA*/pRU1319, and A640 *plyA plyB*/pRU1319. (B) The panels (left to right) show horizontal projected images (*x-y* axis) of A34/pRU1319, the *prsE* mutant A755/pRU1319, and the EPS mutant A550/pRU1319. Magnifications, ×1,000. Size bars indicate 2 μm.

We also examined the progression of the biofilms formed by the EPS-glycanase mutants A600 (*plyB*), A638 (*plyA*), and A640 (*plyA plyB*) labeled with GFP. The biofilm progression (not shown) and the 4-day architecture (Fig. 5A) of the *plyA* mutant showed no marked differences compared with the wild type. This observation was not surprising, since no obvious phenotype had been seen previously with A638 (*plyA*) (18). The times taken to develop microcolonies with both the *plyB* mutant and the *plyA plyB* double mutant were different than the control, A34, and although the phenotype of the double mutant was slightly more severe than that of the *plyB* mutant, an undeveloped biofilm was observed in both mutants after 4 days (Fig. 5A). The biofilms consisted of a high number of small and dense clusters of bacteria. This phenotype was somewhat different from the immature biofilm developed by the *prsD* and *prsE* secretion mutants, which formed a high number of loose aggregates of bacteria (Fig. 4 and 5B). The more severe phenotype of the *plyA plyB* double mutant compared with the *plyB* mutant is consistent with previous data indicating that a synergistic effect on the degree of EPS polymerization was present when both *plyA* and *plyB* were inactive (18). The contribution of PlyA to the *plyA plyB* double-mutant phenotype may explain the reduced attachment of the *plyA* single mutant to the polystyrene plate (Fig. 2). Cloned *plyB* on pIJ7709 restored to normal the biofilm phenotypes of both A600 (*plyB*) and A640 (*plyA plyB*) (data not shown). These observations indicate that (i) the PrsD-PrsE secretion system plays a role in the formation of the biofilm in the bottom of the chamber; (ii) PlyB and PlyA, two of the PrsD-PrsE-dependent extracellular proteins, are responsible in part for the observed *prsD* and *prsE* phenotypes; and (iii) the phenotypic difference between *prsD* and *prsE* mutants and the *plyB plyA* double mutant suggests that additional proteins secreted via the PrsD-PrsE secretion system may play a role in biofilm maturation.

**The acidic EPS is essential for biofilm development.** Mutation of *pssA*, encoding an isoprenylphosphate glycosyl transferase specifically responsible for acidic-EPS biosynthesis, abolished or reduced the formation of a ring of biofilm in shaken cultures and adhesion to glass and polystyrene surfaces in static cultures (Fig. 2). Using CLSM, we observed that the *pssA* mutant (A1077) was totally unable to develop microcolonies or more complex structures (Fig. 4). After 4 days, a flat lawn of bacteria without any architecture was observed on the base of the chamber (Fig. 4). The *pssA* gene cloned in pIJ1427 restored normal EPS production in the *pssA* mutant (A1077) and its ability to develop microcolonies, cell aggregates, and the three-dimensional biofilm on the base of the chamber in static cultures, as well as the ring of biofilm under shaken conditions (not shown). This observation strongly suggests that biofilm formation requires the synthesis of acidic EPS. To obtain more evidence about the role of this EPS in biofilm formation, another acidic-EPS-defective mutant (A550) (60) was analyzed. The A550 mutant has a deletion removing several *pss* genes (other than *pssA*), specifically affecting the biosynthesis, polymerization, and export of the acidic EPS (31–33, 39, 53). The A550 mutant behaved similarly to A1077; thus, the ring of biofilm was greatly reduced (not shown) and no typical architecture was observed by CLSM (Fig. 5B). The *pss* cluster of genes cloned in pIJ7298 restored to A550 normal levels of EPS, a typical mature biofilm in static culture in chamber cover

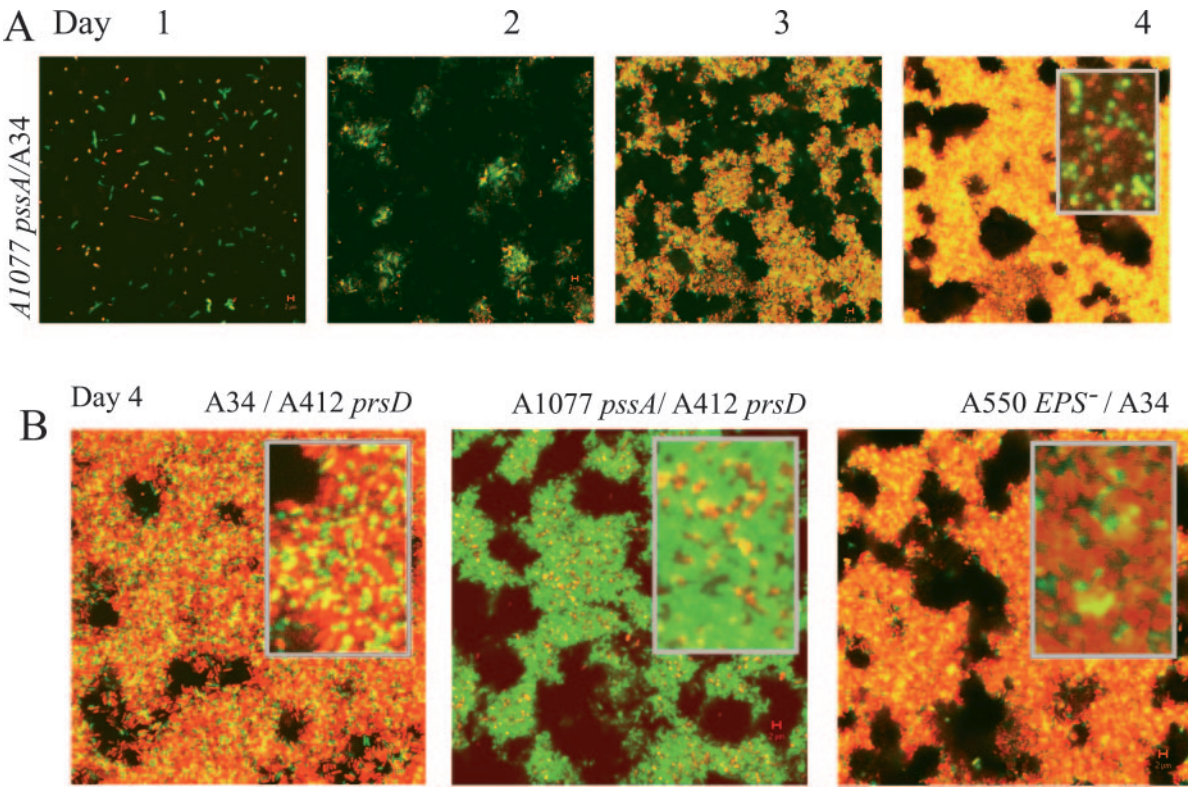


FIG. 6. Incorporation of protein secretion and EPS-defective mutants into mixed biofilms. Dual-color mixed cultures were all inoculated at a 1:1 ratio of bacteria. (A) Mixed cultures of A34/pMP4518 EYFP-labeled (red) and A1077(*pssA*)/pRU1319 GFPuv-labeled (green) were followed during a 4-day experiment. (B) Biofilms formed by mixed cultures after 4 days are shown for the following mixes: A34/pRU1319 (green) and A412(*prsD*)/pMP4518 (red), A1077(*pssA*)/pMP4518 (red) and A412(*prsD*)/pRU1319 (green), and A550 (*pssC-V*)/pRU1319 (green) and A34/pMP4518 (red). The panels show horizontal projected images (*x-y* axis), and zoom images (insets) illustrate tight interactions between dual-color bacteria inside the cluster.

slides, and normal biofilm rings in shaken cultures (not shown). These results show that the synthesis of acidic EPS in *R. leguminosarum* is required for biofilm development.

**Incorporation of biofilm-defective mutants into biofilms containing mixed bacteria.** To determine if the lack of acidic EPS caused bacterial cells to be excluded from the growing biofilms, mixed cultures of differently fluorescent EPS-defective and wild-type bacteria were analyzed over a 4-day time course experiment. Coinoculation of A34 with either the *pssA* mutant A1077 (Fig. 6A) or the A550 mutant, which has a deletion of several *pss* gene (*pssC* to *pssV*) (Fig. 6B) showed that the EPS-defective bacteria could be incorporated into the honeycomb structure developed by the wild-type bacteria. However, the EPS mutants did not form large honeycomb aggregates containing only this bacterial type, whereas many such aggregates containing mostly wild-type bacteria could be observed. This indicates that the ability of the acidic-EPS mutants to incorporate into the biofilms may depend on EPS from adjacent cells. We also analyzed a mixed biofilm of the *prsD* mutant A412 and the wild type, and in this case, there appeared to be more complete integration by the mutant cells (Fig. 6B).

Since abnormal biofilms are produced by both the acidic-EPS mutant and the *prsD* secretion mutant, it was possible to test if extracellular complementation could be observed when mixed cultures of the EPS mutant A1077 (*pssA*) and the secretion mutant A412 (*prsD*) (Fig. 6B) or A755 (*prsE*) (not shown) were analyzed. In this case, a compact honeycomb structure similar to that of the wild type was observed after 4 days (Fig. 6B). However, the *pssA* mutant was again incorporated at a relatively low frequency and was not present in large aggregates. This indicates that efficient rescue of the *prsD* mutant phenotype can be achieved by extracellular complementation from proteins secreted by the *pssA* mutant and confirms the observation that there can be limited extracellular complementation of the *pssA* mutant by EPS from adjacent cells.

**The PrsD-PrsE system is involved in the secretion of three adhesins.** Mutation of *prsD* (17) or *prsE* (Fig. 3B) blocks secretion of other proteins in addition to PlyA and PlyB. In view of the difference in biofilm characteristics between the protein secretion mutants (*prsD* and *prsE*) and the *plyA plyB* double mutant, we set out to identify proteins that are not secreted by the *prsD* mutant. Other proteins predicted to be involved in biofilm formation are the Raps (*R*hizobium *a*dhering *p*roteins), but their mechanism of export is not known (2). Coomassie-blue-stained proteins with apparent molecular masses of 145 kDa, 77 kDa, 59 kDa, 52 kDa, 32 kDa, and 30.5 kDa were found in the culture supernatant of the wild type (A34) but not in that of the *prsD* mutant, A412 (Fig. 7A). The absence of the 145-kDa protein in the *prsD* mutant is difficult to visualize in a 12% acrylamide gel (Fig. 7A), since this protein migrates very

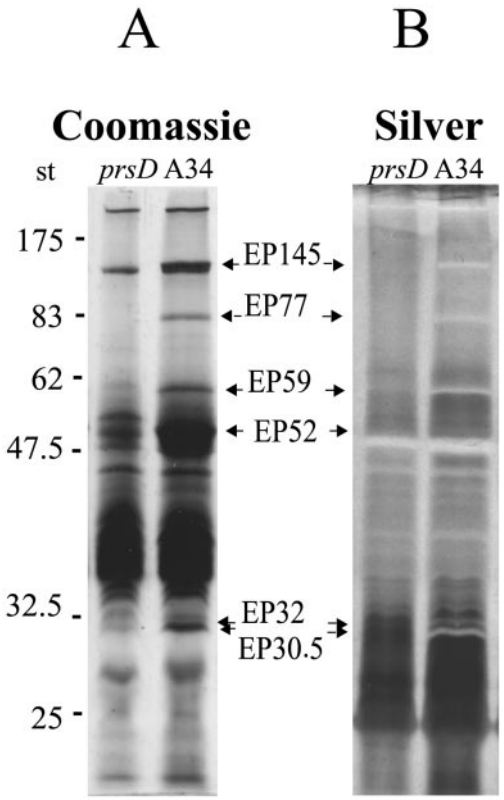


FIG. 7. Analysis of extracellular proteins from the *prsD* mutant and the wild type. Cultures of A34 (WT) or A412 (*prsD*) were grown for 24 h to an $OD_{600}$ of 0.6, and proteins from the culture supernatants were precipitated and analyzed by SDS-12% PAGE. The proteins were stained with Coomassie brilliant blue R-250 (A) or silver (B). The arrows indicate extracellular proteins that were absent in the secretion mutant A412. The numbers indicate the molecular weights of protein standards (st).

close to another secreted protein that is not PrsD-PrsE dependent. However, using lower percentages of acrylamide, both protein bands are well separated, and the absence of the 145-kDa protein in the secretion mutants becomes clear (data not shown). The proteins were also stained with silver; an advantage of silver staining is that some metal-binding proteins, such us calmodulin (which binds $Ca^{2+}$), can inhibit the silver-staining reaction and so appear as negatively stained bands (34). Comparison of the silver-stained extracellular proteins from the WT and the *prsD* mutant revealed the absence of staining in the A34 profile at positions corresponding to 145 kDa, 77 kDa, 59 kDa, 32 kDa, and 30.5 kDa. (Fig. 7B). Since these masses correspond to proteins identified by Coomassie blue staining in the wild type but absent from the *prsD* mutant (Fig. 7A), it is evident that the lack of silver staining at these positions is due to inhibition of the silver-staining reaction (negative staining). This suggests that the 145-kDa, 77-kDa, 59-kDa, 32-kDa, and 30.5-kDa proteins are likely to be metal-binding proteins (Table 2). The strong Coomassie-stained protein band of about 52 kDa (EP52) seen in the wild type was not negatively stained with silver (Table 2), suggesting that this protein may not share some of the structure features with the other PrsD-PrsE secreted proteins. PlyA and PlyB would be expected to migrate at about this position.

We also analyzed proteins that may remain attached to the cell surface. Bacteria were submitted to different treatments, such as 1 M NaCl; 0.2% Tween–50 mM Tris-HCl, pH 7.5; or 0.2% sarcosyl–20 mM EDTA–50 mM Tris-HCl, pH 7.5, and proteins released after these treatments were analyzed by SDS-PAGE. No differences in the protein profiles between the *prsD* mutant, A412, and the wild-type, A34, were observed (even by staining the proteins with silver; data not shown), indicating that there are unlikely to be other PrsD-PrsE secreted proteins that remain attached to the cell surface.

The proteins exported by A34 were transferred to a membrane, and those that were not secreted by the *prsD* and *prsE*

TABLE 2. Proteins secreted by the PrsD-PrsE Type I secretion system

| Protein (homologue) | Determined N-terminal sequence[b] | $Ca^{2+}$ binding | Silver staining[c] | Proposed function |
|---|---|---|---|---|
| PlyB | NA | Possible binding | ND | Extracellular and diffusible β-1, 4-glycanase (18, 60) |
| PlyA | NA | Possible binding | ND | Cell-associated β-1, 4-glycanase (18, 60) |
| NodO[a] | NA | Yes[a] | NA | Pore-forming protein (18, 50) |
| EP145[e] | DPNILDLSGDSAXGTVDND | Yes[a] | Negative image | Unknown |
| EP77 | NDT | ND[d] | Negative image | Unknown |
| EP59 (RapC) | TVLAKPVAVD (MTVLAKPVAVDDVSS ASEKD) | Yes[a] | Negative image | Adhesin (2) |
| EP52 | NDT | ND | Positive image | Unknown |
| EP32 (RapA1) | AVHATDDSATFLET**D**AISGN (MAVHA TDDSATFLET**A**AISGN) | ND | Negative image | Adhesin/agglutinin (2) |
| EP30.5 (RapA2) | ASPIHATDDSATFKETDVIS (MASPIHA TDDSATFKETDVIS) | Yes[a] | Negative image | Adhesin (2) |

[a] Binding of NodO to $Ca^{2+}$ was previously demonstrated (14). NodO is produced only when bacteria are grown in the presence of a flavonoid *nod* gene inducer. Binding to $Ca^{2+}$ of EP145, EP59, and EP30.5 was shown previously (17).

[b] X indicates that no amino acid could be assigned. The boldface letters highlight the nonmatching residues. The predicted sequences of Rap proteins, shown in parentheses, are from genes identified in *R. leguminosarum* bv. trifolii in a previous work (2), and they have been aligned with the determined amino acid sequences. NA, not applicable; NDT, not determined.

[c] The negatively stained bands observed (Fig.7B) suggest metal-binding proteins.

[d] ND, not detected.

[e] The numbers of extracellular proteins (EP) indicate their estimated molecular masses (kDa).

mutants were excised and N-terminally sequenced. With the exception of the 77-kDa protein (EP77), N-terminal sequences were obtained (Table 2). Good matches were found in database searches using the peptide sequences of the 59-kDa (EP59), 32-kDa (EP32), and 30.5-kDa (EP30.5) proteins, which were identical or nearly identical to the amino-terminal sequences of *R. leguminosarum* bv. trifolii R200 predicted proteins RapC, RapA1, and RapA2, respectively (Table 2). The only mismatch was a single-residue difference between RapA1 and the EP32 protein. The predicted RapA1, RapA2, and RapC peptides were identified as *Rhizobium* adhering proteins (2); they share a conserved sequence motif that is also found in PlyB and PlyA that might bind to the EPS (2). The *rapA1* gene was the only *rap* gene that was completely sequenced; it was shown to encode a 30-kDa calcium-binding protein, which attaches to one pole of *R. leguminosarum*, promoting cellular aggregation (2). The predicted molecular mass for RapA1 is similar to that estimated for the homologous extracellular protein (32 kDa).

Only a partial sequence of *rapA2* was published; 20 residues from the N terminus of EP30.5 from A34 were identical to the predicted N terminus of RapA2 from *R. leguminosarum* bv. trifolii R200 (2) (Table 2). The genome sequence of another strain of *R. leguminosarum* (3841) (http://www.sanger.ac.uk/Projects/R_leguminosarum/) revealed an open reading frame that encodes a similar predicted protein that is 25.1 kDa (238 amino acids), significantly smaller than that predicted for EP30.5. Ten amino acids from the N terminus of EP59 from A34 were identical to the predicted N terminus of the RapC partial sequence from the R200 strain (Table 2). The best similarity (95%) obtained with a BLAST search using the available RapC sequence against the translated 3841 genome identified a predicted protein of 22.1 kDa (210 amino acids), which is less than half the mass of EP59. In addition, two other predicted proteins of different masses from the 3841 strain were found to share high similarity (62 and 67%) with the available RapC sequence. In fact, the proteins from the Rap family and its homologues from the A34 and 3841 strains share high similarities with each other (60 to 95%). Taken together, these observations indicate that (i) there are many differences within the family of Rap proteins between different strains of *R. leguminosarum*, (ii) it seems that more than three proteins belonging to the Rap family are present in the genomes of the *R. leguminosarum* strains, and (iii) many of them may be secreted by PrsD-PrsE.

N-terminal sequencing of EP52 revealed mixed sequences, which were difficult to interpret. This observation suggests that multiple PrsD-PrsE-dependent proteins with similar molecular weights corresponding to PlyA and PlyB (18) may comigrate on SDS-PAGE. The N-terminal peptide sequences of EP145 did not have clear homologues in database searches or in the 3841 genome sequence.

The features of the PrsD-PrsE secreted proteins so far identified and the proposed functions are detailed in Table 2. PlyA and PlyB contain a repeat motif, suggesting that they bind calcium (18). Binding to $Ca^{2+}$ of EP145, EP59, and EP30.5 was previously shown (17). Our results indicate that additional proteins, including proteins similar to three putative adhesins (RapA1, RapA2, and RapC) that may bind $Ca^{2+}$, are secreted, along with PlyB and PlyA, by the type I PrsD-PrsE secretion system. Some of these proteins could potentially influence biofilm formation. Since not all the secreted proteins could be identified and several differences were found between R200, A34, and the recently sequenced strain 3841, it will be very difficult to define which secreted proteins are responsible for normal biofilm maturation, especially since some of the gene products are likely to be rather similar and so could be functionally redundant. However, based on the *rapA1* published sequence from the R200 strain, we were able to amplify and clone from A34 a *rapA1*-homologous gene, which shared 90% similarity with *rapA1* from strain R200. An insertional mutation was generated by double recombination in A34 to form strain Z10, but this mutation did not affect the structure of the biofilms observed in shaken cultures or in static culture by CSLM (data not shown). We predict that double and multiple mutants or other approaches will be necessary to have a clear picture of the role of each of the novel PrsD-PrsE candidates.

## DISCUSSION

In this work, we analyzed the structure of biofilms formed by *R. leguminosarum* in vitro to try to understand a process that is probably most relevant to attachment to different type of surfaces in soil. When grown in minimal medium for 4 days, *R. leguminosarum* bv. viciae strain A34 develops a distinctive arrangement of bacteria with a three-dimensional structure containing water-filled channels. This structure is initiated by the formation of microcolonies, followed by the development of more complex structures in which bacteria are aligned side by side in lateral chains rather like a picket fence. There were no chains of bacteria attached end to end, so we assumed that the lateral chains must be formed by migration of the bacteria into these ordered arrays. Using a mixture of bacteria containing different fluorescence markers, we confirmed that the lateral chains contained individual cells of one fluorescent type flanked by bacteria with different fluorescence. This confirms that these chains were produced by incorporation of bacteria rather than simply by cell division. This mode of biofilm growth is rather different from that proposed for *P. aeruginosa*, in which the first steps (mushroom stalks) were formed by clonal growth while later stages (mushroom caps) were formed by aggregation (26). After 4 days, the mature *R. leguminosarum* biofilm contained groups of tightly packed bacteria like hexagonal closely packed arrays, separated by water-filled spaces. At the edges of these arrays, some bacteria appeared to be attached by only one pole, and some aggregate dispersion was seen after several days.

This type of complex biofilm structure was not seen if the bacteria were grown in TY (complex) medium, suggesting that nutrient status is important for such structured biofilms to form. There may be significant differences between different strains of *R. leguminosarum* bv. viciae; Smit et al. (48) showed that strain 248 formed rings of agglutinated cells when grown in TY medium in shake flasks and that this required a high level (6 mM) of $Ca^{2+}$. We observed very poor formation of such rings when strain A34 was grown under the same conditions but did observe clear rings when strain A34 was grown in minimal medium, which contains only 1 mM $Ca^{2+}$. Strain 248 normally synthesizes cellulose fibrils that contribute to attachment to different surfaces (47, 48), but the strain used in our

work (A34) does not produce detectable amounts of cellulose (unpublished results).

It is clear that the acidic EPS of strain A34 is essential for the formation of biofilms on glass and polystyrene; CLSM showed that EPS synthesis was required from the first stages of the development process, and even after 4 days of growth, a flat lawn of bacteria without any architecture was observed in the EPS-defective mutants. The EPS is partially cleaved by two identified extracellular glycanases encoded by *plyA* and *plyB* (18); the altered timing and maturation of biofilm formation in the *plyB* and *plyA plyB* mutants indicate that these glycanases are not essential for biofilm formation, but the size of the EPS chains can modulate the structure and maturation of the biofilm. It is possible that other exopolysaccharides produced by *R. leguminosarum*, such as the cyclic β-1,2-glucans (7), the gel-forming polysaccharide (9, 29, 59), lipopolysaccharide (5, 19, 25), and cellulose (3, 28), may play roles in biofilm formation. Such questions will be better addressed in *R. leguminosarum* bv. viciae strain 3841, whose genome sequence has recently been completed (http://www.sanger.ac.uk/Projects/R_leguminosarum), because it will be relatively easy to make the appropriate mutants in that background. We have already established that strain 3841 forms biofilms identical to those formed by strain A34 (unpublished observations).

PlyA and PlyB are exported via a type I secretion system encoded by *prsD* and *prsE*; additional proteins secreted via this system are clearly important for biofilm formation, because both *prsD* and *prsE* mutants formed abnormal biofilms. The *prsD* and *prsE* mutants were defective for attachment and/or biofilm formation at the air-liquid interface of shaken cultures and on polystyrene surfaces in static cultures, and CLSM revealed an immature structure formed on glass in static cultures. The N-terminal sequences of some of the PrsD-PrsE secreted proteins turned out to be identical (or nearly identical) to the predicted proteins of *R. leguminosarum* bv. trifolii encoded by the *rap* genes. The *rap* genes were initially identified from a phage display library on the basis that the expressed domains bound strongly to *R. leguminosarum* cells. It was demonstrated that one of these *Rhizobium* adhering proteins bound to the polar region of *R. leguminosarum* cells and promoted their agglutination (2). The model proposed that the Rap adhesins would bind to some component of the EPS or the capsular polysaccharide during this process (2).

Type I secretion systems usually secrete one or few related proteins (30). Although we showed that at least nine proteins are exported by the PrsD-PrsE system, this may be due to the fact that they share some structural features (Table 2). All the RapC, RapA1, RapA2, PlyB, and PlyA proteins contain a conserved motif (Ra) that was proposed to bind to some component of the EPS (2). Additionally, there are several lines of evidence suggesting that NodO, PlyB, PlyA, EP145, EP77, EP59, EP32, and EP30.5 are $Ca^{2+}$-binding proteins (Table 2). Our analysis of the secreted proteins was hampered by the fact that the genome sequence of strain A34 is not known, and surprisingly, analysis of the genome of *R. leguminosarum* bv. viciae strain 3841 did not always result in the identification of genes encoding the identified protein sequences. Furthermore, the sizes of some of the proteins identified on the gel did not correspond to the predicted gene products with matching N-terminal sequences based on the sequence of strain 3841 (http://www.sanger.ac.uk/Projects/R_leguminosarum). This means that isolating mutations in genes encoding proteins secreted from strain A34 will be relatively difficult and would be better approached using the recently sequenced strain. This will be particularly important if multiple mutants have to be made. Nevertheless, it does appear from our work with strain A34 that several proteins secreted via the PrsD-PrsE type I secretion system are involved in different aspects of biofilm formation. It is possible that the primary role of the PrsD-PrsE secretion system is to export proteins that are associated with surface attachment and biofilm maturation. If this is the case, perhaps this secretion system has been hijacked to allow export of the nodulation signaling protein NodO.

Although both the EPS-defective mutants (*pssA* and *pssC-V*) and the protein secretion mutants (*prsD* and *prsE*) were defective for the formation of a mature biofilm, mixed cultures of EPS and secretion mutants formed typical biofilms incorporating both types of bacteria. This demonstrates that the defects in the biofilm can be complemented by extracellular components and shows that the defects are not due to autonomous cell effects. The ability of *R. leguminosarum* to develop a biofilm in vitro suggests that rhizobia could have a biofilm lifestyle in their natural environments, in which such extracellular components should be particularly relevant. Living within a biofilm could offer many advantages to rhizobia in comparison with the planktonic state; the biofilm can act as a defense or survival mechanism or it can facilitate cell-to-cell communication and growth in the nutrient-poor soil environment (36).

All the *prsD* (17), *prsE* (data not shown), *plyB*, and *plyA plyB* (18) mutants were not affected for initiation of nodule morphogenesis or infection of peas. We tested if the *plyA plyB* double mutant is less efficient in nodule infection by coinoculating equal numbers of *plyA plyB* and wild-type bacteria (data not shown). The *plyA plyB* mutant was only slightly less represented (43%) in nodules (average obtained from 100 nodules) than the wild type (57%). These observations suggest that, at least in our plant assays on pea, the formation of the typical and mature biofilm observed in vitro is not necessary for infection of roots. In contrast, the *pssA* mutant, which lacks the acidic EPS and does not form microcolonies or aggregates in biofilms, was totally defective for root infection (6). We also found that the other EPS mutant (A550) was defective for infection because it induced only empty nodules (data not shown). Therefore, some kind of biofilm or bacterial aggregation on the roots could be required for root infection. However, this interpretation must be made with caution, since other roles, such as EPS-mediated suppression of plant defense reactions (35) and/or the EPS acting as a specific recognition signal (12), have been proposed, and they could also be related to the defective infection.

It will be important in future work to relate the observations on biofilm formation in vitro to the attachment of rhizobia to root surfaces and to infection events. It is clear that *R. leguminosarum* can attach to roots and that subsequent attachment of bacteria leads to a form of biofilm that aids in infection of legumes (28, 48, 49). This may be similar to or slightly different from the types of in vitro biofilms described here. Biofilm growth in the soil and during attachment to roots and infection of root hairs are all likely to enhance survival and competition of rhizobia. Further genetic and biochemical approaches, com-

bined with in vivo and in vitro studies, will be necessary to develop a more complete model of the structural factors that influence biofilm formation in the different niches occupied by *R. leguminosarum*. However, it is clear that several of the proteins secreted via the PrsD-PrsE type I secretion system are likely to be involved with different facets of biofilm formation, either by processing (cleaving) the EPS or by binding to the EPS and acting as attachment factors.

## ACKNOWLEDGMENTS

We thank our colleagues Adrián Vojnov, Lorena Haurigot, Fernando A. Martín, Alfonso Soler Bistué, Jim Lithgow, and Florence Wisniewski-Dye for helpful discussions; Sofía Lavista Llanos, Martín Radrizzani, and Grant Calder for helping with the microscopy; Marta Bravo, Jimena Ortega, and the staff of the John Innes Centre Genome Centre for DNA sequencing; and Mike Naldret for protein sequencing. Julian Parkhill, Lisa Crossman, and their colleagues at the Sanger Centre kindly gave us access to the annotated genome sequence of *R. leguminosarum* bv. viciae prior to publication. We thank Philip Poole for providing pRU1319 and Guido Bloemberg for pMP4518. We also thank Marcelo E. Tolmasky for critically reading the manuscript and Tomás Santa Coloma for making available the confocal microscope. We are grateful to Dante and Diego Chiarullo for image and graphic design.

This work was supported by the Biotechnology and Biological Sciences Research Council (United Kingdom) via a grant-in-aid and a responsive-mode grant (P19980) to J.A.D.; a financial gift from CERES Inc. to J.A.D. to partially support the visit of A.Z. to the John Innes Centre; PICT 8266 (Agencia de Promoción Científica y Tecnológica, Argentina); and X-811 (UBACyT Programación Científica 2001–2003). A.Z. is a member of CONICET and of the University of Buenos Aires. D.M.R. was supported by CONICET, Argentina; FOMEC-University of Buenos Aires; Fundación Antorchas; and a travel grant (UNESCO-ASM Travel Award) from the American Society for Microbiology to visit the John Innes Centre. A.W. and C.F. were supported by BBSRC studentships.

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