JOURNAL OF BACTERIOLOGY, July 2008, p. 4706–4715 Vol. 190, No. 13
0021-9193/08/$08.00+0 doi:10.1128/JB.01694-07

Glucomannan-Mediated Attachment of *Rhizobium leguminosarum* to Pea Root Hairs Is Required for Competitive Nodule Infection▽

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Received 22 October 2007/Accepted 4 April 2008

The *Rhizobium leguminosarum* biovar viciae genome contains several genes predicted to determine surface polysaccharides. Mutants predicted to affect the initial steps of polysaccharide synthesis were identified and characterized. In addition to the known cellulose (*cel*) and acidic exopolysaccharide (EPS) (*pss*) genes, we mutated three other loci; one of these loci (*gmsA*) determines glucomannan synthesis and one (*gelA*) determines a gel-forming polysaccharide, but the role of the other locus (an *exoY*-like gene) was not identified. Mutants were tested for attachment and biofilm formation in vitro and on root hairs; the mutant lacking the EPS was defective for both of these characteristics, but mutation of *gelA* or the *exoY*-like gene had no effect on either type of attachment. The cellulose (*celA*) mutant attached and formed normal biofilms in vitro, but it did not form a biofilm on root hairs, although attachment did occur. The cellulose-dependent biofilm on root hairs appears not to be critical for nodulation, because the *celA* mutant competed with the wild-type for nodule infection. The glucomannan (*gmsA*) mutant attached and formed normal biofilms in vitro, but it was defective for attachment and biofilm formation on root hairs. Although this mutant formed nodules on peas, it was very strongly outcompeted by the wild type in mixed inoculations, showing that glucomannan is critical for competitive nodulation. The polysaccharide synthesis genes around *gmsA* are highly conserved among other rhizobia and agrobacteria but are absent from closely related bacteria (such as *Brucella* spp.) that are not normally plant associated, suggesting that these genes may play a wide role in bacterium-plant interactions.

Rhizobia have been studied in detail, primarily because of their ability to interact with legume roots producing infected root nodules, within which rhizobia fix N_2 to ammonia, which is then made available to the host plant. There are usually about 100 to 200 nodules on a mature legume, such as a pea, each of which is derived from a clonal infection. After nodule senescence some rhizobia are released back into the soil, where they can reinitiate the cycle of legume infection. However, since pea-nodulating rhizobia are often present at concentrations around 10^2 to 10^6 cells per g of soil, it is clear that most soil rhizobia are unlikely to initiate nodule infection, and so these bacteria must have mechanisms for growing and surviving in soil over prolonged periods. For many bacteria, optimal survival strategies are linked to the formation of aggregates of bacteria, often in the form of biofilms (14). Rhizobia form stable biofilms on inert substrates (25, 49), as well as on legume roots (43).

Up to 20% of total plant photosynthate can be released from roots in the form of mucilage and other carbon sources (22), and strains of *Rhizobium leguminosarum* can use this mucilage from pea roots as a source of carbon and nitrogen for growth (38). Therefore, exudates from roots can be a major source of growth nutrients, and rhizobia have adapted to form biofilms on roots and root hairs. By attaching to and growing on root hairs, rhizobia have the added advantage of possibly being able to initiate nodule infection. In this study, we compared molecular determinants of biofilm formation on an inert surface and on roots, focusing on surface polysaccharides.

Biofilm formation by *R. leguminosarum* requires the initial attachment of individual cells to a surface, followed by growth, aggregation, and accumulation of additional rhizobia (49). The acidic exopolysaccharide (EPS) of *R. leguminosarum* strains is required for attachment to inert substrates; in addition, secreted proteinaceous adhesins play a role in aggregation and biofilm stabilization (4, 49). Attachment to roots and root hairs involves additional specialized mechanisms, partly because plant components also play a role. Plant-made lectins mediate rhizobial attachment to root hairs in many different *Rhizobium*-legume systems (37, 47). The pea lectin involved in attachment of *R. leguminosarum* bv. viciae to root hairs binds the bacteria via a polarly located bacterial polysaccharide called glucomannan (41).

Under slightly alkaline conditions the lectin is released from pea root hairs, significantly reducing this type of attachment (16). However, rhicadhesin, a calcium-binding protein produced by all tested members of the *Rhizobiaceae* (56), can facilitate attachment to root hairs under neutral or alkaline conditions (41). Aggregation of rhizobia following attachment to root hairs is stimulated by, e.g., extracellular adhesins (4) and the production of cellulose fibrils (15, 54). While cellulose-mediated aggregation is not necessary for infection, it may be

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▽ Published ahead of print on 25 April 2008.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
300	*R. leguminosarum* bv. viciae wild type	33
3841	*R. leguminosarum* bv. viciae, streptomycin-resistant derivative of 300	33
A31	Derivative of *R. leguminosarum* 8002 lacking pSYM	19
A34	*R. leguminosarum* bv. viciae A31 carrying pRL1JI	19
A168	Derivative of A34 carrying *pssA1*::Tn*5*	9
A1020	Mutant of 3841, *exoY*::Tn*5*	This study
A1045	Mutant of 3841, *gmsA*::Tn*5*	This study
A1060	Mutant of 3841, *celA*::Tn*5*	This study
A1073	Derivative of 3841 carrying *pssA1*::Tn*5*	This study
A1077	Derivative of A34 carrying *pssA1*::Tn*5*	49
A1090	Mutant of 3841, *gelA*::Tn*5*	This study
A1104	Derivative of 3841 carrying *celA*::Tn*5* (Spc^r) and *pssA1*::Tn*5*	This study
A1208	Derivative of 300 carrying *gmsA*::Tn*5* (Gnt^r)	This study
A1209	Derivative of 300 carrying *exoY*::Tn*5* (Gnt^r)	This study
A1247	Derivative of 300 carrying *gelA*::Tn*5* (Spc^r)	This study
A1248	Derivative of 300 carrying *celA*::Tn*5*	This study
Plasmids		
pRU1319	pOT1-based vector carrying GFPuv (Gnt^r)	2
pHC60	Vector carrying GFP and RK2 stabilization fragment (Tet^r)	13
pPH1	Plasmid for selecting for recombinants	12
pRK2031	Helper plasmid for conjugation	18
pIJ1427	Cosmid carrying *pssA* region	9
pIJ1471	Cosmid carrying *pssA1*::Tn*5*	9

needed for optimal infection of fast-growing root hairs, as opposed to newly emerging root hairs (42).

In addition to the acidic EPS, cellulose, and glucomannan, *R. leguminosarum* bv. viciae produces other surface polysaccharides, including a gel-forming polysaccharide (61) and lipopolysaccharides (35). In this work we used the genome sequence of *R. leguminosarum* bv. viciae strain 3841 (60) to identify genes predicted to be involved in polysaccharide biosynthesis, constructed mutants lacking these polysaccharides, and investigated the role of the polysaccharides in attachment and biofilm formation on glass, roots, and root hairs. This analysis revealed that biofilm formation on glass requires different determinants than biofilm formation on root hairs, that glucomannan-mediated attachment is important for infection, and that under the conditions tested, cellulose-mediated biofilm formation appears not to be essential for competitive nodule infection.

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 (7) or in Y minimal medium (53) containing mannitol (0.2%, wt/vol) as the carbon source and sodium glutamate (6 mM) as the nitrogen source. *Escherichia coli* was grown at 37°C in L medium (51). Bacterial growth was monitored at 600 nm using an MBA 2000 spectrophotometer (Perkin Elmer). Plasmids were mobilized into *R. leguminosarum* by triparental mating using the helper plasmid pRK2013 (18), and transduction was done using RL31 phage (11).

Isolation of Tn*5* mutants. Tn*5* mutagenesis of *R. leguminosarum* bv. viciae 3841 by conjugal transfer of a suicide plasmid was done as described previously (12). The filters from the mating procedures were cut into sectors, and the Tn*5* mutants were selected on Y medium containing streptomycin, kanamycin, and neomycin; this strategy ensured that each pool of mutants was different. A total of 576 sectors were plated, giving about 30 colonies per plate, and a glycerol stock was made from each plate. The stocks of mutants were placed in a 24-by-24 array in six 96-well microtiter plates, and then 100-μl aliquots from each row were combined and 100-μl aliquots from each column were combined, producing 48 2.4-ml pools, and DNA was prepared from each pool. To identify a desired mutant, we designed a gene-specific primer and used a Tn*5*-specific primer to perform PCRs with the 48 pools of DNA. Equal-size PCR products that were the appropriate size from the vertical pools and horizontal pools were used to identify the glycerol stock containing the desired mutation. Single colonies plated from the glycerol stock were screened by single-colony PCR, and the location of the Tn*5* insertion was confirmed by DNA sequencing of the PCR product. The primers used are summarized in Table 2. All of the numbers used in "RL" or "pRL" designations correspond to gene identifiers annotated in the complete genome sequence at http://www.sanger.ac.uk/Projects/R_leguminosarum/.

Generation of *pssA*::Tn*5* mutant strains. Cosmid pIJ1471 (9) carrying *pssA1*::Tn*5* was conjugated into the appropriate parental strain (3841 or A34), and recombinants containing Tn*5* were selected by introduction of the incompatible plasmid pPH1 (12). The resultant EPS-deficient strains were complemented with pIJ1427 carrying an intact *pssA* gene (9), and phage propagated with this complemented mutant was used to transduce *pssA*::Tn*5* into recipients (3841 or A34). To create the *celA pssA* double mutant A1104, the antibiotic resistance carried on *celA*::Tn*5* was switched from kanamycin resistance to spectinomycin resistance as previously described (48), and the resultant strain was transduced to kanamycin resistance with phage carrying *pssA1*::Tn*5*. Surface polysaccharides were isolated and fractionated as previously described (41). Cellulose was measured by the Updegraaf method as described by Fry (24); assays (three biological replicates) were done using cells from 300-ml cultures grown for 5 days in TY medium for each assay.

Biofilm formation. For analysis of biofilm growth, bacteria were grown in TY medium (containing appropriate antibiotics) for 2 days (optical density at 600 nm

TABLE 2. Primers used to identify Tn*5* mutants

Gene	Primer sequence	Size of gene (bp)	Insertion point[a]
RL1646 (*celA*)	5′-CGTCAATCTGGGGTAAC	2,226	1882
RL1661 (*gmsA*)	5′-TATTTCTTAGGGGCTTT CTGG	1,563	504
RL3820 (*exoY*)	5′-CGATTGCCCGTTAGTA GCAT	702	150
RL4404 (*gelA*)	5′-TTTCAAAGTATAAATTT CCCATAAG	738	422

[a] Insertion point relative to the predicted translational start codon.

TABLE 3. BLAST identification of polysaccharide biosynthetic loci

Strain 3841 predicted protein	Query sequence		% Similarity/% identity	E value	Exopolysaccharide Produced
	Protein	Organism (reference)			
RL3752	PssA	*R. leguminosarum* bv. trifolii (32)	96/98	$1.8e^{-133}$	Acidic EPS
RL3751	PssB	*R. leguminosarum* bv. trifolii (32)	96/97	$2.8e^{-135}$	Acidic EPS
RL3653	PssC	*R. leguminosarum* bv. trifolii (40)	96/98	$4.1e^{-175}$	Acidic EPS
RL3654	PssD	*R. leguminosarum* bv. trifolii (40)	86/88	$9.5e^{-64}$	Acidic EPS
RL3655	PssE	*R. leguminosarum* bv. trifolii (40)	91/96	$2.3e^{-76}$	Acidic EPS
RL1646	CelA	*A. tumefaciens* (45)	71/83	$2.1e^{-288}$	Cellulose
RL1647	CelB	*A. tumefaciens* (45)	51/69	$9.2e^{-169}$	Cellulose
RL1648	CelC	*A. tumefaciens* (45)	54/63	$4.4e^{-75}$	Cellulose
RL1649	CelG	*A. tumefaciens* (44)	52/66	0	Cellulose
RL4640	NdvA	*S. meliloti* (21)	77/78	$2e^{-237}$	Cyclic glucan
RL4644	NdvB	*S. meliloti* (30)	69/81	0	Cyclic glucan
RL3820	ExoY	*S. meliloti* (26)	53/66	$4.5e^{-57}$	Not known
RL3823	ExoF	*S. meliloti* (26)	34/56	$3.2e^{-63}$	Not known
RL3821	ExoP	*S. meliloti* (26)	40/57	$3.9e^{-138}$	Not known
RL3816	ExoT	*S. meliloti* (26)	24/46	$1.5e^{-40}$	Not known
RL1661	PssA	*R. leguminosarum* bv. trifolii (32)	48/66	$8.9e^{-48}$	Glucomannan
RL1664	ExoP	*S. meliloti* (26)	25/44	$1.3e^{-42}$	Glucomannan
RL4404	PssA	*R. leguminosarum* bv. trifolii (32)	58/70	$9e^{-68}$	Gel-forming polysaccharide

[OD_{600}], about 1.5), and then the culture was used at a 1:1,000 dilution to inoculate 100 ml Y medium. Rings of biofilm at the air-liquid interface were qualitatively scored after 2 to 9 days of growth in 250-ml conical flasks shaken at 300 rpm in an orbital shaker. Biofilm growth on glass was monitored in static cultures by confocal microscopy as previously described (49).

Nodulation assays. Nodulation tests were performed with peas (*Pisum sativum* L. var. Frisson) as described previously (8), using a minimum of 16 matched plants per test; at least two separate tests were carried out, which produced similar results. To assess competitive nodulation, the relevant mutations were transduced into strain 300, the streptomycin-sensitive parent of strain 3841. Equal numbers of the wild-type and mutant strains were coinoculated onto germinated peas in a vermiculite-sand mixture (50:50, vol/vol). After 4 weeks of plant growth, bacteria were isolated from surface-sterilized nodules and plated onto TY medium containing streptomycin, kanamycin, or both antibiotics. At least 100 nodules from at least five separate plants in each test were checked; less than 1% of the nodules showed dual occupancy, and these nodules were excluded from the analysis.

Root attachment assays. Root attachment was assayed using strains carrying pHC60 or PRU1319, which expresses the green fluorescent protein (GFP). Strains were pregrown in Y medium to an OD_{600} of ~0.7 and then resuspended in 25 mM phosphate buffer at either pH 6.5 or 7.5 to a final OD_{600} of 0.07. Two milliliters of a bacterial suspension was then placed into a modified Fahraeus slide, into which a root (about 1 cm) from a sterile germinated vetch (*Vicia hirsuta*) seedling was inserted. The slides were incubated for 90 min at room temperature, and then root attachment was observed by confocal laser scanning microscopy with a Leica SP microscope using 488-nm argon laser excitation and a 500-nm long-pass emission filter, which allowed observation of GFP-labeled bacteria, and transillumination, which showed root hairs. Images were processed using LCSLite confocal software.

RESULTS

Identification of surface polysaccharide genes in *R. leguminosarum* bv. viciae 3841. Several genes for production of extracellular EPSs have been identified in different strains and biovars of *R. leguminosarum*, but the only polysaccharide mutants described so far for the sequenced strain 3841 are mutants with mutations that affect lipopolysaccharide biosynthesis (1, 34). To identify EPS genes in strain 3841, we performed BLAST searches (3) using protein sequences from known polysaccharide biosynthetic genes from different rhizobia. We focused on clusters of genes containing genes encoding predicted isoprenyl phosphate glycosyl transferases and/or polysaccharide export genes, because these genes are essential for polysaccharide biosynthesis and export. Other genes, such as those encoding glycosyl transferases or epimerases, are poor predictors of surface polysaccharides, because they may be involved in various cellular processes. Our analysis led to the identification of several clusters of genes predicted to be involved in formation of exopolysaccharides other than lipopolysaccharide (Table 3).

Biosynthesis of the acidic EPS is determined by the *pss* genes. As observed for different strains of *R. leguminosarum* (32, 50), two clusters of genes were identified; one cluster (RL3752 and RL3751) contains the *pssA* and *pssB* genes, and the other (RL3642 to RL3663) contains the *pssUSRMLKJIH GFCDE* and *pssPONT* genes (50) separated by genes encoding a polysaccharide lyase (*plyA*) and its secretion system (*prsDE*) (23). The PssA to PssE proteins listed in Table 3 are only representative and are the proteins whose roles have been biochemically defined. PssA catalyzes the first step in biosynthesis of the acidic EPS, transferring UDP-glucose to an isoprenyl phosphate lipid carrier (31), and PssC, PssD, and PssE transfer two glucuronic acid residues onto the lipid-linked glucose (59).

The PssA BLAST search identified RL1661 with a high score, and the adjacent genes are RL1662 encoding a predicted glycosyl transferase (containing the Pfam 00534 glycosyl transferase domain, with an expected value of $4e^{-22}$) and the polysaccharide secretion genes RL1663 and RL1664. The predicted product of RL1663 contains a polysaccharide export conserved protein domain (Pfam 02563, $2e^{-28}$), and the RL1664 protein (Table 3) is most similar to the EPS transport protein ExoP of *Sinorhizobium meliloti* (26). This cluster of four genes (RL1661 to RL1664) is highly conserved in *Rhizobium etli*, *S. meliloti*, *Mesorhizobium loti*, *Agrobacterium tumefaciens*, and *Agrobacterium rhizogenes* but not in *Brucella melitensis*, an animal pathogen which is phylogenetically very closely related to *Rhizobium* and *Agrobacterium* spp. This suggests that these genes form (part of) a polysaccharide biosynthesis cluster that is well conserved among the plant-associated bacteria in this clade. A third predicted protein similar to PssA

TABLE 4. Polysaccharide production by wild-type and mutant strains of *R. leguminosarum* bv. viciae

Strain	Gene (genome identifier)	Polysaccharide production (mg/liter of culture)				
		Acidic EPS	Capsular polysaccharide	Glucomannan	Cyclic glucans	Gel-forming polysaccharide
3841 (wild type)		848 ± 19	217 ± 23	105 ± 13	25 ± 4	174 ± 15
A1020	*exoY* (RL3820)	768 ± 26	243 ± 20	131 ± 15	18 ± 7	172 ± 17
A1045	*gmsA* (RL1661)	975 ± 20	249 ± 16	3 ± 2	23 ± 9	160 ± 12
A1073	*pssA* (RL3752)	17 ± 7	4 ± 4	86 ± 18	27 ± 11	180 ± 20
A1090	*gelA* (RL4404)	911 ± 34	229 ± 22	94 ± 21	24 ± 8	16 ± 9

is the RL4404 protein, but the RL4404 gene is not flanked by genes likely to be involved in polysaccharide biosynthesis. The roles of RL1661 to RL1664 and RL4404 or their orthologues have not been described previously.

In *S. meliloti*, ExoY catalyzes the first step in extracellular succinoglycan biosynthesis, transferring UDP-galactose to the lipid carrier (26); ExoY requires ExoF for its activity (26). A BLAST search with ExoY identified the RL3820 protein as the most similar protein in strain 3841, and the gene is located in a 10-gene cluster (RL3815 to RL3824) encoding ExoY- and ExoF-like proteins (Table 3), four predicted glycosyl transferases, a predicted endoglycanase, two putative EPS export proteins, and a predicted regulator (data not shown).

Four cellulose biosynthesis genes (*celABCG*) predicted to be in a single operon are conserved in the genome sequences of rhizobia and agrobacteria (27, 28, 44, 60). We used the four *A. tumefaciens* C58 *celABCG* cellulose synthesis gene products for BLAST searches and identified the orthologous genes RL1646 to RL1649 (Table 3). Elsewhere (RL1729 and RL1730) there are two genes probably orthologous to the identified *celR1* and *celR2* genes encoding predicted regulators of cellulose production (5).

We identified other clusters of predicted EPS genes, including a cluster of six genes (RL3628 to RL3633) encoding three predicted glycosyl transferases, an epimerase, a possible sugar methyl or acetyltransferase, and a predicted transporter protein. There were also three clusters of known lipopolysaccharide genes. In addition, we identified the RL4640 and RL4644 genes, which are orthologous to the well-characterized *ndvA* and *ndvB* genes (20), whose products synthesize a periplasmic oligosaccharide involved in osmoregulation. We did not identify a gene cluster equivalent to the *S. meliloti rkpABCDEF GHIJ* genes determining production of the K antigen (36), and we did not identify a gene cluster equivalent to the *exs* genes of *S. meliloti* determining production of the galactoglucan (6). Some of the *exs* and *rkp* gene products showed low levels of similarity to the glycosyl transferases identified above, but we concluded that there is no strong evidence for the presence of such components in *R. leguminosarum* bv. viciae strain 3841.

Isolation of strains carrying mutations in predicted polysaccharide biosynthesis genes. To obtain mutants lacking specific polysaccharides, we targeted the genes predicted to encode the first step in polysaccharide biosynthesis, because mutations in such genes are usually specific for individual polysaccharides. As described in Materials and Methods, we first generated an arrayed library of Tn*5* mutants and then screened pools of these mutants by PCR, using gene-specific primers and a Tn*5* primer and checking the locations of Tn*5* in mutants by DNA sequencing from the ends of Tn*5*. In this way we generated strains carrying mutations in the RL3820 (A1020), RL1661 (A1045), RL4404 (A1090), and RL1646 (A1060, *celA*::Tn*5*) open reading frames. We failed to isolate a *pssA* mutant and so used homologous recombination to recombine the *pssA1*::Tn*5* allele (9) into strain 3841 to produce A1073 (*pssA*).

Using established methods (41), we isolated and quantified the acidic EPS from the culture supernatant, the acidic polysaccharide fraction associated with the cells (capsular polysaccharide), the gel-forming polysaccharide, the glucomannan, and the cyclic glucans. As expected, the *pssA* mutant (A1073) essentially lacked both EPS and capsular polysaccharide (Table 4). It was more difficult to confirm that the *celA* mutant lacked cellulose, because Congo red staining (for cellulose) did not distinguish between A1060 (*celA*) and wild-type strain 3841 (Fig. 1A and B), probably because of interference by the large amount of acidic EPS. To overcome this problem, we altered the antibiotic resistance in the *celA* mutant and then transduced the *pssA1*::Tn*5* mutation into the *celA* mutant to form A1104 (*celA pssA*). Comparison of Congo red staining of A1073 (*pssA*) and Congo red staining of A1104 (*pssA celA*) (Fig. 1C and D) revealed that the *celA* mutation reduced staining, indicating that it affected cellulose production. To confirm that the mutation affects cellulose formation, levels of cellulose production were determined; A1073 produced 73 ± 20 ng cellulose per 10^9 cells, whereas the *celA* mutation in A1104 reduced the level to the detection limit of the assay (20 ± 15 ng cellulose per 10^9cells).

Mutations specifically affecting glucomannan and gel-forming polysaccharide formation have not previously been identi-

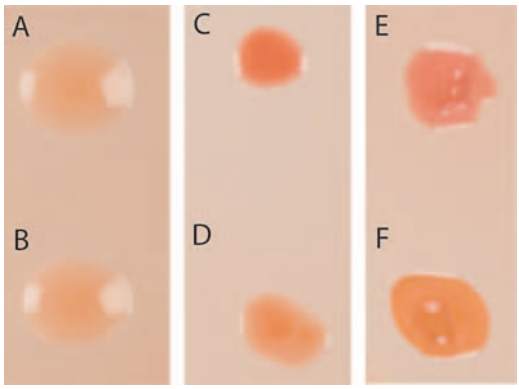

FIG. 1. Bacterial colonies grown on TY agar containing Congo red to stain for cellulose. (A to D) Strain 3841 (wild type) (A) and its derivatives A1060 (*celA*) (B), A1073 (*pssA1*) (C), and A1104 (*pssA1 celA*) (D). (E and F) Colonies of A168 (*pssA1*) (E) and A1077 (*pssA1*) (F), which are derivatives of A34.

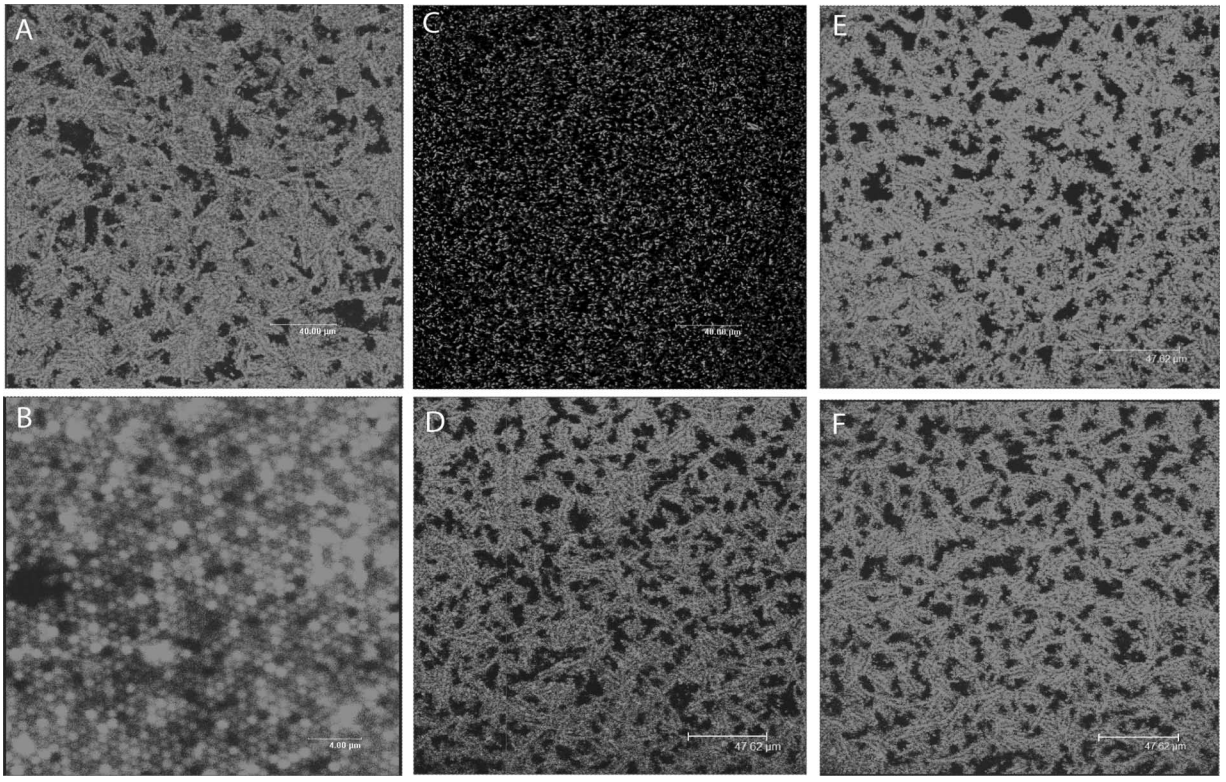

FIG. 2. Effects of mutations affecting surface polysaccharides on formation of biofilms in static culture. (A) Strain 3841 (wild type) formed a distinctive biofilm after 4 days of growth in static culture in Y medium, with microcolonies separated by voids. (B) Like panel A but with ×8 digital zoom, showing that cells within the microcolonies are in a close-packed hexagonal array. (C to F) The *pssA* mutant (C) formed a flat loosely attached lawn, whereas the *celA* (A1060) (D), *gelA* (A1090) (E), and (F) *gmsA* (A1045) (F) mutants formed biofilms indistinguishable from those formed by the wild-type strain.

fied. A1045 (carrying Tn*5* in RL1661) lacks detectable glucomannan, and so we designated the gene involved *gmsA* (glucomannan synthesis) (Table 4). Strain A1090, carrying Tn*5* in RL4404, is specifically affected in the formation of gel-forming polysaccharide (Table 4), and so we designated the gene involved *gelA*. Mutant A1020 carrying Tn*5* in RL3820 was not affected in the production of any of the identified polysaccharides (Table 4), even though the mutation is likely to be polar on four downstream polysaccharide biosynthesis genes (homologues of *exoP* and *exoF*, a predicted glycosyl transferase gene, and a predicted transmembrane protein gene). The lack of an observed phenotype in A1020 may have been because these genes are induced under conditions different from those tested here, or it could have been because there is some problem with the regulation or expression of these genes in the wild-type strain, as has been observed with galactoglucan production in *S. meliloti* (46).

Attachment and biofilm assays with polysaccharide mutants in vitro and on legume roots. We previously analyzed attachment and biofilm growth of a different strain of *R. leguminosarum* bv. viciae grown in in vitro static cultures using confocal microscopy of GFP-labeled strains. We transferred a GFP-expressing plasmid into strain 3841 and all the mutants described in Table 4 and analyzed biofilm formation in slides with glass chambers. The wild-type strain attached and formed a biofilm on glass indistinguishable from the biofilm observed previously (49) for strain A34 (Fig. 2A), with interconnected clusters of cells and open channels. Some of the bacteria were in hexagonal close-packed arrays (Fig. 2B). The *pssA* mutant A1073 formed a flat unstructured biofilm (Fig. 2C) similar to that observed previously for a *pssA* mutant in another background (49). The other mutants, A1060 (*celA*) (Fig. 2D), A1090 (*gelA*) (Fig. 2E), A1045 (*gmsA*) (Fig. 2F), and A1020 (*exoY*) (not shown), formed biofilms indistinguishable from that of the wild type. These mutants were also tested for the formation of biofilm rings in shake flask cultures; as described previously for a different strain of *R. leguminosarum* bv. viciae (49), the *pssA* mutant (A1073) formed only very faint biofilm rings even after 7 days of growth, a time when the wild-type strain (3841) had formed clear biofilm rings. None of the other mutants was significantly different from the wild type with regard to timing or the amount of biofilm rings produced in shaken cultures (data not shown), suggesting that the acidic EPS is a key determinant of biofilm formation by *R. leguminosarum* on a glass surface.

Roles of different EPSs in root attachment and biofilm formation. We analyzed attachment of *R. leguminosarum* bv. viciae to roots using seedlings of its host plant *V. hirsuta* (vetch) by coincubating the bacteria and roots on a covered microscope slide in FP medium at pH 6.5 and 7.5. As observed previously with excised pea roots, we observed attachment of individual wild-type (GFP-labeled strain 3841) bacterial cells, followed by "cap formation," in which additional bacterial cells attached to those bacteria that directly attached to root hairs

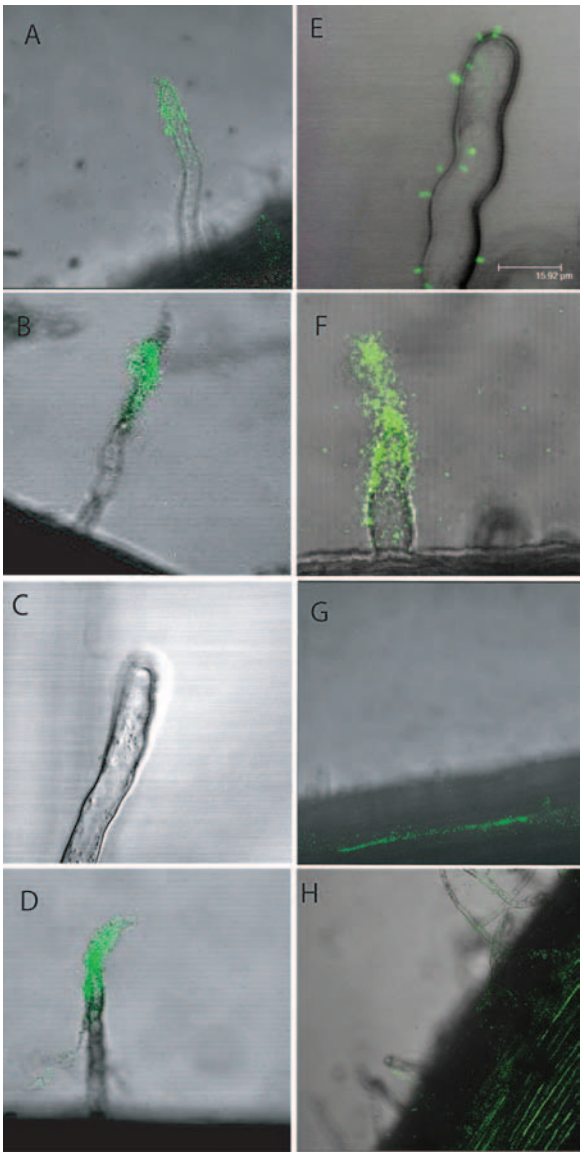

FIG. 3. Role of surface polysaccharides in root hair attachment. Strain 3841 (wild type) forms a root hair cap when it is incubated with vetch root hairs at pH 6.5 (A) or pH 7.5 (B). The glucomannan mutant A1045 (*gmsA*) was unable to attach or form a root hair cap at pH 6.5 (C) but formed root hair caps at pH 7.5 (D). Individual cells of the cellulose mutant A1060 (*celA*) attached to root hairs, but no cap was formed at pH 6.5 (E) or pH 7.5 (not shown). Normal attachment and cap formation was seen with A1090 (*gelA*) at pH 6.5 (F) and pH 7.5 (not shown). The acidic EPS mutant A1073 (*pssA*) did not attach to root hairs, but it did attach at root epidermal cell boundaries (G), as did wild-type strain 3841 (H).

(Fig. 3A and B). In this system, the majority of root hairs (>70%) in the growing root hair zone were coated with *R. leguminosarum* bv. viciae cells.

At pH 6.5, very few cells of the *gmsA* glucomannan mutant (A1045) were attached to root hairs (Fig. 3C), and biofilm caps were present on less than 2% of the root hairs. This is consistent with the prediction (41) that the glucomannan is required for lectin-mediated attachment to root hairs. At alkaline pH, the root lectin which binds the glucomannan is released from the root hairs, and this probably prevents glucomannan-mediated attachment (16, 41). An alternative mechanism of attachment has been proposed to occur at alkaline pH, a mechanism mediated via bacterially made rhicadhesin (56). As shown in Fig. 3D, the glucomannan mutant (A1045) attached and induced cap formation on roots at pH 7.5, demonstrating that glucomannan is not required for root hair attachment at alkaline pH.

Individual cells of the cellulose-deficient (*celA*) mutant A1060 attached to root hairs, but no biofilm caps were formed (Fig. 3E); similar results were obtained at pH 7.5 (data not shown). This confirms the observation (55) that cellulose is required for biofilm cap formation on root hairs. In contrast, normal tight interactions occurred between individual cells of the *celA* mutant in in vitro biofilms (Fig. 2D).

Normal attachment and biofilm cap formation on roots and root hairs were observed with the *gelA* mutant (A1090) defective for gel-forming polysaccharide at both pH 6.5 (Fig. 3F) and pH 7.5 (data not shown), and similar results were obtained with strain A1020 carrying a mutation in a predicted polysaccharide biosynthesis gene with an unknown function (data not shown).

Mutation of *pssA* has been shown to strongly reduce the number of infection foci (58), and we observed little or no root hair attachment or cap formation with the *pssA* mutant (A1073) lacking the acidic EPS. However, the *pssA* mutant did attach to the root surface along the boundary of adjacent root epidermal cells (Fig. 3G), which was also observed with wild-type strain 3841 (Fig. 3H). Similar attachment was seen with the *pssA* mutant and the wild type at pH 7.5 (data not shown), demonstrating that the acidic EPS is involved in root hair attachment but is not essential for attachment at the boundaries of root epidermal cells under both pH conditions tested.

The differences in root hair and in vitro attachment seen with the glucomannan and cellulose mutants implies that the structure of the root hair cap biofilm is different from that of the in vitro biofilms, suggesting that in addition to the acidic EPS cellulose fibrils and glucomannan play roles in root hair attachment but not in in vitro attachment.

Roles of different EPSs in legume infection and nodulation. Pea seedlings inoculated with *R. leguminosarum* bv. viciae 3841 formed white nodules, which first appeared 7 days after inoculation and became pink after another 3 to 4 days, at which point they could fix nitrogen based on assays of acetylene reduction (data not shown). Previously, the *pssA1*::Tn*5* allele preventing acidic EPS production was reported to block nodule development and infection (10). In contrast, the A1073 mutant carrying the same *pssA1*::Tn*5* allele formed white nodules after 7 days; the nodules did not become pink within the next 10 to 14 days, and 21 days after inoculation the plants showed signs of nitrogen deficiency (reduced growth and yellowed leaves) and no significant acetylene reduction was detected (the reduction was less than 1% of the wild-type acetylene reduction). At this time point the number of nodules was increased compared to the wild type (115 nodules compared with 90 nodules), which is often observed with plants inoculated with *R. leguminosarum* bv. viciae mutants unable to fix nitrogen. By about 28 days after inoculation, many of the nodules induced by the *pssA* mutant had turned slightly pink, and acetylene reduc-

tion assays showed that nitrogen fixation had begun, with the rate reaching about 35% of the maximal rates seen with roots inoculated with the wild type. This demonstrated that infection had occurred, and bacteria isolated from the pink nodules induced by A1073 (*pssA*) had a colony morphology typical of the *pssA* mutant and induced small white nodules when they were reinoculated onto peas, showing that a suppression/reversion event had not occurred.

In order to investigate the discrepancy between the phenotypes of A1073 (*pssA*) and the *pssA* mutant 8401/pRL1JI::*pssA1* described previously (9), we used strain A1077, in which the *pssA* mutation in 8401/pRL1JI::*pssA1* was transduced back into the parental strain 8401/pRL1JI. Inoculation of 8401/pRL1JI::*pssA1* onto peas confirmed that it did not induce nodule morphogenesis, whereas the supposedly identical strain A1077 induced production of small white nodules, which eventually turned pink, similar to the nodules induced by strain A1073 (*pssA*). It was also apparent that when 8401/pRL1JI::*pssA1* was grown in liquid culture, it flocculated to a much greater extent than A1077. Such flocculation can be caused by increased production of cellulose (5). When plated onto TY agar containing Congo red to stain for cellulose, 8401/pRL1JI::*pssA1* stained significantly more intensely than the other *pssA* mutants, A1077 and A1073 (Fig. 1); several other transductants carrying the *pssA1*::Tn*5* allele exhibited little Congo red staining (data not shown). This suggests that strain 8401/pRL1JI::*pssA1* used previously has a background mutation which increases cellulose production. High levels of cellulose production in an EPS-deficient strain have previously been shown to cause *R. leguminosarum* bv. viciae to become entrapped in infection threads, preventing successful invasion of root hairs (42), and this may be why the 8401/pRL1JI::*pssA1* mutant is defective for infection even after prolonged periods. The *gmsA* (A1005), *exoY* (A1020), *gelA* (A1090), and *celA* (A1060) mutants all induced production of normal numbers of pink nodules, and the plants showed no signs of nitrogen stress (data not shown).

In order to test for their ability to compete with the wild type during nodule infection, the *gmsA*, *exoY*, *gelA*, and *celA* mutations were tested to determine their effects on competitive nodulation in coinoculation experiments with wild-type strain 3841. In order to have different selectable markers for each strain, the mutations were transduced into strain 300 (the streptomycin-sensitive parent of strain 3841). Four weeks after inoculation of peas with mixed cultures of the wild type and each mutant, nodules were excised, and ex nodule bacteria were plated on selective media. At least 100 nodules from at least five separate plants used in each test were checked. Less than 1% of the nodules showed dual occupancy, and such nodules were excluded from the analysis. The *celA*, *gelA*, and *exoY* mutants were as competitive as the wild type for nodule occupancy, whereas the glucomannan (*gmsA*) mutant was strongly outcompeted (Fig. 4).

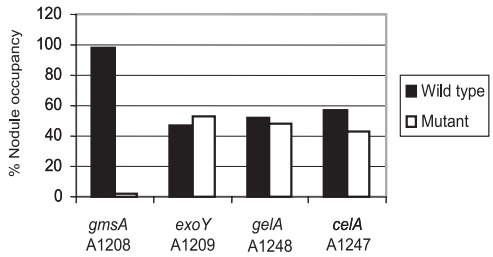

FIG. 4. Nodulation competitiveness of surface polysaccharide mutants. Each of the mutants shown was coinoculated onto peas with equal numbers of wild-type strain 3841 cells. The nodule occupancy of each mutant is expressed as a percentage of the bacteria recovered from individual nodules based on scoring using antibiotic resistance (streptomycin for the wild type, gentamicin for A1208 [*gmsA*] and A1209 [*exoY*], spectinomycin for A1247 [*gelA*], and kanamycin for A1248 [*celA*]). The *gmsA* mutant is significantly less competitive than the wild type, but the other mutants are not significantly different from the wild type, based on a chi-square test.

DISCUSSION

Using the *R. leguminosarum* bv. viciae strain 3841 genome sequence, we identified likely exopolysaccharide biosynthesis loci and isolated mutations in key genes. The PCR-based screen that we used to identify mutations was chosen because (i) we wanted to make many targetted mutations in this strain, whose sequence was recently completed; (ii) once established, the pools of mutants could be screened for gene-specific mutations by PCR more quickly than constructs for double-crossover mutations could be generated; (iii) Tn*5* mutations are very stable in *R. leguminosarum* bv. viciae (much more stable than single-crossover integration events); and (iv) we established a resource that could be used repeatedly over a long period of time.

Isolation of strains carrying mutations in different predicted polysaccharide biosynthesis loci resulted in identification of the *gmsA* and *gelA* genes, which are specifically required for the synthesis of glucomannan and gel-forming polysaccharides, respectively. Mutants specifically lacking these polysaccharides had not previously been described. A pleiotropic *exoB* mutant (defective for UDP-galactose formation due to loss of UDP-glucose-4-epimerase activity) lacked glucomannan, had a greatly reduced level of acidic EPS, had altered lipopolysaccharide (41, 52), and probably lacked the galactose-rich gel-forming polysaccharide. The sequence conservation of *gmsA*, the conserved location of this gene adjacent to predicted glycosyl transferase and polysaccharide secretion genes, and the conservation of the genes in rhizobia and agrobacteria but not in the closely related organism *B. melitensis* suggest that glucomannan or a similar neutral polysaccharide is produced by various different rhizobia and agrobacteria that interact with plants.

In contrast, the *gelA* gene that we identified here as a gene that is required for biosynthesis of gel-forming polysaccharide is not adjacent to other genes predicted to be required for polysaccharide synthesis. Other genes must be required for the production of the hexasaccharide repeat (containing mannose, glucose, and four galactose residues) that constitutes the gel-forming polysaccharide (61), but such genes were not identified in this study.

In addition, we mutated another locus (*exoY*) predicted to determine an extracellular polysaccharide, but we were unable to identify which polysaccharide is determined by this region,

possibly because the polysaccharide may be expressed under conditions not tested here.

As described previously (49), *R. leguminosarum* bv. viciae grown in static culture in minimal medium produces biofilms on glass with interconnected clusters of cells interspersed with water-filled channels. Blocking the formation of the acidic EPS by mutation of *pssA* abolishes the formation of such biofilms. In contrast, the formation of these biofilms by mutants lacking glucomannan (*gmsA*), gel-forming polysaccharide (*gelA*), or cellulose (*celA*) was not affected.

A key question was whether the in vitro biofilms are similar to the biofilms formed on root hairs or, in other words, whether the molecular determinants of stress survival are similar to the molecular determinants required for colonization of a favorable niche. The observation that the cellulose and glucomannan mutants were defective for root hair colonization but not for in vitro biofilm production suggests that the interactions between the bacteria and the glass surface are different from the interactions occurring during root cap formation. This may be related to the absence of plant components, such as lectins or cellulose, on an inert surface.

It was clear from the competitive nodule infection tests that glucomannan-mediated attachment is important for legume infection. This fits well with the observations and predictions of Laus et al. (41), who demonstrated that polar attachment to root hairs occurs as a result of root hair lectin binding to the polarly located glucomannan. It also fits with the model postulating that enhanced rhizobial attachment to transgenic legume roots expressing pea lectin can enhance nodulation capacity (17, 57). Plant lectins may also induce additional effects because they can modify rhizobia to enhance their nodulation capacity (29), possibly as a result of changes to surface polysaccharides (39).

We were surprised that cellulose, which plays such a significant role in formation of biofilm caps on root hairs, did not play a significant role in in vitro biofilm formation. We were also surprised that under our test conditions the cellulose-deficient mutant was as infective as the wild type. Biofilm cap formation on root hairs is a two-step process consisting of attachment followed by aggregation (15, 55, 56). The glucomannan is important for the attachment, whereas the cellulose is important for the aggregation. Our observations suggest that the cellulose-mediated aggregation that was observed on root hairs shortly after inoculation is relatively unimportant for competitive nodule infection under the conditions tested. It seems likely that cellulose fibrils is associated primarily with the growth of *R. leguminosarum* bv. viciae on roots rather than with infection. A cellulose-deficient mutant has been shown to was reduced in its ability to infect older root hairs of vetch, although it infected young root hairs normally (42). The way that cap formation is established may be critical, because in addition to the formation of caps mediated by cellulose, lectins can also promote the formation of caps by some rhizobia, and this type of cap formation is correlated with enhanced infection by these bacteria (37). Furthermore, under soil conditions, increasing the rhizobial rhizosphere population by enhancing growth on roots or root hairs is likely to have an indirect effect on infection by simply increasing the numbers of bacteria, and so such enhanced growth may be brought about by cellulose- and/or lectin-glucomannan-mediated attachment.

In mutants lacking the acidic EPS, cellulose has been shown to interfere with infection by *R. leguminosarum* bv. viciae (41). We demonstrated that the lack of nodulation by the *pssA* mutant used previously (9) is due to a second mutation causing increased cellulose production, corroborating the previous observations (42). The *pssA* mutant of strain 3841 has a very low level of cellulose production, and it seems likely that the *pssA* mutant bacteria identified along the intercellular boundaries may account for the observed nodule infection events.

R. leguminosarum bv. viciae has different modes of attachment and biofilm formation. On roots at pH values less than 7 there is glucomannan-lectin mediated attachment, and at pH values greater than 7 there is rhicadhesin-mediated attachment; the glucomannan-mediated attachment to roots is required for competitive nodule infection. It is clear that attachment to glass is glucomannan independent, and since attachment to glass can occur at acidic pH values at which rhicadhesin is solubilized, it is unlikely that rhicadhesin mediates attachment to glass. However, the presence of the acidic EPS is required for attachment to both glass and root hairs. Following attachment biofilm structures develop on roots and on glass, but the fact that cellulose biosynthesis genes are required for the former but not for the latter implies that the biofilms are different. It should be noted that the biofilms formed in vitro are formed during growth, whereas the biofilms formed on roots develop within a relatively short time.

Given the likely diverse ecological niches that rhizobia occupy and the different timing of events relevant for survival in these niches, it makes sense that these bacteria can adapt to different situations by producing different types of biofilms. The tight-packed biofilms on glass may reflect a mechanism for survival in soil.

ACKNOWLEDGMENTS

We thank Grant Calder for advice on confocal microscopy, Nick Tucker for help with separation of polysaccharides by column chromatography, Anne Edwards for help with strains and plasmids, the staff at the John Innes Genome Centre for help with DNA sequencing, Philip Poole for providing pRU1319, and Paul Barrat and Paul Derbyshire for advice concerning cellulose analysis. We are indebted to Jan Kijne for his thoughtful and critical comments on the manuscript.

This work was supported by the Biotechnology and Biological Sciences Research Council via a grant-in-aid, by response-mode grants P19980 and BB/ED17045, and by a studentship (to A.W.); by a John Innes Foundation award to M.K.; by a financial gift from CERES Inc. to J.A.D. used to partially support a visit by A.Z. to the John Innes Centre; and by a UNESCO-ASM travel award from the American Society for Microbiology (to D.R.).

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