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The Role of Glycosyl Linkages of Exopolysaccharide of *Rhizobium meliloti* in Nodule Cell Invasion

Rola wiązań glikozydowych egzopolisacharydu *Rhizobium meliloti* w procesie infekcji komórek brodawek

The formation of N_2-fixing nodules on the roots of leguminous plants is a highly complex interaction between gram-negative soil bacteria of the genus *Rhizobium* and a limited range of host plants (7, 8). This partnership is specific: a particular bacterium invades and forms nodules only on some host plants and not on others. Thus, *Rhizobium meliloti* nodulates only *Medicago*, *Melilotus* and *Trigonella* species (10). The normal steps in the *Medicago sativa*–*Rhizobium meliloti* symbiosis leading to the formation of nodule can be described as follows: attachment of the bacteria to the root surface, root hair deformation, infection thread formation within the root hairs, nodule initiation, releasing of rhizobia from the infection thread into the nodule cells and differentiation of bacteria into nitrogen fixing bacteroids.

Effective nodulation of legumes by many *Rhizobium* species requires extracellular polysaccharides (EPS), which are produced by these bacteria (2, 3, 6, 10–12, 14, 15). During the last few years mutants of *R. meliloti*, that are unable to produce an exopolysaccharide or produce changed EPS have been isolated (3, 6, 10, 11, 14, 15). These mutants induced ineffective (Fix^-) nodules not infected by bacteria (Inf^-). They were isolated on the basis of their failure to fluoresce under UV light on medium containing Calcofluor, the stain for exopolysaccharide of *R. meliloti* (6, 9).

In this paper we report the ^1H NMR analysis of EPS of *R. meliloti* wild type strain L5.30 and its mutant RM152 which forms nodules noninfected by bacteria (9).

MATERIALS AND METHODS

Bacterial strains and growth conditions. In our experiments we have used wild type strain L5.30 of *R. meliloti*, which forms effective nodules on *Medicago sativa* (4) and its ineffective mutant RM152, which on alfalfa forms nodules noninfected by bacteria ($Nod^+Inf^-Fix^-$) (9). Mutant RM152 does not fluoresce on agar medium with Calcofluor (9). The *R. meliloti* strains were maintained and grown on liquid medium "5" (5) and agar medium "79" (1).

Isolation of EPS. The supernatant fluid obtained after the removal of cells by centrifugation was concentrated by rotary evaporation at 42°C. Exopolysaccharide was next precipitated from the culture supernatant with 3 volumes of ethanol, dialyzed, clarified by centrifugation and lyophilized (13).

Proton NMR spetroscopy. Each sample of exopolysaccharide (approximately 3 mg) was dissolved in water, sonicated to aid in dissolution and decrease viscosity and next exchanged several times with D_2O. Spectra were obtained with JEOL GX270 500 MHz instrument with probe heated to 60°C .

RESULTS AND DISCUSSION

In order to analyze the role of *R. meliloti* exopolysaccharide in nodule cell invasion we have studied by proton spectroscopy, the structure of EPS produced by wild type strain L5.30 of *R. meliloti* and its Inf^- mutant RM152, which forms "empty" nodules without bacteria. The spectra obtained with EPS of both strains are shown in Fig. 1.

The ^1H NMR spectral signals of ethanol precipitate from the supernatant of *R. meliloti* strain L5.30 was found to be almost identical to that of the precipitate from the mutant RM152. This indicates, that exopolysaccharide from Calcofluor dark $Nod^+Inf^-Fix^-$ mutant is extremely similar to that produced by its Inf^+Fix^+ parent. Both preparations contained peaks coresponding to the acetyl (1.9–2.1 ppm), succinyl (2.2 and 2.4 ppm) and 1-carboxy-ethylidene (1.3–1.5 ppm) groups (Fig. 1).

On the basis of proton NMR analysis we have also determined the anomeric configuration of the glycosyl linkages in the exopolysaccharides of *R. meliloti*. The glycosyl linkages in the EPS of *R. meliloti* wild type strain L5.30 was shown to be in α (signals at 5.2 and 5.4 ppm) and β configuration (peaks at 4.3 and 4.8 ppm) whereas in the case of the exopolysaccharide of noninfective (Inf^-) mutant RM152 no signals from the α anomeric proton was present.

It is known that the structural features of the exopolysaccharide of *R. meliloti* are important for their symbiotic function (6, 10, 11, 14, 15). One of these features is the presence of succinyl groups. In 1987 Leigh et al. (6) described mutants of *R. meliloti* 1021, that failed to succinylate

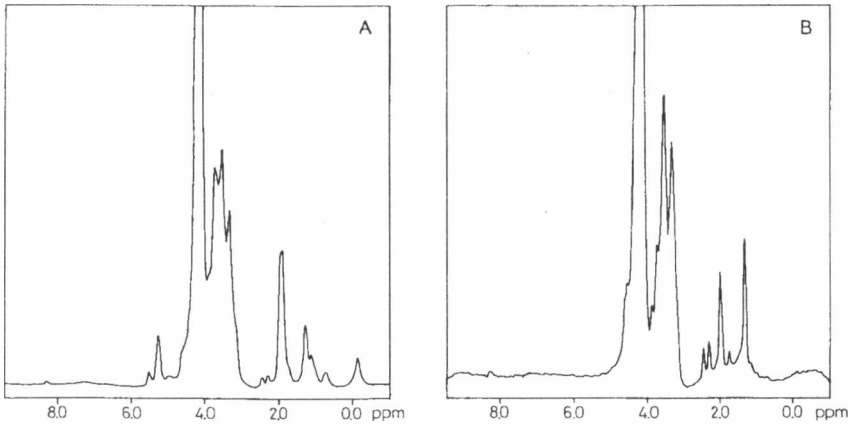

Fig. 1. ^{1}H NMR spectra of EPS produced by wild type strain L5.30 of *R. meliloti* (A) and Inf$^-$ mutant RM152 (B)

EPSI and formed empty, ineffective nodules. Empty nodules formed also Inf$^-$ mutant RM152 (9) with only β-anomeric glycosyl linkages in the exopolysaccharide. These effects of exopolysaccharides on the host suggests to us that many specific structural features of EPS are required for its function in symbiosis. One of these functions is to help the bacteria to avoid plant defense responses. Another possible role of exopolysaccharide is to act as signal molecule, thus causing the plant to perform a correct nodule development.

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STRESZCZENIE

Badano spektrum protonowego rezonansu magnetycznego (^{1}H NMR) egzopolisacharydu wytwarzanego przez szczep dziki L5.30 *R. meliloti* i jego mutanta RM152, który indukował nieefektywne brodawki pozbawione bakterii (Inf$^-$). W obu przypadkach spektra były niemal identyczne. Obserwowano bowiem w egzopolisacharydzie zarówno szczepu dzikiego, jak i mutanta Inf$^-$ sygnały pochodzące z grup acetylowych (1,9–2,1 ppm), bursztynianowych (2,2 i 2,4 ppm) i pirogronianowych (1,3–1,5 ppm).

Podstawowa różnica w spektrum ^{1}H NMR egzopolisacharydu szczepu L5.30 i RM152 *R. meliloti* dotyczy konfiguracji wiązań glikozydowych. EPS syntetyzowany przez szczep dziki L5.30 *R. meliloti* posiada wiązania glikozydowe zarówno typu α (sygnały przy 5,2 i 5,4 ppm), jak i β (sygnały przy 4,3 do 4,8 ppm), podczas gdy egzopolisacharyd mutanta Inf$^-$ RM152 wytwarza sygnały w regionie β-anomerycznym (4,3 do 4,8 ppm), a nie daje w regionie α (5,2–5,4 ppm), co potwierdza występowanie wiązań glikozydowych jedynie typu β.