Structural and putative regulatory genes involved in cellulose synthesis in *Rhizobium leguminosarum* bv. *trifolii*

Nora Ausmees,1 Hans Jonsson,1 Stefan Höglund,2 Hans Ljunggren1 and Martin Lindberg1

Author for correspondence: Nora Ausmees. Tel: +46 18 67 32 06. Fax: +46 18 67 33 92.
e-mail: nora.ausmees@mikrob.slu.se

Six genes involved in cellulose synthesis in *Rhizobium leguminosarum* bv. *trifolii* were identified using Tn5 mutagenesis. Four of them displayed homology to the previously cloned and sequenced *Agrobacterium tumefaciens* cellulose genes *celA*, *celB*, *celC* and *celE*. These genes are organized similarly in *R. leguminosarum* bv. *trifolii*. In addition, there were strong indications that two tandemly located genes, *celR1* and *celR2*, probably organized as one operon, are involved in the regulation of cellulose synthesis. The deduced amino acid sequences of these genes displayed a high degree of similarity to the *Caulobacter crescentus* DivK and PleD proteins that belong to the family of two-component response regulators. This is to our knowledge the first report of genes involved in the regulation of cellulose synthesis. Results from attachment assays and electron microscopic studies indicated that cellulose synthesis in *R. leguminosarum* bv. *trifolii* is induced upon close contact with plant roots during the attachment process.

**Keywords:** *Rhizobium leguminosarum* bv. *trifolii*, cellulose synthesis, two-component regulation

INTRODUCTION

Cellulose production has been documented in several bacterial genera such as *Acetobacter*, *Agrobacterium*, *Rhizobium* and *Sarcina* (Ross et al., 1991). *Acetobacter xylinus* (previously *Acetobacter xylinum*) has served as a model organism in studies of bacterial cellulose synthesis at the molecular and biochemical level (Wong et al., 1990; Saxena et al., 1990, 1991; Saxena & Brown, 1995). Two operons from *Agrobacterium tumefaciens* with genes encoding proteins involved in cellulose synthesis (*celABC* and *celDE*) have also been characterized (Mathysse et al., 1993a, b). The results suggest that cellulose synthesis in these two organisms involves at least two homologous genes, a cellulose synthase and an endoglucanase. The other three genes in the *Ag. tumefaciens cel* operons show no homology to known genes in *Ac. xylinus*, thus reflecting differences in the mechanisms of cellulose synthesis in these two bacteria. Mathysse et al. (1995b) have suggested a model pathway for cellulose production in *Ag. tumefaciens*. In this model, cellulose synthesis proceeds via several lipid-linked intermediates, involving the CElE and CElB proteins. A cellulose synthase (the product of the *celA* gene) adds additional glucose moieties to these lipid-glucose intermediate products. The incorporation of the last intermediate into cellulose requires the CElC protein. The exact function of all the *cel* genes is not yet clear.

Here we report the cloning of genes involved in cellulose synthesis (the *cel* genes) from *Rhizobium leguminosarum* bv. *trifolii*. The primary sequence and the organization of the genes are similar to that of *Ag. tumefaciens* (Mathysse et al., 1995a). In addition, we have identified two putative regulator genes, *celR1* and *celR2*, probably organized as one operon and most likely controlling cellulose synthesis. According to the sequence composition, these genes belong to the family of two-component response regulators (Parkinson & Kofoid, 1992). Surprisingly, they exhibit highest sequence homology to the *Caulobacter crescentus* pleD

**Abbreviations:** Amp, ampicillin; Km, kanamycin; Sm, streptomycin; Tet, tetracycline.

The GenBank accession numbers for the *celABC* and *celR1*-*celR2* sequences determined in this work are AF121340 and AF121341, respectively.

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1 Swedish University of Agricultural Sciences, SLU, Department of Microbiology, Box 7025, S-75007 Uppsala, Sweden

2 Uppsala University, Department of Biochemistry, Box 576, S-75123 Uppsala, Sweden
and divK genes, which are the key components in interconnecting polar morphogenesis and cell division events in this bacterium (Sommer & Newton, 1989; Hecht et al., 1995; Hecht & Newton, 1995).

The identification of genes involved in the synthesis of cellulose fibres opens the possibility to more precisely investigate the biological significance of these structures in *Rhizobium*.

**METHODS**

**Bacterial strains, plasmids and growth media.** The wild-type strain R200 is a spontaneous streptomycin- and rifampicin-resistant (Sm Rif) mutant of *R. leguminosarum* bv. *trifolii* strain 2046 from the culture collection at the Department of Microbiology, Swedish University of Agricultural Sciences. Strain R201 is a cellulose-overproducing mutant obtained after NTG treatment of strain R200. Strains R204, R205, R206, R207, R211 and R214 are cellulose-negative Tn5 mutants generated from strain R201. *Escherichia coli* strains XL-1 Blue and DH5<sub>x</sub> were used for cloning. The pUC18 and Bluescript plasmids were used for cloning and sequencing. Vector p RK404A was used as a broad-host-range plasmid in complementation experiments. It is a derivative of plasmid pRK404 (Ditta et al., 1985) with a unique EcoRI site in the multilinker, constructed by W. Buikema at the University of Chicago, USA. Plasmid pRK2013 (Figurski & Helinski, 1979) was used as a helper plasmid in bacterial conjugations. Plasmid pSUP2021 (Simon et al., 1983) was used for Tn5 mutagenesis.

**Rhizobium** strains were grown in TY medium (1:1:1 g tryptone, g yeast extract and g NaCl): or YMB medium (1:1:1 g K<sub>2</sub>HPO<sub>4</sub>, g NaCl, g mannitol, 0.4 g yeast extract and 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O), supplemented with Congo red (25 mg l<sup>-1</sup>) when needed. *E. coli* strains were grown in Luria–Bertani liquid (LB) or agar (LA) media. When appropriate, antibiotics were added at the following concentrations: ampicillin (Amp), 50 µg ml<sup>-1</sup>; kanamycin (Km), 20 µg ml<sup>-1</sup>; tetracycline (Tet), 10 µg ml<sup>-1</sup>; and streptomycin (Sm), 20 µg ml<sup>-1</sup>.

**Mutagenesis and isolation of mutants.** Washed cells of *R. leguminosarum* bv. *trifolii* strain R200 (exponential-growth phase) were suspended in 10 ml 0-1 M citrate buffer pH 5-8 to a cell density of 5 × 10<sup>8</sup> c.f.u. ml<sup>-1</sup>. Freshly dissolved NTG was added to a final concentration of 100 µg ml<sup>-1</sup>. After incubation the seedlings were washed twice with equal volumes of Fahraeus nitrogen-free medium (Fahraeus, 1937) and diluted five times with the same medium before being added to clover seedlings. The seedlings were produced from seeds of red clover cv. Vivi incubated on water agar for 48-52 h in the dark. The binding assays were performed in two different ways, essentially as described by Smit et al. (1986) or Dazzo et al. (1984), respectively. In the first case five seedlings were incubated in 10 ml bacterial suspension in a 100 ml flask at room temperature under slow shaking. After the incubation the seedlings were washed three times in 10 ml Fahraeus medium under vigorous shaking. The attachment of the bacteria to the root hairs was evaluated under a phase-contrast microscope. All binding experiments were repeated at least three times.

**Electron microscopy.** Bacterial suspensions were analysed by different techniques: positive and negative staining and shadow casting to select optimal electron microscopic contrast. Since the diameter of the cellulose microfibrils produced by the bacteria should correspond approximately to that of double-stranded nucleic acid, the contrasting technique, which is used for DNA or RNA (Holmgren & Ohagen, 1997), was employed. Bacterial suspensions (taken directly from the growth medium or washed gently in 0-1 M Tris buffer pH 7-5) were adhered to carbon-coated grids, stained with 10<sup>-4</sup> M uranyl acetate and rinsed with 95% ethanol. Rotary shadowing with platinum was then employed at low angle (8°) to allow visualization of thin fibres. Specimens were analysed with a Zeiss CEM 902 electron microscope, equipped with a N2(1) trap, at an accelerating voltage of 80 kV.

**Nodulation test.** Seeds of red clover cv. Brita were immersed for 5 min in a chlorohexidine/ethanol (60%) solution (5 mg ml<sup>-1</sup>), washed in three changes of sterile water and distributed onto the surface of water agar plates, which were then incubated upside down for 2 d in the dark at room temperature. Seedlings with 5-7 mm roots were inoculated with 1 ml bacterial suspension prepared from 10 ml YMB agar plates containing Sm, Tet and Congo red.

**Attachment assay.** *R. leguminosarum* bv. *trifolii* strains were grown in TY medium until early stationary phase, washed twice with equal volumes of Fahraeus nitrogen-free medium (Fahraeus, 1937) and diluted five times with the same medium before being added to clover seedlings. After incubation the seedlings were washed three times in 10 ml Fahraeus medium under vigorous shaking. The attachment of the bacteria to the root hairs was evaluated under a phase-contrast microscope. All binding experiments were repeated at least three times.

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sterile tap water with 2 loopfuls from 5-d-old colonies grown on YMB agar. After inoculation early nodulation was followed for 3 weeks.

RESULTS AND DISCUSSION

Characterization of cellulose production in the overproducing mutant R201

Most strains of Rhizobium lack the ability to absorb the dye Congo red. However, there are exceptions to this generalized behaviour, as reported by Zevenhuizen et al. (1986). These authors showed that a few Rhizobium strains produced red to deep-red colonies on medium with Congo red and had cellulose contents of 2–10% of their dry weights. Binding of Congo red was positively correlated to the presence of microfibrils as observed under the electron microscope and, furthermore, these fibrils disappeared upon treatment with cellulase. These data suggested to us that binding of Congo red could be used in screening for cellulose-overproducing mutants. Such strains with an increased cellulose production would be a basis for generating cellulose-negative mutants since most wild-type strains do not produce enough cellulose to acquire significant colour in the Congo-red-based plate assay.

The wild-type strain R200 (as most Rhizobium strains) forms white colonies on YEM plates with Congo red. After mutagenesis of this strain with NTG, deep-red-coloured colonies appeared at a frequency of approximately $5 \times 10^{-4}$. One such mutant, here called R201, was selected for further studies. In comparison to the wild-type strain, the mutant strain R201 flocculated heavily when grown in YEM broth and produced a pellicle in static cultures. It also produced an alkali- and acid-insoluble substance that was degraded by cellulase but not by protease treatment (data not shown).

Electron microscopy of cells from strain R201 showed an abundance of fibres or bundles of fibres surrounding the bacteria. Negative staining visualized the fibres as relatively thick bundles or a meshwork of these bundles. It was difficult to determine any specific localization of these structures or the site of emergence from the cell membrane (Fig. 1a). To avoid these problems and to see if the appearance of the fibres was dependent on the method of preparation, rotation shadowing was used, a technique suitable for visualizing thin structures (Högland & Ohagen, 1997). Furthermore, the bacteria were not washed before application onto the grid to avoid aggregation of the cellulose. By this technique, much thinner microfibrils were seen emerging radially from all parts of the cell surface (Fig. 1b). A clear difference between these fibrils and the flagella was obvious (Fig. 1c). With reference to the thickness of flagella of 13–14 nm (Madigan et al., 1997), the diameter

![Fig. 1. Cellulose fibrils produced by *R. leguminosarum* bv. *trifolii* mutant strain R201. (a) Thick bundles of cellulose surrounding the cells (negative staining technique, washed cells). (b) Numerous thin cellulose fibrils (indicated by C) are seen emerging radially from the cells (rotary shadowing technique, unwashed cells). (c) Part of a bacterium showing a flagellum (F) and cellulose fibres (C). Bar, 100 nm.](image-url)
of the microfibrils ranged from approximately 1 to 2 nm. The fibrils are produced in the late exponential or early stationary phase since cells in early exponential phase were almost devoid of these fibrils, whereas most bacteria from an early-stationary-phase culture were heavily fibrillated. Cellulase treatment of the bacteria resulted in total disappearance of the fibrils.

According to Brown et al. (1976) and Wong et al. (1990), the newly polymerized subelementary cellulose strands in Ac. xylinus are extruded through pores in the outer membrane and crystallized into cellulose microfibrils by hydrogen bonding. The diameter of these microfibrils was reported to range between 1.6 and 3.8 nm. The very thin fibres of R. leguminosarum bv. trifolii protruding from the cell membrane (Fig. 1b, c) have a diameter of 1–2 nm and resemble the microfibrils described by Brown et al. (1976). The considerably thicker cellulosic structures visualized by negative staining (Fig. 1a) are probably aggregates of entwined fibrils.

Screening of 50 individual cells of the wild-type strain under the electron microscope did not detect any fibrillated cells. Likewise, no fibrils were detected on the cells of the cellulose-negative mutants R204 (celA::Tn5) and R205 (celR2::Tn5) (see below). These strains formed white colonies on agar plates with Congo red, confirming that binding of the stain by the mutant R201 resulting in red colonies is correlated to high production of cellulose fibrils. Although it has been reported that Congo red also can bind to outer-membrane proteins of various Gram-negative, animal pathogenic bacteria (Theisen et al., 1993), our data support the observation by Zevenhuizen et al. (1986) that in Rhizobium the Congo red binding is related to the cellulose content of the bacteria. Thus in all subsequent experiments (e.g. the complementation of the cellulose-negative phenotype), the binding of Congo red was taken as a marker for cellulose production, without additional confirmative tests.

Identification of genes involved in cellulose synthesis by Tn5 mutagenesis

The cellulose-overproducing strain R201 was subjected to Tn5 mutagenesis. The screening of cellulose non-producing mutants was carried out on agar plates containing Km and Congo red. Eleven white colonies were isolated and total DNA from them prepared. The DNA was cleaved with EcoRI and subjected to Southern blot hybridization. In order to detect all possible insertion events, the whole pSUP2012 plasmid was cleaved with HindIII and used as template in the random priming labelling procedure to create a radioactive probe. All mutant strains showed a single radioactive band on the autoradiography plates. After prolonged cultivation some of the mutants exhibited weakly red or segmented colony types and were not investigated further. Six isolates, R204, R205, R206, R207, R211 and R214, gave stable white colonies on Congo red plates and were chosen for further analysis. The EcoRI fragments carrying the Tn5 insertions from all six strains were cloned into pUC18. Since Tn5 does not contain any EcoRI cleavage sites, the cloned fragments contained flanking rhizobial DNA. Nucleotide sequences obtained from these clones were compared to sequences in the nucleic acid and protein databases. The results showed that three mutants, R204, R206 and R214, had Tn5 inserted in a region showing high sequence homology to the Ag. tumefaciens celA gene, encoding a cellulase synthase (Matthysse et al., 1995a). Certain regions of the deduced protein sequence also showed homology to the BscA, Axcs and Acsai proteins, all three characterized as cellulose synthases in Ac. xylinus (Wong et al., 1990; Saxena et al., 1990; Saxena & Brown, 1995). Tn5 flanking sequences from strains R207 and R211 showed strong homology to the Ag. tumefaciens cellulose genes celE and celB, respectively, suggesting that the cellulose synthesis pathway in Rhizobium might be similar to that of Ag. tumefaciens (Matthysse et al., 1995a, b). The CеД protein in Ag. tumefaciens is proposed to attach UDP-glucose to a lipid carrier, forming a lipid intermediate product. The celB gene product was also shown to be involved in the synthesis of lipid-linked intermediates, although the exact function of the protein is not known (Matthysse et al., 1995b).

Sequences obtained from strain R205 did not show homology to the Ag. tumefaciens cel operon. Instead, they indicated that the Tn5 insertion was located in a regulatory gene. The involvement of this gene in cellulose synthesis is described below.

Organization of the cel genes is conserved in Rhizobium and Agrobacterium

To establish the organization of the R. leguminosarum bv. trifolii cel genes, we took advantage of the sequence homology to the Ag. tumefaciens cel genes as revealed by the database search. Partial EcoRI cleavage products of total DNA from strains R207 and R211 were ligated into pUC18. The ligation mixtures were electroporated into E. coli DH5α and transformants were selected on LA plates with Amp and Km. From these clones, inserts from isolated plasmids, containing the putative flanking sequences of the previous EcoRI fragments, were analysed by EcoRI cleavage and sequencing (Fig. 2a–c). Comparison of the resulting DNA sequence with the homologous region in Ag. tumefaciens showed a similar organization of the corresponding genes in R. leguminosarum bv. trifolii (Fig. 2b). The sequence homology is limited to the region where the cellulose genes are located. Upstream of the celA and celE genes the sequences of R. leguminosarum bv. trifolii and Ag. tumefaciens diverge. The R. leguminosarum bv. trifolii celABC operon was completely sequenced. Alignment of the deduced R. leguminosarum bv. trifolii and Ag. tumefaciens proteins indicated that the CelA proteins of the two species showed 69.5% identity and 80% similarity, the CelB proteins showed 49% identity and 62% similarity, and the CelC proteins showed 45% identity and 35% similarity in their amino acid
sequences. The sequence of the celA gene offers an ORF with three alternative start sites. No obvious Shine-Dalgarno (SD) sequence is present upstream of the first and second ATG in the ORF, while the third ATG is preceded by a classical SD motif. This yields a deduced protein of 730 amino acids. Similarly, the celA gene from Ag. tumefaciens also has two alternative initiation codons (GenBank accession no. L38609). Alignment of the deduced CelA proteins from Ag. tumefaciens and R. leguminosarum bv. trifolii showed sequence homology starting from the second and the third methionine, respectively. The CelA sequence was also similar to several cellulose synthases from other organisms, for example Ac. xylinus, Ag厌fex and Arabidopsis, and also to a hypothetical protein of unknown function from E. coli and to NodC proteins (N-acetylglucosaminyltransferase, involved in the synthesis of nod factors) from several Rhizobium species, as revealed by a BLAST database search. The celB gene begins at a start codon overlapping the celA stop codon. The deduced R. leguminosarum bv. trifolii CelB protein (822 amino acids) was somewhat longer than Ag. tumefaciens CelB (698 amino acids). Alignment of the two CelB proteins showed a highly conserved part near the C terminus, displaying 78% amino acid sequence similarity between amino acids 410 and 640. In the N-terminus of both proteins there was a domain of 110 amino acids rich in prolines, although there was no high score in pairwise alignment. The stop codon for R. leguminosarum bv. trifolii celB overlapped the start codon for celC and was situated approximately 320 bp downstream of the proposed site for the Ag. tumefaciens celC start codon. Thus the same pattern of overlapping stop and start codons was observed for these three genes in R. leguminosarum bv. trifolii, indicating that the synthesis of the proteins is coupled. CelC protein showed significant similarity sequence similarity to several bacterial endoglucanases, among them the Ac. xylinus CMCax protein, encoded by a gene linked to the cellulose synthase operon (Standal et al., 1994). The involvement of a cellulase thus seems to be a common feature in the cellulose production pathway in Acetobacter, Agrobacterium and Rhizobium. R. leguminosarum bv. trifolii is also reported to possess extracellular, cell-associated cellulase activity (Mateos et al., 1992). However, at present there are no data available to correlate this activity to CelC.

The celE region was partially sequenced. The sequence data indicated the presence of a gene with considerable sequence similarity to Ag. tumefaciens celE, located downstream of the celC gene. Sequences obtained from the DNA region located immediately upstream of the celE gene did not display similarity to Ag. tumefaciens celD.

Interestingly, a gene with strong homology to sensor histidine kinases is situated just upstream of the celA gene (Fig. 2b). In a BLAST database search, the FixL protein from R. leguminosarum showed the highest score. FixL is an oxygen sensor and transduces the low oxygen signal by controlling the phosphorylation of FixJ protein (a transcriptional activator). Sequence similarities covered only the C-terminal kinase part of the FixL protein (Lois et al., 1993). No similarities were detected between the membrane-spanning region and the haem-binding domains of FixL and the N-terminal sequence of the putative kinase. The possibility that this gene encodes the cognate kinase for the putative response regulators CelR1 and CelR2, involved in the regulation of the cellulose synthesis (see below), will be tested in the future.

**Identification of putative components of a signalling pathway involved in cellulose synthesis**

Sequence comparisons showed that the gene inactivated by Tn5 in strain R205 is homologous to several response regulators belonging to the so-called two-component regulatory systems. We named the new regulator gene celR2. Besides the similarity to the conserved N-terminal parts of several response regulators, the deduced amino acid sequence of celR2 shows high sequence homology to C. crescentus PleD (Hecht & Newton, 1995) throughout its entire length (Fig. 3). In C. crescentus, the regulatory genes pleC (a histidine kinase) and pleD and divK (the cognate response regulators), are the key elements in coupling shifts in polar morphogenesis to cell cycle events (Sommer & Newton, 1989; Hecht et
al., 1995; Hecht & Newton, 1995; Wang et al., 1993). The divK and pleD genes in *C. crescentus* are situated immediately next to each other (Hecht & Newton, 1995). Interestingly, sequencing the upstream region of *celR2* revealed a gene with very high sequence homology to *divK* (Fig. 3). In *R. leguminosarum* bv. *trifolii* these two genes are also located close to each other, being separated by only 18 bp. The *R. leguminosarum* bv. *trifolii* divK homologue, named *DivK*, encodes a protein of 123 amino acids, and *celR2* encodes a protein of 457 amino acids. In *C. crescentus*, both DivK and PleD regulators are phosphorylated by the same histidine kinase, PleC. DivK is an essential cell division regulator (Hecht et al., 1995) and PleD is required in the regulation of polar behaviors (Wang et al., 1993). The alignment was carried out using the PGO/GENE program PALIGN. Bold letters indicate identical amino acids, dots indicate similar amino acids and asterisks represent conserved amino acids involved in phosphorilation in response regulators. The DivK and DivK proteins exhibit 74% identity and 83.7% similarity, and the PleD and CelR2 proteins exhibit 50.2% identity and 64.3% similarity, in their amino acid composition.

However, this polar behavior is so far not explained at the molecular level. It is an intriguing possibility that the *celR1*–*celR2* regulatory system might in some way be involved in maintaining the polarity in dividing *Rhizobium* cells. There may also be other traits that are co-regulated with cellulose synthesis.

Cellulose synthesis in strain R201 is not polar. The electron microscopy study clearly showed that the cell surface of the overproducing strain was completely covered by cellulose. However, the attachment of these cells to the root hairs was clearly polar as seen under the phase-contrast microscope (data not shown). The same polar attachment was also observed both for the wild-type and the cellulose-negative strain R204 (*celA::Tn5*).

The cloned *celR1*–*celR2* genes complement the R205 *Cel*+ phenotype and trigger cellulose overproduction in wild-type R200

To confirm the involvement of *celR* regulators in cellulose synthesis, complementation of the *celR2* mutation in strain R205 (*celR2::Tn5*) was carried out with the functional regulator region cloned from the over-producing strain R201. A HindIII fragment of approxi-
Cellulose synthesis genes in *R. leguminosarum*

The complementation analysis supported the requirement for the *celR1–celR2* tandem gene structure in the activation of cellulose synthesis in *R. leguminosarum* bv. *trifolii*. The fact that the pReg2 construct, lacking the proximal region of the *celR1* gene, only showed weak and delayed complementation, indicates that *celR1* and *celR2* are organized in an operon. Although the transconjugants received an intact *celR2*, this was not sufficient for effective complementation. The reason for the weak expression after prolonged incubation in our transconjugants, despite the lack of the *celR1* promoter, is unclear, but might be explained by spurious transcription from within the vector sequence. In *C. crescentus*, these two genes are coupled and transcription is initiated from a single promoter upstream of *divK*. A construct containing the promoterless *divK* and *pleD* region failed to complement the *pleD* defect (Hecht & Newton, 1995).

Interestingly, the construct pReg1 also caused cellulose overproduction in the wild-type R200 when this strain was used as a recipient in the conjugation experiments. As the pReg1 construct, containing the *reg* region from the overproducing strain R201, might carry a mutation, the corresponding HindIII fragment (wild-type *reg* region) was cloned from strain R200 and designated pRegW. The conjugative transfer of plasmid pRegW into strains R205 and R200 yielded bright red transconjugant colonies in both cases. No visual difference was observed between transconjugant colonies harbouring pReg1 or pRegW on Congo red plates. This shows clearly that the enzymic machinery for cellulose production is fully functional in the wild-type R200, and can be triggered by increasing the gene dosage of *celR1–celR2*. This result further strengthens our hypothesis that *celR1* and *celR2* are involved in the regulation of cellulose synthesis. An example of a similar gene dosage effect was reported concerning the regulation of virulence (*vir*) genes in *A. tumefaciens*, where increasing the gene dosage of a response regulator gene (*virG*) resulted in increased *vir* gene expression and elevation of virulence (Jin et al., 1987; Rogowsky et al., 1987).

**Fig. 4.** Complementation of the *Cel* phenotype of R205 (*celR2::Tn5*) with different DNA fragments from the *reg* region. The *reg* region contains a ~6.5 kb HindIII fragment containing both regulator genes *celR1* and *celR2* and flanking DNA. The corresponding fragments were cloned into pRK404A, generating the clones pReg1–pReg4 and then conjugated into R205. The restriction map of the *reg* region and the location of the fragments is shown. + indicates full complementation (bright red colonies on Congo red plates after conjugation), −/+(+) indicates incomplete or delayed complementation (originally white colonies turning pale red after a prolonged incubation), and − indicates no complementation (white colonies).

Nodulation assay

All *R. leguminosarum* bv. *trifolii* strains described in this paper were capable of nodulating red clover under the assay conditions used (data not shown). This result agrees with data published by Smit et al. (1987) who also compared cellulose-producing strains and cellulose-negative mutants for their ability to nodulate, without seeing any differences in this respect.

**Cellulose synthesis is probably an induced event in *R. leguminosarum* bv. *trifolii* as indicated by attachment assays**

Since cellulose fibres were undetectable by electron microscopy of wild-type *R. leguminosarum* bv. *trifolii* cells, we tested the different strains for their ability to
attach to the root hairs of clover seedlings. Earlier reports showed that the firm attachment and especially the formation of large aggregates of bacteria on root hair tips (so-called 'cap formation') is an attribute of the cellulose fibres (Napoli et al., 1975; Matthysse et al., 1981; Smit et al., 1986, 1987, 1992). According to Smit et al. (1987), the caps are formed during the first 2 h of the attachment process.

We carried out binding assays essentially as described by Smit et al. (1986). The strains tested were wild-type R. leguminosarum bv. trifolii R200, the cellulose-overproducing mutant R201 and the cellulose-negative Tn5 mutants R204 (celA::Tn5) and R205 (celR2::Tn5). After 2 h incubation with R201, virtually all root hairs had large clumps of adhered bacteria (caps) around the root hair tips and along the root hairs. Besides aggregates, we also observed single cells attached polarly to the different parts of the root. Strains R200, R204 and R205 behaved differently from strain R201. After 2 h incubation, 90% of the root hairs were covered with single cells attached to the tip and stem region. No bacterial aggregates were seen on the root hairs of the seedlings incubated with these bacteria. Even after prolonged incubation times of up to 16 h, no visible caps were formed. All three strains displayed this single cell type of attachment. Assuming a direct correlation between cap formation and the presence of cellulose fibres, this result indicated that the wild-type R200 did not produce cellulose under these conditions as its attachment behaviour was indistinguishable from that of the cellulose-negative mutants. This conclusion is strengthened by the fact that we failed to see cellulose fibres on the R200 cells from a liquid culture under the electron microscope (see above).

An alternative strategy for studying attachment was described by Dazzo et al. (1984). These authors observed the synthesis of extracellular fibrils only after 12 h incubation in an undisturbed hydroponic slide culture. We repeated this experiment with the wild-type and the cellulose-negative mutants. After 2 h incubation, only single cell attachment was seen for all strains. However, after overnight incubation, some aggregation of the wild-type bacteria around the root hair tips was observed, whilst the binding pattern of the cellulose-negative strains remained unchanged.

Smit et al. (1992) presented a two-step attachment model in which the protein rhicadhesin was responsible for specific binding of the bacteria to plant roots and the cellulose fibres were required for the second stage of firm anchoring and floc or cap formation. Cellulose production in R. leguminosarum was considered to be constitutive by these authors (Smit et al., 1987). On the other hand, cellulose synthesis in Ag. tumefaciens has been shown to be induced by plant extracts (Matthysse et al., 1981). Earlier data presented by Dazzo et al. (1984) also indicated that cellulose synthesis in R. leguminosarum bv. trifolii was induced by some compounds in root exudates. Our data are in agreement with Dazzo et al. (1984) and indicate that cellulose production in R. leguminosarum bv. trifolii is induced by contact between roots and bacteria. The identification of genes probably involved in the regulation of synthesis of cellulose (see above) strengthens that observation. The deduced structure of these regulators suggests that they are involved in a signalling pathway. The data obtained so far allow us to hypothesize that some plant-derived compound(s) may act as signal(s) for turning on cellulose synthesis in R. leguminosarum bv. trifolii, and that CelR1 and CelR2 are likely components of such a signal transduction pathway.

The attachment mechanisms are considered to be similar in Rhizobium and Agrobacterium, proceeding according to the two-step attachment model (Smit et al., 1992). The protein rhicadhesin, mediating the first stage in the attachment process, is compatible between members of the family Rhizobiaceae (Smit et al., 1989; Swart et al., 1993). Cellulose fibres are involved in the second attachment stage to ensure the firm binding and aggregation of bacteria around a possible infection site. A similar model was also proposed for the attachment behaviour of Azospirillum bacteria (Michiels et al., 1991; Vande Broek & Vanderleyden, 1995), where a flagellar protein mediates the first binding step and a Calcofluor-binding polysaccharide is responsible for the second firm attachment step. In Ag. tumefaciens, the cellulose fibrils serve to bind the bacteria tightly to the plant host cells and mutants which cannot synthesize cellulose are reduced in virulence, but still capable of infection, at least under laboratory conditions (Matthysse et al., 1981; Minnemeyer et al., 1991). Similarly, Smit et al. (1987) showed that fibril-negative as well as fibril-overproducing mutants of R. leguminosarum showed a normal nodulation behaviour, which was confirmed for R. leguminosarum bv. trifolii in the present study. Thus although the second binding step seems to be conserved among different bacteria it is not a prerequisite for a successful interaction.

The structural and putative regulatory genes described in this report will be the basis for making suitable reporter gene constructs to further investigate the environmental signalling process leading to cellulose production in these bacteria.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Swedish Council for Forestry and Agricultural Research (32.0371/96) and Carl Trygger's Foundation for Scientific Research to M.L.

We thank Professor Gerhart Wagner for helpful discussions and critical reading of the manuscript.

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Received 16 November 1998; revised 1 February 1999; accepted 4 February 1999.