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Interaction of *Azospirillum lipoferum* with Wheat Germ Agglutinin Stimulates Nitrogen Fixation

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In vitro, the nitrogen fixation capability of *A. lipoferum* is efficiently increased in the presence of wheat germ agglutinin (WGA). A putative WGA-binding receptor, a 32-kDa protein, was detected in the cell capsule. The stimulatory effect required *N*-acetyl-D-glucosamine dimer ($GlcNAc_{di}$) terminated sugar side chains of the receptor and was dependent on the number of $GlcNAc_{di}$ links involved in receptor-WGA interface. Binding to the primary sugar binding sites on WGA had a larger stimulatory effect than binding to the secondary sites. The WGA-receptor complex generated stimulus led to elevated transcription of the *nifH* and *nifA* genes and of the *glnBA* gene cluster but not of the *glnA* gene from its own promoter. There may well be a signalling cascade contributing to the regulation of nitrogen fixation.

Biological nitrogen fixation is one of the most important processes in the natural environment: it is the major pathway for the reduction of dinitrogen molecules from air to give ammonia and subsequently glutamine and other nitrogen-containing molecules. Diazotrophic *Azospirillum lipoferum* enters into associative symbiosis with the roots of several cereals (rice, maize, wheat, and sorghum), tomato, legumes, etc. (16). Under conditions of low oxygen tension and ammonia limitation, bacteria fix nitrogen both in association with roots and in the free-living state (35). *A. lipoferum* is considered as a potential phytostimulator, since it significantly increases plant growth. However, this is mostly due to production of phytohormone-like substances (16), and only a small amount of the fixed nitrogen is transferred from the bacterium to the plant (4).

Lectins are proteins that recognize and reversibly bind to specific sugar chains of glycosylated molecules (polysaccharides, glycoproteins, etc.) (22). The sugar chains on the cell surfaces play important roles as signals in various cell-to-cell recognition events (reviewed in reference 26). During binding to their cognate cell surface receptors, lectins can cross-link and aggregate these receptors, which can, in turn, lead to a variety of biological responses (reviewed in reference 34).

Lectins exposed on plant roots may contribute to the contact with bacteria in the rhizosphere, due to their ability to distinguish between sugar moieties of the bacterial cell wall. There is evidence that root lectins of leguminous plants are involved in the recognition and subsequent binding to rhizobia (13, 23). Bacterial lectin-binding receptors (cell wall polysaccharide antigens) have been found on the cell surface of *Rhizobium trifolii*, and the involvement of *nod* genes in the regulation of their biosynthesis was reported (8). However, the molecular basis of the interaction between the lectins of gramineous plants and *Azospirillum*, during the process of root association, is unknown. Wheat germ agglutinin (WGA) (29) is the best studied of the closely related grass lectins. WGA is present on whole surface of wheat seedlings and on root tips of adult wheat plants (37). It may therefore be a specific attachment site for the putative receptors (including capsular glycoproteins, polysaccharides, fimbriae, and flagella) of the bacterium and could contribute to bacterial adhesion to the root surface, leading to colonization of wheat roots by *Azospirillum* (reviewed in reference 9).

WGA is a protein of two identical 18-kDa subunits, with two primary and two secondary independent sugar-binding sites (1, 38). It exhibits sugar binding specificity for two types of N-acetylated sugars: for terminal β(1→4)-linked *N*-acetyl-D-glucosamine dimers ($GlcNAc_{di}$) and for α(2→3)- and α(2→6)-linked terminal *N*-acetylneuraminic acid (NeuNAc) residues. The primary sugar binding sites link either $GlcNAc_{di}$ or NeuNAc residues (but never both at the same time), and the secondary binding sites only recognize $GlcNAc_{di}$ (49, 50).

The regulation of nitrogen fixation in *A. lipoferum* is less well documented than that in *A. brasilense*. The regulation is both transcriptional and posttranslational (19) in both species. In *A. brasilense*, expression of the *nifHDK* operon (the structural genes of the nitrogenase enzyme) is positively controlled by NifA. *nifA* is expressed under conditions both compatible and incompatible with nitrogen fixation (30). NifA activity is modulated by the P_{II} protein (encoded by *glnB*), the intracellular signal transmitter, in response to the nitrogen status of the cell. *glnB* is clustered with *glnA*, the structural gene of glutamine synthetase (GS), required for ammonia assimilation. *glnBA* is expressed under control of three nitrogen-regulated promoters, *glnBp1*, *glnBp2*, and *glnAp*, active in nitrogen access, nitrogen fixation, and ammonia assimilation, respectively (reviewed in reference 11). In *A. lipoferum*, the *nifHDK* operon under NifA-like control has been identified (17), and the *glnB* gene, contiguous with *glnA*, has been described. However, the P_{II} protein has not yet been characterized (reviewed in reference 15).

In this report, we describe the specificity of the recognition event between WGA and the WGA-binding receptor on the cell surface of *A. lipoferum*. We also report evidence of an enhanced nitrogen fixation capacity of the bacterium, as a consequence of this interaction. Several target genes of the

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TABLE 1. Plant lectins

Lectin	Plant source	Recognized terminal sugar residue of the cognate receptor	Reference or description
WGA	*Triticum vulgaris*	$GlcNAc_{di}$, α(2→3)- or α(2→6)-linked NeuNAc	1, 38
WGA-Neu	None	$GlcNAc_{di}$	WGA primary sugar-binding sites saturated with NeuNAc[a]
WGA-Glc	None	None	WGA primary and secondary sugar-binding sites saturated with GlcNAc[a]
DSA	*Datura stramonium*	GlcNAc	6
SNA	*Sambucus nigra*	NeuNAcα(2→6)Gal	44
MAA	*Maackia amurensis*	NeuNAcα(2→3)Gal	47
GMA	*Glycine max*	GalNAc, α-lactosyl-	31
LCA	*Lens culinaris*	D-Mannosyl-, D-glucosyl-	25
UEAI	*Ulex europaeus*	L-Fucosyl-α(1→2)Gal	22

[a] See Materials and Methods. DSA, *Datura stramonium* agglutinin; SNA, *Sambucus nigra* agglutinin; MAA, *Maackia amurensis* agglutinin; GMA, *Glycine max* agglutinin; LCA, *Lens culinaris* agglutinin; UEAI, *Ulex europaeus* agglutinin I; GalNAc, *N*-acetyl-D-galactosamine; Gal, D-galactase.

WGA-induced stimulus (*nifH*, *nifA*, and *glnB*) were identified by enhanced expression of the corresponding promoter-*lacZ* fusions. A preliminary characterization of the putative capsular WGA-binding receptor is reported.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *A. lipoferum* SpBr17R is rifampin-resistant derivative of SpBr17, the wild-type strain (46). SpBr17R::Tn*2706* (Rif^r Cm^r) is a WGA-nonbinding (WGA^-) mutant strain. *A. brasilense* Sp7 (46), *Azotobacter vinelandii* UW136 (14), and *Klebsiella pneumoniae* UNF122 (24) are wild-type strains. pAW1142::Tn*2706* (Cm^r Tc^r) (40) was used as a transposon donor for mutagenesis. pRK2013 (Km^r) is a helper plasmid for conjugative transposon transfer (18). pAB358 (*nifH-lacZ* Tc^r Km^r), pAB576 (*nifA-lacZ* Tc^r Km^r) (30), pAB904 (*glnB-lacZ* Tc^r), and pAB912 (*glnA-lacZ* Tc^r) (12) carry *A. brasilense* promoter-*lacZ* transcriptional fusions. pAB53 (Km^r) (30) and pAB914 (Tc^r) (12) carry the *nifA* and *glnB* genes, respectively, of *A. brasilense*. Complete medium for *Escherichia coli* and *A. lipoferum* was Luria-Bertani broth (42). Minimal medium for *A. lipoferum* (39) contains 29 mmol of KH_2PO_4, 26 mmol of K_2HPO_4, 37 mmol of malic acid, 75 mmol of NaOH, 20 mmol of NH_4Cl, 1.7 mmol of NaCl, 0.81 mmol of $MgSO_4$, 0.13 mmol of $CaCl_2$, 0.01 mmol of $MnSO_4$, 0.02 mmol of Fe_2SO_4, 0.02 mmol of Na_2EDTA, 0.004 mmol of Na_2MoO_4, and 4 mmol of biotin. Cell numbers of *A. lipoferum* cultures were measured by determining the number of CFU per milliliter in plating assays. Chloramphenicol, tetracycline, rifampicin, and kanamycin were used at concentrations of 10, 20, 20, and 50 μg/ml, respectively.

Random Tn*2706* mutagenesis. pAW1142::Tn*2706* was introduced into *A. lipoferum* SpBr17R by triparental plate mating using the pRK2013 helper plasmid. A WGA^- strain was selected from among the fluorescein isothiocyanate (FITC)-WGA-stained Cm^r Rif^r transconjugants for lack of fluorescence by epifluorescence microscopy (10).

Differential lectin binding assay. Binding of plant lectins to *A. lipoferum* was tested with FITC-labeled lectins (Sigma) (Table 1). Three-day-old cells, grown on solid minimal medium, were suspended in phosphate-buffered saline (PBS), incubated with lectins (40 μg of lectin/10^8 cells/ml) for 1 h at 30°C, and then washed in PBS. Lectin binding was quantified (Perkin-Elmer MPF 44B fluorimeter) by measuring mean epifluorescence at 525 nm (E_{525}) (10^7 cells/ml). WGA-Neu and WGA-Glc were obtained by incubation of WGA with a molar excess of NeuNAc or GlcNAc at 30°C for 1 h, and unbound ligand was removed by molecular sieving on Sephadex G-25 gels.

Detection of capsular WGA-binding components. Capsules of 3-day-old cells, grown on solid minimal medium, were solubilized by shaking bacteria in 20 mmol of HEPES–1% Triton X-100 (pH 7.0) for 18 h at 4°C. Protease inhibitors (protease inhibitors set; Boehringer) were added. Capsule-free cells were removed by centrifugation at 8,000 rpm for 20 min at 4°C and discarded. The supernatant was concentrated with a Centricon-10 centrifugal concentrator (molecular weight [MW] cutoff, 10,000), and the protein content was measured as previously described (33). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel) as described by Laemmli (28). Gels were stained with Coomassie brilliant blue R-250. Proteins were transferred to a nitrocellulose membrane and subsequently tested for WGA binding with digoxigenin (DIG)-conjugated WGA (DIG Glycan Differentiation kit; Boehringer).

Isolation of capsular WGA-binding components by WGA affinity chromatography. Capsular fraction was prepared as described above. The solubilized capsular fraction was applied to WGA-agarose matrix (Vector Laboratories, Burlingame, Calif.) and washed with 10 mmol of HEPES–150 mmol of NaCl–0.1 mmol of $CaCl_2$ (pH 7.5). The bound of material was eluted with 500 mmol *N*-acetyl-D-glucosamine–10 mmol HEPES–150 mmol NaCl (pH 3.0). Flow-through and eluted fractions were analyzed by SDS-PAGE (10% gel) (28). Gels were stained with Coomassie brilliant blue R-250.

Assay of nitrogenase activity in the presence of lectins. Cultures were subjected to 2 h of nitrogenase derepression (in nitrogen-free medium at 30°C under N_2-O_2 [99.5:0.5]), and lectin (1 μg/10^7 cells/ml) and acetylene (10% [vol/vol]) were then added anaerobically. After a further 4 h of incubation (or various times for kinetic experiments), nitrogenase activity was measured by gas chromatography as ethylene production (3).

Transfer of *nifH-lacZ*, *nifA-lacZ*, *glnBA-lacZ*, and *glnA-lacZ* fusions of *A. brasilense* into *A. lipoferum* and β-galactosidase assay under lectin stimulus. pAB358, pAB576, pAB904, and pAB912 were transferred into *A. lipoferum* wild-type and WGA^- strains by conjugation (45). Transconjugants were selected on minimal medium containing appropriate antibiotics. Nitrogenase was derepressed as described above, and β-galactosidase activity (36) was measured 4 h after addition of lectins.

Both nitrogenase and β-galactosidase assays were performed with two parallel cultures from each of three independent experiments.

RESULTS

Isolation of a WGA^- strain of *A. lipoferum*. A WGA^- strain defective for FITC-WGA binding was isolated from a Tn*2706*-mutagenized population of *A. lipoferum* SpBr17R. FITC-WGA-stained preparations of the wild-type and mutant strains are shown in Fig. 1A and B. In the wild-type cells, binding of FITC-WGA produced fluorescence mostly coupled, without polarity, to the cell capsules. Up to 96% of the cell population was positively stained. In contrast, the WGA^- strain showed an FITC-WGA dark phenotype, with less than 1% of the cell population showing dim fluorescence. The mutant strain showed wild-type calcofluor staining (data not shown), indicating the presence of intact β(1→3)- and β(1→4)-linked glucans on the cell surface (48).

Stimulation of nitrogenase activity by WGA. The effect of purified WGA in vitro on nitrogen fixation of the wild-type and WGA^- strains was investigated (Fig. 1C and D). In the absence of WGA, a similar optimum for nitrogen fixation was determined at 0.5% oxygen, both in the wild type and in the WGA^- strain. Under lower oxygen tensions, the nitrogen-fixing capacity decreased. The optimum oxygen requirement for nitrogen fixation was not modified by addition of WGA (data not shown).

In the presence of WGA, wild-type bacteria produced a maximal nitrogenase activity, 4.7-fold higher than that in the absence of WGA. The greatest stimulation was during the exponential phase of growth. The stimulatory effect of WGA was dose dependent, and maximal stimulation was obtained

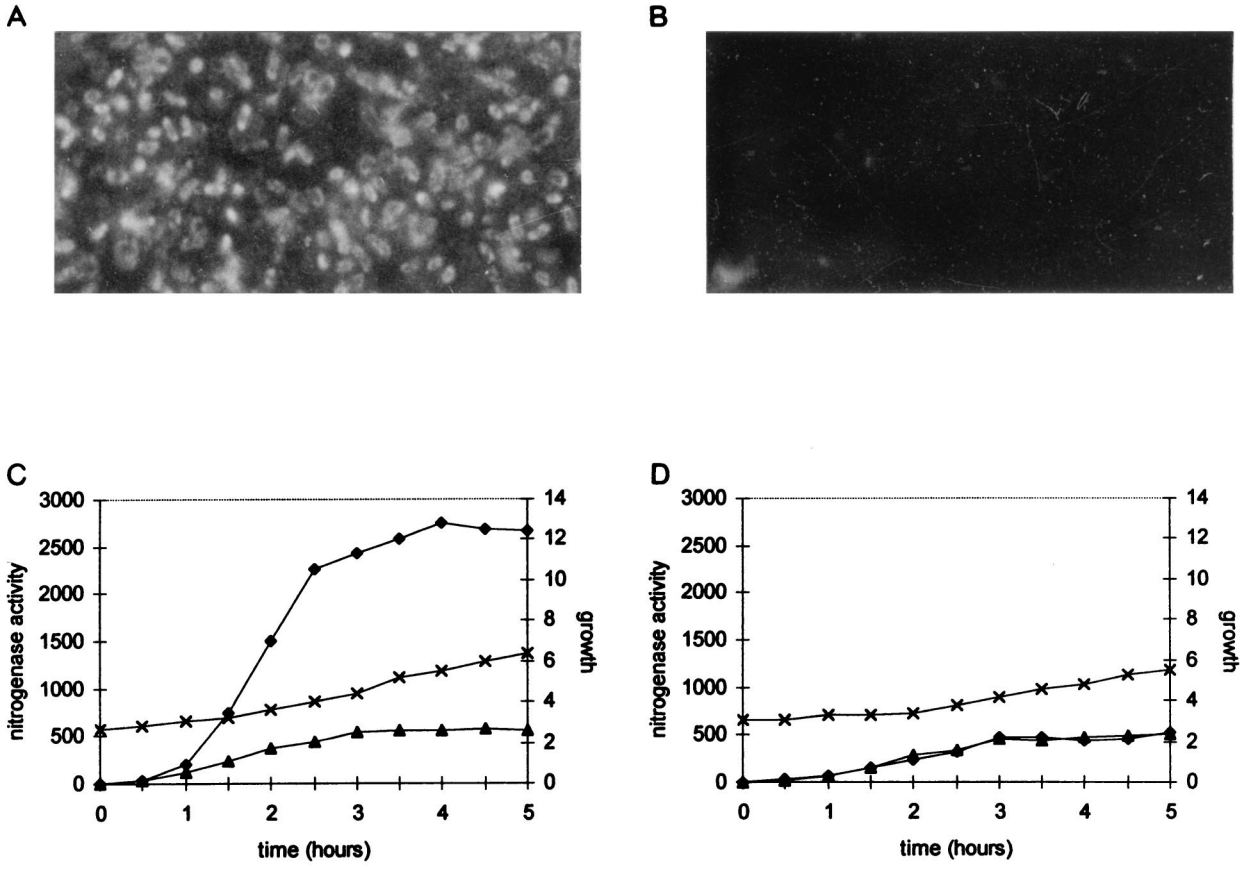

FIG. 1. Binding of WGA to *A. lipoferum* cells (A and B) and effect of WGA stimulus on nitrogen fixation (C and D). (A and B) FITC-WGA-stained wild-type (A) and WGA$^-$ (B) cells visualized by epifluorescence microscopy (approximately 3×10^2). (C and D) Time course of nitrogenase activity in the presence of WGA. After 2 h of nitrogenase derepression in nitrogen-free medium at 0.5% of oxygen, WGA (1 μg/ml) and acetylene (10% [vol/vol]) were added (time zero). Nitrogenase activity (nanomoles of $C_2H_4/10^7$ cells/milliliter) in the presence (◆) and absence (▲) of WGA. ×, growth curve (growth is expressed in 10^7 cells/milliliter). The standard deviation was less than 12% of the value in each case. The reported values are means of three independent assays.

with 1 μg of WGA/10^7 cells/ml (data not shown). Stimulation with WGA did not increase the nitrogen-fixing capacity of the WGA$^-$ strain. Under conditions of nitrogen fixation, the wild-type and WGA$^-$ strains grew similarly but slowly, and their growth rate was not affected by addition of WGA (Fig. 1C and D). Presumably, the poor growth was due to the oxygen-depleted conditions of the cultures.

Involvement of terminal sugar residues of the cell surface in the stimulation of nitrogen fixation by lectin. Binding of lectins to the bacterium is essentially mediated by terminal sugar residues of the corresponding receptors on the cell surface. Terminal sugar residues on the surface of the wild-type and WGA$^-$ cells were surveyed by a differential lectin binding assay, using FITC-labeled lectins with different sugar-binding specificities (Table 1). All lectins tested except UEAI, were able to attach to the wild-type cells, as assessed by epifluorescence microscopy, and no agglutination of cells was observed (at 1 μg of lectin/10^7 cells/ml) (data not shown). Consequently, terminal $GlcNAc_{di}$, terminal α(2→3)- and/or α(2→6)-linked NeuNAc, GalNAc, and/or α-lactosyl-, α-mannosyl-, and/or α-glucosyl- but not L-fucosyl-α(1→2)-linked D-galactose (Gal) residues are present on the cell surface. The specific fluorescence intensities ($E_{525}/10^7$ cells/ml) of the cells treated with WGA, SNA, and MAA were comparable (Table 2). The fluorescence levels obtained with DSA and WGA-Neu were 23 and 66% lower, respectively, than that with WGA. No fluorescence was observed with WGA-Glc, as expected. The cell surface of the WGA$^-$ strain was similarly probed with FITC-lectins. WGA, WGA-Neu, WGA-Glc, and DSA were not able to bind to the cells, whereas SNA and MAA showed a level of

TABLE 2. Lectin-binding capacities of wild-type and WGA$^-$ strains

Strain	Specific fluorescence intensity[a] ($E_{525}/10^7$ cells/ml)					
	WGA	WGA-Neu	WGA-Glc	DSA	SNA	MAA
SpBr17R (wild type)	924 ± 74	318 ± 29	27 ± 3	712 ± 62	1,025 ± 68	907 ± 56
WGA$^-$	35 ± 4	93 ± 11	25 ± 2	13 ± 1	852 ± 99	983 ± 98

[a] Three-day-old cells grown on solid minimal medium were stained with FITC-labeled lectins (at 40 μg/10^8 cells/ml). In the absence of lectin, the background fluorescence intensity was 15. The values are means of three independent assays.

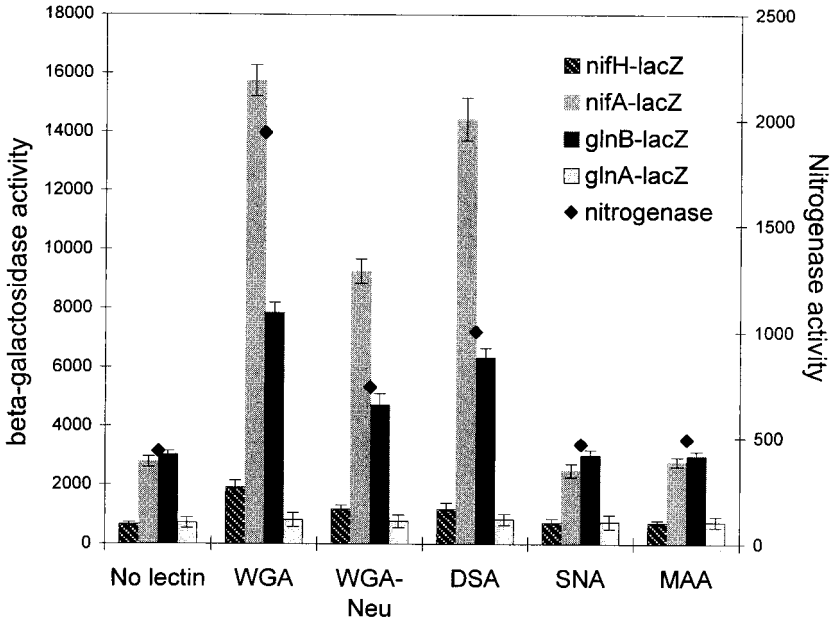

FIG. 2. Nitrogenase activity and expression of N-regulated genes in the wild-type strain in the presence of lectins. Lectins (at 1 μg/10^7 cells/ml) were added after 2 h of nitrogenase derepression. Nitrogenase activity (nanomoles of C_2H_4/10^7 cells/milliliter) and β-galactosidase activity (Miller units/milligram of protein) were measured 4 h after addition of WGA. Error bars indicate the standard deviation of the mean. The standard deviation of nitrogenase activity was less than 13% of the value in each case. The values are means of three independent experiments.

binding comparable to that obtained with the wild-type strain (Table 2).

In view of its specificity for sugar moieties (Table 1), WGA is linked at both sugar-binding sites to $GlcNAc_{di}$ on the wild-type strain. Note that at the primary sugar-binding sites, linkage to terminal NeuNAc can also be established. WGA-Neu and DSA attach exclusively to GlcNAc, and SNA and MAA attach to terminal NeuNAc residues. As the WGA^- strain interacts with SNA and MAA but not with WGA, WGA-Neu, and DSA (Table 2), it probably possesses structurally modified or no GlcNAc, but has intact terminal NeuNAc residues on the cell surface.

Next, we attempted to identify which lectin-terminal sugar contact is involved in the stimulation of nitrogen fixation. Nitrogenase activities of the wild-type strain induced by the different lectin stimuli were determined (Fig. 2). The highest (4.4-fold) increase of nitrogenase activity was obtained by WGA, and lower but significant effects were observed after WGA-Neu and DSA stimulation (1.7- and 2.7-fold, respectively). SNA and MAA had no stimulatory effect on the nitrogenase activity. These results suggest that the $GlcNAc_{di}$ residue of the cell surface is most probably involved in the stimulation of nitrogen fixation. Moreover, binding of $GlcNAc_{di}$ to the secondary sugar binding site of WGA alone (tested with WGA-Neu) stimulated the nitrogen fixation, but to only 38% of the level resulting from WGA attached by both sugar-binding sites to the cell surface (Fig. 2). In addition, no subsequent increase in nitrogen fixation was produced by GMA and LCA, i.e., through GalNAc-/α-lactosyl- and α-mannosyl-/α-glucosyl-linkages, respectively (data not shown).

In the WGA^- strain, stimulation with neither WGA (Fig. 1D) nor WGA-Neu and DSA (data not shown) increased the nitrogen fixation. Thus, the defect in lectin binding (Table 2) was consistent with the absence of nitrogenase stimulation.

In addition to *A. lipoferum*, the effects of WGA, DSA, and SNA on the nitrogen fixation of *A. brasilense*, *Azotobacter vinelandii*, and *K. pneumoniae* were assayed. The nitrogenase stimulation rate and lectin-binding capacity of *A. brasilense* Sp7 for WGA, DSA, and SNA were similar to those of *A. lipoferum* (Table 3). In contrast, no change in nitrogenase activity of *Azotobacter vinelandii* UW136 and *K. pneumoniae* UNF122 was detected with the same lectin stimuli (data not shown).

Stimulation of *nifH*, *nifA*, *glnB*, and *glnA* expression by lectin binding. Next, we tested whether the lectin stimuli exert a regulatory effect through modulation of the transcription of *nifH*, *nifA*, *glnB*, and *glnA* genes. Promoter fusions *nifH-lacZ*, *nifA-lacZ*, *glnBA-lacZ*, and *glnA-lacZ* (plasmids pAB358, pAB576, pAB904, and pAB912, respectively) (12, 30) were introduced into the wild-type and WGA^- strains. The β-galactosidase activities of the plasmid-borne fusions were monitored upon stimulation with WGA, WGA-Neu, DSA, SNA, and MAA, under conditions of nitrogen fixation. For the wild-type strain (Fig. 2), the lectins linked to GlcNAc residues of the

TABLE 3. Nitrogen fixation in the presence of lectins and lectin binding capacity of *A. brasilense* Sp7

Lectin	Nitrogenase activity[a] (nmol of C_2H_4/10^7 cells/ml)	Specific fluorescence intensity[b] (E_{525}/10^7 cells/ml)
None	1,018 ± 100	36 ± 4
WGA	3,767 ± 452	1,116 ± 129
DSA	2,239 ± 300	1,328 ± 185
SNA	928 ± 102	1,273 ± 162

[a] Assayed as described elsewhere (21). Lectins (at 1 μg/10^7 cells/ml) were added after 2 h incubation under conditions required for nitrogen fixation, and activity was measured 4 h after addition of lectins.

[b] Three-day-old cells grown on solid medium were stained with FITC-labeled lectins (at 40 μg/10^8 cells/ml). The values are means of three independent assays.

TABLE 4. Nitrogen fixation in the presence of WGA in wild-type and WGA$^-$ strains carrying plasmid-borne extra copies of *nifA* (pAB53) and *glnB* (pAB914) genes

Strain	Nitrogenase activity[a] (nmol of C_2H_4/10^7 cells/ml)	
	−WGA	+WGA
SpBr17R (wild type)	716 ± 55	2,019 ± 170
SpBr17R(pAB53)	1,520 ± 142	1,600 ± 122
SpBr17R(pAB914)	1,704 ± 105	1,533 ± 86
WGA$^-$	663 ± 61	624 ± 43
WGA$^-$(pAB53)	1,459 ± 157	1,436 ± 162
WGA$^-$(pAB914)	1,597 ± 127	1,653 ± 113

[a] Measured in the absence of WGA or 4 h after addition of WGA (at 1 μg/10^7 cells/ml) after 2 h of nitrogenase derepression in nitrogen-free medium at 0.5% oxygen. The values are means of three independent assays.

cell surface (WGA, WGA-Neu, and DSA) increased the expression of *nifH-lacZ*, *nifA-lacZ* and *glnBA-lacZ* fusions. β-Galactosidase activities obtained with WGA, WGA-Neu, and DSA stimuli were 2.9-, 1.8-, and 1.8-fold (*nifH-lacZ*), 5.7-, 3.3-, and 5.2-fold (*nifA-lacZ*), and 2.6-, 1.5-, and 2.1-fold (*glnBA-lacZ*), respectively, higher than the corresponding unstimulated β-galactosidase activities. Expression of *glnA-lacZ* was not enhanced by any lectin stimuli. Neither of the NeuNAc-specific lectins (SNA and MAA) had an effect on the transcription of any fusion. No increased transcription of the promoter fusions by the lectin stimuli was detected in the WGA$^-$ strain, as expected (data not shown). These results are in agreement with those obtained for the stimulation of nitrogenase activity (Fig. 2). In a control experiment, extra copies of functional *nifA* and *glnB* genes (provided by pAB53 and pAB914, respectively) (12, 30) were introduced into the wild-type strain (Table 4). In the absence of WGA stimulus, nitrogenase activity was 2.1 (*nifA*) and 2.4 (*glnB*) times higher than in the parental strain with only a single chromosomal copy of the corresponding genes. Addition of WGA led to no further increase. Significantly, the overexpressed *nifA* and *glnB* led to levels of nitrogenase activity comparable to that induced by the WGA stimulus in the parental strain. Similarly, in the *nifA* and *glnB* merodiploid derivatives of the WGA$^-$ strain, a level of nitrogen-fixing capacity comparable to the wild-type background level was produced, both in the presence and in the absence of WGA (Table 4).

Detection and isolation of capsular WGA-binding components. Observation of WGA-stained wild-type cells under an epifluorescence microscope (Fig. 1A) (10) showed that WGA was mainly attached to the cell capsules. We attempted to identify the capsular proteins including the WGA-binding components. The solubilized capsular material was separated by SDS-PAGE (Fig. 3A). The protein profiles of the wild-type and WGA$^-$ strains were comparable. Six distinct polypeptides were revealed. On the basis of its intensity, the 32-kDa band might correspond to a doublet of two closely comigrating polypeptides. The 45- and >116-kDa polypeptides are comparable in size to the 45- and 110-kDa protein components of the lateral and polar flagella of *A. brasilense*, respectively (5). A Western blot of the protein patterns was probed with DIG-conjugated WGA (Fig. 3B). WGA-binding signal was detected only in the wild-type and not in the WGA$^-$ preparation. This single WGA-binding component was identified as a 32-kDa polypeptide.

The WGA-binding component from capsule preparation of the wild-type strain was isolated by WGA affinity column chromatography. Both flowthrough and eluted fractions of the capsule preparation, separated by WGA-agarose matrix, were analyzed by SDS-PAGE (Fig. 3C). In agreement with the results of the DIG immunodetection experiment, a capsule component of a 32-kDa polypeptide was specifically retained in eluted fractions 2 and 3 but was no longer detected in the flowthrough fraction. In contrast, the 32-kDa protein of the capsule preparation of WGA$^-$ strain was obtained in the flowthrough fraction, without any specific retention by the WGA matrix (Fig. 3C). Consequently, the 32-kDa polypeptide may be a component of the WGA-binding receptor on the cell surface of *A. lipoferum*.

DISCUSSION

In this report we demonstrate that two GlcNAc-specific lectins, WGA and DSA, are able to stimulate the nitrogen-fixing capacity of *A. lipoferum*, presumably after formation of a complex with the corresponding receptor on the bacterium cell surface. The stimulus, which led to the up-regulation of nitrogen fixation, is absent when the lectin-bacterium interaction is prevented, as in the WGA$^-$ strain.

Moreover, the stimulus is efficient with plant-associative bacteria, such as *A. lipoferum* and *A. brasilense*, but not with the free-living diazotrophs *Azotobacter vinelandii* and *K. pneumoniae*. Interestingly, not only WGA but also other lectins of a few additional plants, colonized by *Azospirillum* (16), bind also to NAcGlc (41).

The WGA stimulus elicited an increased expression of *nifH-lacZ*, *nifA-lacZ*, and *glnBA-lacZ* fusions and consequently a higher nitrogenase activity (Fig. 2). Presumably, the stimulus

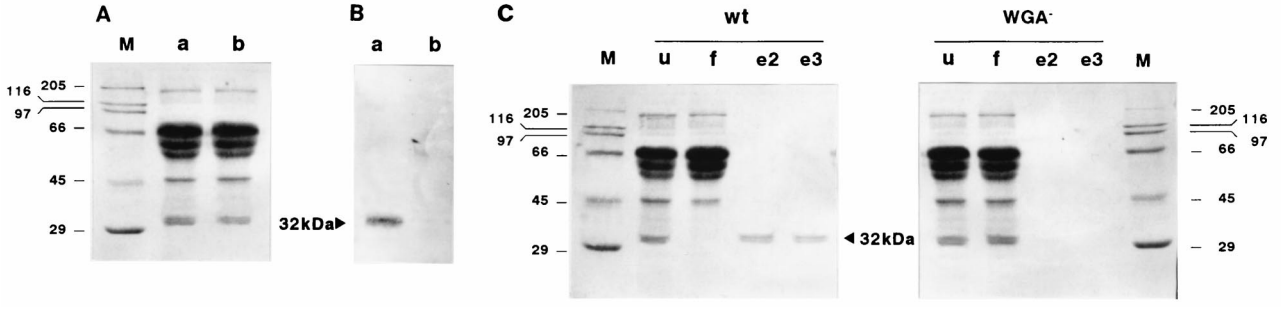

FIG. 3. Detection and isolation of the putative WGA-binding receptor. (A and B) Migration patterns of capsular proteins in SDS-PAGE. (A) Protein profiles; (B) Western blot probed with DIG-labeled WGA. Lanes: a, wild-type strain; b, WGA$^-$ strain. (C) Isolation of the receptor protein by WGA affinity chromatography. Protein migration patterns were determined by SDS-PAGE of unfractionated capsule (lanes u), flowthrough (lanes f), and eluted fractions (lanes e2 and e3) of capsule preparation from the wild-type (wt) and WGA$^-$ strains; lane M, protein molecular weight standards (in thousands).

from the WGA-receptor complex enhances the transcription of *nifA*. The elevated level of NifA could, in turn, increase *nifH* expression. Indeed, extra copies of *nifA*, introduced into wild-type cells, were also able to enhance the nitrogenase activity (Table 4). Similarly, the WGA-receptor-elicited signal would also trigger the transcription of *glnB*. An increased level of the P_{II} protein would improve the conversion of excess NifA protein to its active form. Accordingly, when *glnB* was overexpressed in the wild-type cells, nitrogenase activity was enhanced (Table 4). Note that the plasmid-borne *glnA* promoter did not respond to the WGA stimulus (Fig. 2). However, the surplus ammonia, produced by WGA-triggered nitrogen fixation, could be assimilated by an elevated level of GS (12), since the *glnBA* mRNA is synthesized in larger amounts. Both an enhanced nitrogen fixation, induced by the WGA stimulus, and subsequently an increased level of glutamine synthetase (GS) have been reported in *A. brasilense* (2).

No information is available about lectin-induced bacterial signalling pathways in plant-bacterium associations. In mammalian cells, the binding of WGA triggers cellular recognition, glucose transport and lipolysis, cell growth inhibition, control of morphological states and activation of transcriptional factors, and several lectin-induced signal transducing pathways have been reported (7, 43, 51). The sugar residues of the lectin binding receptors are believed to transmit the signal (20, 27).

The binding specificity of the lectins is generally determined by analyzing terminal sugars of the carbohydrate side chains of the corresponding receptors (22). Our data contribute to understanding the specific chemical recognition between WGA and the putative receptor of *A. lipoferum*. The $GlcNAc_{di}$ residues are the specific receptor components at the WGA-receptor binding interface and are responsible for the biologically active stimulus for the nitrogen fixation machinery. Although NeuNAc residues are also present on the cell surface (evidenced by the binding of SNA and MAA) (Table 2), presumably they are not required for the WGA-binding: the WGA^- strain binds with SNA and MAA but not WGA, WGA-Neu and DSA. Furthermore, the NeuNAc termini are not sufficient for the stimulation of nitrogen fixation, as shown in the case of the SNA-receptor and MAA-receptor interactions, both in the wild type and WGA^- strains (Fig. 2). Moreover, binding to the primary and secondary sugar binding sites on WGA were differently effective in elucidating the biologically active stimulus. The receptor, linked exclusively by the secondary sites of WGA (WGA-Neu–receptor interaction), produced only 38% of the total stimulatory effect (Fig. 2). This agrees with the reduced WGA-Neu-binding capacity of the bacterium (Table 2). The lower level of stimulation may be due to a poorer accessibility of the secondary binding sites in the WGA molecule for the cognate sugar residues of the receptor (49).

The number of molar GlcNAc links between the receptor and WGA modulates several WGA-triggered biological functions (32). From hemagglutination inhibition assays, we estimate that DSA possesses two GlcNAc sites (unpublished data) whereas the WGA molecule has four. Thus, the different stimulation strengths of WGA and DSA (Fig. 2) may be explained by the different number of GlcNAc moieties in the WGA-receptor and DSA-receptor interfaces.

A 32-kDa polypeptide was isolated in vitro, as a putative capsular glycoprotein of WGA-binding receptor. The defective receptor protein of the WGA^- strain has a molecular weight similar to that of the wild type. Thus, the structural gene encoding the receptor protein is most probably not inactivated by the mutation in the WGA^- strain. Possibly the glycosylation pattern of the protein is affected preventing the cognate linkage with the sugar-binding sites on the WGA molecule.

The interaction between *Azospirillum* and WGA may contribute to the adhesion of the bacteria to the root surface and to establishing a nitrogen-fixing association of improved efficiency with the wheat host. Presumably, the elevated nitrogen fixation capacity of the bacterium is supported by enhanced carbon and energy supply from the host plant.

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REFERENCES

1. **Allen, A. K., A. Neuberger, and N. Sharon.** 1973. The purification, composition and specificity of wheat germ agglutinin. Biochem. J. **131:**155–162.
2. **Antonyuk, L. P., O. R. Fomina, M. A. Galkin, and V. V. Ignatov.** 1993. The effect of wheat germ agglutinin on dinitrogen fixation, glutamine synthetase activity and ammonia excretion in *Azospirillum brasilense* Sp 245. FEMS Microbiol. Lett. **110:**285–290.
3. **Burris, R. H.** 1972. Nitrogen fixation—assay methods and techniques. Methods Enzymol. **24B:**415–431.
4. **Christiansen-Weniger, C., and J. A. Van Veen.** 1991. NH_4-excreting *Azospirillum brasilense* mutants enhance the nitrogen supply of a wheat host. Appl. Environ. Microbiol. **57:**3006–3012.
5. **Croes, C. L., S. Moens, E. van Bastelaere, J. Vanderleyden, and K. Michiels.** 1993. The polar flagellum mediates *Azospirillum brasilense* adsorption to wheat roots. J. Gen. Microbiol. **139:**2261–2269.
6. **Crowley, J. F., I. J. Goldstein, J. Arnarp, and J. Lonngren.** 1984. Carbohydrate binding studies on the lectin from *Datura stramonium* seeds. Arch. Biochem. Biophys. **231:**524–533.
7. **Danilov, Y. N., and C. M. Cohen.** 1989. Wheat germ agglutinin but not concanavalin A modulates protein kinase C-mediated phosphorylation of red cell skeletal proteins. FEBS Lett. **257:**431–434.
8. **Dazzo, F. B., and G. L. Truchet.** 1983. Interactions of lectins and their saccharide receptors in the *Rhizobium*-legume symbiosis. J. Membr. Biol. **73:**1–16.
9. **Del Gallo, M., and I. Fendrik.** 1994. The rhizosphere and *Azospirillum*, p. 57–75. *In* Y. Okon (ed.), *Azospirillum*/plant associations. CRC Press, Boca Raton, Fla.
10. **Del Gallo, M., M. Negi, and C. A. Neyra.** 1989. Calcofluor- and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. J. Bacteriol. **171:**3504–3510.
11. **de Zamaroczy, M.** 1995. Genetic control of nitrogen assimilation and nitrogen fixation in free living *Azospirillum brasilense*, p. 77–89. *In* I. Fendrik, M. Del Gallo, J. Vanderleyden, and M. de Zamaroczy (ed.), *Azospirillum* VI and related microorganisms. NATO ASI Series, vol. 37. Springer-Verlag, Berlin, Germany.
12. **de Zamaroczy, M., A. Paquelin, and C. Elmerich.** 1993. Functional organization of the *glnB-glnA* cluster of *Azospirillum brasilense*. J. Bacteriol. **175:** 2507–2515.
13. **Diaz, L. C., L. S. Melchers, P. J. J. Hooykaas, B. J. J. Lugtenberg, and J. W. Kijne.** 1989. Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. Nature **338:**579–581.
14. **Dingler, C., J. Kuhla, H. Wassink, and J. Oelze.** 1988. Levels and activities of nitrogenase proteins in *Azotobacter vinelandii* grown at different dissolved oxygen concentrations. J. Bacteriol. **170:**2148–2152.
15. **Elmerich, C., W. Zimmer, and C. Vieille.** 1991. Associative nitrogen fixing bacteria, p. 212–258. *In* G. Stacey, H. Evans, and R. Burris (ed.), Biological nitrogen fixation. Chapman and Hall, New York, N.Y.
16. **Fages, J.** 1994. *Azospirillum* inoculants and field experiments, p. 87–109. *In* Y. Okon (ed.), *Azospirillum*/plant associations. CRC Press, Boca Raton, Fla.
17. **Fahsold, R., M. Singh, and W. Klingmuller.** 1985. Cosmid cloning of nitrogenase structural genes of *A. lipoferum*, p. 30–40. *In* W. Klingmuller (ed.), *Azospirillum* III: genetics, physiology, ecology. Springer-Verlag, Berlin, Germany.
18. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origen-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
19. **Fu, H. A., A. Hartmann, R. G. Lowery, W. P. Fitzmaurice, G. P. Roberts, and R. H. Burris.** 1989. Posttranslational regulatory system for nitrogenase activity in *Azospirillum* spp. J. Bacteriol. **171:**4679–4685.
20. **Gabius, H. J.** 1991. Detection and functions of mammalian lectins—with emphasis on membrane lectins. Biochim. Biophys. Acta **1071:**1–18.
21. **Galimand, M., B. Perroud, F. Delorme, A. Paquelin, C. Vieille, H. Bozouklian, and C. Elmerich.** 1989. Identification of DNA regions homologous to nitrogen fixation genes *nifE*, *nifUS* and *fixABC* in *Azospirillum brasilense* Sp7. J. Gen. Microbiol. **135:**1047–1059.
22. **Goldstein, I. J., R. C. Hughes, M. Monsigny, T. Osawa, and N. Sharon.** 1980.

What should be called a lectin? Nature **285:**66.
23. **Halverson, L. J., and G. Stacey.** 1986. Signal exchange in plant-microbe interactions. Microbiol. Rev. **50:**193–225.
24. **Holtel, A., and M. Merrick.** 1988. Identification of the *Klebsiella pneumoniae glnB* gene: nucleotide sequence of wild-type and mutant alleles. Mol. Gen. Genet. **215:**134–138.
25. **Howard, I. K., H. J. Sage, and C. S. Horton.** 1972. Studies on the appearance and location of hemagglutinins from a common lentil during the life cycle of the plant. Arch. Biochem. Biophys. **149:**323–326.
26. **Kobata, A.** 1992. Structures and functions of the sugar chains of glycoproteins. Eur. J. Biochem. **209:**483–501.
27. **Kornberg, L. J., H. S. Earp, C. E. Turner, C. Prockop, and R. L. Juliano.** 1991. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of β_1 integrins. Proc. Natl. Acad. Sci. USA **88:**8392–8396.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
29. **LeVin, D., M. J. Kaplan, and P. J. Greenaway.** 1972. The purification and characterization of wheat germ agglutinin. Biochem. J. **129:**847–856.
30. **Liang, Y. Y., P. A. Kaminski, and C. Elmerich.** 1991. Identification of a *nifA*-like regulatory gene of *Azospirillum brasilense* Sp7 expressed under conditions of nitrogen fixation and in the presence of air and ammonia. Mol. Microbiol. **5:**2735–2744.
31. **Lis, H., and N. Sharon.** 1972. Soy-bean (*Glycine max*) agglutinin. Methods Enzymol. **28:**360–368.
32. **Lovrien, R. E., and R. A. Anderson.** 1980. Stoichiometry of wheat germ agglutinin as a morphology controlling agent and as a protective agent for the human erythrocyte. J. Cell Biol. **85:**534–548.
33. **Lowry, O. H., M. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193:**265–275.
34. **Mandal, D. K., and C. F. Brewer.** 1993. Lectin-glycoconjugate cross-linking interactions, p. 117–128. *In* H. J. Gabius and S. Gabius (ed.), Lectins and glycobiology. Springer-Verlag, Berlin, Germany.
35. **Michiels, K., J. Vanderleyden, and A. Van Gool.** 1989. *Azospirillum*-plant root associations: a review. Biol. Fertil. Soils **8:**356–368.
36. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Mishkind, M. L., B. A. Palevitz, and N. V. Raikhel.** 1983. Localization of wheat germ agglutinin-like lectins in various species of the *Gramineae*. Science **220:**1290–1292.
38. **Nagata, Y., and M. M. Burger.** 1974. Wheat germ agglutinin: molecular characteristics and specificity for sugar binding. J. Biol. Chem. **249:**3116–3122.
39. **Okon, Y., S. L. Albrecht, and R. H. Burris.** 1976. Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*. J. Bacteriol. **127:**1248–1254.
40. **Olasz, F., R. Stalder, and W. Arber.** 1993. Formation of the tandem repeat $(IS30)_2$ and its role in IS30-mediated transpositional DNA rearrangements. Mol. Gen. Genet. **239:**177–187.
41. **Pusztai A.** 1991. Plant lectins, p. 9–14. Cambridge University Press, Cambridge, England.
42. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **San José, E., A. Benguria, and A. Villalobo.** 1990. A novel adenylylation process in liver plasma membrane-bound proteins. J. Biol. Chem. **265:**20653–20661.
44. **Shibuya, N., I. J. Goldstein, W. F. Broekart, M. Nsimba-Lubaki, B. Peeters, and W. J. Peumans.** 1987. The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(2-6)Gal/GalNAc sequence. J. Biol. Chem. **262:**1596–1601.
45. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
46. **Tarrand, J. J., N. R. Krieg, and J. Döbereiner.** 1978. A taxonomic study of the *Spirillum lipoferum* group with description of a new genus, *Azospirillum* gen. nov., and two species *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24:**967–980.
47. **Wang, W. C., and R. D. Cummings.** 1988. The immobilized leukoagglutinin from the seeds of *Maackia amurensis* binds with high affinity to complex-type Asn-linked oligosaccharides containing terminal sialic acid-linked α-2,3 to penultimate galactose residues. J. Biol. Chem. **263:**4576–4585.
48. **Wood, P. J., and R. G. Fulcher.** 1978. Interaction of some dyes with cereal β-glucans. Cereal Chem. **55:**952–966.
49. **Wright, C. S.** 1980. Crystallographic elucidation of the saccharide binding mode in wheat germ agglutinin and its biological significance. J. Mol. Biol. **141:**267–291.
50. **Wright, C. S.** 1984. Structural comparison of the two distinct sugar binding sites in wheat germ agglutinin isolectin II. J. Mol. Biol. **178:**91–104.
51. **Wright, C. S.** 1992. Crystal structure of a wheat germ agglutinin/glycophorin-sialoglycopeptide receptor complex. J. Biol. Chem. **267:**14345–14352.