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β-Glucan Synthesis in *Bradyrhizobium japonicum*: Characterization of a New Locus (*ndvC*) Influencing β-(1→6) Linkages†

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***Bradyrhizobium japonicum* synthesizes periplasmic cyclic β-(1→3),β-(1→6)-D-glucans during growth in hypoosmotic environments, and evidence is growing that these molecules may have a specific function during plant-microbe interactions in addition to osmoregulation. Site-directed Tn*5* mutagenesis of the DNA region upstream of *ndvB* resulted in identification of a new gene (*ndvC*) involved in β-(1→3),β-(1→6)-glucan synthesis and in nodule development. The predicted translation product was a polypeptide (ca. 62 kDa) with several transmembrane domains. It contained a sequence characteristic of a conserved nucleoside-sugar-binding motif found in many bacterial enzymes and had 51% similarity with a β-glucanosyltransferase from *Candida albicans*. *B. japonicum* carrying a Tn*5* insertion in *ndvC* resulted in synthesis of altered cyclic β-glucans composed almost entirely of β-(1→3)-glycosyl linkages. The mutant strain was only slightly sensitive to hypoosmotic growth conditions compared with the *ndvB* mutant, but it was severely impaired in symbiotic interactions with soybean (*Glycine max*). Nodulation was delayed by 8 to 10 days, and many small nodule-like structures apparently devoid of viable bacteria were formed. This finding suggests that the structure of the β-glucan molecule is important for a successful symbiotic interaction, and β-glucans may have a specific function in addition to their role in hypoosmotic adaptation.**

Pathogenic and symbiotic interactions with host plants are a characteristic of microorganisms from the family *Rhizobiaceae* (19, 38, 40). All species from this family examined so far have been shown to synthesize periplasmic cyclic β-glucans (10, 58, 60). Most studies on β-glucans have been done with *Rhizobium meliloti* and *Agrobacterium tumefaciens*, which produce cyclic β-(1→2)-D-glucans composed of 17 to 25 glucose residues which may be substituted with phosphoglycerol and/or succinate or methylmalonate (3, 4, 31). It is generally accepted that cyclic β-(1→2)-glucans function not only during plant-microbe interactions but also during bacterial adaptation to hypoosmotic stress (13, 22, 44, 48, 59). Two genes from *Rhizobium* sp. and *Agrobacterium* sp. that code for the synthesis and transport of cyclic β-(1→2)-D-glucans have been identified (11, 23). The nodule development (*ndvB*) gene from *R. meliloti* (chromosomal virulence [*chvB*] gene in *A. tumefaciens*) encodes a large membrane protein, estimated at 319 kDa by nucleotide sequence analysis (33) and 235 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (60). The NdvB protein is thought to be a multifunctional enzyme that catalyzes the synthesis of the cyclic β-glucan molecules (33). The second gene, *ndvA* (*chvA* in *A. tumefaciens*), appears to encode an ABC-type transporter (26) that allows the cyclic β-glucans to move from the cytoplasm to the periplasmic space (12, 47, 54). The nodule development (*ndv*) mutants of *R. meliloti* in previous studies exhibited pleiotropic phenotypes such as loss of motility, increased resistance to bacteriophage infection, and increased sensitivity to certain antibiotics (13, 22).

Bradyrhizobium japonicum does not synthesize cyclic β-(1→2)-glucans. Instead, this organism produces cyclic β-(1→3),(1→6)-glucans composed of 11 to 13 glucosyl residues (15, 42, 50) which may be substituted with phosphocholine. These structurally different molecules appear to be functionally equivalent to the cyclic β-(1→2)-glucans for hypoosmotic adaptation (9, 41, 48). Molecules with various proportions of β-(1→3) and β-(1→6) linkages have been observed (21, 42, 50, 55). This mixture of linkages suggests that the pathway for cyclic β-(1→3),(1→6)-glucan synthesis may be different from and more complex than that for the synthesis of cyclic β-(1→2)-glucan molecules.

Using the hypothesis that the different species of β-glucans were functional analogs for osmoregulation, we identified a clone from a *B. japonicum* gene library (cosmid clone p5D3) which complemented the *ndvB* mutant of *R. meliloti* for hypoosmotic tolerance. This clone carried the genetic information for synthesis of β-(1→3),(1→6)-linked glucans and complemented an *ndvB* mutant of *R. meliloti* for effective symbiotic interaction with alfalfa (9). Subsequently we identified an *ndvB*-like locus on a subclone, p115. Site-directed Tn*5* mutagenesis and homogenotization of this mutation into the genome of *B. japonicum* USDA 110 resulted in a hypoosmotically sensitive strain, AB-14, which was unable to synthesize cyclic β-(1→3),(1→6)-linked glucans (7).

In this paper, we report discovery of an additional gene involved in *B. japonicum* glucan synthesis which we have named *ndvC* and which appears to be involved in the synthesis of β-(1→6) linkages. With a Tn*5* insertion in this locus, *B.*

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TABLE 1. Bacterial strains, cosmids, and vectors used

Strain, cosmid, or vector	Description	Reference
Bacterial strains		
B. japonicum USDA 110	Wild-type strain	55
B. japonicum AB-14	*ndvB*::Tn*5* mutant of *B. japonicum* USDA 110	7
B. japonicum AB-1	*ndvC*::Tn*5* mutant of *B. japonicum* USDA 110	This study
R. meliloti 102F34	Wild-type strain	23
R. meliloti TY7	*ndvB*::Tn*5* mutant of *R. meliloti* 102F34	33
Cosmids		
p5D3	Glucan synthesis locus from *B. japonicum* USDA 110 gene library	9
p115	Subclone of p5D3 with internal *Hin*dIII deletions	7
Vector		
pSUP202	Mobilizable suicide vector; Ap^r Cm^r Tc^r	18

japonicum synthesized glucans with predominantly β-(1→3) linkages. This new class of glucan molecule appears to function effectively in osmoregulation but not in supporting symbiotic effectiveness in soybean. Thus, segregation of the pleiotropic phenotypes as a result of mutations in the glucan synthesis locus was possible for the first time.

MATERIALS AND METHODS

Bacteria and culture conditions. The bacterial strains and vectors used in this study are described in Table 1. *B. japonicum* strains were cultured at 28°C in arabinose-gluconate (AG) growth medium (16). *R. meliloti* strains were cultured at 28°C in gluconate-yeast extract-mannitol growth medium (22). *Escherichia coli* was grown at 37°C in LB medium (52). All media were supplemented with antibiotics when appropriate. The growth rate in AG medium was monitored turbidimetrically at A_{410}, using 1% of inoculum from stationary-phase cultures. Motility was determined 3 days after spot inoculation on AG medium containing 0.35% Difco agar.

Mutagenesis. Fragment-specific Tn*5* mutagenesis of cosmid p115 was carried out as described previously (18). The location of each Tn*5* insertion was determined by restriction endonuclease analysis. Insertions were mobilized to *R. meliloti* TY7 by triparental mating (18). For homologous recombination at the *ndvC* locus, a 2.05-kbp *Eco*RI fragment plus Tn*5* insertion 1 (total of 7.8 kbp) was subcloned into pSUP202 and mobilized to *B. japonicum* USDA 110 by triparental mating. The exconjugants were selected on AG agar medium containing antibiotics (kanamycin and streptomycin; each at 200 μg/ml), and colonies were screened for loss of pSUP202 essentially as described by Fu and Maier (27). Total genomic DNA was isolated from selected colonies (7), and the position of Tn*5* in the chromosome was determined by Southern blot analysis.

Assays for glucan synthesis. Glucans from *B. japonicum* and *R. meliloti* were extracted from cell pellets with perchloric acid and were isolated and purified on a C_{18} silica reverse-phase column as previously described (8, 50). Extracellular glucans were isolated from the medium after pelleting of the cells. The extracellular polysaccharides were precipitated by adding 3 volumes of ethanol, the supernatant was evaporated to 1/10 of the original volume, and 10 volumes of ethanol was added. The mixture was kept at −20°C for 2 to 3 h, and extracellular glucans were collected by centrifugation. The crude extracellular glucans were dissolved in water and dialyzed (molecular mass cutoff, 1,500 Da) against distilled water before C_{18} silica reverse-phase column purification. Samples of glucans (100 to 150 μg) for glycosidic linkage analysis were permethylated, subjected to acid hydrolysis, acetylated, and analyzed by capillary gas chromatography-mass spectrometry (9). For in vitro glucan synthesis, cell-free membranes were prepared and assayed by using UDP-[U-^{14}C]glucose (266 mCi/mmol) as previously described (6). To isolate glucans synthesized in vitro, 10 mg of cell-free membranes was suspended in 30 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH buffer (10 mM, pH 7.5) containing 5 mM UDP-glucose and 10 mM $MnCl_2$. After denaturing of the enzyme at 100°C for 2 min and centrifugation at 14,000 × *g* for 20 min, the supernatant was dialyzed (molecular mass cutoff, ca. 1,500 Da) against water for 48 h at 4°C. The neutral fraction was collected by passing the supernatant over a DEAE-cellulose column (2.5 by 8 cm) followed by elution with water. Glucans were purified by C_{18} silica reverse-phase column chromatography as described above.

Plant tests. Seeds of soybean (*Glycine max* cv. Williams) and alfalfa (*Medicago sativa* cv. Sarnac) were surface sterilized (56) and sown in modified Leonard jars filled with vermiculite-perlite (3:1). Jars were moistened with N-free nutrient solution (56) and autoclaved before use. Plants were maintained in a greenhouse with low-pressure Na illumination to supplement natural light. Nodules were harvested at 5 weeks after inoculation, and samples were prepared for freeze-fracture scanning electron microscopy (57).

DNA sequence analysis. Overlapping restriction fragments of the *ndvC* region were subcloned into the M13 vectors mp18 and mp19, and single-stranded templates were isolated (52). Nucleotide sequence determinations were performed with a TaqTrack sequencing kit (Promega Corp.) and Sequenase 2.0 kit (Amersham Corporation, Arlington Heights, Ill.), using a universal 17-mer M13 sequencing primer. Sequencing reactions were run with three labeling mixes, dGTP, dITP, and 7-deaza-dGTP, to resolve regions of compression on the gels. Sequencing gels were made by using the HydroLink DNA sequencing system (AT Biochem, Malvern, Pa.). The DNA sequence analysis was done with the Wisconsin sequence analysis program (28a).

Nucleotide sequence accession number. The sequence reported is recorded as GenBank accession number U49784.

RESULTS

Identification of a new *ndv* locus. p115 (Fig. 1) is a subclone of cosmid p5D3 which originated from a *B. japonicum* USDA 110 gene library. This clone previously was shown to contain the genetic information for complementing *R. meliloti* TY7 (*ndvB*) for osmoregulation and symbiosis (7, 9). The glucans synthesized by *R. meliloti* TY7(p5D3) were cyclic and contained predominantly β-(1→3)-linked glucose, in contrast to the cyclic glucans produced by wild-type USDA 110, which are composed of β-(1→3),(1→6)-linked glucose. Strain TY7(p5D3) was symbiotically effective with alfalfa (9). In an attempt to locate additional genes involved in glucan synthesis and function, several Tn*5* insertions upstream of the *ndvB* locus were generated on p115 and individually mobilized to *R. meliloti*

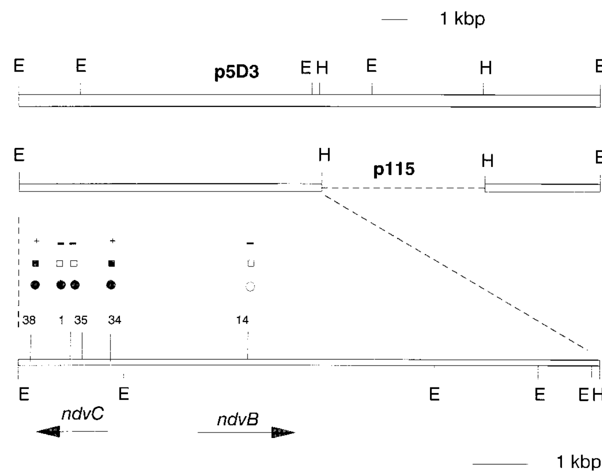

FIG. 1. Cosmid clone p5D3 and subclone p115. Individual Tn*5* insertions on p115 were mobilized to *R. meliloti* TY7 (*ndvB*) to examine glucan synthesis, motility, and symbiotic phenotype with alfalfa. E, *Eco*RI; H, *Hin*dIII. Symbols represent synthesis (■) or absence (□) of cellular (in vivo) glucans and synthesis (●) or absence of (○) in vitro glucans. +, symbiotically effective; −, symbiotically ineffective. The locations and the directions of transcription of *ndvC* and *ndvB* are shown by arrows.

TABLE 2. Glucan synthesis by *R. meliloti* transconjugant strains

Strain	Glucan synthesis[a]	
	In vitro[b] (nmol of glucose incorporated/h/mg of protein)	In vivo[c] (μg of glucan/mg of protein)
102F34	2.67	37.2
TY7	0.01	2.5
TY7(p5D3)	1.85	25.4
TY7(p115)	2.72	29.7
TY7(p115::Tn*5*#34)	2.46	26.2
TY7(p115::Tn*5*#35)	1.44	4.4
TY7(p115::Tn*5*#1)	1.44	2.1
TY7(p115::Tn*5*#38)	1.33	21.6

[a] Average of two experiments.
[b] UDP-[^{14}C]glucose was used as the substrate.
[c] β-Glucans were isolated from cells grown in gluconate-yeast extract-mannitol broth for 3 days.

TY7. These strains, carrying individual p115::Tn*5* insertions, were analyzed for in vitro and in vivo glucan synthesis and for symbiotic competence with alfalfa. As shown in Table 2, two strains with Tn*5* insertions 1 and 35 did not synthesize significant amounts of cellular glucans in vivo, but membrane preparations were active in vitro at about 50% of the rate of the wild type. The constructs were symbiotically ineffective (Fix$^-$) on alfalfa, producing white pseudonodules (Fig. 1). Tn*5* insertions 34 and 38 had essentially the wild-type phenotype and therefore apparently delimited the locus.

To create this mutation in *B. japonicum*, the *Eco*RI fragment from p115 carrying Tn*5* insertion 1 was cloned into pSUP202 and was mobilized to wild-type *B. japonicum* USDA 110, generating a site-specific mutation. The site of the mutation and the absence of vector sequences were confirmed by Southern blot analysis (data not shown). The resulting strain was designated *B. japonicum* AB-1.

Symbiotic phenotype of strain AB-1. On the basis of the Fix$^-$ phenotype of *R. meliloti* TY7(p115::Tn*5*#1) and TY7(p115::Tn*5* #35) on alfalfa, we expected AB-1 to be Fix$^-$ in soybean, and this was confirmed. Strain AB-1 produced numerous small immature nodule-like growths (pseudonodules) on soybean roots from which no viable bacteroids could be isolated. Formation of these pseudonodules was much delayed and was visible only 15 to 18 days after inoculation. At 4 weeks after inoculation, the host plants were visibly nitrogen deficient (yellow in color). Scanning electron microscopy of freeze-fractured pseudonodules (Fig. 2) revealed empty but differentiated host cells with comparatively thickened cell walls and dense endoplasmic reticulum compared with wild-type nodules. No infection threads were visible in these pseudonodules (44a).

Glucan synthesis by *B. japonicum* strain AB-1. As shown in Table 3, there did not appear to be a significant difference in the quantities of intracellular and extracellular glucans produced by AB-1 and wild-type USDA 110. The amount capsular polysaccharide produced by strain AB-1 was comparable to that synthesized by USDA 110 (data not shown). The membrane preparations from both the strains synthesized neutral β-glucans in vitro at about the same rate. These differences do not appear to be sufficient to explain the Fix$^-$ phenotype of strain AB-1; therefore, we examined the glucans produced by strain AB-1 for their glycosyl linkage composition. As shown in Table 4, the glucans from strain AB-1 were found to be predominantly β-(1→3) linked, strikingly different from glucans from the wild-type strain. The linkage pattern of extracellular glucans secreted into the culture medium was similar to that for the cellular glucans. Further characterization of the glucan synthesized by strain AB-1 (49) indicated a molecule structurally different from any β-glucan molecules previously reported to be produced by wild-type strains.

We studied the linkage composition of the β-glucans produced by cell-free membrane preparations. The glucans produced by strain AB-1 were similar to those produced in vivo. Curiously, the composition of the molecules produced by wild-type strain USDA 110 was different from that of the molecules produced in vivo. Although the explanation for this observation is not apparent, the data closely resemble the composition of the model proposed by Rolin et al. (50).

Growth and motility characteristics of strain AB-1. Growth and motility characteristics of strain AB-1 in low-osmolarity medium (AG broth [16]) were only slightly different from those of wild-type strain USDA 110 (Fig. 3 and 4). Compared with strain AB-14 (*ndvB*), strain AB-1 had a lag period similar to that of wild-type USDA 110 and a growth rate only slightly lower than that of the wild type (Fig. 3). In semisolid AG medium (0.35% agar) (Fig. 4), strain AB-1 was almost as motile as the wild type, whereas AB-14 was nonmotile.

Sequence analysis of the *ndvC* locus. To gain insight into the nature of the product encoded by this locus, the 2.05-kbp *Eco*RI DNA fragment (Fig. 1) flanking Tn*5* insertion 1 was sequenced. We identified one open reading frame within the sequenced region on the basis of potential start codons. The putative translational start site at the first available ATG codon of the open reading frame results in a polypeptide with 556 amino acid residues and a predicted molecular mass of 62 kDa. A search of the protein sequence database by using BLAST (1) revealed some regions in the putative NdvC that were strongly similar to regions in β-(1→3)-glucanases from tobacco (39) and *Saccharomyces cerevisiae* (35, 46) and a glucanosyltransferase from *Candida albicans* (30, 36). The deduced amino acid sequence of NdvC showed 27% identity (and up to 51% similarity, allowing conservative substitutions) with enzymes involved in β-glucan metabolism from yeast species (Fig. 5a).

A Kyte-Doolittle plot (20, 37) of the predicted translation product revealed several potential transmembrane domains (Fig. 6), consistent with a membrane location for the putative product.

DISCUSSION

Previously we identified a DNA locus which complemented an *ndvB* mutant of *R. meliloti*. We constructed a strain of *B. japonicum*, AB-14, with a Tn*5* insertion in this locus, and the resulting mutant had a phenotype similar to that of *ndvB* mutants of *R. meliloti* (7); i.e., it lacked in vivo and in vitro glucan synthesis and was hypoosmotically sensitive and symbiotically ineffective. In the present study, analysis of an adjacent DNA region revealed the presence of another gene involved in β-(1→3),(1→6)-glucan synthesis and in nodule development. This gene, which we have designated *ndvC*, appears to be involved in the synthesis or the control of the synthesis of β-(1→6) linkages in the glucan molecule. *ndvC* appears to encode a 62-kDa polypeptide with several transmembrane domains (Fig. 6). In a preliminary report, on the basis of the Fix$^-$ symbiotic phenotype of strain AB-1 and the strain's ability to synthesize glucans from UDP-[^{14}C]glucose in vitro, we postulated that the Tn*5* insertions 1 and 35 may be in a locus similar to that in *ndvA* (5), which is postulated to code for a transporter protein. This view was not supported by our subsequent work with strain AB-1, which revealed that glucans were syn-

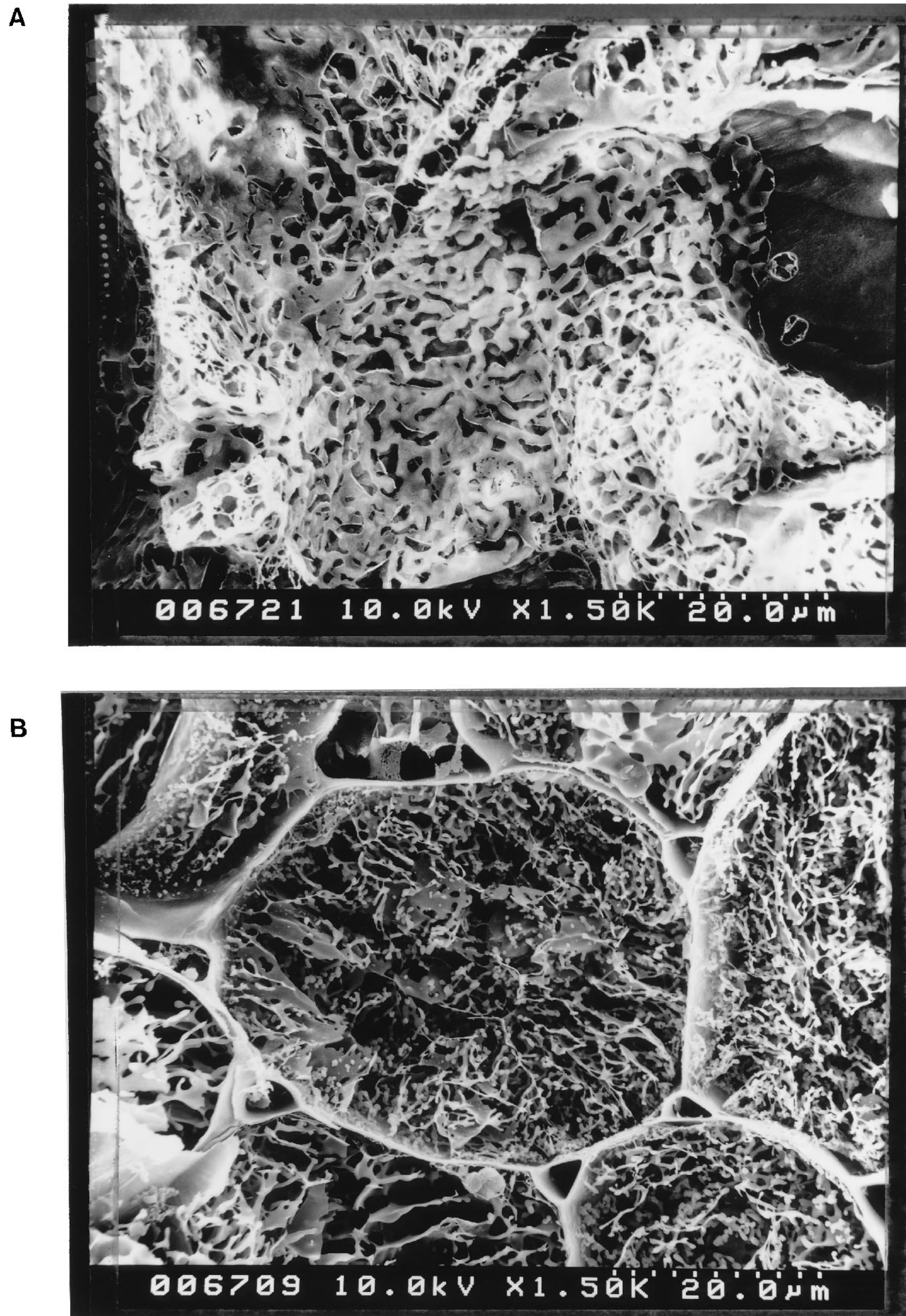

FIG. 2. Freeze-fracture scanning electron micrographs of soybean nodules. Nodules were selected for microscopy (57) from plants 5 weeks after inoculation with *B. japonicum* USDA 110 (A) and AB-1 (B). The dotted bar represents 20 μm.

thesized and excreted into the medium (Table 3). Linkage analysis of the glucans illustrated that this strain produced molecules with almost entirely β-(1→3) linkages (Table 4), suggesting that the product of *ndvC* is involved in the synthesis of the β-(1→6) linkages of *B. japonicum* glucans. A very small amount (less than 4 mol%) of β-(1→6) linkages was found in the analysis, which we assume at this stage may be a minor contamination in the glucan fraction. How β-glucans are cyclized is not fully understood, and occurrence of a β-(1→6) linkage on the branch glucose molecule in glucans of strain

TABLE 3. Glucan synthesis by *B. japonicum* USDA 110 and AB-1

Strain	In vivo glucan synthesis[a] (μg of glucan/mg of protein)		In vitro glucan synthesis (nmol of [^{14}C]glucose incorporated/h/mg of protein by cell-free membrane preparations)
	Intracellular	Extracellular	
USDA 110	76	71	5.85
AB-1	51	86	5.01

[a] Average of two experiments. β-Glucans were isolated from cells grown in AG broth for 4 days. Cell pellets were extracted with perchloric acid to obtain cellular glucans. Extracellular glucans were isolated from the medium after pelleting of the cells.

AB-1 could possibly result from an unknown mechanism of cyclization. Cellular glucan produced by strain AB-1 quantitatively was comparable to the wild-type amount, and export to the extracellular medium appeared to be unaffected (Table 3). We observed identical glycosyl linkage composition from glucans synthesized in vivo and in vitro by strain AB-1 (Table 4).

Glucans synthesized in vitro in cytoplasmic membrane preparations from the wild-type strain had essentially equimolar ratios of β-(1→6) to β-(1→3) linkages. Several researchers have analyzed the β-(1→3),(1→6)-glucans from *B. japonicum* USDA 110 (50, 55), and considerable variation has been reported in the ratio of β-(1→3) to β-(1→6) linkages (Table 4). Even greater variation was reported earlier for the glucans from *B. japonicum* 3I1b71a (21). Whether this observation reflects a possible regulatory mechanism in β-glucan synthesis and whether it has biological significance are not apparent at this stage in our understanding. The glucans isolated from wild-type cells are a mixture of molecules with different degrees of polymerization (ca. 10 to 14) (42, 50, 55). Thus, variation in glycosyl linkage ratios may be expected from various preparations in which different growth, isolation, and purification procedures were used, but this cannot explain the gross variation found in strain AB-1. Thus, we hypothesize that the altered structure of the β-glucan in strain AB-1 is the primary factor causing the nodule development phenotype.

Although the β-(1→3)-glucans produced by strain AB-1 are not effective in supporting nodule development, they are fairly effective in providing osmoprotection during growth on low-osmolarity media. Strain AB-1 was almost as motile as the wild type in semisolid agar (Fig. 4), and the growth rate on low-osmolarity medium was only slightly lower than that of wild-type USDA 110 (Fig. 3).

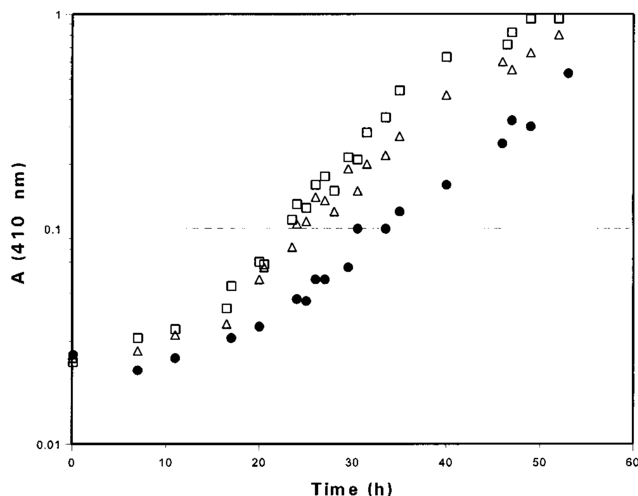

FIG. 3. Growth of *Bradyrhizobium* strains in AG medium. Stationary-phase cells from AG medium were inoculated into a fresh AG medium and incubated at 28°C on a rotary shaker. Growth was monitored by measuring A_{410}. Data are from three experiments. □, USDA 110; △, AB-1; ●, AB-14.

Two hypotheses can be derived from these results: (i) the locus containing the Tn*5* insertion in AB-1 somehow influences or controls the synthesis of β-(1→6) linkages of the β-glucans, and (ii) there is specificity for the type of glucan required to support an effective symbiosis. The observation that the β-(1→3)-linked glucans support an effective symbiosis in alfalfa but not in soybean also is consistent with a structural specificity of glucans for symbiotic function. However, why p115, which contains *ndvC*, synthesized only β-(1→3)-linked glucans is not understood. One possibility which is under investigation is that *ndvC* is not transcribed efficiently in the *R. meliloti* genetic background.

An alignment of the putative *ndvC* translation product with sequences found in the protein database (GenBank, release 92) revealed similarity to other enzymes involved in β-glucan metabolism. Of particular interest are endo-β-(1→3)-glucanases from *S. cerevisiae* (46) and glucanosyltransferase from

TABLE 4. Linkage analysis of β-glucans synthesized in vivo and in vitro from *B. japonicum* and *R. meliloti* strains

Strain	Glucans produced	Source[a]	Glucose linkages (mol%)				Reference
			(1,6)	(1,3)	(1,3)(1,6)	Terminal	
B. japonicum							
USDA 110	In vivo	Cellular	71	10	13	5	This work
		Extracellular	73	10	10	7	This work
	In vitro	Cell-free membranes	37	40	9	14	This work
AB-1	In vivo	Cellular	4	68	17	11	This work
		Extracellular	0	69	15	16	This work
	In vitro	Cell-free membranes	2	67	16	15	This work
USDA 110	In vivo	Cellular	68	9	18	7	55
			59	35	ND[b]	ND	41
			46	38	8	8	50[c]
3I1b71a	In vivo	Extracellular	27	56	8	9	21
R. meliloti TY7(p5D3)	In vivo	Cellular	5	62	20	13	9

[a] β-Glucans were isolated as described in the footnote to Table 3. In vitro glucans were synthesized by using cell-free membranes and unlabeled UDP-glucose as the substrate. The glucans were purified by DEAE-cellulose and C_{18} reverse-phase column chromatography (50).
[b] ND, not determined.
[c] Based on model structure proposed.

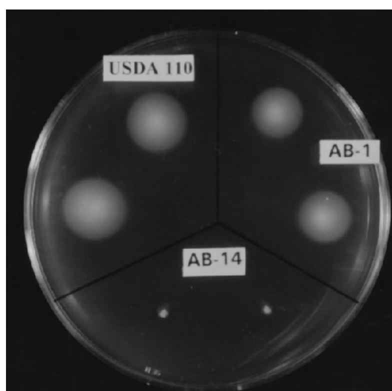

FIG. 4. Motility of *B. japonicum* strains in semisolid AG medium. Stationary-phase inoculum was spot inoculated on semisolid (0.35% agar) AG medium. Plates were photographed after 3 days of incubation at 28°C.

C. albicans (30, 36), to which NdvC had up to 51% similarity, allowing for conservative substitutions (Fig. 5a). This level of similarity is not in itself outstanding except that it includes two potential substrate-binding motifs: (i) a conserved nucleotide sugar-binding motif found in bacterial and yeast proteins, -Lys-Val-Gly-Gly- (25, 28), is found in the predicted NdvC Lys-115-Val-Gly-Gly-118, and (ii) the putative NdvC protein had a region of similarity (Glu-248-Ile-Leu-Ile-Gly-Glu-253) in the vicinity of a conserved active-site domains of other enzymes involved in β-glucan metabolism, e.g., yeast β-glucan synthases KRE6p and SKN1p (51), β-(1→3)-glucan clotting factor FGA from horseshoe crab (53), and β-(1→3)-glucanase from tobacco (39) (Fig. 5b). These similarities provide support for the idea that NdvC has a role in synthesis of β-(1→3)(1→6)-glucans, perhaps in controlling the addition of β-(1→6)-linked units.

The cyclic β-(1→3),(1→6)-glucans of *B. japonicum* share structural features with branched β-(1→3),(1→6)-linked glucan fragments derived from the mycelial walls of fungal pathogens of the soybean plant, which have been shown to be potent elicitors of plant defense compounds such as phytoalexins (24). It has been suggested that one way by which pathogens and symbionts can avoid plant defenses is by secreting suppressor compounds that prevent these defense responses (14, 19, 40). Suppressors have been isolated from various fungal pathogens and characterized (2, 34). Synthesis of cyclic β-glucan continues throughout soybean nodule development, and the levels within *B. japonicum* bacteroids isolated from mature soybean nodules were similar to levels present within aerobic, free-living cultures (29, 50). Although cyclic β-glucans of *B. japonicum* are very weak elicitors of glyceollin production in soybean cotyledons (43, 45), it is probably more important that during coincubation of fungal and bradyrhizobial glucans with soybean cotyledons, induction of a host defense response was suppressed by the bradyrhizobial glucans in a concentration-dependent manner (45). In assays with a ^{125}I-labeled photoaffinity conjugate of the hepta-β-glucoside elicitor and solubilized soybean root membrane receptor protein (17, 24), the cyclic β-(1→3),(1→6)-glucans of *B. japonicum* competitively inhibited binding of the elicitor (45). The absence of bacteroid development as well as thickened cell walls observed in the cortex of pseudonodules may be indicative of plant defense responses during interaction with strain AB-1. Thus, failure to develop a symbiotic association by strain AB-1 may in part be due to the inability of the structurally altered β-(1→3)-glucans to suppress the induction of inducible plant defenses.

FIG. 5. Similarity of NdvC to yeast and bacterial enzymes involved in β-glucan metabolism. (a) Comparison of the deduced amino acid sequence of the *ndvC* gene product with sequences of endo-β-(1→3)-glucanase from *S. cerevisiae* (46) (GenBank accession number p15703) and glucanosyltransferase from *C. albicans* (30) (GenBank accession number p43070). Alignment was performed by using the Wisconsin sequence analysis package (28a). Residues conserved with NdvC are boxed. (b) Similarity of NdvC to the bacterial β-glucanase active-site sequence and other β-glucan-binding proteins. The conserved residues are boxed. An asterisk identifies the proposed catalytic nucleophile (32). Accession numbers for the proteins are M80657 (KRE6p, β-glucan synthase from *S. cerevisiae*); L18859 (SKN1p, β-glucan synthase from *S. cerevisiae*); U04836 [BglA, β-(1→3)-glucanase from *Rhodothermus marinus*]; D16622 [FGA, β-(1→3)-glucan clotting factor from horseshoe crab]; and C38257 [cl101, β-(1→3)-glucanase from tobacco].

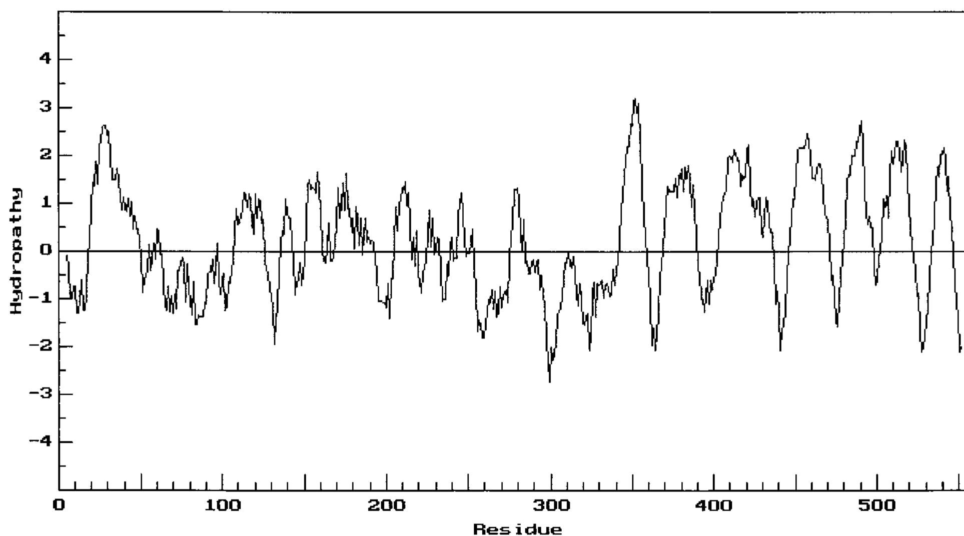

FIG. 6. Hydrophobic profile of NdvC. The Kyte-Doolittle plot (37) is of the derived amino acid sequence of NdvC, using a window of 10 residues. The vertical axis indicated relative hydrophobicity (positive numbers) or hydrophilicity (negative numbers).

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