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An Osmorevertant of a *Rhizobium meliloti ndvB* Deletion Mutant Forms Infection Threads but is Defective in Bacteroid Development

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A defined deletion mutant in the *ndvB* locus of *Rhizobium meliloti* that was identified by its Inf$^-$ phenotype lacked the β-1,2-glucans and showed osmotic instability. The Inf$^-$ phenotype was restored by a spontaneous mutation causing osmotic tolerance. This osmorevertant still lacked the β-1,2-glucans but induced Fix$^-$ nodules containing infection threads. Release of bacteria from the infection thread was observed, but bacterial multiplication in the plant cell and development into bacteroids was blocked. In contrast to the original *ndvB* mutant, the osmorevertant was able to establish Fix$^+$ nodules following mixed inoculation with a Nod$^-$ mutant. Thus, osmotic intolerance seems to be the reason for the Inf$^-$ phenotype of *ndvB* mutants. Reisolation experiments revealed that Fix$^+$ nodules were occupied mainly by the Nod$^-$ mutant, indicating that the partially osmotic-tolerant osmorevertant still lacked functions that could not be complemented on the cellular level.

Additional keywords: bacteroid development, symbiosis.

The soil bacterium *Rhizobium meliloti* is able to form nitrogen-fixing nodules on the roots of its leguminous host alfalfa. Nodule formation can be subdivided into three main stages, namely nodule induction (Nod), bacterial infection (Inf), and conversion of bacteria into nitrogen fixing bacteroids (Fix). The infection process of alfalfa nodules has been intensively analyzed by the isolation of *R. meliloti* mutants blocked in the formation of infection threads (Müller *et al.* 1988). The *R. meliloti* Inf$^-$ mutants could be subdivided into two groups according to their capacity to form the acidic exopolysaccharide succinoglucan. One class, designated Inf$^-$ EPS$^-$, did not produce succinoglucan any more; the other class, originally designated Inf$^-$ EPS*, however, produced even more EPS than the wild-type. The mutation of the Inf$^-$ EPS$^-$ mutants was found to map on a 7.8-kb DNA fragment of the *R. meliloti* megaplasmid 2, whereas the mutation of the Inf$^-$ EPS* class could be located on a 6.4-kb DNA fragment of the *R. meliloti* chromosome. Preliminary hybridization experiments showed that mutants of the Inf$^-$ EPS* class are related to *R. meliloti ndvB* mutants (Müller *et al.* 1988) first described by Dylan *et al.* (1986). The *R. meliloti ndvB* gene was originally identified by its homology to the *Agrobacterium tumefaciens* virulence gene *chvB* (Zorteguieta *et al.* 1986; Dylan *et al.* 1986), which was found to be necessary for plant tumor formation (Puvanesarajah *et al.* 1985; Douglas *et al.* 1985).

Several defects and alterations accompanying the Inf$^-$ phenotype of *ndvB* mutants have been reported. *R. meliloti ndvB* mutants no longer produce β-1,2-glucans. They show a decrease in motility and in bacteriophage sensitivity. On the other hand, their sensitivity to hydrochloric antibiotics and to low osmolarity is drastically increased (Ielpi *et al.* 1990).

In this paper we now confirm that the Tn*5* insertions of *R. meliloti* Inf$^-$ EPS* mutants described by Müller *et al.* (1988) are located in the *ndvB* locus. We also describe the construction of defined *ndvB* deletion mutants and the isolation of an osmorevertant. With the help of the osmorevertant we will show that the increased osmotic sensitivity of *ndvB* mutants is responsible for the inability to infect alfalfa nodules. Additionally, we found that the Tn*5* insertions in *ndvB* were not responsible for the lack of the pyruvate residue of the EPS (EPS*) as was described by Müller *et al.* (1988). Therefore, *ndvB* mutants overproducing wild-type EPS are designated EPS^{++}.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The *Escherichia coli* and *R. meliloti* strains, plasmids, and phages used in this study are listed in Table 1.

Media and growth conditions. *E. coli* strains were cultivated at 37° C in Luria-Bertani (LB) medium (Miller 1972), *R. meliloti* strains at 30° C in LB medium. For the selection of osmorevertants, osmotic sensitive strains were grown on tryptone-yeast (TY) medium at 30° C. Antibiotics were added at the following concentrations (per liter): 50 mg of kanamycin (Km); 10 mg of gentamicin (Gm) for *E. coli*, 50 mg for *R. meliloti*; 10 mg of tetracycline (Tc); 600 mg of streptomycin (Sm); 100 mg of neomycin (Nm); 50 mg of chloramphenicol (Cm); 100 mg of spectinomycin (Sp) for *E. coli*, 200 mg of for *R. meliloti*; 150 mg of ampicillin (Ap).

DNA biochemistry. DNA isolation, restriction enzyme analysis, agarose gel electrophoresis, and cloning procedures were performed using established techniques (Maniatis *et al.* 1982). Restriction endonucleases, T4 DNA ligase, and Klenow polymerase were purchased from Bethesda Research Laboratories (Gaithersburg, MD). All enzymatic

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reactions were carried out as recommended by the manufacturers.

DNA sequencing. Various derivatives of pUC plasmids with different multiple cloning sites (Arnold and Pühler 1988) were used to clone distinct fragments of the 6.4-kb *Eco*RI fragment from pRmPM582 (Fig. 1). In addition, a set of ordered deletions within the *Eco*RI fragment was performed by partial digestion with *Sau*3A. The sequence was determined by the dideoxy chain termination method (Sanger *et al.* 1977) using a T7 sequencing kit (Pharmacia, Freiburg, Germany). The DNA of the plasmids was prepared as described by Arnold and Pühler (1988).

Interposon mutagenesis. Interposon mutagenesis was performed as described by Masepohl *et al.* (1988).

In addition, the *npt*I-*sac*B/R cartridge (Reed and Collmer 1987) was cloned into suitable restriction sites (e.g., *Bam*H1, *Bgl*II, or *Sal*I) of the vectors. Plating the mating mixture on LB agar containing 5% sucrose and the appropriate antibiotic homogenotized strains could be directly selected. Deletions (Fig. 1) were verified by using the nonradioactive digoxigenin DNA labeling and detection kit as recommended by the manufacturer (Boehringer-Mannheim, Mannheim, Germany). Cosmid pRmPM582 was used as a hybridizing probe.

Plant tests. Nodulation of *Medicago sativa* L. 'Du Puits' was tested as described by Hynes *et al.* (1986). Nitrogenase activity was measured by the acetylene reduction assay. Coinoculation experiments were performed as described by Kapp *et al.* (1990). Reisolation experiments were carried out according to the method of Klein *et al.* (1988).

Light microscopy. For fixation, specimens were immersed immediately in cold 2.5% glutaraldehyde in 0.05 M phosphate buffer pH 7.2 for 4 hr. Fixation and all following steps were carried out in an ice bath. Fixed tissues were washed in the same buffer for 4 hr. After dehydration with increasing ethanol concentrations, the samples were embedded in LR White (London Resin). Polymerization was done at 60° C for 48 hr. Semithin sections were stained with toluidine blue 0.

For fast differentiation between Inf^+ and Inf^- nodules, Feulgen staining and phenol clearing were used (Niehaus and Pühler 1988).

Analysis of exopolysaccharides (EPS). The culture conditions, isolation and purification were as described by Kapp *et al.* (1990). *R. meliloti ndvB* mutants were stabilized by adding 200 mM NaCl to the TY medium.

^{1}H-NMR spectroscopy. About 20 mg of freeze-dried EPS was used to carry out ^{1}H-NMR spectroscopy in D_2O at 300 MHz on a Bruker (Billerica, MA) spectrometer.

Identification of β-1,2-glucans. The isolation and puri-

Table 1. Strains, plasmids, and phages used in this study

Name	Relevant genotypic and phenotypic characteristics	Source
Strains		
Rm2011	Fix^+ EPS^+; Sm^r	Cassé *et al.* (1979)
Rm101.45	Inf^-	Müller *et al.* (1988)
Rm688	Inf^-	Müller *et al.* (1988)
Rm0540	Inf^- EPS^-	Müller *et al.* (1988)
Rm2526	Nod^-	Müller *et al.* (1988)
RmAH1	12-kb deletion of chromosomal DNA; Tc^r	This work
RmAH2	7.5-kb deletion of chromosomal DNA; Nm^r	This work
RmHQ50	28-kb deletion of chromosomal DNA; Inf^-; Gm^r/Sp^r	This work
RmJQ60	0540 transduced into RmHQ50, Inf^- EPS^-	This work
RmJQ61	10-kb deletion of chromosomal DNA; Inf^-; Tc^r	This work
RmHQ50oR	Spontaneous growing colony of RmHQ50 on TY	This work
JM83	*ara*, Δ*lac-pro*, Sm^r, *thi*, Φ80d*lac*ZΔM15	Vieira and Messing (1982)
S17-1	*hsdR pro recA*, containing an RP4 derivative integrated into the chromosome	Simon *et al.* (1983)
Plasmids		
pRmPM582	Cosmid containing *ndvB* sequences	Müller *et al.* (1988)
pRmPM584	Cosmid containing *ndvB* sequences	Müller *et al.* (1988)
pAH1	12-kb deletion, containing Tc^r of pSUP101Tn*5*Tc (Simon *et al.* 1983)	This work
pAH2	7.5-kb deletion, containing Km^r of pHPΩKm (Fellay *et al.* 1987)	This work
pHQ50	28-kb deletion, containing Gm/Sp of Tn*1696* (Hirsch *et al.* 1986)	This work
pJQ61	10-kb deletion, containing Tc^r of Tn*1771* (Schöffl and Pühler 1979)	This work
pHQ2	Sp^r *Pst*I-*Bam*HI fragment of pHQ3 cloned into *Pst*I-*Bcl*I fragment of PSUP203	This work
pSUP203	Ap^r Cm^r Tc^r, *mob*	Simon *et al.* (1983)
pHQ3	Sp^r *Eco*RI-*Bgl*II fragment of pJQ42 cloned into *Eco*RI-*Bam*HI sites of pK18	This work
pJQ42	Sp^r *Bam*HI fragment of pHP45Ω substituting *Bam*HI-*Bcl*I of pSLE75	This work
pK18	Km^r mcs	Pridmore (1987)
pSLE75	Km^r *Hin*dIII-*Bam*HI fragment of Tn*5* cloned into pUC8 (Vieira and Messing 1982)	G. Muth, Bielefeld
pHP45Ω	Ap^r Sp^r Sm^r	Fellay *et al.* (1987)
pHP45ΩKm	Ap^r Km^r	Fellay *et al.* (1987)
pJQ47	*Bam*HI fragment containing *mob*- site cloned into *Bgl*II site of pK18	This work
pSUP202-1	pBR325-*mob*	Simon *et al.* (1983)
pSUP101Tn*5*Tc	Ap^r; Km^r; Tc^r	Simon *et al.* (1983)
pSVB28 - 31	Sequencing vectors with various mcs	Arnold and Pühler (1988)
Phage		
M12	*R. meliloti* transducing phage	Finan *et al.* (1984)

fication of β-1,2-glucans were performed according to Breedveld *et al.* (1990). Thin-layer chromatography was used to identify the β-1,2-glucans (Zevenhuizen *et al.* 1990).

Transduction. Transductions were carried out as described by Finan *et al.* (1984) using the *R. meliloti* phage M12. The mutations of the transduced strains were verified by growth on selective agar and also by testing on plates containing Cellufluor white (Polysciences, Warrington, PA) as described by Müller *et al.* (1988).

RESULTS

Identification of the *ndvB* locus on a 48.7-kb DNA fragment of *R. meliloti* and construction of defined deletion mutants. In a previous paper, it was reported that two Tn*5*-induced *R. meliloti* mutants, Rm101.45 and Rm688, unable to infect alfalfa nodules were complemented by the two overlapping cosmids pRmPM582 and pRmPM584 (Müller *et al.* 1988). We established the restriction map of the DNA region covered by the cosmids. Southern hybridization experiments with subcloned DNA fragments confirmed that the 48.7-kb DNA region presented in Figure 1 could be considered as a contiguous part of the *R. meliloti* chromosome. A common 6.4-kb *Eco*RI DNA fragment of both cosmids was sufficient to complement the Tn*5*-induced mutants Rm101.45 and Rm688. As shown in Figure 1, we established the sequence of a 1.7-kb subfragment within the 6.4-kb *Eco*RI DNA fragment containing the Tn*5* insertions and determined the insertion sites. By comparing our sequence data with those recently published by Ielpi *et al.* (1990), we confirmed that the Tn*5* insertion sites of the mutants Rm101.45 and Rm688 are located in the coding region of *ndvB*.

To test whether further fragments of this DNA region play a role in nodule infection, we constructed defined deletion mutants. As shown in Figure 1, different fragments of the 48.7-kb DNA region were subcloned in mobilizable

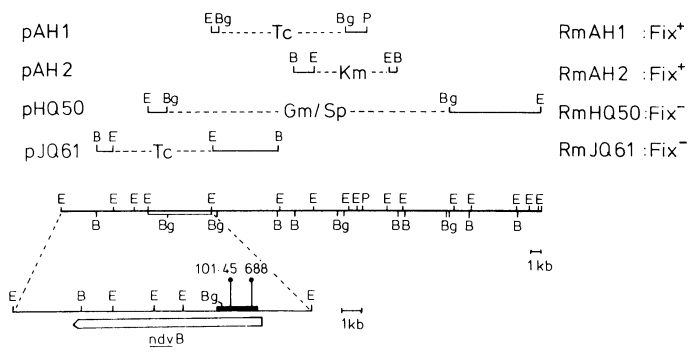

Fig. 1. Deletion mutagenesis of a *Rhizobium meliloti* chromosomal DNA fragment carrying the *ndvB* gene. The restriction map of a 48.7-kb of DNA fragment carrying the *ndvB* gene is presented. Open bar indicates the 6.4-kb *Eco*RI fragment. The *ndvB* locus is enlarged. The solid bar indicates the sequenced region the Tn*5* insertion sites of the *R. meliloti* mutants Rm101.45 and Rm688 (Müller *et al.* 1988) are given. The open arrow shows the *ndvB* gene as published by Ielpi *et al.* (1990). The fragments shown above the restriction map of the 48.7-kb fragment were used to construct deletion mutants. Solid lines correspond to fragments given in the restriction map, while broken lines indicate the region that is replaced by an antibiotic resistance gene. Abbreviations: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Pst*I (P).

nonreplicating *E. coli* vectors. By using suitable restriction sites, large deletions within the insert were obtained. Different cassettes carrying antibiotic resistance markers for selection in *R. meliloti* were cloned to replace the deletions of the plasmids resulting in pAH1, pAH2, pHQ50, and pJQ61 (Fig. 1). The plasmids were mobilized into strain Rm2011 to select for a double crossing-over, resulting in a deletion in the *ndvB* gene and the insertion of the antibiotic resistance marker. The resulting deletion strains designated RmAH1, RmAH2, RmHQ50, and RmJQ61 were selected by loss of the antibioic resistance markers of the plasmid vectors. Homogenotization was also verified by hybridization experiments. Plant tests revealed Fix^+ nodules for the strains RmAH1 and RmAH2, whereas the strains RmHQ50 and RmJQ61 induced Fix^- nodules which were spherical, small, and white. Similar nodules were first described to be induced by *R. meliloti* mutants Rm101.45 and Rm688. The data obtained indicated that a long stretch of DNA upstream of the *ndvB* gene defined by the deletions in the mutants RmAH1 and RmAH2 was not essential for nodule infection. In contrast, the mutants RmHQ50 and RmJQ61 characterized by deletions in the *ndvB* gene could not infect alfalfa nodules.

Characterization of the *ndvB* deletion mutant RmHQ50 and its osmorevertant RmHQ50oR. A main reason for constructing defined *R. meliloti* deletion mutants was the observed instability of the original Tn*5*-induced *R. meliloti* mutants Rm101.45 and Rm688. We continued the work with the deletion mutant RmHQ50 and with the spontaneous osmorevertant RmHQ50oR. This mutant was selected with a frequency of 10^{-6} by growing RmHQ50 on

Table 2. Comparison of the wild-type Rm2011 and the *ndvB* mutants RmHQ50 and RmHQ50oR

Analyzed feature	Rm2011	RmHQ50	RmHQ50oR
β-1,2-glucans[a]	+	−	−
EPS-pyruvylation[b]	+	+	+
Proton NMR	WT	WT	nt[c]
Growth in TY[d]	++	−	−
Growth in TY + 200 mmol NaCl	++	++	++
Growth on TY[e]	++	−	+
Growth on TY + 200 mmol NaCl	++	++	++
Motility on TY[f]	++++	−	+
Motility on TY + 200 mmol NaCl	+++	++	++
Plant test[g]	Fix^+	Inf^-	Fix^-

[a] Thin-layer chromatography of isolated polysaccarides. β-1,2-glucans visible: +; β-1,2-glucans not detectable: −.

[b] Pyruvate determination in the acidic exopolysaccharide by an enzymatic assay. Pyruvylation like wild type: +; no pyruvylation detectable: −.

[c] Not tested.

[d] Growth in liquid TY medium. Growth like wild-type: ++; No growth: −.

[e] Growth on TY agar medium. Growth like wild-type: ++; growth reduced: +; No growth: −.

[f] Swarming observed on TY soft agar. Motility like wild-type: ++++; intermediate motility: +++ and ++; very little motility: +; no motility observed: −.

[g] Plant tests were performed for 3 wk. Fix^+: plants were tall and green, nodules cylindrical and pink, acetylene reduction test positive; Inf^-: plants were stunted and yellow, nodules spherical, acetylene reduction test negative; Fix^-: plants were stunted and yellow, nodules cylindrical and white, acetylene reduction test negative.

TY medium. The mutant RmHQ50oR retained the Gm/Sp resistance of the inserted cassette, indicating that no alteration of the deletion in mutant RmHQ50 had occurred. This could also be confirmed by hybridization experiments. Table 2 presents information on the different properties of the strains Rm2011, RmHQ50, and RmHQ50oR. Isolation of polysaccharides and thin-layer chromatography revealed that RmHQ50 and RmHQ50oR did not produce β-1,2-glucans (see also Fig. 2). The lack of β-1,2-glucans is considered to be responsible for osmosensitivity (Dylan *et al.* 1990a). Osmosensitivity was tested by growing the *R. meliloti* strains on TY agar and in liquid TY broth. Whereas RmHQ50 grew neither on solid nor in liquid medium, RmHQ50oR showed growth on TY agar but was still unable to grow in liquid TY broth. The discrepancy of reported EPS structures (pyruvylated/unpyruvylated) of *ndvB* mutants led us to test the pyruvylation of the acidic exopolysaccharide EPS of deletion mutant RmHQ50. This was carried out by an enzymatic assay. The acidic exopolysacchride of the wild-type strain Rm2011 as well as that of the *ndvB* mutants RmHQ50 and RmHQ50oR were found to carry pyruvate residues. This was in contrast to the finding of Müller *et al.* (1988), who reported that the EPS of the original Tn*5*-induced mutant Rm101.45 was unpyruvylated. We investigated the EPS of our *ndvB* mutant RmHQ50 further by ^{1}H-NMR spectroscopy. The signal for the pyruvate residue could clearly be detected. The ^{1}H-NMR spectrum of the EPS of mutant RmHQ50 was found to be identical to that obtained with the EPS of the *R. meliloti* wild-type strain 2011 (data not shown).

RmHQ50 was found to be nonmotile on TY soft agar, whereas RmHQ50oR exhibited a certain motility. By adding 200 mmol NaCl, neither mutant strain gained wild-type motility, although normal growth could be achieved on TY agar stabilized with NaCl. The plant nodulation test with 100 plants revealed that RmHQ50 induced only spherical nodules of the Inf$^-$ phenotype. On the contrary, its osmorevertant RmHQ50oR exhibited cylindrical nodules like the wild-type Rm2011. But these nodules were white and could not fix nitrogen.

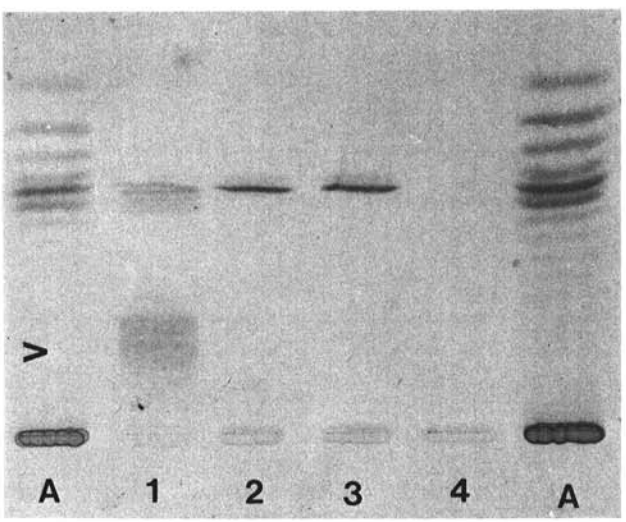

Fig. 2. Thin-layer chromatography of polysaccharides from different *Rhizobium meliloti* strains Rm2011 and RmHQ50. The *R. meliloti* strains were grown in 300-ml cultures, and the cell pellets were extracted with 10% TCA as described in Material and Methods. A, Dextran 20 as marker substance; 1–4, TCA-extracts of 1) Rm2011, 2) RmHQ50, 3) RmHQ50oR, and 4) Rm7Q60. The arrow indicates the expected position of β-1,2-glucans according to the publication of Zevenhuizen *et al.* (1990).

The osmorevertant RmHQ50oR invades alfalfa nodules and allows the formation of effective nodules following mixed inoculation with a nodulation mutant. The osmorevertant RmHQ50oR showed different behavior in plant nodulation tests, compared to the original deletion mutant RmHQ50. Therefore, the *R. meliloti* mutants RmHQ50, RmHQ50oR, and RmJQ60 were analyzed in more detail for their capacity to infect alfalfa nodules and to form effective nodules after mixed inoculation with the Tn*5*-induced nodulation mutant Rm2526. The Tn*5* insertion of Rm2526 is located within the *nodC* coding region. This was confirmed by sequencing the DNA adjacent to the Tn*5* insertion site and comparing the nucleotide sequence with the published *R. meliloti nodC* sequence (Jacobs *et al.* 1985). The results of the inoculation tests are summarized in Table 3. While the deletion mutant RmHQ50 only induced spherical pseudonodules, RmHQ50oR produced cylindrical white nodules. No bacteria could be isolated from pseudonodules induced by RmHQ50. Interestingly, only very few ($<10^3$) colony-forming bacteria were isolated from nodules induced by RmHQ50oR. These nodules, exhibiting the Fix$^-$ morphology, differed markedly when compared with *nifH* mutants. From nodules induced by *nifH* mutants bacteria could be reisolated at the same frequency as from wild-type nodules (10^6 colony-forming bacteria).

For further investigation, we tested the behavior of the *ndvB* deletion mutants in mixed inoculation experiments, because the original Inf$^-$ mutant Rm101.45 could not induce effective nodules in mixed inoculation experiments with a Nod$^-$ mutant (Müller *et al.* 1988). The same result was obtained for the *ndvB* mutant RmHQ50 in mixed inoculation experiments with the Nod$^-$ strain Rm2526. The nodules produced were small, spherical, and white. No bacteria could be reisolated.

To investigate if the exopolysaccharide of the *ndvB* mutant inhibits mixed infection as speculated by Müller *et al.* (1988), we introduced a second mutation into

Table 3. Single and mixed inoculations with *Rhizobium meliloti ndvB* mutants[a]

Strain I	Strain II	Nodule phenotype[b]	N_2-fixation[c]	Reisolated colonies	
				Strain I	Strain II
Rm2011	−	A	+	5×10^5	−
Rm2526	−	D	−	0	−
RmHQ50	−	C	−	0	−
RmHQ50	Rm2526	C	−	0	0
RmHQ50oR	−	B	−	$< 10^3$	−
RmHQ50oR	Rm2526	A	+	$<10^3$	5×10^5
RmJQ60	−	C	−	0	−
RmJQ60	Rm2526	C	−	0	0

[a] Nodules were squashed in LB media and plated on selective media.
[b] A: wild type, cylindrically shaped, pink; B: cylindrically shaped, white, short infection threads; C: spherically shaped, white; D: no nodules.
[c] Plants green and healthy, acetylene reduction test positive: + Plants yellow and stunted, acetylene reduction test negative: −.

RmHQ50. Via transduction, the Tn*5* insertion of the EPS$^-$ mutant Rm0540 was added to the genome of RmHQ50. The resulting strain, designated RmJQ60, lacked EPS production as demonstrated by precipitation and Cellufluor white staining. RmJQ60 induced only Inf$^-$ nodules in a plant test and remained Inf$^-$ when inoculated together with the nodulation mutant Rm2526. No bacteria could be reisolated. Inf$^-$ mutant Rm0540, in contrast, formed a well-established Fix$^+$ symbiosis when coinoculated with Nod$^-$ mutant Rm2526. Both partners colonized the inner cells of the root nodule and performed N_2 fixation (Kapp *et al.* 1990). Therefore, we concluded that in mixed inoculation experiments only the defects related to the *ndvB* locus were responsible for the Inf$^-$ phenotype.

When mixed inoculation experiments with RmHQ50oR and Rm2526 were performed, only Fix$^+$ nodules were observed and the alfalfa plants were green and healthy. When bacteria were reisolated from these Fix$^+$ nodules, the nodulation mutant Rm2526 dominated. Only very few RmHQ50oR colonies could be detected. Evidently, RmHQ50oR could help the Nod$^-$ mutant to invade the nodule and to form Fix$^+$ nodules. RmHQ50oR in contrast still lacked functions necessary for later stages of nodule formation and which could not be provided *in trans* on a cellular level by the Nod$^-$ partner.

The osmorevertant RmHQ50oR is blocked in multiplication within the plant cell and is defective in bacteroid development. To further investigate the cylindrical nodules induced by the osmorevertant RmJQ50oR, the nodules were analyzed by semithin sectioning and light microscopy. Sections of root nodules induced by the *R. meliloti* wild-type Rm2011, the *ndvB* deletion mutant RmHQ50 and the derived osmorevertant RmHQ50oR, were prepared 3 wk after inoculation of the plants. The cylindrical root nodule induced by the wild-type Rm2011 showed a fully infected central tissue. The typical zonation in meristem, infection zone, symbiotic zone, and a hardly perceptible senescence zone were observed (Fig. 3A). The *ndvB* deletion mutant RmHQ50 on the contrary induced a spherical pseudonodule, lacking infection threads (Fig. 3B). The central tissue is composed of small, noninfected parenchymatic cells. Additionally, more than one meristem could be detected, giving rise to the typical multilobed structure of pseudonodules. The osmorevertant RmHQ50oR (Fig. 3C) of the *ndvB* deletion mutant again induced infected cylindrical nodules, which showed a zonation dramatically changed when compared to a wild-type nodule. The infection zone seems to be enlarged, while a small symbiosislike zone is followed by an enlarged senescence zone.

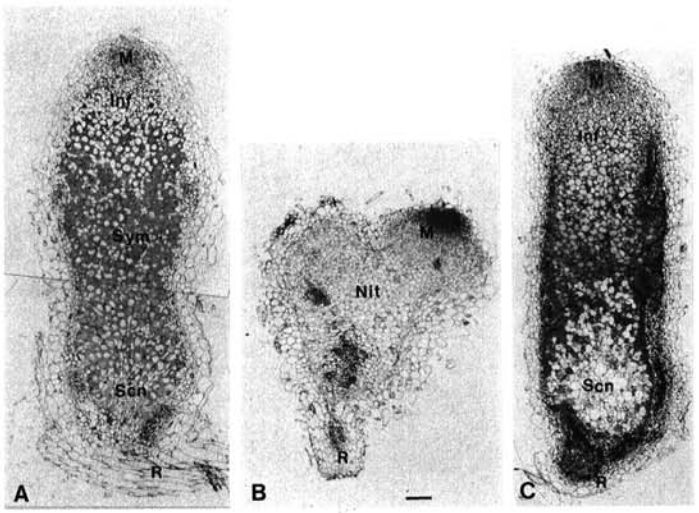

Fig. 3. Comparison of semithin sections of alfalfa root nodules induced by *Rhizobium meliloti* wild-type and *ndvB* deletion mutants. Three weeks after inoculation, semithin sections were prepared from alfalfa nodules induced by **A,** the *R. meliloti* wild-type Rm2011; **B,** the *ndvB* deletion mutant RmHQ50; and, **C,** the osmorevertant RmHQ50oR. In contrast to the cylindrical and fully infected wild-type nodule induced by Rm2011, the *ndvB* mutant RmHQ50 induced a spheroid pseudonodule which lacked infection threads and bacteroids. The osmorevertant RmHQ50oR leads to the formation of a cylindrical white nodule. Bar = 100 μm. Abreviations: meristem (M), noninfected tissue (Nit), senescence zone (Scn), symbiotic zone (Sym), root (R), infection zone (Inf).

A detailed analysis of 3-wk-old nodules induced by the osmorevertant RmHQ50oR resulted in the detection of released bacteria (Fig. 4A). The meristematic and the early infection zone are evidently identical to those seen in wild-type nodules. The early symbiotic zone is composed of many infected and enlarged plant cells containing only a few bacteria (Fig. 4B). This zone is more expanded than in the wild-type counterpart. An obvious difference exists between the symbiotic zones of nodules induced by the osmorevertant RmHQ50oR (Fig. 4C) and the wild-type Rm2011 (Fig. 4D). The plant cells within the symbiotic zone of nodules induced by RmHQ50oR are poorly infected and contain a large amount of starch granules. The released bacteria are small in size and evidently not differentiated into bacteroids.

From these observations it can be concluded that the osmorevertant of the *ndvB* deletion mutant has regained the capacity to infect alfalfa nodules, but this infection does not culminate in an effective symbiosis, because the bacteria released from the infection threads are blocked in multiplication and differentiation into bacteroids.

DISCUSSION

In our previous work we studied *ndvB* mutants of *R. meliloti* induced by Tn*5* mutagenesis (Müller *et al.* 1988). These mutants turned out to be very unstable, since in plant tests they often reverted back to the wild-type form, because of an exact excision of the inserted transposon Tn*5* (an observation that was also made by Dylan *et al.* [1990a]). We therefore used in this paper molecular genetic methods to construct a defined *ndvB* deletion mutant that was supposed to be more stable in plant experiments.

As far as the properties tested (Table 2), we could demonstrate that a deletion eliminating the promoter and the proximal part of the *ndvB* gene has the same effects as Tn*5* insertions in the first half of the *ndvB* coding region (Ielpi *et al.* 1990). These effects are the loss of β-1,2-glucans and occurrence of osmotic sensitivity in a medium of low osmolarity. We have also tested the major acidic EPS of the mutant RmHQ50. The ^{1}H-NMR spectrum and the enzymatic test revealed that the EPS is unaltered. This agrees with the finding of Ielpi *et al.* (1990) who reported the EPS of *ndvB* mutants to be like wild-type EPS.

Transduction of the Tn*5* of strain Rm101.45 to wild-type strain Rm2011 led to a *ndvB* mutant whose isolated EPS is pyruvylated (data not shown). Thus we speculate that the altered (unpyruvylated) EPS structure of strain Rm101.45 reported by Müller *et al.* (1988) is due to a second mutation in this strain. Additional deletions were introduced upstream of the *ndvB* coding region. No altered phenotype regarding infection, growth properties, and swarming could be identified within the adjacent 20-kb.

Thus, *ndvB* and the 2-kb downstream-located *ndvA* (Dylan *et al.* 1986) seem to be the only loci affecting osmolarity or nodule infection within a 40-kb region of the *R. meliloti* chromosomal DNA.

The use of a defined deletion mutant made it unlikely that any kind of reversion can occur in the original *ndvB* locus. Nevertheless we examined the deletion locus of RmHQ50oR by hybridization and found it to be unaltered (data not shown). This finding indicates that RmHQ50oR probably harbors an additional mutation, the locus of which has not been identified. The defects of the original *ndvB* mutant RmHQ50 altered by the new mutation are only partially restored. We call RmHQ50oR an osmorevertant because it can infect alfalfa nodules and exhibits growth on low osmolar medium but still lacks β-1,2-glucans. Nevertheless, it differs remarkably from those osmorevertants that were described by Dylan *et al.* (1990b). It neither achieved full symbiosis like the class of symbiotic revertants nor the motility as described for the motile revertants. It remains to be discovered which physiological alterations have led to the new phenotype. We speculate that either an osmolyte not yet detected can partially substitute for the β-1,2-glucans or that a structural change might allow the cells to tolerate the osmotic stress. We measured the osmolarity of our plant test medium, which turned out to be very low (0.01 osmol/kg) in contrast to LB medium (0.26 osmol/kg). The ability of RmHQ50oR to tolerate low osmolarity in contrast to its parental strain might be the reason for the infection of alfalfa nodules. On the other hand osmotic adaption in general seems not to be the key for infection because Dylan *et al.* (1990b) reported their symbiotic revertants to remain osmotically sensitive to some extent.

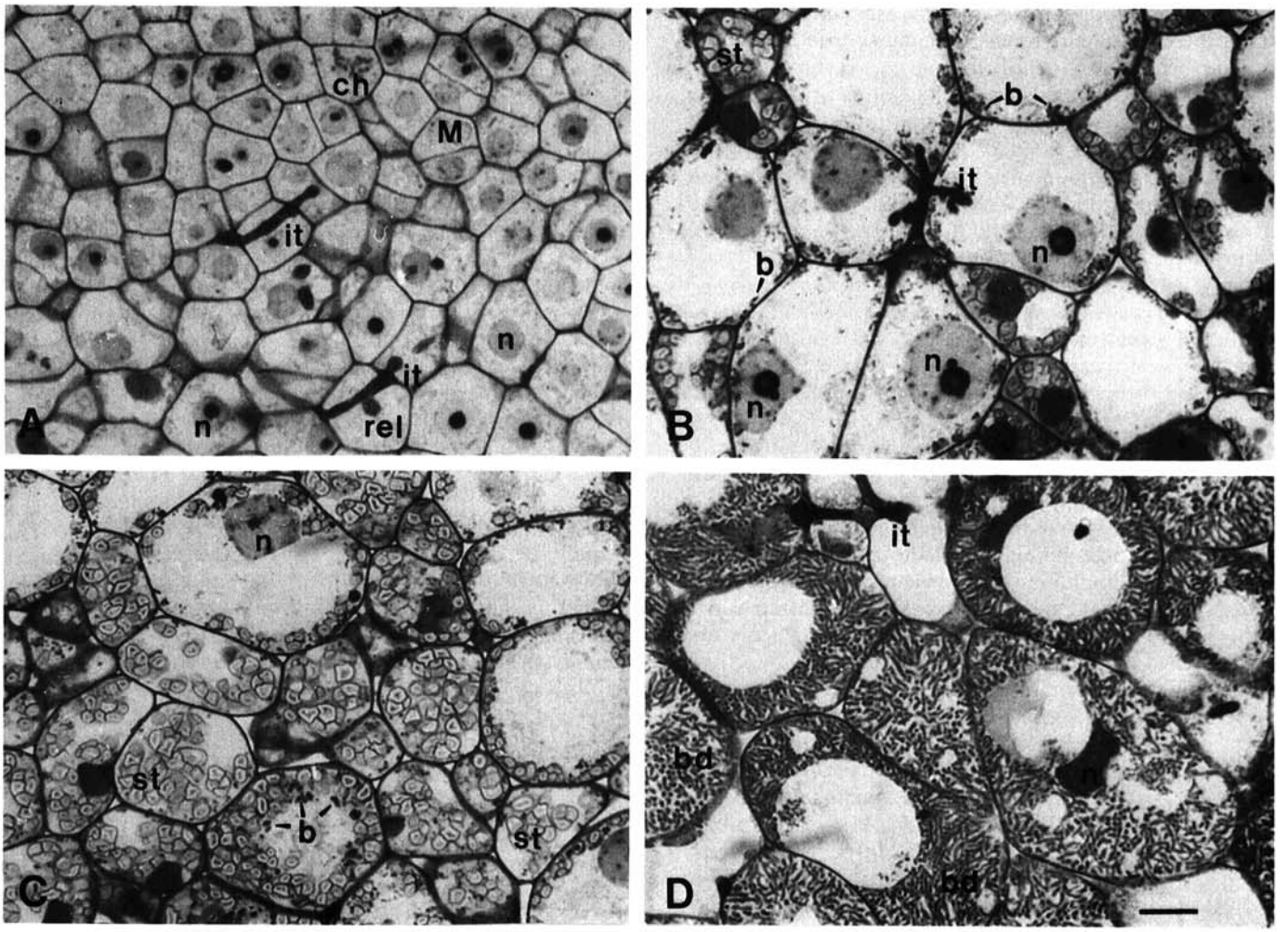

Fig. 4. Detailed light microscopic analysis of a root nodule induced by the osmorevertant RmHQ50oR 3 wk after inoculation. **A,** A detailed view of the meristematic zone. Infection threads and released bacteria can be seen. **B,** A part of the early symbiotic zone. Infected cells with poor colonization can be observed. **C,** Poorly infected cells of the symbiotic zone with large amounts of starch granules. **D,** The symbiotic zone of the wild-type nodule shows a massive infection and no starch granules. The bar represents 10 μm. Abreviations: bacteria (b), bacteroids (bd), chromosomes (ch), infection thread (it), meristem (M), nucleus (n), release of bacteria (rel), starch granules (st).

The osmorevertant RmHQ50oR is able to induce an infected root nodule. Nevertheless, after the release from the infection thread only a few bacteria were detectable within the plant cytoplasm. The enlarged infection zone indicates that RmHQ50oR fails to multiply after the release from the infection thread. It is as yet unclear whether the parameters within the plant cytoplasm are not conducive or whether the bacteria are themselves incapable of undergoing a specific differentiation step leading to multiplication and differentiation into bacteroids. An interesting question is why RmHQ50oR remains Fix$^-$. It is known that mutation of *ndvB* also leads to several alterations in the cell surface (Dylan *et al.* 1990a). One of these may prevent RmHQ50oR from colonizing the infected nodule properly, from developing into bacteroids and from fixing nitrogen. The ability of RmHQ50oR to survive within the nodule seems to be severely reduced because only very few colonies could be found in reisolation experiments. We cannot exclude that RmHQ50oR is still capable of fixing N_2, which might be undetectable because of the small number of viable bacteria within the nodule.

The missing β-1,2-glucans of RmHQ50oR led to the conclusion that these β-1,2-glucans are not essential for nodule infection. As demonstrated by symbiotically competent revertants that still lack the β-1,2-glucans, these are also not required in later steps of development (Dylan *et al.* 1990b).

Although reduced, the ability of RmHQ50oR to invade alfalfa nodules seems to be the reason that Fix$^+$ nodules are formed in mixed inoculation experiments. The Nod$^-$ partner is able to colonize the nodule and to fix nitrogen, in contrast to mixed infections with RmHQ50. This is probably because RmHQ50 is not able to survive in the infection thread. As shown by Kapp *et al.* (1990) for mixed inoculation experiments with EPS$^-$/Nod$^-$ mutants, both partners have to be present in close contact in an early stage of nodule development. Our experiments reveal that also in the system of mixed inoculation of Nod$^-$ mutant and RmHQ50oR, close contact of both partners is necessary in the infection thread. The Nod$^-$ mutant cannot form infected nodules even when a wild-type *R. meliloti* is added but separated from the roots by a filter. In a later stage of nodule development, the inoculation partners seem to be no longer strictly dependent on each other because the ratio of bacteroids of a Nod$^-$ mutant and its partner could be found to differ in a wide range within single infected plant cells.

Our experiments, where only a few bacteria of RmHQ50oR were released from the infection thread, demonstrate the independence of the Nod$^-$ mutant Rm2526 from Nod functions in later stages of nodule infection, release, and nitrogen fixation. On the other hand, the Nod$^-$ mutant cannot help RmHQ50 to infect alfalfa nodules or provide functions that would allow RmHQ50oR to carry out successfully later steps of nodule invasion. Thus, β-1,2-glucans or other molecules that are provided at a distance and that might function as signals are not the crucial deficiency in *ndvB* mutants nor in early or later steps of nodule infection. On the contrary, the results reported in this paper support the idea that alterations of the cell envelope are responsible for the inability of *R. meliloti ndvB* mutants to propagate infection threads. In RmHQ50oR, these alterations might have been only partially restored or substituted by the secondary mutation, thus allowing infection thread formation and invasion but still preventing the proper colonization of the inner cortex and subsequently the effective nitrogen fixation.

Future investigation should focus on cell surface components to identify the crucial phenotype of pleiotropic *R. meliloti ndvB* mutants that is responsible for the failure to infect alfalfa nodules.

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