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RESEARCH LETTER

Cyclic-β-glucans of *Rhizobium* (*Sinorhizobium*) sp. strain NGR234 are required for hypo-osmotic adaptation, motility, and efficient symbiosis with host plants

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Abstract

Cyclic-β-glucans (CβG) consist of cyclic homo-polymers of glucose that are present in the periplasmic space of many Gram-negative bacteria. A number of studies have demonstrated their importance for bacterial infection of plant and animal cells. In this study, a mutant of *Rhizobium* (*Sinorhizobium*) sp. strain NGR234 (NGR234) was generated in the cyclic glucan synthase (*ndvB*)-encoding gene. The great majority of CβG produced by wild-type NGR234 are negatively charged and substituted. The *ndvB* mutation abolished CβG biosynthesis. We found that, in NGR234, a functional *ndvB* gene is essential for hypo-osmotic adaptation and swimming, attachment to the roots, and efficient infection of *Vigna unguiculata* and *Leucaena leucocephala*.

Introduction

Symbiotic nitrogen-fixing bacteria, collectively named rhizobia, interact with the legume family of plants. In this mutualistic interaction, the symbiotic bacteria locate in plant-derived structures called 'nodules' where they differentiate into 'bacteroids' and fix atmospheric nitrogen. To reach their symbiotic niche, rhizobia engage in a complex molecular dialogue with the plant, which eventually leads to infection and nodule colonization. During this interaction, rhizobia undergo many physiological changes and may have to overcome stressful conditions (Perret *et al.*, 2000).

Surface and cell envelope polysaccharides are important to protect bacteria from their surrounding environment and are often essential for functional legume–rhizobia symbioses (Fraysse *et al.*, 2003). Cyclic β-1,2-glucans (CβG) are found in the periplasmic space of several Gram-negative bacteria. The CβG backbone consists of 16–25 glucose residues that are polymerized by a cyclic glucan synthase, a large multi-domain enzyme (Ciocchini *et al.*, 2007). First identified in the phytopathogen *Agrobacterium tumefaciens*, these polysaccharides are essential for survival and infection in several Eukaryote – microbe interactions including legume-rhizobia symbioses between *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Mesorhizobium loti*, and their respective host legumes (Dylan *et al.*, 1986; Geremia *et al.*, 1987; Ielpi *et al.*, 1990; Bhagwat *et al.*, 1992; Breedveld & Miller, 1994; D'Antuono *et al.*, 2005; Crespo-Rivas *et al.*, 2009).

CβG of *Brucella abortus* are essential for intracellular survival and replication by preventing phagosome–lysosome fusions (Arellano-Reynoso *et al.*, 2005). In a similar fashion, CβG produced by the phytopathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) are neces-

sary for bacterial survival on tobacco leaves where they suppress systemic plant immune responses (Rigano *et al.*, 2007). In *S. meliloti*, NdvB and NdvA are responsible for CβG synthesis and translocation to the periplasmic space, respectively, roles that are essential for nodulation (Breedveld & Miller, 1994). The effects of mutated *ndvA* and *ndvB* may not be direct however and could be related to a combination of pleiotropic disturbances associated with the absence of CβG, hypo-osmotic adaptation, motility, attachment and infection (Dylan *et al.*, 1990). As CβG are present in bacteroids (Gore & Miller, 1993) of *Bradyrhizobium japonicum*, CβG might also be important within functional nodules.

Rhizobium (*Sinorhizobium*) sp. strain NGR234 (hereafter NGR234) has the largest known host range of legumes (Pueppke & Broughton, 1999). NGR234 synthesizes cyclic β-1,2-glucans, and previous chemical analyses showed that more than 90% of CβG are substituted with anionic *sn*-1-phosphoglycerol residues (Batley *et al.*, 1987). In this study, the NGR234 cyclic glucan synthase encoded by *ndvB* was identified and functionally characterized by mutational analysis to observe its role on nodule formation..

Materials and methods

Bacterial strains, growth conditions, and plasmids

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in Luria–Bertani medium (Sambrook *et al.*, 1989). NGR234 and derivative strains were grown at 27 °C in tryptone/yeast medium (TY) (Beringer, 1974) or in the hypo-osmotic minimal GYM medium (Dylan *et al.*, 1986) to which NaCl was added at final concentrations of 25, 50, or 100 mM. If necessary, antibiotics were added to the media at the following concentrations: gentamycin (Gm) and tetracycline (Tc), 20 μg mL^{-1}; kanamycin (Km) and spectinomycin (Sp), 50 μg mL^{-1}; rifampicin (Rif), 100 μg mL^{-1}.

Mutant construction

To generate the *ndvB* mutant, a fragment of 2779 bp was amplified by PCR using the specific primers (5 -CTGCCGCATACCAGGAAGGG-3 and 5 -TCGTCAGGCTGAAGATGTAAGG-3) and cloned into the SmaI site of pBluescript KS(+), creating pGF01. The fragment cloned included 290 bp of the upstream intergenic space and 2489 bp of the 5 end of *ndvB*. An Ω interposon conferring spectinomycin resistance was excised from pHP45Ω (Fellay *et al.*, 1987) and inserted into the BspEI restriction sites of pGF01, generating a deletion at the 5 end of *ndvB* (from bases 488 to 860) producing pGF02. This was digested with ApaI and NotI, and then the DNA fragment containing the truncated *ndvB* fragment and the spectinomycin resistance Ω interposon was transferred to the suicide vector pJQ200SK (Quandt & Hynes, 1993) using the same restriction sites, generating pGF03. Finally, a tri-parental mating procedure with the helper plasmid pRK2013 (Figurski & Helinski, 1979) was used to transfer pGF03 into NGR234. Growth on TY agar plates supplemented with sucrose (5% w/v), and spectinomycin allowed selection for the *ndvB* mutant (named NGRΔ*ndvB*).

Construction of promoter-*gfp* fusions and production of green fluorescence protein (GFP)-tagged strains

The *ndvB* promoter region was amplified using the following primer pair: 5 -GCGAATTCATCAGCGAGCAGGT-3 and 5 -TTTCTAGACACGGTCATGTGTCCC-3 . The resulting fragment was digested with EcoRI and XbaI to enable cloning into pBluescript pSK+ resulting in pALQ09. The *ndvB* promoter region of pALQ09 was then transferred into the PstI and ClaI sites of pBDG116 creating pALQ12. In turn, *ndvB* promoter was inserted into the HindIII restriction site of pPROBE-GT (generating pALQ27). The *flaC* promoter region was amplified by PCR using the following primer pair: 5 -CGGAATTCTGGTGCGCTCCTTC-3 and 5 -GGTCTAGATGCGGTTCTGCG-3 , digested using EcoRI–XbaI and cloned into pBluescript pSK+ generating pALQ24. The insert was transferred into the KpnI-SacI sites of pPROBE-GT-producing pALQ28.

All constructed plasmids were sequenced to confirm PCR fidelity. The final constructs containing the *ndvB* and the *flaC* promoters fused to the GFP-encoding gene (pALQ27 and pALQ28, respectively), or empty vectors were mobilized into recipient strains using tri-parental mating as described previously.

To generate GFP-tagged strains, the broad host-range vector pHC60 (Cheng & Walker, 1998) which constitutively expresses GFP was mobilized into NGR234 and the ndvB mutant by tri-parental mating.

Isolation and detection of cellular cyclic-β-glucans

Extractions of CβGs were performed using the following protocol, based on a method developed by Inon de Iannino *et al.* (1998). Briefly, strains were cultivated in 50 mL TY for 2 days to a stationary growth phase (i.e., a final $OD_{600\,nm}$ of 2.0–2.5). Cells were centrifuged for 10 min at 10 000 ***g***, 10 °C and washed twice with water. Pellets were resuspended in 1 mL of 70% ethanol, incubated for 1 h at 37 °C, and further centrifuged for 2 min at 9000 ***g***. The supernatants were finally desiccated by speed-vacuum and

Table 1. Strains and plasmids used

	Features	Reference or source
Strains		
E. coli DH5α	hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44 DlacU169 (f80lacZDM15)	Hanahan (1983)
E. coli DH10B	F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ M15 ΔlacX74 recA1 endA1 araΔ139 Δ(ara, leu)7697 galU galK λ- rpsL (Str^R) nupG	Invitrogen
NGR234	Rifr derivative of *Rhizobium* (Djordjevic, *et al.*) sp. strain NGR234, isolated from *Lablab purpureus* (Rif^R)	Stanley *et al.* (1988)
NGRΔ*ndvB*	*ndvB* mutant obtained by inserting an omega interposon into the BspEI sites of *ndvB* (Rif^RSp^R)	This study
Plasmids		
pBluescript KS (+) or SK (+)	Cloning vector (Amp^R)	Stratagene, La Jolla, CA
pHP45ΩSpec	Vector containing an Ω interposon (Sp^R)	Fellay *et al.* (1987)
pJQ200SK	pACYC184-derived (p15A) suicide vector (Gm^R)	Quandt & Hynes (1993)
pRK2013	Helper plasmid containing the ColE1 replicon with RK2 tra genes (Km^R)	Figurski & Helinski (1979)
pProbe-GT′	pVS1-derived (p15a) vector, *gfp* (Gm^R)	Miller *et al.* (2000)
pGF01	pBluescript KS(+) containing a fragment of *ndvB* cloned in the SmaI site	This study
pGF02	pGF01 containing a spectinomycin resistance Ω interposon in the BspEI sites of the *ndvB* fragment	This study
pGF03	pJQ200SK carrying a fragment of *ndvB* truncated by a spectinomycin resistance Ω interposon in ApaI and NotI sites	This study
pALQ09	pBluescript SK(+) containing the promoter region of *ndvB* cloned in EcoRI and XbaI sites	This study
pALQ12	pBDG116 containing the promoter region of *ndvB* cloned in PstI-blunt ClaI sites upstream eYFP	This study
pALQ24	pBluescript SK(+) containing the promoter region of *flaC* cloned in HindII site	This study
pALQ27	pProbe-GT′ carrying *ndvB* promoter cloned in HindIII site upstream of *gfp*	This study
pALQ28	pProbe-GT′ carrying *flaC* promoter cloned in KpnI and SacI sites upstream of *gfp*	This study
pBDG116	pBluescript SK(+) containing the *eYFP* in SmaI	Deakin WJ (unpublished)
pHC60	pSW213 derived vector expressing constitutively the gfp (Tc^R)	Cheng & Walker (1998)

Amp^R, Gm^R, Km^R, Rif^R, Sp^R, Str^R, Tc^R resistance to ampicillin, gentamicin, kanamycin, rifampicin, spectinomycin, streptomycin and tetracycline, respectively.

resuspended in 20 μL of 70% ethanol. Aliquots (5 μL) of each extract were separated by thin-layer chromatography (Cromatofolios AL TLC – Silicagel 60F) using n-butanol–ethanol-dH_2O (v/v/v of 5 : 5 : 4), and CβGs were visualized by spraying the plates with 5% sulfuric acid in ethanol, followed by heating at 120 °C 10 min.

Swimming tests

Swimming plates were produced by adding 0.2% agar to GYM medium supplemented with various amounts of NaCl. The plates were inoculated by injecting 2 μL of 2-day-old TY saturated cultures ($OD_{600\,nm}$ of 2.0–2.5). Photographs were taken after 6 days of growth at room temperature.

Nodulation tests

Seeds were obtained from the suppliers listed by Pueppke & Broughton (1999). Seeds of *Leucaena leucocephala* and *Vigna unguiculata* were surface-sterilized, planted, and inoculated as described previously (Broughton & Dilworth, 1971; Lewin *et al.*, 1990). Plants were harvested 6 weeks after inoculation. At harvest, the aerial portion of the plant was collected and weighted. The total number of active (pink) nodules and their fresh weight were determined.

Root adhesion tests

Stationary-phase bacterial cultures in TY were washed twice with 25 mM phosphate buffer (pH 7.5) and equilibrated to an optical density of 0.7. Adhesion tests were performed on roots of 6-day-old *L. leucocephala* and *V. unguiculata* plants using an established procedure (Albareda *et al.*, 2006). Results were expressed as colony-forming units (CFU) per mg of root tissue.

Transcriptional analyses

Bacterial strains carrying the promoter-pPROBE constructs were grown on TY agar plates supplemented with the appropriate antibiotics. Using sterile toothpicks, fresh

colonies were transferred to sterile 8-tube strips containing 100 μL of GYM supplemented with 100 mM of NaCl. Cells were homogenized by repeatedly drawing through a fine pipette, and for each transcriptional assay, equal quantities of bacteria were used to inoculate 1 mL of GYM supplemented with 0, 25, or 100 mM NaCl in 96-deep well plates. The plates were incubated at 27 °C with shaking at 200 r.p.m. Optical density (595 nm) and fluorescence (excitation filter at 485 nm and emission filter at 535 nm) from 100 uL of cultures were recorded 48 h post-inoculation using a Plate CHAMELEON Multilabel Detection Platform (Hidex Oy, Turku, Finland). A minimum of three transcriptional assays were performed for each bacterial strain carrying the constructs. Optical density and fluorescence values were first corrected with the values obtained from the media alone. Corrected fluorescence values were then normalized to the average optical density.

Fluorescence microscopy

Leucaena leucocephala and *V. unguiculata* seeds were surface-sterilized, germinated, and planted as described previously. Two-day-old seedlings were inoculated with NGR 234 derivatives containing pALQ27 or pHC60. Plants were harvested at different times post-inoculation and their roots screened with an epifluorescence microscope Leica DMIRE2 [Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland] using GFP filter cubes (excitation BP 470/40 nm; emission BP 525/50 nm). Images were recorded with a Leica DC300F digital camera.

Results and discussion

Identification of the NGR234 cyclic glucan synthase (*ndvB*) and subsequent phenotypic characterization

The nucleotide sequence from *S. meliloti* 1021 of the *ndvB* gene was used to search the genome of NGR234 (Schmeisser *et al.*, 2009). A putative *ndvB* homolog was identified (NGR_c32910). The predicted cyclic glucan synthase protein of NGR234 shares 98% and 90% identity with NdvB proteins of *S. fredii* and *S. meliloti* 1021, respectively. To test whether the NGR234 *ndvB* homolog is involved in CβG biosynthesis, we created a mutant NGRΔ*ndvB* (see Materials and methods).

Cyclic glucans isolated from NGR234 and NGRΔ*ndvB* were analyzed by thin-layer chromatography (TLC) (Fig. 1a). As expected, extracts from the wild-type bacterium show a predominant, strongly stained band in the area where anionic, phosphoglycerol-substituted CβG are expected to migrate, as well as lower amounts of neutral CβG (Batley *et al.*, 1987) (lane 1). Mutation of *ndvB* abolished CβG biosynthesis (lane 2), showing that this gene is essential for CβG biosynthesis in NGR234.

Growth of the *ndvB* mutant was compared to that of NGR234 in hypo-osmotic GYM medium. Maximal growth (OD600 nm) of the mutant was significantly reduced as compared to the wild type in GYM medium, while growth was completely restored with GYM medium containing NaCl at 100 mM final concentration (Fig. 1b), indicating that the growth of NGRΔ*ndvB* is impaired only in hypo-osmotic media. Cell motility is also affected in *ndvB* mutants of *S. meliloti* (Dylan *et al.*, 1990). We tested the motility of NGRΔ*ndvB* using 0.2% agar plates. While NGR234 swam significantly in GYM medium, NGRΔ*ndvB* was nonmotile (Fig. 1c). Supplementing GYM medium with 25 mM NaCl led to a partial recovery of the swimming ability of NGRΔ*ndvB* (Fig. 1d). The results obtained here agree with findings obtained with *ndvB* mutants of other Rhizobiaceae (Breedveld *et al.*, 1994). Final NaCl concentrations of 100 mM reduced motility in both NGR234 and NGRΔ*ndvB* (Fig. 1e), suggesting that salt affects flagella assembly, stability or interferes with chemotactic signaling in NGR234.

Expression of *ndvB* and *flaC* in media of varying osmotic strength

Expression of *flaC* (encoding flagellin, the major structural component of the flagellar filament) and *ndvB* using the GFP reporter system were used as proxies to study the effect of osmotic strength on the regulation of bacterial motility as well as CβG synthesis (Fig. 2). Fluorescence was significantly higher in strains carrying promoter-*gfp* fusions (Fig. 2a, b and d) as compared to the empty vector controls (Fig. 2c and e), indicating that *flaC* and *ndvB* in NGR234 and *flaC* in NGRΔ*ndvB* are transcribed under the conditions studied. Nevertheless, and in agreement with the phenotypes observed in motility tests (Fig. 1c and e), expression of *flaC* was significantly reduced after 48 h in the presence of 100 mM NaCl for NGR234 (Fig. 2a). While *flaC* expression was observed in the *ndvB* mutant in all media tested (Fig. 2b), its transcription levels remained low compared to the wild-type strain. Interestingly, these levels were comparable to those obtained for *flaC* expression in NGR234 grown in the presence of 100 mM NaCl which leads to a nonmotile phenotype. These results suggest that reduced *flaC* transcription is correlated to the nonmotile phenotype, and possibly that the presence and/or absence of CβGs somehow affect *flaC* transcriptional regulation. In contrast, expression of *ndvB* was not significantly affected by

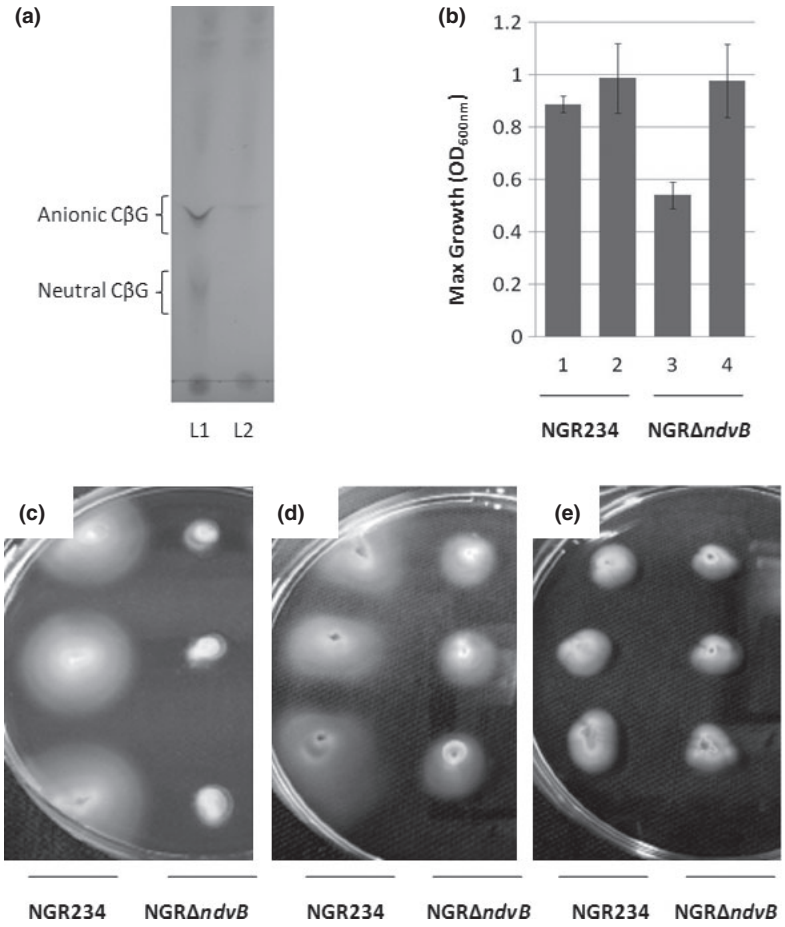

Fig. 1. Effects of the *ndvB* mutation on the production of cyclic-β-glucans (CβG) and on growth and swimming after an osmotic shock. (a) TLC showing CβG extracted from NGR234 (lane 1) and NGRΔ*ndvB* (lane 2). (b) Maximal growth expressed as OD_{600} for NGR234 (1 and 2) or the mutant NGRΔ*ndvB* (3 and 4) obtained in GYM medium (1 and 3) or GYM containing 100 mM NaCl (2 and 4) are reported (error bars correspond to + and − standard deviations calculated from three independent replicates). (c–e) Swimming tests of NGR234 and NGRΔ*ndvB* were performed in 0.2% agar plates containing (c) GYM, (d) GYM + 25 mM NaCl, or (e) GYM + 100 mM NaCl. NGR234 and the *ndvB* mutant were spotted in triplicates on the plates.

changes in osmolarity of the growth medium. This suggests that despite its role in hypo-osmotic adaptation, production of CβGs is constitutive and not osmo-regulated. Our data agree with a transcriptome study of osmo-adaptation in *S. meliloti* (Dominguez-Ferreras *et al.*, 2006), which showed that many genes involved in flagellum biosynthesis and function are repressed in response to increased osmolarity and that transcription of *ndvB* is not significantly regulated by the osmotic strength of the medium. Interestingly, in response to an osmotic downshift, the *S. meliloti* CβG transporter *ndvA* was induced, however (Dominguez-Ferreras *et al.*, 2006), suggesting that although CβG synthesis is not regulated, the transport of CβG from the cytoplasmic compartment to the periplasmic space is osmo-regulated.

Importance of NGR234 cyclic glucans for root adhesion and symbiosis

The capacity of NGRΔ*ndvB* to attach to the roots and develop a functional symbiosis with legume plants producing either determinate (*V. unguiculata*) or indeterminate (*L. leucocephala*) types of nodules was compared to that of the wild-type strain. As expected, we found that adhesion to the roots and nodulation of both plant species were strongly affected by mutation of *ndvB* (Table 2). These results are consistent with previous studies made with CβG mutants in other rhizobia (Breedveld & Miller, 1994; Crespo-Rivas *et al.*, 2009). When *L. leucocephala* which forms indeterminate nodules was tested, the mutant produced mostly pseudonodules and one pink nodule for every 20 plants indicating that nodulation was

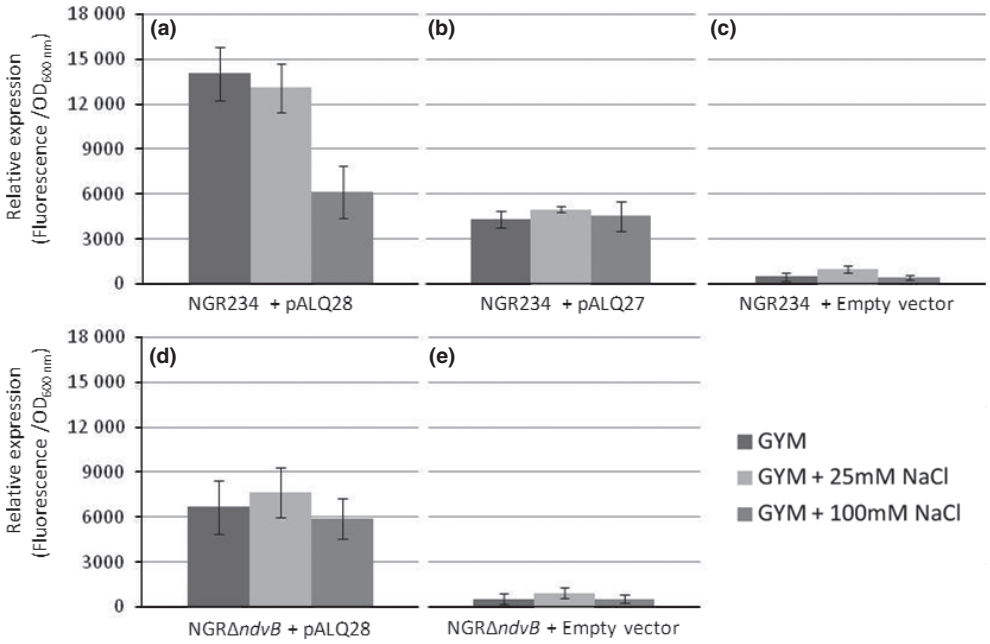

Fig. 2. Effects of osmotic strength on *ndvB* and *flaC* expression using the promoter::*gfp* fusion reporter system. Fluorescence and absorbance were recorded from cell cultures of NGR234 (a–c) or NGRΔ*ndvB* (d, e) carrying the gene encoding GFP fused to the *flaC* promoter (pALQ28) (a and d) or to the *ndvB* promoter (pALQ27) (b) as well as strains carrying the empty vector (c and e). All derivative strains were grown in GYM, GYM + 25 mM NaCl, or GYM + 100 mM NaCl. The relative expression of GFP after 48 h of incubation are shown on the *Y* axes and correspond to the fluorescence normalized to the optical density (calculated from a minimum of three biological replicates with error bars corresponding to + and − standard deviations).

not fully inhibited. On the other hand, neither nodules nor pseudonodules were observed on *V. unguiculata* roots when inoculated with the CβG mutant, suggesting that nodule development is impaired at an early stage in this plant. These results confirm that in *V. unguiculata*, nodulation is aborted early in the nodulation process when a CβG mutant is tested as showed for *S. fredii* (Crespo-Rivas *et al.*, 2009). To further investigate the importance of cyclic glucans in the symbiosis, the transcriptional activity of *ndvB* was studied during nodule development, and the early infection process was followed using GFP-tagged strains.

Roots of *V. unguiculata* and *L. leucocephala* were inoculated with NGR234 carrying the *ndvB* promoter cloned upstream of *gfp*. *ndvB* expression was observed in both young/developing nodules as well as mature (nitrogen-fixing) nodules (Fig. 3a, b, d, and e). This suggests that CβG of NGR234 are produced in nodules, supporting a role for cyclic glucans in invaded nodule cells, as suggested for *B. japonicum* (Gore & Miller, 1993). However, the pleiotropic effects shown by the mutant and the expression of *ndvB* in all conditions tested make it difficult to assess the role of CβG at this later stage of symbiosis development and during the functional symbiosis. We wanted to explore the effect cyclic glucans had on the early stage of symbiosis development. To know whether the nodulation defect was directly linked to the low plant root adhesion capacity of the *ndvB* mutant (Table 2) or if the mutation altered the normal infection process notably in *V. uniguiculata* which never formed nodules, we screened plant root systems using GFP-tagged NGR234 and NGRΔ*ndvB* strains. To facilitate the visualization of these derivative strains and study the early infection development, we used the pHC60 vector which constitutively expresses GFP to screen for rare infection events on root systems. While the presence of bacteria inside nodule cells could be observed when the GFP derivatives were used to inoculate *Leucaena* (data not shown), which was, despite its rarity, easy to detect macroscopically, we were not able to observe typical infection threads in this plant species. This may result from the low nodulation frequency observed with this plant species. A much greater number of plant root systems screened may enable the characterization of this early infection step.

In contrast, despite the absence of nodulation by NGRΔ*ndvB* on *Vigna*, using this mutant, infected root hairs could be detected, suggesting that bacteria were able to enter plant cells. While the wild-type bacterium triggered normal root hair curling and typical infection

Table 2. Impact of a mutation in *ndvB* of NGR234 on adhesion to the roots of *Leucaena leucocephala* and *Vigna unguiculata* and nodulation of the plants

Plant	Strain	CFU/mg of roots	Nodule number per plant	Nodule fresh weight per plant (g)	Shoot fresh weight per plant (g)
Vigna unguiculata	NGR234	130 (28)	73 (9)	0.6 (0.2)	10.0 (4.2)
	NGRΔ*ndvB*	7 (2)	0	0	0.65 (0.1)
Leucaena leucocephala	NGR234	290 (91)	12 (5)	0.120 (0.09)	0.80 (0.2)
	NGRΔ*ndvB*	3 (5)	0.05 (0.07)	0.003 (0.004)	0.4 (0.1)

Average values are indicated with standard deviations in parentheses. CFU = colony forming units.

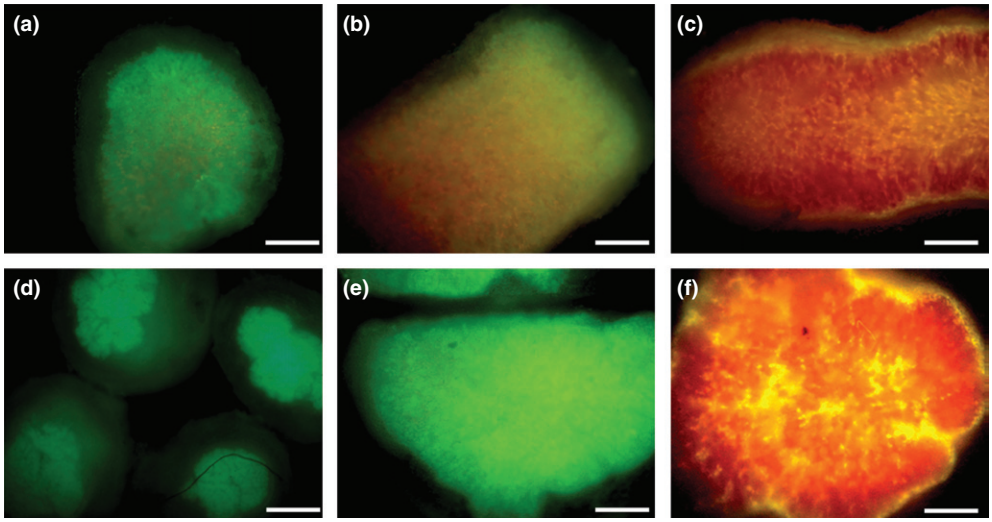

Fig. 3. Expression of *ndvB* during nodule development. NGR234 carrying pPROBE with the promoter region of *ndvB* (a, b, d, and e) or a promoter-less vector (c and f) were inoculated onto the roots of *Leucaena leucocephala* (a–c) and *Vigna unguiculata* (d–e). Fluorescence obtained with an epifluorescence microscope shows that the *ndvB* gene is expressed in young (a, d) as well as in functional (b, e) nodules of plants possessing both indeterminate and determinate nodules. Bars correspond to 500 μm.

threads (Fig. 4a), the CβG mutant triggered root hair curling but then showed abnormal infection of the *Vigna* root hair cells that apparently lacked typical plant-derived infection threads (Fig. 4b). Surprisingly, we found that the mutant bacteria completely invaded infected root hair cells (Fig. 4c). This phenotype was reproducible and and became more pronounced with longer growth periods (Fig. 4d). This suggests that lack of cyclic glucans alters early infection thread development in *Vigna* and causes a release of bacteria in the plant root hair cell cytoplasm. Such a phenotype could result from apoptosis of the root hair cell as part of a defense response which would lead to invasion by bacteria through intracellular replication. It should be noted that we never observed the infection of surrounding root cells, suggesting that the plant restricts bacteria to the infected cells and aborts very early the normal nodule primordium development. Our results corroborates previous work on *S. fredii* HH103 (Crespo-Rivas *et al.*, 2009) and confirm the importance of this polysaccharides for proper infection thread development in *V. unguiculata*. The exact role of cyclic glucans in the infection thread initiation remains to be addressed.

Conclusions

Taken together, our results show that CβG production in NGR234 requires the cyclic glucan synthase NdvB. Mutation of *ndvB* causes deficiencies in motility, hypo-osmotic adaptation, as well as nodule development. We show that the expression of *ndvB* is constitutively expressed regardless of the osmolarity of the growth medium and is active during nodule development. The pleiotropic effects observed upon *ndvB* mutation suggest that cyclic glucans play a major role in the adaptation of NGR234 to the changing environments that confront free-living bacteria (in soils) in their transition to symbionts (inside nodules). Finally, we show that the nodulation of *V. unguiculata* by NGRΔ*ndvB* is aborted very early in the

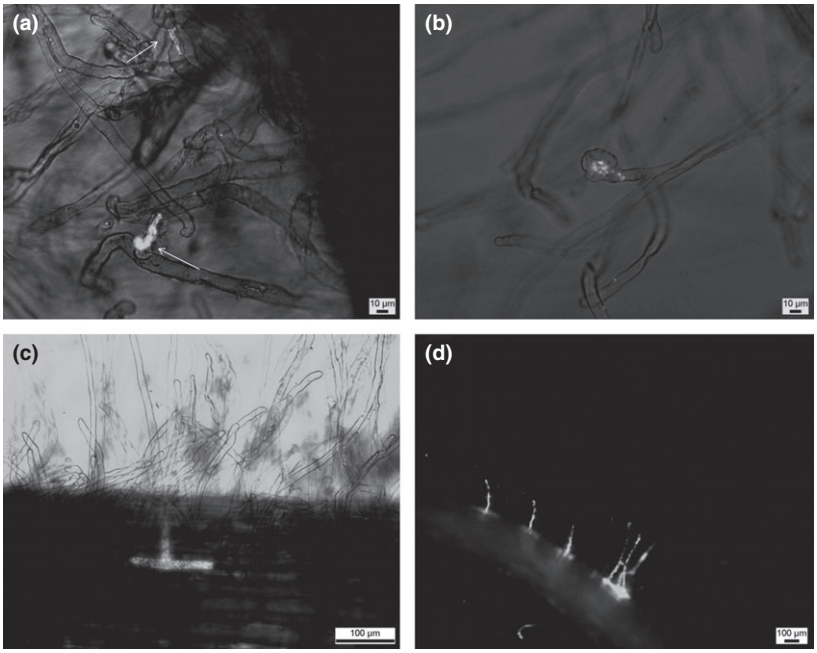

Fig. 4. Effect of the *ndvB* mutation on infection thread development in *Vigna unguiculata*. NGR234 (a) and NGRΔ*ndvB* (b–d) carrying pHC60 were inoculated onto the roots of *V. unguiculata* seedlings. Plants were harvested at different time post-inoculation. After 2 weeks (a and b), curled root hairs infected with fluorescent bacteria were already visible for both isolates, and typical infection thread were only visible in the wild-type NGR GFP-tagged strain (arrows). After 1 month post-inoculation (c) and up to 2 months (d), root hair cells were totally filled up when infected by the NGRΔ*ndvB* derivative.

infectious process and most probably results from a loss of integrity of the infection thread.

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References

Albareda M, Dardanelli MS, Sousa C, Megias M, Temprano F & Rodriguez-Navarro DN (2006) Factors affecting the attachment of rhizospheric bacteria to bean and soybean roots. *FEMS Microbiol Lett* **259**: 67–73.

Arellano-Reynoso B, Lapaque N, Salcedo S, Briones G, Ciocchini AE, Ugalde R, Moreno E, Moriyon I & Gorvel JP (2005) Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. *Nat Immunol* **6**: 618–625.

Batley M, Redmond JW, Djordjevic SP & Rolfe BG (1987) Characterisation of glycerophosphorylated cyclic [beta]-1,2-glucans from a fast-growing *Rhizobium* species. *Biochim Biophys Acta* **901**: 119–126.

Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**: 188–198.

Bhagwat AA, Tully RE & Keister DL (1992) Isolation and characterization of an ndvB locus from *Rhizobium fredii*. *Mol Microbiol* **6**: 2159–2165.

Breedveld MW & Miller KJ (1994) Cyclic beta-glucans of members of the family *Rhizobiaceae*. *Microbiol Rev* **58**: 145–161.

Breedveld MW, Yoo JS, Reinhold VN & Miller KJ (1994) Synthesis of glycerophosphorylated cyclic beta-(1,2)-glucans by *Rhizobium meliloti ndv* mutants. *J Bacteriol* **176**: 1047–1051.

Broughton WJ & Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. *Biochem J* **125**: 1075–1080.

Cheng HP & Walker GC (1998) Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *J Bacteriol* **180**: 5183–5191.

Ciocchini AE, Guidolin LS, Casabuono AC, Couto AS, de Iannino NI & Ugalde RA (2007) A glycosyltransferase with a length-controlling activity as a mechanism to regulate the size of polysaccharides. *P Natl Acad Sci USA* **104**: 16492–16497.

Crespo-Rivas JC, Margaret I, Hidalgo A *et al.* (2009) *Sinorhizobium fredii* HH103 cgs mutants are unable to nodulate determinate- and indeterminate nodule-forming legumes and overproduce an altered EPS. *Mol Plant Microbe Interact* **22**: 575–588.

D'Antuono AL, Casabuono A, Couto A, Ugalde RA & Lepek VC (2005) Nodule development induced by *Mesorhizobium loti* mutant strains affected in polysaccharide synthesis. *Mol Plant Microbe Interact* **18**: 446–457.

Dominguez-Ferreras A, Perez-Arnedo R, Becker A, Olivares J, Soto MJ & Sanjuan J (2006) Transcriptome profiling reveals the importance of plasmid pSymB for osmoadaptation of *Sinorhizobium meliloti*. *J Bacteriol* **188**: 7617–7625.

Dylan T, Ielpi L, Stanfield S, Kashyap L, Douglas C, Yanofsky M, Nester E, Helinski DR & Ditta G (1986) *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *P Natl Acad Sci USA* **83**: 4403–4407.

Dylan T, Helinski DR & Ditta GS (1990) Hypoosmotic adaptation in *Rhizobium meliloti* requires beta-(1—2)-glucan. *J Bacteriol* **172**: 1400–1408.

Fellay R, Frey J & Krisch H (1987) Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. *Gene* **52**: 147–154.

Figurski DH & Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *P Natl Acad Sci USA* **76**: 1648–1652.

Fraysse N, Couderc F & Poinsot V (2003) Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. *Eur J Biochem* **270**: 1365–1380.

Geremia RA, Cavaignac S, Zorreguieta A, Toro N, Olivares J & Ugalde RA (1987) A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form beta-(1—2) glucan. *J Bacteriol* **169**: 880–884.

Gore RS & Miller KJ (1993) Cyclic [beta]-1,6 -1,3 glucans are synthesized by *Bradyrhizobium japonicum* bacteroids within soybean (*Glycine max*) root nodules. *Plant Physiol* **102**: 191–194.

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557–580.

Ielpi L, Dylan T, Ditta GS, Helinski DR & Stanfield SW (1990) The *ndvB* locus of *Rhizobium meliloti* encodes a 319-kDa protein involved in the production of beta-(1—2)-glucan. *J Biol Chem* **265**: 2843–2851.

Inon de Iannino N, Briones G, Tolmasky M & Ugalde RA (1998) Molecular cloning and characterization of *cgs*, the *Brucella abortus* cyclic beta(1-2) glucan synthetase gene: genetic complementation of *Rhizobium meliloti ndvB* and *Agrobacterium tumefaciens chvB* mutants. *J Bacteriol* **180**: 4392–4400.

Lewin A, Cervantes E, Wong C-H & Broughton WJ (1990) *nodSU*, two new nod genes of the broad host range *Rhizobium* strain NGR234 encode host-specific nodulation of the tropical tree *Leucaena leucocephala*. *Mol Plant Microbe Interact* **3**: 317–326.

Miller WG, Leveau JH & Lindow SE (2000) Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol Plant Microbe Interact* **13**: 1243–1250.

Perret X, Staehelin C & Broughton WJ (2000) Molecular basis of symbiotic promiscuity. *Microbiol Mol Biol Rev* **64**: 180–201.

Pueppke SG & Broughton WJ (1999) *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *Mol Plant Microbe Interact* **12**: 293–318.

Quandt J & Hynes MF (1993) Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. *Gene* **127**: 15–21.

Rigano LA, Payette C, Brouillard G *et al.* (2007) Bacterial cyclic beta-(1,2)-glucan acts in systemic suppression of plant immune responses. *Plant Cell* **19**: 2077–2089.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbour Laboratory Press, New York.

Schmeisser C, Liesegang H, Krysciak D *et al.* (2009) *Rhizobium* sp. strain NGR234 possesses a remarkable number of secretion systems. *Appl Environ Microbiol* **75**: 4035–4045.

Stanley J, Dowling DN & Broughton WJ (1988) Cloning of hemA from *Rhizobium* sp. NGR234 and symbiotic phenotype of a gene-directed mutant in diverse legume genera. *Mol Gen Genet* **215**: 32–37.