New NodW- or NifA-Regulated *Bradyrhizobium japonicum* Genes

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A cluster of genes coding for putative plant cell-wall degrading enzymes (i.e., genes for two endoglucanases [*gunA* and *gunA2*], one pectinmethylesterase [*pme*], and one polygalacturonase [*pgl*]) was identified by sequence similarities in the symbiotic region of the *Bradyrhizobium japonicum* chromosome. In addition, a systematic screen of the region revealed several genes potentially transcribed by the σ^{54} -RNA polymerase and activated by the transcriptional regulator NifA (i.e., genes for proteins with similarity to outer membrane proteins [*id117* and *id525*] and a citrate carrier [*id331* or *citA*] and one open reading frame without similarity to known proteins [*id747*]). Expression studies using transcriptional *lacZ* fusions showed that *gunA2* and *pgl* were strongly induced by the isoflavone genistein in a NodW-dependent manner, suggesting a role of the gene products in early events of the nodulation process; by contrast, *gunA* and *pme* expression was very weak in the conditions tested. The *gunA2* gene product was purified and was shown to have cellulase activity. β -Galactosidase activity expressed from transcriptional *lacZ* fusions to *id117*, *id525*, and *id747* in the wild type and in *nifA* and *rpoN* mutant backgrounds confirmed that their transcription was dependent on NifA and σ^{54} . Despite the presence of a –24/–12-type promoter and a NifA binding site upstream of *citA*, no regulation could be demonstrated in this case. Null mutations introduced in *gunA*, *gunA2*, *pgl*, *pme*, *citA*, *id117*, *id525*, and *id747* did not impair the symbiosis with the host plants.

Additional keywords: nitrogen fixation, *nod* genes.

The sequencing of the so-called symbiotic gene region of the *Bradyrhizobium japonicum* chromosome was completed recently in our laboratories (Göttfert et al. 2001). This chromosomal region of about 400-kb length comprises all of the known nodulation (*nod*) and nitrogen fixation (*nif*) genes, which are involved in the nitrogen-fixing interaction between *B. japonicum* and its host plants (Kündig et al. 1993). Analysis of the nucleotide sequence revealed that 51% of the open reading frames (ORFs) were homologs to genes in the databases (35% from rhizobia), 20% were related to DNA mobilization events, whereas 29% of the ORFs did not uncover any similarity. Therefore, mutational and gene expression analyses of the whole region might increase our knowledge about genes required at different steps of the symbiotic process. The experiments described in this report are related to two different aspects of this symbiosis: i) the penetration

of the rhizobia into the root hair cell, and ii) the adaptation of the rhizobia to the symbiotic life style, which requires the induction of a number of symbiotic genes in response to the micro-oxic conditions prevailing in the central nodule tissue.

One of the early events in the rhizobial infection of root hairs implies a localized degradation of the root hair cell wall to allow penetration by the rhizobia. This suggests the involvement of plant cell wall-degrading enzymes. The role of such enzymes is well established in pathogenesis. Polygalacturonase (Pgl), pectinmethylesterases (Pme) and pectate lyases, which degrade the pectate network, are glycoside hydrolases or polysaccharide lyases. Cellulases or endoglucanases, which degrade the cellulose network, belong to the hydrolase group. Many organisms secrete multiple isoforms of the same enzymes. Strains of *Erwinia* spp., the bacterial soft-rot pathogens, produce a set of extracellular enzymes including both pectinolytic enzymes (PehA, PehB, and PehC) and cellulases (CelS, CelV, and CelVI) (Saarilahti et al. 1990a, 1990b, 1992; Mae et al. 1995; Shevchik et al. 1996, 1997). In addition, they also secrete extracellular proteases (Marits et al. 1999). However, none of these enzymes are absolutely required for infection and pathogenicity. *Ralstonia solanacearum* causes bacterial wilt on an extensive range of host plants, including over 450 plant species. Generally, the bacterium enters host plant roots through wounds, colonizes the root cortex, and invades the xylem vessels. In this process, an endoglucanase (Egl), three polygalacturonases (PehA, PehB, and PehC) and one Pme play important roles but are not absolutely essential for pathogenesis (Roberts et al. 1988; Schell et al. 1988; Tans-Kersten et al. 1998). Thus, plant pathogens possess a multiplicity of plant cell wall-degrading enzymes as virulence factors, but if one considers them individually, they are of limited relevance in the development of a disease.

By analogy with their function in pathogen-plant interactions, pectinolytic and cellulolytic enzymes might play a role in symbiosis. Two highly similar glycanases with cellulolytic activity (PlyA and PlyB) were isolated from *Rhizobium leguminosarum* bv. *viciae* (Finnie et al. 1997, 1998; Zorreguieta et al. 2000). However, these enzymes are not required for nodule formation and symbiotic nitrogen fixation in the host plant vetch. The existence of a third glycanase was postulated due to the residual cellulase activity of the double mutant *plyA-plyB*. Pectinolytic and cellulolytic activities were also detected in cell extracts of *R. leguminosarum* bv. *trifolii* (Mateos et al. 1992). Recently, the same authors (Mateos et al. 2001) showed that rhizobia penetrate into the legume root hair through a hole created by localized enzymatic degradation of the plant cell wall. Finally, cellulase activity was found in several rhizobial species, including *Sinorhizobium meliloti*, *Mesorhizobium loti*, *B. japonicum*, and different *R. leguminosarum* strains

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(Jimenez-Zurdo et al. 1996). Nevertheless, the role of such enzymes in symbiosis remained unclear.

Once bacteria have successfully penetrated into the plant root nodule cells, they divide and differentiate into nitrogen-fixing bacteroids. Activation of *fix* and *nif* genes in *B. japonicum* depends on two largely independent regulatory cascades, which both respond to the low-oxygen conditions existing in the host cells (Fischer 1994, 1996). In the FixLJ-FixK₂ cascade, the low-oxygen signal is detected and is transduced by the FixLJ two-component regulatory system to the transcriptional regulator FixK₂. FixK₂ can operate as a repressor or as an activator depending on the target promoter. In the RegSR-NifA cascade, the transcription activator NifA is the oxygen sensor (Fischer 1996). The signal for the RegSR two-component regulatory system is unknown at present (Bauer et al. 1998). NifA activates transcription of *nif* and *fix* genes, which are essential for symbiotic nitrogen fixation and comprise the genes for nitrogenase biosynthesis, for synthesis and incorporation of the nitrogenase cofactors, and for regulatory proteins. Transcriptional activation of target genes by NifA involves a specialized RNA polymerase holoenzyme containing the alternative σ factor RpoN (σ 54), which enables recognition of characteristic -24/-12-type promoters. NifA usually binds to a conserved upstream activator sequence (UAS) of *nif* genes.

By sequence analysis, a cluster of genes coding for homologs of secreted plant cell wall-degrading enzymes (i. e., Egl, dipeptidylpeptidase (Dpp), Pgl, and Pme and 32 ORFs possessing -24/-12-type promoters were identified in the sym-

biotic region of *B. japonicum*. The purpose of this work was to find out whether such genes play a role in symbiosis.

RESULTS

Identification of a gene cluster for plant cell wall-degrading enzymes in *B. japonicum*.

Sequence analysis of the symbiotic region of *B. japonicum* suggested that *id568*, *id587*, *id599*, *id636*, and *id637* might encode enzymatic functions in the hydrolysis of plant cell wall components. Interestingly, these five ORFs map in relatively close vicinity in the genome (Fig. 1) (Göttfert et al. 2001). The predicted protein product of *id568* shows significant similarity (between 43 and 26%) to cellulases or endoglucanases (GunA) from *Streptomyces coelicolor*, *Aspergillus* spp., and *Erwinia carotovora* (CelS) (Fig. 2). The conserved domain of the glycosyl hydrolase family 12 was detected at the C-terminal end of the protein (Henrissat et al. 1993). Protein Id587 shared a weak similarity (26%) with a Dpp of *Xanthomonas maltophilia*. Similarity was higher between protein Id636 and polygalacturonase (Pgl2) from *E. carotovora* (36%) and between Id637 and Pme of *Lycopersicon esculentum* (30%). Moreover, Id599 showed 25% similarity to a methyltransferase of *Pyrococcus horikoshii*. All these five putative plant cell wall-degrading enzymes were predicted to contain N-terminal signal sequences supporting the hypothesis that these proteins have extracellular functions (Göttfert et al. 2001). The possible function and the regulation of *id568* (*gunA*), *id636* (*pgl*), and *id637* (*pme*) were studied in greater detail with the following experiments.

Fig. 1. Physical map of the *Bradyrhizobium japonicum* gene cluster coding for plant cell wall-degrading enzymes. Nucleotide positions correspond to the sequence deposited in the EMBL database (accession number AF322013). Black arrows refer to the location and orientation of the kanamycin (*aphII*) or gentamicin (*genA*) resistance gene cassettes that were used for construction of the mutants indicated. The dashed lines represent the positions of the pSUP202pol4 plasmid insertions in the specific genes. The structures of the transcriptional *lacZ* fusions of *gunA2*, *gunA*, *pgl*, and *pme*, which were integrated into the chromosome, are depicted. Numbers of the mutant strains are indicated.

Identification of two *B. japonicum* *gunA* genes.

When the polymerase chain reaction (PCR)-generated *gunA* gene (*id568*) was used to probe a Southern blot of restricted *B. japonicum* genomic DNA under low-stringency conditions, the hybridization signal suggested the presence of a second *gunA*-like gene, which was cloned and named *gunA2* (data not shown). Incidentally, the *gunA2* gene was found in a previously sequenced segment downstream of the *nodVW* genes in the symbiotic region (M. Göttfert, *unpublished data*). The deduced amino acid sequences of GunA and GunA2 were 73.8% identical. The N-terminal part of both proteins did not share much similarity (Fig. 2). Indeed, in contrast to GunA, no N-terminal signal sequence was predicted by computer analysis for GunA2. Southern blot experiments using *pgl* or *pme* as probes did not show any additional signal, indicating that *pgl* and *pme* might be present as a single copy in the *B. japonicum* genome.

Analysis of *gunA*, *gunA2*, *pgl*, and *pme* expression with transcriptional *lacZ* fusions.

To study *gunA*, *gunA2*, *pgl*, and *pme* expression, transcriptional *lacZ* fusions to the four genes were constructed (described below; Fig. 1) and integrated into the chromosome of the *B. japonicum* wild type. The *gunA2-lacZ* fusion was also integrated into the chromosomes of the regulatory mutants 613 (*nodW* mutant) and $\Delta 1267$ (*nodD*₁₊₂ mutant) (Göttfert et al. 1990a, 1992); for unknown reasons, integration of the *pgl-lacZ* fusion was successful only in *B. japonicum* 613 but not in $\Delta 1267$. Cells of all strains were grown in peptone-salts-yeast extract (PSY) medium in the presence of 0.1% plant cell wall

components (carboxymethylcellulose, polygalacturonic acid, or pectin) and different concentrations of genistein (100 nM, 500 nM, 1 μ M, and 10 μ M). β -Galactosidase activity was determined 48 h after inoculation of the cultures (Fig. 3). The *gunA* and *pme* genes were weakly expressed in any tested conditions. By contrast, *gunA2* and *pgl* were substantially expressed in all conditions and were strongly induced in the presence of 10 μ M genistein (Fig. 3A and C). In the case of *gunA2*, this induction was almost completely abolished in the regulatory mutants 613 (*nodW*) and $\Delta 1267$ (*nodD*₁₊₂) (Fig. 3B). Similarly, there was no *pgl* induction in the regulatory mutant 613 (Fig. 3D). Hence, expression of *gunA2* and *pgl* is clearly flavonoid-inducible, and transcription of these genes depends on NodW plus NodD for *gunA2* and at least on NodW for *pgl*. The *pgl* and *pme* genes are oriented in the same direction and are separated by only 221 bp, which might imply that both genes form an operon and, therefore, that expression of *pme* also depends on the flavonoid-inducible promoter upstream of *pgl*. This assumption was strongly supported by the fact that reverse transcription-PCR products with correct lengths could be generated from total RNA isolated from *B. japonicum* wild type in the exponential phase of growth, using four different pairs of primers that allowed amplification of the intergenic region between *pgl* and *pme* (data not shown).

Symbiotic properties of the *gunA*, *gunA2*, *pgl* and *pme* mutants.

Mutations in the genes for the putative plant cell wall-degrading enzymes were constructed as described below (Fig. 1). The potential roles of gene products Pgl, Pme, and

Fig. 2. Amino acid sequence alignment of the *Bradyrhizobium japonicum* (Bj) GunA and GunA2 proteins with cellulase proteins of *Streptomyces coelicolor* (Sc), *Aspergillus kawachii* (Ak), and *Erwinia carotovora* (Ec). The probable cleavage site of the GunA signal sequence is marked by an arrow. Conserved amino acids are shaded as follows: black for amino acids conserved in all five proteins, dark gray for amino acids conserved in four proteins, and light gray for amino acids conserved in three proteins.

GunA/GunA2 in symbiotic nitrogen fixation were studied by infection tests with the respective mutant strains, using soybean, cowpea, and mung bean as the host plants. Nodulation and nitrogen fixation activity of the plants were evaluated three weeks after infection. The size, morphology, N₂-fixing abilities (Table 1), and the interior color of nodules elicited by all the mutant strains did not differ significantly from those that were formed by the wild-type strain. Thus, the products of *pgl*, *pme*, *gunA*, and *gunA2* are not essential for an effective *B. japonicum*–host plant symbiosis. In addition, a triple mutant, *gunA2-pgl-pme* (strain 1585+1622), and three large deletion mutants (mutant strains 1588, 1593, and 1621) were constructed (discussed below; Fig. 1) and were tested in plant assays using

soybeans. The results in Table 1 show that the nodulation and nitrogen fixation activities of all kinds of mutants did not differ significantly from those of the wild type. Hence, even the combination of several mutations did not impair the ability of the mutant strains to invade host cells.

Cellulase activity in *B. japonicum*.

A plate assay was used in an attempt to detect in vivo cellulase activity in the *B. japonicum* wild type. *E. carotovora* was included as a positive control. No area of carboxymethyl cellulose (CMC) hydrolysis was observed around *B. japonicum* colonies as it was seen with *E. carotovora* (data not shown). A CMC–sodium dodecyl sulfate (SDS) polyacrylamide gel elec-

Fig. 3. Expression of chromosomally integrated, transcriptional *lacZ* fusions. **A**, *gunA-lacZ* (1589) and *gunA2-lacZ* (1599) fusions in *Bradyrhizobium japonicum* 110*spc*4; **B**, *gunA2-lacZ* in *B. japonicum* 613 and Δ 1267 (W1599 and D1599); **C**, *pgl-lacZ* (1612) and *pme-lacZ* (1617) fusions in *B. japonicum* 110*spc*4; and **D**, *pgl-lacZ* in *B. japonicum* 613 (W1612). Cells were grown under the conditions indicated. Aerobic cultures were grown for 48 h in peptone-salts-yeast extract (PSY) medium supplemented with carboxy-methylcellulose (0.1 % CMC), polygalacturonic acid (0.1% PGA), pectin (0.1% PE), and different concentrations of genistein (G). β -Galactosidase activities of two cultures were determined in duplicate.

trophoresis also failed to demonstrate the presence of cellulases in crude-extract protein of *B. japonicum* cells that had been induced by genistein (data not shown). Both methods were obviously not sensitive enough to detect cellulase activity, and these results are in line with the weak expression observed with the *lacZ* fusions. The results are somewhat similar to those obtained previously with *R. leguminosarum*, where it was suggested that glycanases responsible for the degradation of CMC remained cell-bound (Finnie et al. 1998). We therefore changed strategies and tried to overexpress a signal sequence-less GunA variant and the intact GunA2 protein from expression vector pET24b. The hexahistidine-tagged proteins were purified by affinity chromatography on a Ni-NTA column. On an SDS-polyacrylamide gel, they migrated as bands with apparent molecular masses of 20 to 30 kDa (data not shown), in agreement with the molecular masses calculated from the deduced amino-acid sequences; these are 23.65 kDa for GunA (without signal peptide) and 29.48 kDa for GunA2. Cellulose degradation activity was monitored by measuring the production of reducing sugars, using the sensitive 2,2'-bicinchoninate method (Waffenschmidt et al. 1987). One mg of recombinant GunA2 produced 110.4 ± 27.0 pmol glucose per min (average of three experiments). This value compared reasonably well with the activity of commercially available cellulase of *Aspergillus niger* that produced 199.1 ± 85.1 pmol glucose per min per mg of protein. In conclusion, biochemical evidence for the identity of GunA2 as a cellulase was thus obtained. No cellulase activity was observed with recombinant GunA. Whether or not the N-terminal deletion or the histidine tag has possibly affected GunA function was not further investigated.

Identification of genes in the symbiotic region that are transcribed in either a NifA- or RpoN-dependent manner or both.

For the identification of potential NifA-regulated and RpoN-dependent promoters, we searched for the sequences TGT-N₁₀-ACA (consensus NifA UAS) and TGGCAC-N₅-TTGCT/A (σ^{54} consensus promoter), in which N₁₀ and N₅ represent stretches of ten or five variable nucleotides (Fischer 1984). A maximal deviation from the consensus sequence at two positions was accepted; however, no deviation at the strictly conserved positions (nucleotides underlined in Fig. 4) in *B. japonicum* was allowed for the -24/-12 promoter. A systematic screen of the symbiotic region of *B. japonicum* revealed the presence of over 30 ORFs possessing -24/-12-like promoters, implying their

potential transcription by the σ^{54} RNA polymerase (Göttfert et al. 2001). In front of many but not all of these promoters, the presence of putative NifA binding sites also indicated possible transcriptional activation by NifA. Figure 4 shows the RpoN and NifA binding sites of those ORFs that were studied here. The proteins encoded by *id117* and *id525* exhibit amino acid sequence similarity to outer membrane proteins (OMPs), which might have a transport function relevant for the plant-bacteria interaction. The product of *id331* (or *citA*) shares convincing similarity with citrate-proton symport proteins from *Salmonella typhimurium*, *Klebsiella pneumoniae*, and a plasmid-mediated citrate transporter from *Escherichia coli*. The amino acid sequence identity among all these citrate carriers is about 36%. The hydropathy profiles reflect that these carriers, including CitA from *B. japonicum*, have 12 hydrophobic segments that span the membrane bilayer. No particular similarity of *id747* to other known proteins in the databases was uncovered.

Regulatory and mutational studies on *citA*, *id117*, *id525*, and *id747*.

Transcriptional *lacZ* fusions to *citA*, *id117*, *id525*, and *id747* were constructed as described below in order to quantify expression of these genes. The fusions were not only integrated into the chromosome of *B. japonicum* wild type but also into mutants A9 (*nifA*) and N50-97 (*rpoN*₁₊₂). Cells were grown under aerobic or anaerobic conditions, and β -galactosidase activity was determined (Table 2). Regardless of the genetic background, no significant or only very weak expression of the *lacZ* fusions was detected when cells had been grown aerobically. By contrast, β -galactosidase activity derived from the fusions to *id117*, *id525*, and *id747*, but not from the fusion to *citA* was strongly induced in the wild-type background under anaerobic conditions. Residual or no activities were measured in the *nifA* and *rpoN*₁₊₂ mutant strains grown under these conditions. In conclusion, expression studies with *id117*, *id525*, and *id747* confirmed that their transcription is dependent of NifA and σ^{54} . However, despite the presence of a -24/-12-type promoter and a potential NifA binding site upstream of *citA*, neither regulation by NifA nor dependency on σ^{54} was demonstrated.

The potential function of *citA*, *id117*, *id525*, and *id747* in symbiosis was addressed by testing corresponding mutants (Table 3) in a plant assay using soybean as the host plant. The mutants showed a wild-type phenotype with regard to nodule number, size, and morphology of nodules and acetylene reduction activity within the root nodules (Table 1).

Table 1. Symbiotic properties of *Bradyrhizobium japonicum* wild type and mutants

Strain	Nodulation	N ₂ fixation (% of wild type) ^a		
		Soybean	Mung bean	Cowpea
110 <i>spc4</i>	+	100 ± 26	100 ± 23	100 ± 21
1570	+	83 ± 30	109 ± 37	85 ± 14
1572	+	105 ± 34	95 ± 29	115 ± 58
1574	+	134 ± 23	105 ± 22	95 ± 26
1585	+	129 ± 28	96 ± 15	98 ± 17
1585+1574	+	92 ± 29	108 ± 19	91 ± 20
1585+1622	+	95 ± 23	ND	ND
1588	+	114 ± 27	ND	ND
1590	+	103 ± 31	ND	ND
1593a	+	115 ± 31	ND	ND
1593b	+	88 ± 19	ND	ND
1615	+	118 ± 34	ND	ND
1621	+	113 ± 28	ND	ND
1624a	+	100 ± 14	ND	ND
1624b	+	92 ± 19	ND	ND
1634	+	115 ± 61	ND	ND

^a For six to eight individual plants. Fixation activity was measured as the amount of C₂H₂ reduced per minute per g (dry weight) of nodule. ND = not determined.

DISCUSSION

Partial chromosome sequencing of the nitrogen-fixing *B. japonicum* led to the identification of potentially new genes involved in symbiosis. Genes coding for homologs of secreted plant cell wall-degrading enzymes were mutated to test their role in the invasion of root hair cells. None of the genes (*gunA*, *gunA2*, *pgl*, and *pme*), considered either individually or in combination, was critical for nodulation and nitrogen fixation in symbiosis. One explanation for this result might be the redundancy of genes for these enzymes, which could functionally replace each other and act in a network. These genes are clustered in relatively close vicinity within the symbiotic region, but even large deletions did not impair the symbiotic properties of the mutants. In the genome databases of *Sinorhizobium meliloti*, *Mesorhizobium loti* MAFF303099, and R7A, no homologs of *gunA/A2*, *pgl*, and *pme* of *B. japonicum* were found. No rhizobial genes coding for pectinolytic enzymes were reported in the literature, but cellulase activity was found in several rhizobial strains (Jimenez-Zurdo et al. 1996; Mateos et al. 1992, 2001) and also in *B. japonicum* 110*spc4* (this study). Two genes coding for glycanases (*plyA* and *plyB*) have been sequenced so far in a symbiotic bacterium, and their products were not required to establish an effective symbiosis (Finnie et al. 1998). Furthermore, expression of a cellulase from the pathogenic organism *E. carotovora* in *Rhizobium fredii* did not influence nodulation of cowpea (Krishnan and Puepke 1994). In addition, one cannot exclude the role of plant cell wall-degrading enzymes produced by the host in the infection process. Legume plants excrete similar enzymes that could also be involved in symbiosis. A polygalacturonase was identified in the legume *Medicago sativa* that was specifically expressed in roots and nodules induced by *S. meliloti*, but its role in symbiosis was not analyzed (Munoz et al. 1998). Although partial hydrolysis of the plant cell wall would seem to be a prerequisite for the penetration of the rhizobia into the root cells, such a mechanism is not yet proven.

In pathogenic bacteria, plant cell wall-degrading enzymes (pectinases, cellulases, proteases, phospholipases, and xylanases) act as virulence factors and enable the invasion of the host cell followed by its death (Barras et al. 1994; Collmer and Keen 1986). In the symbiotic relationship, the involvement of such enzymes would require a tight regulation of their activity so that it remains localized exclusively at the site of infection, in order to avoid destruction of the root hair.

Transcriptional *lacZ* fusions to *gunA2*, *pgl*, and *pme* were found to be weakly expressed in free-living cultures (10-fold above the β -galactosidase activity level of *B. japonicum* containing a promoterless *lacZ* gene), and *gunA* was not expressed at all in the tested conditions. Interestingly, expression of *gunA2* and *pgl* was strongly induced in medium containing genistein. Genistein and other flavonoids from host legumes are known to stimulate in rhizobia the synthesis of nodulation (*nod*) factors, which are involved in events prior to the infection of the root hairs (Göttfert 1993). In *B. japonicum*, *nod* gene transcription depends on the NodD or NodW transcription regulators, or both, which specifically bind to so-called *nod*-box regulatory sequences. In this context, it was interesting to note that expression of *gunA2* and *pgl* was abolished in *nodW* and *nodD*₁₊₂ regulatory mutants, suggesting an involvement of these regulators in the control of *gunA2* and *pgl*; intriguingly, however, neither of the two putative target genes contained a possible promoter region with a *nod*-box consensus sequence. One possible explanation could be the existence of a hierarchical regulatory cascade in which NodW, NodD or both control a yet unidentified regulatory gene whose product, in turn, controls *gunA2* and *pgl*. An alternative type of flavonoid-dependent regulation not associated with *nod* boxes was already postulated in the transcriptional analysis of the symbiotic plasmid of *Rhizobium* sp. NGR234 (Perret et al. 1999).

Plant cell wall-degrading enzymes must be secreted by the bacteria to reach their site of action. By sequence analysis, peptide signals for secretion were detected in GunA, Pgl, and Pme, but not in GunA2, which does not necessarily imply that GunA2 is not secreted. In former studies, examples of isoflavone-inducible proteins that were secreted without N-terminal processing have been described in two species of rhizobia, NGR234 and *S. fredii* USDA257 (Krishnan 2002; Krishnan et al. 1995; Viprey et al. 1998). These proteins were exported via a type III secretion system that has recently been proven to play a role in specific symbioses (Marie et al. 2001). One should also mention here that the two glycanases of *R. leguminosarum*, PlyA and PlyB, do not possess any potential N-terminal secretion signals and are secreted via a type I secretion system (Finnie et al. 1998). However, they seemed to be activated by a bacterial expolysaccharide-related component (Zorreguieta et al. 2000). At present, the secretion pathway used for GunA2 remains speculative.

ORF	RpoN binding site	NifA binding site
<i>id117</i>	TGGAAC-N₅-TTGCT	TGT-N₁₀-ACA (2x)
<i>id331</i>	AGGCAT-N₅-TTGCA	TGT-N₁₀-TCA
<i>id525</i>	TGGCAC-N₅-TTGCA	TGT-N₁₀-ACA
<i>id747</i>	TGGCAT-N₅-TTGCT	CGT-N₁₀-ACA
Consensus	TGGCAC-N₅-TTGCT_A	TGT-N₁₀-ACA

Fig. 4. Putative RpoN and NifA binding sites detected upstream of *id117*, *id331*, *id525*, and *id747*. Bold characters refer to the conserved nucleotides in the consensus sequence depicted in the last line.

In the second part of this study, transcriptional *lacZ* fusions were used to investigate the regulation of four ORFs containing putative RpoN and NifA binding sites in the symbiotic region of *B. japonicum*. β -Galactosidase activity produced from *id117*, *id525*, and *id747* was clearly induced in anaerobic conditions and was largely absent in both NifA- and RpoN-deficient backgrounds. This indicates that NifA is the activator of these genes whose transcription depends on the σ^{54} -RNA polymerase. By contrast, expression of a *citA*-like gene did not depend on RpoN and NifA, despite the presence of a -24/-12-type consensus sequence containing only two mismatches in not absolutely conserved positions and a possible NifA-binding site with one mismatch at a conserved position. By comparison, the putative NifA binding site of *id747*, a gene that was shown to be controlled by NifA and RpoN (discussed above), had also one mismatch at a conserved position but only one mismatch at one less-conserved position in the RpoN consensus binding site. In conclusion, it is difficult to predict solely from sequence gazing whether or not a gene that carries these consensus sequences is actually subject to NifA and RpoN control. Interestingly, *id117* has similarity to i) *msi158* from *M. loti* R7A, which possesses a putative NifA binding site (Sullivan et al. 2002); ii) *y4mb* from *Rhizobium* sp. NGR234, which is associated with a NifA/ σ^{54} -type promoter and is strongly expressed in nodules (Perret et al. 1999); and iii) *omp21* from the soil bacterium *Comamonas acidovorans*, which is characterized by an enhanced expression in oxygen-depleted conditions (Baldermann et al. 1998).

The fact that NifA plays a crucial role in the symbiotic nitrogen fixation process as a key regulatory protein for many *nif* and *fix* genes initially led us to believe that any newly identified NifA target gene might also be involved in symbiosis. We now know that this assumption is not correct. The question regarding the potential function in symbiosis of *id117*, *525*, and *747* was addressed by phenotypic analyses of appropriate mutants in a plant assay. The results clearly showed that these three NifA-controlled genes are not essential for nodulation and nitrogen fixation in symbiosis with soybean. It has been shown previously that the family of genes that are targets of NifA also includes anaerobically induced genes not directly related to nitrogen fixation, such as *groESL₃*, *hemN₁*, *ndp*, *nrgA*, and *nrgBC* (Fischer et al. 1993, 2001; Nienaber et al. 2000; Weidenhaupt et al. 1993). One could argue that both OMPs encoded by the *id117* and *id525* genes are redundant or are re-

placed by potential homologs in the respective mutants. However, the amino acid sequences of proteins Id117 and Id525 do not share any similarity. Southern blot hybridization experiments under low-stringency conditions with a suitable *id117* probe did not reveal any additional bands (data not shown), whereas *id525* does share some similarity with *id352*, *id693*, and *id877*, which are also localized in the symbiotic gene region (Göttfert et al. 2001). However, two of these homologs (*id352* and *id693*) do not possess putative NifA and RpoN binding sites, suggesting a different kind of regulation for these genes. Gene *id747* shared no similarity with known sequences in the databases and was dispensable in symbiosis; therefore, its function and the nature of the gene product remain unclear.

Regarding the role of *citA*, a gene that is also not essential for symbiosis and that was shown not to be controlled by NifA and RpoN, there is evidence to suggest the existence of additional citrate uptake systems, because the *citA* mutant was still able to grow on citrate as the sole carbon source; yet, no closely related gene was detected by Southern blot hybridization of restricted chromosomal DNA using *citA* as a probe (data not shown).

In conclusion, the question of whether or not plant cell wall-degrading enzymes of bacterial origin function in symbiosis between *B. japonicum* and its host plants (soybean, cowpea, and mung bean) could not be answered. Nevertheless, this study has identified one genuine cellulase (GunA2) and one putative polygalacturonase (Pgl) induced by the flavonoid genistein, a compound that is known to be an important *nod* gene inducer in the early events of the symbiotic process. Sequence analysis of the symbiotic region has revealed three potential NifA- or RpoN-regulated genes, whose expression was indeed dependent on these regulators and a low-oxygen status of the cell, but they did not correlate with a function in symbiosis. Thus, the family of NifA-regulated genes that are symbiotically irrelevant has grown by three members.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

The bacterial strains and plasmids used in this work are listed in Table 3. Luria-Bertani (LB) medium was used for growth of *Escherichia coli* and *E. carotovora*. When appropriate, antibiotics were used at the following concentrations (μ g per ml): kanamycin, 30; tetracycline, 10; and ampicillin, 200. PSY medium (Regensburger and Hennecke 1983) supplemented with 0.1% L-arabinose was used for aerobic cultures of *B. japonicum*, whereas yeast extract-mannitol medium (Daniel and Appleby 1972) supplemented with 10 mM KNO₃ was used for anaerobic *B. japonicum* cultures. Anaerobic cultures were kept under argon in rubber-stoppered serum bottles. The following concentrations of antibiotics were used for *B. japonicum* cultures (μ g per ml): spectinomycin, 100; kanamycin, 100; tetracycline, 50 (solid media) or 25 (liquid media); streptomycin, 50; and gentamicin, 150.

DNA work and sequence analysis.

Recombinant DNA work and Southern blotting were performed according to standard protocols (Sambrook and Russell 2001). Digoxigenin-labeled DNA probes generated by PCR were used for Southern hybridization (DIG DNA labeling kit; Roche Diagnostics, Rotkreuz, Switzerland). *B. japonicum* chromosomal DNA was isolated as described (Hahn and Hennecke 1984). For computer-assisted analyses of DNA and protein sequences, we used the software package (version 10.0) of the Genetics Computer Group (University of Wisconsin, Madison, WI, U.S.A.). Homology searches were performed using the National Center for Biotechnology Information BLAST network server.

Table 2. Aerobic and anaerobic expression^a

Strain	Relevant genotype	β -Galactosidase activity (Miller units) ^b	
		Aerobic growth ^c	Anaerobic growth ^d
1600	<i>citA-lacZ</i>	76.6 $\pm$ 2.4	53.2 $\pm$ 2.8
A1600	<i>citA-lacZ nifA</i>	72.8 $\pm$ 2.6	39.6 $\pm$ 0.2
N1600	<i>citA-lacZ rpoN₁₊₂</i>	76.7 $\pm$ 3.8	49.2 $\pm$ 1.8
1613	<i>id747-lacZ</i>	25.5 $\pm$ 3.9	309.4 $\pm$ 21.0
A1613	<i>id747-lacZ nifA</i>	43.5 $\pm$ 1.1	20.8 $\pm$ 3.4
N1613	<i>id747-lacZ rpoN₁₊₂</i>	27.4 $\pm$ 5.8	34.1 $\pm$ 2.4
1614	<i>id525-lacZ</i>	6.5 $\pm$ 2.0	893.6 $\pm$ 92.8
A1614	<i>id525-lacZ nifA</i>	9.0 $\pm$ 2.3	5.7 $\pm$ 2.7
N1614	<i>id525-lacZ rpoN₁₊₂</i>	4.6 $\pm$ 0.8	9.3 $\pm$ 1.6
1623	<i>id117-lacZ</i>	18.9 $\pm$ 1.9	596.9 $\pm$ 77.3
A1623	<i>id117-lacZ nifA</i>	18.3 $\pm$ 0.9	10.2 $\pm$ 0.3
N1623	<i>id117-lacZ rpoN₁₊₂</i>	21.1 $\pm$ 1.2	13.9 $\pm$ 1.5

^a Expression of *citA*-, *id117*-, *id525*-, *id747-lacZ* fusions chromosomally integrated in different *Bradyrhizobium japonicum* strains.

^b Numbers are mean values $\pm$ standard errors of four independent cultures of individual strains, which were assayed in duplicate.

^c Aerobic cultures were grown for 48 h in peptone-salts-yeast extract medium.

^d Anaerobic cultures were grown for 4 to 5 days in yeast extract-mannitol medium containing 10 mM KNO₃.

Construction of chromosomally integrated *lacZ* fusions.

To construct transcriptional *lacZ* fusions to the *gunA*, *gunA2*, *pgl*, *pme*, *id117*, *citA*, *id525*, and *id747* genes, fragments of approximately 500 bp of the noncoding 5' end of each gene were ligated to the appropriate linearized vector pRJ5631 containing a promoterless *lacZ* gene and a ribosome binding site preceded by three stop codons. The oligonucleotide sequences used to amplify the fragments are available from the authors on request. The correct sequence of all inserts was confirmed by sequencing. These constructs were mobilized into the *B. japonicum* wild type (strain 110*spc4*) and into suitable regulatory mutants (Table 3 and Fig. 1). The correct genomic integration was verified by colony PCR or Southern blot hybridization.

Construction of *gunA*, *gunA2*, *pgl*, *pme*, *citA*, *id117*, *id525*, and *id747* mutants.

Insertion-inactivation mutagenesis of *gunA*, *pgl*, *pme*, *citA*, and *id747* genes was achieved by subcloning internal fragments of the target genes into the vector pSUP202*pol4*, which cannot replicate in *B. japonicum*. These constructs were integrated into the *B. japonicum* 110*spc4* chromosome by conjugation, resulting in the mutant strains 1570, 1572, 1574, 1590, and 1615 (Fig. 1 and Table 3). Conjugants carrying plasmid insertions were selected on tetracycline-contain-

ing plates. The *gunA2* gene was mutagenized by insertion of a 1.2-kb *SacI* kanamycin-resistance cassette (*aphII*), isolated from pBSL15 (Alexeyev 1995) into its unique *SacI* site. The *id117* gene was mutated by deleting a 0.527-kb *SacI* fragment that was replaced by a *SacI* *aphII* cassette. Similarly, the *id525* gene was mutated by deleting a 1.4-kb *PstI* fragment, replacing it with a *PstI* *aphII* cassette. Appropriate DNA fragments containing the region to be mutated were cloned into the vector pSUP202*pol4* and were mobilized into *B. japonicum* 110*spc4* by conjugation, resulting in the mutant strains 1585, 1634, and 1624 (Table 3). For construction of the double-mutant *gunA-gunA2* (strain 1585+1574), the plasmid pRJ1574, containing an internal fragment of *gunA*, was integrated by homologous recombination into the chromosome of mutant strain 1585. To construct *B. japonicum* 1585+1622, the *pgl* and *pme* genes were mutagenized by deleting a 1.8-kb *ApaI/XhoI* fragment, replacing it with a 1.6-kb *ApaI/XhoI* gentamicin cassette isolated from pBSL142 (Alexeyev et al. 1995). The DNA fragment containing the mutated *pgl* and *pme* was cloned into pSUP202*pol4* and was mobilized into *B. japonicum* 1585 (Fig. 1). The strategy used to construct deletion mutants 1588, 1593, and 1621 of *B. japonicum* was as follows: two approximately 1.5-kb DNA fragments flanking the *aphII* cassette from pBSL15 were

Table 3. Bacterial strains and plasmids used in this work

Strain	Relevant genotype or phenotype	Source or reference
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ψ 80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR1</i> <i>recA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	BRL, Gaithersburg, U.S.A.
S17-1	Sm ^r Sp ^r <i>hdsR</i> (RP4-2 <i>kan::Tn7</i> <i>tet::Mu</i> integrated in the chromosome)	Simon et al. 1983
BL21 (DE3)	Cm ^r F ⁺ <i>ompT</i> <i>lon</i> <i>hsdS_B</i> (<i>r_B⁻</i> <i>gal</i> <i>dcm</i> (DE3) ; T7-RNA polymerase gene with <i>lacUV5</i> -promoter	Studier et al. 1986
<i>Bradyrhizobium japonicum</i>		
110 <i>spc4</i>	Sp ^r , wild type	Regensburger and Hennecke 1983
613	Sp ^r Sm ^r <i>nodW::</i> Ω	Göttfert et al. 1990a
Δ 1267	Sp ^r Km ^r Δ (<i>nodD1-nodD2</i>):: <i>aphII</i>	Göttfert et al. 1992
A9	Sp ^r Km ^r <i>nifA::aphII</i>	Fischer et al. 1986
N50-97	Sp ^r Km ^r Sm ^r <i>rpoN₂::aphII</i> <i>rpoN₂::</i> Ω	Kullik et al. 1991
1570	Sp ^r Tc ^r <i>pme::</i> pSUP202 <i>pol4</i>	This work
1572	Sp ^r Tc ^r <i>pgl::</i> pSUP202 <i>pol4</i>	This work
1574	Sp ^r Tc ^r <i>gunA::</i> pSUP202 <i>pol4</i>	This work
1585	Sp ^r Km ^r <i>gunA2::aphII</i> (in the opposite orientation)	This work
1585+1574	Sp ^r Tc ^r Km ^r <i>gunA::</i> pSUP202 <i>pol4</i> , <i>gunA2::aphII</i>	This work
1585+1622	Sp ^r Km ^r Gm ^r <i>gunA2::aphII</i> , <i>pgl-pme::genA</i>	This work
1588	Sp ^r Km ^r Δ (<i>id549-id585</i>):: <i>aphII</i>	This work
1589	Sp ^r Tc ^r <i>gunA-lacZ</i> chromosomally integrated	This work
1590	Sp ^r Tc ^r <i>citA::</i> pSUP202 <i>pol4</i>	This work
1593 (a+b)	Sp ^r Km ^r Δ (<i>id588-id606</i>):: <i>aphII</i> (both orientations; a: same orientation; b: opposite orientation)	This work
1599 ^a	Sp ^r Tc ^r <i>gunA2-lacZ</i> chromosomally integrated	This work
1600 ^b	Sp ^r Tc ^r <i>citA-lacZ</i> chromosomally integrated	This work
1612 ^a	Sp ^r Tc ^r <i>pgl-lacZ</i> chromosomally integrated	This work
1613 ^b	Sp ^r Tc ^r <i>id747-lacZ</i> chromosomally integrated	This work
1614 ^b	Sp ^r Tc ^r <i>id525-lacZ</i> chromosomally integrated	This work
1615	Sp ^r Tc ^r <i>id747::</i> pSUP202 <i>pol4</i>	This work
1617	Sp ^r Tc ^r <i>pme-lacZ</i> chromosomally integrated	This work
1621	Sp ^r Km ^r Δ (<i>id619-id637</i>):: <i>aphII</i>	This work
1623 ^b	Sp ^r Tc ^r <i>id117-lacZ</i> chromosomally integrated	This work
1624 (a+b)	Sp ^r Km ^r <i>id525::aphII</i> (both orientations; a: same orientation, b: opposite orientation)	This work
1634	Sp ^r Km ^r <i>id117::aphII</i> (in the same orientation)	This work
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
Plasmids		
pET24b	Km ^r	Novagen
pBSL15	Ap ^r Km ^r	Alexeyev 1995
pBSL142	Gm ^r	Alexeyev et al. 1995
pSUP202 <i>pol4</i>	Tc ^r (pSUP202) part of polylinker from pBluescript II KS+ between <i>EcoRI</i> and <i>PstI</i>	Fischer et al. 1993
pRJ5631	Tc ^r (pSUP202 <i>pol4</i>) <i>lacZ</i>	From this laboratory
pRJ1605	Kmr (pET24b) <i>gunA</i> (coding sequence of <i>gunA</i> without the leader peptide-coding region)	This work
pRJ1606	Kmr (pET24b) <i>gunA2</i>	This work

^a The *gunA2-lacZ* was also integrated into the chromosome of *B. japonicum* 613 and Δ 1267. The *pgl-lacZ* was integrated exclusively into the chromosome of *B. japonicum* 613. The resulting strains were given the same numbers 1599 or 1612 preceded by W (613 derivative) or D (Δ 1267 derivative).

^b The *citA-lacZ*, *id117-lacZ*, *id525-lacZ*, and *id747-lacZ* were also integrated into the chromosome of *B. japonicum* A9 and N50-97. The resulting strains were given the same numbers preceded by A (A9 derivative) or N (N50-97 derivative).

cloned into the vector pSUP202pol4. These constructs were integrated into the *B. japonicum* wild-type 110*spc4* chromosome by double crossing-over (Fig. 1 and Table 3). The chromosomal regions comprising nucleotide positions 60,153 to 70,059 in the mutant 1588, from nucleotide 71,558 to 80,229 in strain 1621, and from nucleotide 81,631 to 94,489 in strain 1593 (nucleotide position numbers refer to the EMBL database entry under accession number AF322013) were deleted. The correct genomic structures of all mutants were confirmed by colony PCR or Southern blot hybridization.

β -Galactosidase assays.

β -Galactosidase activity assays were performed as described previously (Fischer et al. 1993).

GunA and GunA2 expression and purification.

Plasmids pRJ1605 and pRJ1606 were constructed for the production of GunA and GunA2 by respective cloning of the corresponding genes into the *Nde*I and *Xho*I sites and the *Nde*I and *Not*I sites of expression vector pET24b (Novagen, Schwalbach, Germany). Cellulases were expressed in the host strain *Escherichia coli* BL21(DE3)pLysS. Overexpression cultures were grown at 37°C. Once they reached an optical density at 600 nm of 0.6, production of the recombinant proteins was induced by addition of 0.5 mM IPTG. After 3 h of induction, bacterial cells were harvested, were resuspended in binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole), and were frozen at -30°C. Lysis was performed by cell thawing and sonication for 15 min. Soluble crude extracts were prepared by centrifugation at 15,000 g for 30 min at 4°C. The supernatant was cleared by ultracentrifugation at 45,000 g for 60 min at 4°C. Purification of hexahistidine-tagged proteins was performed by Ni-NTA affinity chromatography under native conditions with Ni-NTA resin from Qiagen (Basel, Switzerland). The column was equilibrated with binding buffer. After application of the crude extract, the column was washed with a solution of 25 mM imidazole and, subsequently, with a solution of 30 mM imidazole. Proteins were eluted in 2 steps, first with 150 mM imidazole and, thereafter, with 300 mM imidazole.

Cellulase activity in vivo and in vitro.

For plate assays, bacteria were directly streaked on PSY plates containing 0.1% CMC. After seven days of growth at 30°C, plates were stained with 0.1% Congo Red for 30 min and were rinsed with 1 M NaCl for 30 min (Teather and Wood 1982). For quantitative enzyme assays, the reaction mixture containing 0.4 ml of substrate (2% CMC in 50 mM citrate buffer, pH 4.8) and 0.4 ml of recombinant enzyme (in 50 mM citrate buffer, pH 4.8). A 0.4-ml sample (considered as $t = 0$ h) was frozen at -80°C. The rest of the sample was incubated at 30°C overnight. Product formation was measured by using the 2,2'-bicinchoninate method (Waffenschmidt et al. 1987). Samples were mixed with 0.1 ml of water and 0.5 ml of 2,2'-bicinchoninate reagent and were heated at 100°C for 15 min. Their optical density was measured at 540 nm. A standard curve for reducing sugars was prepared with glucose in the range of 0 to 50 nmol. Commercially available cellulase from *Aspergillus niger* (Sigma, Buchs, Switzerland) was used as a positive control.

Plant infection test.

The symbiotic phenotype of the knock-out mutants was determined in plant infection tests using soybean, cowpea, and mung bean as host plants, and nitrogenase activity was measured in an acetylene reduction assay (Göttfert et al. 1990b; Hahn and Hennecke 1984).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- INRA genome sequencing project database for *Sinorhizobium meliloti*: sequence.toulouse.inra.fr/meliloti.html.
- INRA genome sequencing project database for R7A: sequence.toulouse.inra.fr/msi.
- Kazusa DNA Research Institute genome database for rhizobia (for *Mesorhizobium loti* MAFF303099): www.kazusa.or.jp/rhizobase.
- National Center for Biotechnology Information BLAST network server: www.ncbi.nlm.nih.gov/BLAST.