The *typA* Gene is Required for Stress Adaptation as Well as for Symbiosis of *Sinorhizobium meliloti* 1021 with Certain *Medicago truncatula* Lines

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In this article, we describe the *typA* gene of *Sinorhizobium meliloti*, the orthologue of *typA/bipA* genes found in a wide range of bacteria. We found that *typA* was required for survival of *S. meliloti* under certain stress conditions, such as growth at low temperature or low pH and in the presence of sodium dodecyl sulfate (SDS). The cold-sensitive phenotype of both *Escherichia coli* *bipA* and *S. meliloti* *typA* mutants were cross-complemented, indicating that the two genes are functionally equivalent. *typA* was indispensable for symbiosis on *Medicago truncatula* Jemalong and F83005.5 and contributes to the full efficiency of symbiosis on other host plant lines such as DZA315.16 or several cultivars of *M. sativa*. Hence, the symbiotic requirement for *typA* is host dependent. Interestingly, the symbiotic defect was different on Jemalong and F83005.5 plants, thus indicating that *typA* is required at a different stage of the symbiotic interaction.

Many bacteria establish intimate associations with eukaryotic hosts. This interaction can be either pathogenic, resulting in damage or death of the respective host, or symbiotic, which is beneficial for both partners.

The soil bacteria rhizobia are able to establish a nitrogen-fixing symbiosis with leguminous plants by inducing the formation of a new organ, the nodule, on the roots of the host plants. Bacteria penetrate into the developing nodules via host-derived tubular structures, called infection threads. Upon release from the infection threads, bacteria arrive into intracellular membrane compartments of plant origin called symbiosomes where they differentiate into bacteroids capable of fixing atmospheric nitrogen which is provided to the plants in exchange for carbon and amino acid compounds (Gage and Margolin 2000; Lodwig et al. 2003; Oke and Long 1999b; Spaink 2000; Viprey et al. 2000).

Although the outcome is different, the establishment of the rhizobium–legume symbiosis is similar to pathogenesis in several respects. In both cases, bacteria have to penetrate the plant tissues, avoid the host defenses, and adapt to the physiological conditions found in the new environment, suggesting that common mechanisms may exist in pathogenic and symbiotic

bacteria to support successful colonization of the host cells (Goebel and Gross 2001; Hentschel et al. 2000; Tsois 2002; Van Sluys et al. 2002).

Analysis of the complete *Sinorhizobium meliloti* genome sequence (Galibert et al. 2001) revealed the presence of approximately 200 genes exhibiting overall sequence similarity to genes known to be essential for establishment or maintenance of infection of plant or animal cells by pathogens, including homologues of virulence determinants, cell-surface components, adhesins, outer-membrane proteins, pili proteins, regulators, and so on. In order to evaluate the role of these genes during symbiosis, we recently initiated transcriptome and systematic mutagenesis experiments. The expression pattern of the candidate genes was analyzed under different symbiotic and free-living conditions, revealing the differential expression of several classes of genes upon the symbiotic interaction (Ampe et al. 2003). Side by side with transcriptome experiments, the construction of mutants from a subset of the genes either exhibiting enhanced expression in nodules or showing especially high sequence similarity to infection genes of pathogens was started.

One of the candidate genes was found to be of special interest, because its close homologue *bipA/typA* controls environmental stress as well as virulence-related functions in enteropathogenic bacteria. In *Salmonella typhimurium*, BipA was strongly induced by the cationic host defense protein BPI (Qi et al. 1995). In *Escherichia coli*, BipA affects the phosphorylation of the universal stress protein UspA during starvation, causing growth arrest (Freestone et al. 1997), and it controls bacterial survival under a variety of stress conditions (Freestone et al. 1998). BipA is required for temperature-dependent regulation of capsular polysaccharide production (Rowe et al. 2000) as well as for growth at low temperature (Pfennig and Flower 2001). In enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli* strains, BipA is necessary not only for general housekeeping functions but it also is involved in several important pathogen–host interactions. BipA confers resistance to BPI (Grant et al. 2003), regulates flagella-mediated cell motility, and is involved in the actin rearrangements within the infected host cells (Farris et al. 1998; Grant et al. 2003).

Based on their conserved sequence motifs, BipA proteins belong to ribosome-binding GTPases. Indeed, it was shown that BipA from the EPEC strain possesses GTPase activity (Farris et al. 1998) and data indicate that purified BipA protein binds to ribosomes (Grant et al. 2003). That latter fact suggests that BipA represents a new type of regulator, which acts at the level of protein synthesis. Recent findings indicate that BipA is

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involved in diverse regulatory cascades and coordinates the expression of pathogenicity islands and other virulence-associated factors (Grant et al. 2003).

In this article, we present evidence that *bipA* of *E. coli* and *typA* of *Sinorhizobium meliloti* can functionally complement each other. We show that *typA* contributes to stress adaptation of *S. meliloti* and also is required for the establishment of nitrogen-fixing symbiosis with certain *Medicago truncatula* lines; however, it is not indispensable for symbiosis on other lines and on *M. sativa*.

RESULTS

The TypA protein sequence is highly conserved in bacteria.

Blast search of databases with the *S. meliloti* TypA sequence revealed more than 100 homologues in a wide range of bacteria, including proteobacteria, gram-positive bacteria, and cyanobacteria. The highest level of homology with *S. meliloti* TypA (55 to 60% amino acid identity, 70 to 75% amino acid similarity) was found in α -proteobacteria, including plant and animal

pathogens such as *Agrobacterium* and *Brucella*, respectively, and plant symbionts like *Mesorhizobium loti* and *Bradyrhizobium japonicum*. Three regions typify TypA proteins, which, indeed, are present in the putative amino acid sequence from *S. meliloti*. An ATP/GTP-binding motif and a GTP-binding elongation factor signature are located in the amino-terminal part of these proteins, whereas the carboxy-terminal part harbors a putative ribosome-binding domain (Fig. 1). Several tyrosine residues are conserved in TypA proteins, which is of interest because, in enteropathogenic *E. coli* strains, BipA phosphorylation of tyrosine residues is required for invasion of the host cells (Farris et al. 1998; Freestone et al. 1998) (Fig. 1). Interestingly, BipA homologues are present and share the same domain structure and pattern of conserved tyrosine residues in both pathogenic and nonpathogenic bacteria, suggesting that the protein originally might have a housekeeping role and subsequently acquired virulence-related functions in certain bacteria.

To investigate whether *typA* plays a role during invasion of the symbiotic nodules, a *typA* null mutant derivative (EK257) of *S. meliloti* 1021 (Rm1021) as well as strains overexpressing

Fig. 1. The TypA protein sequence is highly conserved in bacteria. Sequence comparisons of the deduced amino acid sequences of the *typA*-homologue proteins. TypA_Sm = TypA of *Sinorhizobium meliloti* (GenBank: NP_387196; RhizoBase SmC03242), TypA_AtC58 = TypA of *Agrobacterium tumefaciens* C58 (AF2903), TypA_Ml = TypA of *Mesorhizobium loti* (NP_105062), TypA_Bs = TypA of *Brucella suis* (NP_700043), BipA_EPEC = BipA of enteropathogenic *Escherichia coli* (CAC20136). Black and gray boxes represent amino acid identity and similarity, respectively, conserved in all five proteins. Motifs indicated in the sequence: I, ATP/GTP binding site; II, GTP-binding elongation factor signature; III, putative ribosome binding domain. Triangles show tyrosine residues conserved in all five proteins.

the gene in the wild-type and *typA* background (EK273 and EK275, respectively) were constructed.

TypA is required for growth of *S. meliloti* at low temperature, low pH, and in the presence of sodium dodecyl sulfate.

Gene *bipA* of *E. coli* is involved in stress-related responses (Freestone et al. 1997) as well as in the temperature-dependent regulation of cell surface polysaccharides (Rowe et al. 2000); therefore, we tested whether the *typA* mutant of *S. meliloti* had an altered sensitivity to different environmental stresses.

To test whether *typA* was required for growth of *S. meliloti* at low temperature, the Rm1021 and *typA* mutant bacteria carrying the empty vector (pFAJ1700) or the complementing plasmid harboring the *typA* coding sequence (pEK264), respectively, were grown on Luria-Bertani (LB) plates at 30 and 14°C (Table 1). At 30°C, the four strains formed single colonies after 2 days. However, after 5 to 10 days of incubation at 14°C, only the Rm1021 and the mutant complemented by the *typA* gene were able to grow, whereas the *typA* null mutant did not form single colonies. These results indicate that the *S. meliloti typA* mutant, similarly to the *E. coli bipA* mutant (Pfennig and Flower 2001), is cold sensitive.

In order to assess the pH sensitivity of the *typA* mutant, serial dilutions of bacteria were spread on plates at a range of pH values (pH 7 to 5.5). At pH 7 and pH 6.5, all strains formed colonies after 2 days of incubation at 30°C. At pH 6, the *typA* mutant showed a reproducible delay in colony formation compared with the wild type. The wild-type colonies appeared on day three and four, whereas the *typA* mutant colonies only appeared after day five to seven. At pH 5.5, none of the strains could grow.

The wild-type strain and the *typA* mutant showed the same viability under oxidative (H₂O₂, paraquat) stress conditions and in the presence of deoxycholic acid (DOC), NaCl, ZnSO₄, and ethanol in plating assays (data not shown). Rm1021 and the *typA* mutant were equally sensitive to sodium dodecyl sulfate (SDS) in filter disks assays (25-mm zone of inhibition), whereas overexpression of *typA* in the Rm1021 background (EK273) showed increased resistance against SDS (20-mm zone of inhibition). The difference in SDS sensitivity was more pronounced in plating assays. In the presence of SDS at 0.05 g/liter, the *typA* null mutant formed 10⁴ to 10⁵ times fewer colonies compared with Rm1021. Although there was no significant difference between the numbers of colonies produced, EK273 overexpressing *typA* formed colonies 2 days earlier than Rm1021.

S. meliloti typA and *E. coli bipA* complement each other.

To test whether *typA* from *S. meliloti* was able to substitute for the *E. coli bipA* gene, the pEK264 plasmid carrying *S. meliloti typA* was introduced into AF600 *E. coli* strain lacking *bipA*. The bacteria were grown on LB plates at 37 and 20°C. At 37°C, all the strains formed single colonies after overnight incubation. However, at 20°C after 3 days of inoculation, only the mutants carrying either the *typA* (pEK264) or the *bipA* (pEK287) genes on a plasmid were able to grow, whereas mutants lacking the functional *bipA* gene did not form single

colonies. Hence, the *S. meliloti* gene complemented the cold-sensitive phenotype of the *E. coli* mutant. Conversely, the plasmid-borne *bipA* open reading frame (ORF) (pEK287) complemented the cold sensitive phenotype of the *S. meliloti typA* mutant (Table 1).

Lipopolysaccharide composition of the *typA* mutant.

Lipopolysaccharide (LPS) mutants of *S. meliloti* often were found to be sensitive to detergents such as SDS or DOC (Ferguson et al. 2002; Kereszt et al. 1998; Lagares et al. 1992; Oke and Long 1999a). The SDS-sensitive phenotype of the *typA* mutant raised the possibility that the mutation may have some impact on the cell surface composition of the bacterium. To test this possibility, the surface polysaccharide content of the *typA* mutant (EK257), the *typA* overexpressing strain (EK273), and the wild-type Rm1021 were investigated. DOC-PAGE analysis of the capsular polysaccharides (KPS) and LPSs demonstrated no significant changes in regard to size or distribution affecting the polysaccharides (data not shown). More detailed analysis was performed on LPSs. The composition of lipid A (Fig. 2) showed no differences between EK257 and EK273 compared with Rm1021. Furthermore, no significant variation could be observed on the sugar composition of the polysaccharides (data not shown). Thus, the rough LPS composition of the three strains is fully consistent with the previous publication by Ferguson and associates (2002). Hence, *typA* does not grossly affect the LPS composition of *S. meliloti* 1021.

Fig. 2. Gas chromatograph with flame ionization detection traces of lipid A constituents obtained after acidic methanolysis and silylation. **A**, Rm1021, **B**, EK257, **C**, EK273; 1, 4, and 5 = methyl-trimethylsilyl-glucosamine, 2 = nC16 fatty acid methyl ester (FAME), 3 = 3-OH-C14 FAME, 6 = nC18 FAME, 7 = 3-OH-C16 FAME, 8 = 3-OH-C18 FAME, and 9 = 27-OH-C28 FAME.

Table 1. Cross complementation of the cold-sensitive phenotype of the *typA* and *bipA* mutants

Complementing plasmid	Bacteria ^a		
	Rm1021 (wild type)	EK257 (Rm1021 <i>typA</i>)	AF600 (<i>Escherichia coli bipA</i>)
pFAJ1700 (empty plasmid)	+	–	–
pEK264 (<i>typA</i>)	++	+	+
pEK287 (<i>BIP</i>)	++	+	+

^a Growth at low temperature: +, like wild type; ++, colonies appeared earlier; –, no single colonies.

TypA is required for normal nodule development on certain *M. truncatula* lines.

The symbiotic phenotype of the *S. meliloti typA* mutant was compared with that of the wild-type Rm1021 strain on five different *M. sativa* cultivars and as well as on three *M. truncatula* lines (Fig. 3). *M. sativa* plants inoculated with the *typA* mutant and the Rm1021 strain were indistinguishable and we found no difference in either nodulation kinetics or nodule morphology (data not shown). However, dry weight measurements of the shoots showed approximately 20% decrease in the case of

three cultivars (Magali, 18%; Mercedes, 21%; and Sitel, 21%) inoculated with the mutant.

Similarly to *M. sativa*, the *M. truncatula* line DZA315.16 (Thoquet et al. 2002) established a nitrogen-fixing symbiosis with *typA* mutant bacteria (Fig. 3A). Although the average number of nodules was twofold higher on the *typA*-inoculated plants, they were smaller, and dry weight measurements of the shoots showed 25% decrease compared with plants inoculated with the Rm1021 bacteria (Table 2). *M. truncatula* F83005.5 and Jemalong plants inoculated with the *typA* mutant exhibited a Fix⁻ phenotype. In both cases, plants were small and chlorotic, indicating that they were starved for nitrogen (Fig. 3B and C). Jemalong plants possessed small, round-shaped and white Fix⁻ nodules, whereas F83005.5 plants carried elongated, slightly pink nodules. However, F83005.5 plants inoculated with the *typA* mutant did not show acetylene-reduction activity (data not shown), indicating that, despite their nodule morphology similarity to the wild type, nitrogen fixation did not occur. These data suggest that the *typA* mutation affects differently the symbiotic efficiency on these two *M. truncatula* lines.

In order to test the complementation of the *typA* mutation under symbiotic condition, *M. truncatula* Jemalong, DZA315.16, and F83005.5 plants were inoculated by the Rm1021 and *typA* mutant bacteria carrying the intact *typA* ORF on the pFAJ1700 broad host range plasmid (strains EK273 and EK275, respectively). Thirty days after inoculation, the dry weight of the plant shoots was compared. The presence of the complementing plasmid (pEK264) in the mutant background (EK275) resulted in well-developed plants (Fig. 3) with dry weights similar to that of the wild type in each case, indicating that the complementation of the *typA* mutation was successful on all three lines (Table 2). Overexpression of *typA* in the Rm1021 background had no effect on the symbiotic efficiency of the bacteria (data not shown).

typA mutants fail to colonize

M. truncatula Jemalong nodules; however, they occupy the nodules of F83005.5 plants.

For monitoring the infection process, the pXLGD4 plasmid carrying the *hemA-lacZ* fusion (Leong et al. 1985) was introduced into the Rm1021 and the *typA* mutant bacteria (EK18 and EK99 strains, respectively). Then, the plant test was repeated with these strains on *M. truncatula* Jemalong and F83005.5 plants. Root and nodule samples were collected 6 days and 4 weeks after inoculation for *lacZ* staining. On Jemalong plants, 6-day-old nodules induced by the Rm1021 and the *typA* mutant bacteria were comparable in size; however, *typA* nodules were only weakly stained, indicating that they were weakly colonized (Fig. 4A and B). Thirty days postinoculation (dpi), Rm1021 nodules were elongated and showed typical developmental zones (Vasse et al. 1990). However, the *typA*-induced nodules were round shaped, without visible meristem and separated zones. They had almost the same size as the 6-day-old nodules possessing only a few bacteria inside, indicating that the bacteria either were impaired in the invasion of the nodules or were unable to survive within the plant (Fig. 4C and D). On F83005.5 plants, no obvious difference was found between the Rm1021 and the *typA*-induced nodules with respect to nodule invasion at either 6 days or 4 weeks after inoculation (Fig. 4E through H).

typA expression under free-living conditions and in planta.

To determine *typA* expression in a free-living environment, the mutant strain carrying a wild-type *typA* allele (EK275) was exposed to different stress conditions such as oxygen-limitation, cold stress, and presence of different concentrations of

Fig. 3. *Medicago truncatula* lines inoculated with the wild-type (left), *typA* mutant (middle), and *typA* mutants carrying the complementing plasmid (right) **A**, DZA315.16, **B**, F83005.5, and **C**, Jemalong.

NaCl or H₂O₂, as well as low pH. After 1 h of stress treatment, the enzyme activity of the *typA*–GUS fusion was measured. Under all the conditions tested, the enzyme activities were low (35 to 45 Miller units) and none of the stresses applied resulted in more than ± 5 units changes as compared with the nonchallenged level (data not shown).

In planta, the expression pattern of *typA* was determined by GUS staining of longitudinal nodule sections of *M. sativa* cv. Europe, and *M. truncatula* Jemalong, F83005.5, and DZA315.16 plants. We found similar expression patterns in all of the examined plants. In young, developing nodules (6 days dpi), *typA* expressed strongly (Fig. 5A). In mature nodules (18 dpi), *typA* expression started at the interzone II–III, where bacteria begin to differentiate into bacteroids and continued in the nitrogen-fixing zone (III) as well as in the senescent zone (IV) of the nodule. However, no expression was detected in the invasion zone (II) (Fig. 5B). A lower level of *typA* expression was detected in *M. sativa* nodules, which is in accordance with our previous observation (based on transcriptome data) showing that *typA* expressed at a lower level in *M. sativa* compared with *M. truncatula* (Ampe et al. 2003).

DISCUSSION

In this article, we describe the chromosomal *typA* gene of *S. meliloti* and show that it is the orthologue of *bipA* found in a wide range of bacteria. In *E. coli*, BipA has a dual role. In all strains tested so far, it is required for general housekeeping functions such as flagella-mediated cell motility and growth at low temperature (Grant et al. 2003; Pfennig and Flower 2001). In enteropathogenic (EPEC and EHEC) strains, BipA also is required for virulence functions (Farris et al. 1998; Grant et al. 2003). Similarly to BipA, we have found that *S. meliloti* TypA has a dual function: it is required for housekeeping functions and improves survival of *S. meliloti* under some stress conditions (growth at low temperature, low pH, or in the presence of SDS), and it is required as well for the establishment of a nitrogen-fixing symbiosis with certain host plants.

Likewise in *E. coli*, the *S. meliloti* *typA* mutant was found to be cold sensitive. In both bacteria, the cold-sensitive phenotype of the mutants could be complemented by introduction of either the *bipA* gene of *E. coli* or the *S. meliloti* *typA* on a multicopy plasmid. The *typA* mutant of the Rm1021 strain showed delayed colony formation at pH 6 compared with the wild type. Moreover, the mutant exhibited increased sensitivity to SDS that may indicate a weakened cell envelope, which often

is due to alteration of LPS production. Furthermore, *S. meliloti* mutants altered in their cell envelope, such as the *lpsB* and *bacA* mutants, were not able to establish chronic infection within the host plants (Campbell et al. 2002; Ferguson et al. 2002). Despite their SDS sensitivity and similar symbiotic phenotype, biochemical analysis of the cell surface polysaccharides LPS and KPS of the *typA* null mutant or the *typA* overexpressing strain did not reveal, so far, any significant difference in the amount or in the composition of polysaccharides produced compared with the wild type. Interestingly, the *typA* overexpressing strain EK273 was more resistant to SDS and grew faster at low temperature than the wild type. These data suggest that overexpression of *typA* may be advantageous for the bacteria under certain stress conditions.

Our data showed that TypA plays an essential role during symbiosis on certain plants; however, its function is not strictly required on other hosts. On *M. sativa* and *M. truncatula* DZA315.16, *typA* had only quantitative effects on plant growth. On the other two *M. truncatula* lines tested, *typA* is essential for symbiosis; however, it plays a role at two different stages. On Jemalong, *typA* is required for the successful colonization of the nodules. In contrast, on F83005.5, the *typA* mutant is fully infective but does not perform nitrogen fixation. In addition, the plant responses to *typA* mutant bacteria also differ on the two *M. truncatula* lines. Development of Jemalong nodules terminated at a very early stage; in contrast, F83005.5 nodules induced by the *typA* mutants were well differentiated. The genetic background of this difference on the plant side is under investigation.

The full role of the *typA* gene in *S. meliloti* is not yet completely understood. The pleiotropic phenotype of the *typA* mutant suggests that it codes for a regulatory protein. One of the possibilities is that *typA* is not specific to symbiosis and only mediates adaptation to certain stress responses that are encountered both under the free-living state as well as in symbiosis with some host plants. During nodule invasion and bacteroid differentiation, rhizobia are exposed to new physiological conditions (pH, osmotic concentration, oxidative stress, and defense responses), supposedly different in each host plant. This would explain why *typA* is not required equally on all hosts. On Jemalong plants, for example, the *typA* mutant may be unable to adapt to the environment found in the host; consequently, it is incapable of sustaining long-term intracellular infection.

In EPEC or EHEC strains, *bipA* not only mediates stress adaptation but also coordinates pathogenicity-related gene cluster expression (Grant et al. 2003). Similarly, it is possible

Table 2. Nitrogen fixation efficiency of *S. meliloti* strains on different host plants in the presence or absence of the functional *typA* gene

Line, strain ^a	Nodule number (average/plant $\pm$ SD)	Shoot dry weight (mg/plant $\pm$ SD)	Phenotype
Jemalong			
Noninoculated	0 $\pm$ 0	6.45 $\pm$ 1.6	
Rm1021 (wt)	17.6 $\pm$ 4.9	26.4 $\pm$ 6.6	Fix ⁺ ^d
EK257 (<i>typA</i> -)	33 $\pm$ 7.8	10.9 $\pm$ 3.2 ^b	Fix ⁻ ^d
EK275 (<i>typA</i> +))	24.2 $\pm$ 4	21.1 $\pm$ 2.1 ^c	Fix ⁺
DZA315.16			
Noninoculated	0 $\pm$ 0	7.07 $\pm$ 2.2	
Rm1021 (wt)	5 $\pm$ 0.9	31.9 $\pm$ 2.1	Fix ⁺ ^d
EK257 (<i>typA</i> -)	11.7 $\pm$ 2.1	23.9 $\pm$ 2.5 ^b	Fix ⁺ ^d
EK275 (<i>typA</i> +))	5 $\pm$ 1.6	30 $\pm$ 2.2 ^c	Fix ⁺
F83005.5			
Noninoculated	0 $\pm$ 0	7.02 $\pm$ 1.5	
Rm1021 (wt)	8 $\pm$ 1.6	32.7 $\pm$ 3.5	Fix ⁺ ^d
EK257 (<i>typA</i> -)	8.1 $\pm$ 1.9	7.3 $\pm$ 1.4 ^b	Fix ⁻ ^d
EK275 (<i>typA</i> +))	7 $\pm$ 1.4	31.4 $\pm$ 0.3 ^c	Fix ⁺ ^d

^a *Medicago truncatula* line and bacterial strain (genotype).

^b Significantly different from the wild-type value; $n = 10$, $P = 0.05$.

^c No significant difference compared with wild type.

^d Verified by acetylene reduction assay.

that, beyond its housekeeping role, *S. meliloti typA* has some symbiosis-specific function and may play a direct role in plant infection, bacteroid maturation or persistence, or nitrogen fixation. Although we could not get direct evidence for this, it is

possible that *S. meliloti typA* is affected on some cell surface component that is crucial for proper interaction with (some) host plants. Identifying the targets of *typA* should help in testing this possibility.

Fig. 4. *lacZ* staining of nodules inoculated by pXLGD4-containing bacteria. **A** and **B**, Jemalong 6 days postinoculation (dpi), **C** and **D**, Jemalong 30 dpi, **E** and **F**, F83005.5 6 dpi, **G** and **H**, F83005.5 30 dpi; **A**, **C**, **E**, and **G**, inoculated with Rm1021 and **B**, **D**, **F**, and **H**, inoculated with the *typA* mutant. Bar = 100 μ m (**A**, **B**, **D**, **E**, and **F**) and 200 μ m (**C**, **G**, and **H**).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids used in this study are described in Table 3. *E. coli* DH5 α (Gibco BRL) was used in the cloning procedures. *S. meliloti* and *E. coli* strains were grown at 30 and 37 °C respectively, in Luria-Bertani (LB) medium supplemented with the following concentration of antibiotics when required: ampicillin (100 μ g ml⁻¹), chloramphenicol (30 μ g ml⁻¹), tetracycline (10 μ g ml⁻¹), streptomycin (200 μ g ml⁻¹), and neomycin (200 μ g ml⁻¹). Plasmids were transferred into *S. meliloti* by triparental mating (Glazebrook and Walker 1991) using the pRK600 helper plasmid (Finan et al. 1986).

DNA manipulations.

Standard procedures, including DNA isolation, restriction enzyme digestion, radioactive labeling of DNA, Southern hybridization, agarose gel electrophoresis, DNA ligation, and transformation of *E. coli* were performed using conventional methods (Sambrook et al. 1989) or as recommended by the suppliers.

Computer-assisted sequence analysis.

Amino acid homology searches were performed against the “nonredundant” database of NCBI BLAST Network Service using the BLASTX program (Altschul and Lipman 1990). The multiple sequence alignment was performed using the Multalin program (Corpet 1988).

Fig. 5. Localization of *typA* expression in *Medicago truncatula* DZA315.16 nodules **A**, 6 days postinoculation (dpi), bar = 100 μ m and **B**, 18 dpi. II = Invasion zone, III = nitrogen fixation zone, and IV = senescent zone. Bar = 200 μ m.

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	References
Plasmids		
pRK600	ColE1 replicon with RK2 transfer region Cm ^r	Finan et al. 1986
pEK243	pVO155 carrying the <i>typA</i> PCR fragment, Nm ^r Ap ^r	This work
pFAJ1700	Broad host range cloning vector, Tc ^r	Dombrecht et al. 2001
pEK264	pFAJ1700:: <i>typA</i> , cloned <i>typA</i> gene of <i>Sinorhizobium meliloti</i> Tc ^r	This work
pEK287	pFAJ1700:: <i>bipA</i> , cloned <i>bipA</i> gene of <i>Escherichia coli</i> K12 Tc ^r	This work
pPLP7	pBR322:: <i>bipA</i> , Tc ^r	Pfennig and Flower 2001
pXLGD4	<i>hema-lacZ</i> fusion, Tc ^r	Leong et al. 1985
Strains		
<i>S. meliloti</i>		
Rm1021	Wild type, Nod ⁺ Fix ⁺ , Sm ^r	Meade et al. 1982
EK18	Rm1021(pXLGD4)	This work
EK99	Rm1021 <i>typA</i> (pXLGD4)	This work
EK257	Rm1021 <i>typA</i> ::pVO155	This work
EK273	Rm1021(pEK264)	This work
EK274	Rm1021(pFAJ1700)	This work
EK275	EK257(pEK264)	This work
EK276	EK257(pFAJ1700)	This work
EK290	EK257(pEK287)	This work
<i>E. coli</i>		
DH5 α	F ⁻ <i>supE44</i> <i>ΔlacU169</i> (Φ 80 <i>dlacZDM15</i>) <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Boyer and Roulland-Dussoix 1969
MT616	MT607 (pRK600)	Finan et al. 1986
AF600	<i>E. coli</i> K12 <i>bipA</i> ⁻	Pfennig and Flower 2001

^a Tc, tetracycline; Sm, streptomycin; Ap, ampicillin; Nm, neomycine, Cm, chloramphenicol.

Construction of the *typA* mutant Rm1021 strain.

For insertional inactivation of the *typA* gene, the plasmid-integration mutagenesis strategy was used as previously described (Oke and Long 1999a). Briefly, a 284-bp-long internal DNA fragment was amplified from Rm1021 genomic DNA by polymerase chain reaction (PCR) using the 5'-CGATGCCC GTCACGAGGAAGT-3' and 5'-AATGAATGCGCCCGGTGA TGAT-3' primers. The PCR fragment was ligated into the pCR2.1 TOPO vector (Invitrogen, San Diego, CA, U.S.A.) and then subcloned into the VO155 suicide vector carrying the promoterless *uidA* gene, resulting in the pEK243 plasmid, which was introduced into the Rm1021 genome by triparental mating. Single recombination events resulting in the integration of pEK243 into the *typA* ORF were selected using the neomycin resistance marker of the plasmid. Integration of the pEK243 plasmid disrupted the *typA* ORF and generated a transcriptional GUS fusion. Disruption of the *typA* ORF was verified by Southern hybridizations using *typA* and vector-specific PCR fragments as probes. The stability of the mutation was verified by testing the neomycin resistance of bacteria i) diluted back and grown up again five times consecutively from stationary phase liquid cultures and ii) isolated back from 6-week-old nodules. Both experiments confirmed that the mutation is stable without antibiotic selection pressure under the experimental conditions tested.

Construction of the complementing plasmids.

The wild-type *typA* gene was amplified from the Rm1021 genome by PCR using 5'-AGATGACGGTGCGGCTGATGG-3' and 5'-TCGCTCCGGGGTTTGTAGAT-3' primers. A 2,340-bp-long PCR fragment was ligated into the pCR2.1 TOPO vector (Invitrogen) and then subcloned into pFAJ1700 (Dombrecht et al. 2001) as a *HindIII*-*XbaI* fragment, resulting in the plasmid pEK264. The *bipA* gene (2,267-bp *Sau3A1* fragment in pBR322 *Bam*HI site) was subcloned from the pPLP7 plasmid (Pfennig and Flower 2001) to pFAJ1700 in two steps. First, *bipA* was isolated from pPLP7 as a *Bam*HI-*XhoI* fragment (2,135 bp) and ligated into pOK12 vector (Vieira and Messing 1991) (*Bam*HI-*SalI*) sites. Then the gene was isolated from pOK12 as a *Bam*HI-*EcoRI* fragment and ligated into pFAJ1700, resulting in the plasmid pEK287. pEK264 and pEK287 were introduced into Rm1021 and EK257 by triparental mating.

Sensitivity assays.

For all assays, *S. meliloti* strains were grown to an optical density at 600 nm (OD_{600}) = 1 in LB medium supplemented with antibiotics when required. For disk assays, 100- μ l aliquots of cells adjusted to OD_{600} = 0.2 were mixed with 0.7% soft agar and poured onto LB plates. Then, sterile paper disks (6 mm in diameter) (Schleicher & Schuell, Dassel, Germany) placed onto the plates were spotted with 5 μ l of 10% SDS, 3% H_2O_2 , or 0.1 M paraquat (methyl viologen, Sigma, St. Louis), respectively. After 3 days of incubation at 30°C, the zone of inhibition was measured. In plating experiments, series of dilutions (10^{-1} to 10^{-6}) of bacteria were spread onto LB plates with or without the addition of DOC at 1 to 2 g/liter, SDS at 0.05 to 0.1 g/liter, 2 to 8% (vol/vol) ethanol, 100 to 400 μ M $ZnSO_4$, and 0.1 to 0.7 M $NaCl_2$, respectively. For assaying the effect of pH, LB medium was supplemented with 35 mM 2-(N-morpholino) ethanesulfonic acid and pH was adjusted as requested (pH 7 to 5.5) with NaOH. Bacterial strains were challenged with 2 to 8 mM H_2O_2 as described (Santos et al. 2000). The numbers of colonies on plates from treated and nontreated samples were compared.

typA expression assays.

Expression of the *typA* gene was monitored, taking advantage of the fact that the insertion of the pVO155 plasmid gen-

erated a mutant and a transcriptional GUS fusion at the same time. In order to analyze *typA* expression in a wild-type background, the enzyme activity of the *typA*-GUS fusion was measured in the EK275 strain carrying the wild-type *typA* gene on a complementing plasmid. Exponential-phase cultures (OD_{600} = 0.5) grown in LB medium containing tetracycline were subjected to the following stresses for 1 h: cold shock at 10°C, osmotic stress (0.1 to 0.5 M NaCl), oxidative stress (2 to 8 mM H_2O_2), low pH (pH 7 to 5.5), and low oxygen (2% oxygen). In the latter case, 4 h of induction was applied. β -Glucuronidase activities were measured by a standard protocol using p-nitrophenyl β -D-glucuronide as a substrate (Jefferson and Wilson 1991). Values were obtained using triplicate samples from three independent experiments.

Cultured-cell polysaccharide isolation.

Media were centrifuged at 12,000 $\times$ g for 20 min. The EPS-containing supernatant was removed, evaporated, and differentially precipitated into low and high molecular fractions. The bacteria were extracted by the hot phenol/water method. The water layer (200 ml) was added to 100 ml of phenol and stirred for 15 min at 65°C. The mixture then was centrifuged at 7,500 $\times$ g at 5°C for 20 min. The aqueous supernatant was dialyzed in tube (cutoff: 10 kDa) against two 6-liter changes of deionized water in order to remove the phenol. It then was concentrated to 50 ml, made 10 mM in $MgSO_4$ and 50 mM in Tris-HCl, and incubated with 1 mg of RNase and 1 mg of DNase overnight under stirring. Proteinase K (Sigma) (1 mg) was added and the solution was stirred again at 37°C for 4 h. The sample was dialyzed a second time and freeze dried. The freeze-dried aqueous phase obtained consisted of a crude extract, 20 mg of which was passed over a 1.6-by-23-cm polymyxin column (Sigma) in order to separate the KPS from the LPS, as described earlier (Forsberg and Carlson 1998). After addition of the aqueous sample solution, the column was washed with 4% ammonium carbonate in aqueous solution to elute the KPS. LPSs were eluted with 2% deoxycholic acid in H_2O . The two KPS- and LPS-containing fractions were dialyzed, evaporated under vacuum, and freeze-dried.

Polysaccharide identification and analysis.

The polyacrylamide gel electrophoresis (PAGE) protocol has been described previously (Reuhs et al. 1993). PAGE was performed on the phenol-water extracts or purified polysaccharides using 18% polyacrylamide gels with deoxycholic acid as detergent; the gels either were silver stained to detect LPS (Tsai and Frasch 1982) or Alcian blue/silver stained to check for the presence of all polysaccharides (Corzo et al. 1991).

Polysaccharide monosaccharide analysis.

PS glycosyl compositions were determined by preparation of the alditol acetates (York et al. 1985). The sample was dissolved in 200 μ l of 2M trifluoroacetic acid (TFA) in a screw-cap Teflon vial, and heated for 2 h at 120°C for hydrolysis. The samples were evaporated at 60°C under a stream of filtered air. The addition of 3 $\times$ 200 μ l of isopropyl alcohol results in more complete removal of the TFA. The sugars then were reduced to give the corresponding alditols by the addition of a 1 M ammonia solution containing sodium borohydride at 10 mg/ml, purchased from Sigma (1 h at room temperature). The reaction then was quenched by the addition of two drops of pure acetic acid. The mixture was evaporated; meanwhile, 6 $\times$ 200 μ l of MeOH is added. The alditols were peracetylated by treatment with 100 μ l of pyridine and 100 μ l of acetic anhydride for 30 min at 80°C. The pyridine is evaporated and the mixture is injected for gas chromatography (GC) in solution in chloroform. The products

were identified and quantified by comparison with authentic standards in GC and GC-mass spectroscopy (MS).

The GC chromatograms were performed on a Chrompack CP9000 gas chromatograph with on-column injection, using an OV-1 capillary column (50 m by 0.25 μ m by 0.1 μ m). The temperature program was 60°C (initial temperature) to 110°C (10°C min⁻¹), then up to 300°C (final temperature) at 3°C min⁻¹. The vector gas was helium at 110 kPa.

GC-MS analysis was recorded on a Finnigan mat Trace MS 2000 series in the positive EI mode (ionization energy: 70 eV) using the following parameters: source, 200 or 250°C; splitless injector at T° 250°C; gas, He, 0.7 bar; and temperature program, 110°C for 2 min, 110°C up to 310°C (3°C min⁻¹), and 310°C for 10 min on an Optima MS capillary column (50 m $\times$ 0.25 μ m $\times$ 01 μ m) (Macherey-Nagel, Hoert, France).

Lipid A study.

The lipid A moiety was isolated from the LPS using mild hydrolysis with 1% acetic acid for 1 h. Then, the sample was submitted to centrifugation and the precipitate was washed twice with water. Lipid A was subjected to acidic methanolysis (3 M MeOH and HCl), and the resulting fatty acids (monomethyl ester derivatives) were silylated with 100 μ l of HMDS and 40 μ l TMCS (purchased by Sigma) in pyridine (100 μ l) at 70°C.

Plant growth conditions.

Assays for the symbiotic properties of the mutant *S. meliloti* strains were carried out with *M. sativa* (cvs. Site1, Europe, Gemini, Mercedes, and Magali) and *M. truncatula* (lines Jemalong, DZA315.16, and F83005.5) plants. Seed were surface sterilized by subsequent treatment with H₂SO₄ (2 min) and H₂O (five times), then 5% bleach (4 min) followed by washing with sterile water (five times). Seed were germinated on water-agar (0.7%) plates supplemented with 1 μ M Gibberellin A3 (Sigma) at 14°C for 2 to 3 days. The seedlings were grown on nitrogen-free agar slants (Fahraeus 1957) either in test tubes or in plates at 22°C with day and night cycle of 16 and 8 h, respectively. Plantlets were inoculated with *S. meliloti* strains resuspended in liquid Fahraeus medium (100 μ l/plant) at an OD₆₀₀ = 0.1 after 48 h of growth on agar slants.

Histochemical localization of bacteria.

For visualization of the bacteria in nodules, plants were inoculated with either the Rm1021 or the *typA* mutant strains carrying the pXLGD4 plasmid, which provides a high level of *lacZ* expression (Leong et al. 1985). Samples were fixed in phosphate-buffered saline (PBS) pH 7.2 containing 2.5% glutaraldehyde and stained for β -galactosidase activity (Teeri and Herrera-Estrella 1991). For localization of the *typA* gene activity in *M. sativa* and *M. truncatula* F83005.5 and DZA315.16 plants, on which the *typA* mutants were fully effective in terms of nodule occupancy, β -glucuronidase staining of roots and nodules induced by the *typA* mutant harboring the promoterless *uidA* genes within the integrated pVO155 plasmid was carried out using a standard protocol (Jefferson and Wilson 1991). In order to detect *typA* expression in *M. truncatula* Jemalong nodules (which are not invaded in the absence of a functional *typA* copy), the mutant strain carrying the wild-type *typA* gene on a plasmid (EK275) was used. For both types of staining, root and nodule samples were collected 6, 18, or 30 days after inoculation. Six-day-old nodules and roots were studied as intact organs. From developed nodules, 80- μ m longitudinal sections were made with a microcut H1200 (Bio-Rad, Munich). Microscopic analysis was carried out with an Axiophot 2 light microscope (Carl Zeiss, Oberkochen, Germany) coupled with a Leica DC200 digital camera.

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