Identification of Genes Relevant to Symbiosis and Competitiveness in *Sinorhizobium meliloti* Using Signature-Tagged Mutants

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Sinorhizobium meliloti enters an endosymbiosis with alfalfa plants through the formation of nitrogen-fixing nodules. In order to identify *S. meliloti* genes required for symbiosis and competitiveness, a method of signature-tagged mutagenesis was used. Two sets, each consisting of 378 signature-tagged mutants with a known transposon insertion site, were used in an experiment in planta. As a result, 67 mutants showing attenuated symbiotic phenotypes were identified, including most of the *exo*, *fix*, and *nif* mutants in the sets. For 38 mutants in genes previously not described to be involved in competitiveness or symbiosis in *S. meliloti*, attenuated competitiveness phenotypes were tested individually. A large part of these phenotypes was confirmed. Moreover, additional symbiotic defects were observed for mutants in several novel genes such as infection deficiency phenotypes (*ilvI* and *ilvD2* mutants) or delayed nodulation (*pyrE*, *metA*, *thiC*, *thiO*, and *thiD* mutants).

Sinorhizobium meliloti is a gram-negative α -proteobacterium that enters an endosymbiosis with plants of the genera *Medicago*, *Melilotus*, and *Trigonella* through the formation of specialized plant organs called root nodules. Inside the nodules, bacteria fix atmospheric nitrogen, converting it to ammonia that is transported to the plant. In turn, the plant provides the bacteria with dicarboxylic acids as carbon sources. Symbiosis between *S. meliloti* and its host plants is a complex and fragile process that requires participation of many gene products from both symbiotic partners. So far, most of the genes that are specific and crucial for establishment of a successful symbiosis or nitrogen fixation probably have been discovered. However, many of the genes that influence symbiosis in a more subtle way still are not identified. In order to discover genes which are specifically expressed in symbiosis, novel high-throughput methods such as transcriptomics (Ampe et al. 2003; Barnett et al. 2004; Becker et al. 2004; Capela et al. 2006), proteomics (Djordjevic et al. 2003), and in vivo expression technology (Oke and Long 1999; Zhang and Cheng 2006), were successfully applied to *S.*

meliloti. Data from these experiments, however, provide no information about the involvement of the identified genes in survival and competitiveness of *S. meliloti* in the host.

Usually, to study the function of genes in symbiosis and competitiveness in detail, gene disruption or deletion with subsequent analysis of symbiotic phenotypes is applied. This method is efficient if one or a few genes have to be tested. However, it is very laborious and time consuming when used for screening of hundreds or thousands of mutants. A preselection of mutants in defined free-living conditions often is performed in order to reduce the number of mutants that have to be tested in planta (Milcamps et al. 1998; Summers et al. 1998; Trzebiatowski et al. 2001; Uhde et al. 1997). At the same time, such a preselection reduces the chances to find unknown genes involved in symbiosis if these genes are not expressed in the tested free-living conditions. In contrast, signature-tagged mutagenesis (STM) allows for screening of hundreds of mutants in one passage through the host; thus, large libraries of mutants can be screened quite easily.

STM is based on a collection of mutants split into sets, where each mutant is modified by one or more different signature tags (Hensel et al. 1995). The tags are short DNA segments that are unique for each mutant in a set. Tagged mutants from the same set are pooled prior to an experiment, and each mutant in the mixture can be identified based on the unique tag in its genome. In order to integrate signature tags into a genome, a polymerase chain reaction (PCR) targeting strategy (Shoemaker et al. 1996; Winzeler et al. 1999) or a strategy based on libraries of tag-carrying transposons can be used. In the transposon-based STM (Hensel et al. 1995), a library of transposons carrying signature tags is generated, and these transposons then are used to perform the mutagenesis.

In a previous study (Pobigaylo et al. 2006), we described an improved STM approach in which a large library of tagged transposons was constructed and two-channel microarrays were used to detect signature tags in the input and output pools. A large library of *S. meliloti* mutants was generated using these signature-tagged transposons, and transposon insertion sites were mapped for more than 5,000 mutants. Here, we describe the screening of a part of this transposon library in planta in order to identify genes involved in symbiosis and competitiveness.

RESULTS

Screening of signature-tagged mutants in symbiotic conditions.

To identify genes important for survival and competitiveness in symbiosis, two sets of *S. meliloti* signature-tagged mutants, each containing 378 mutants (Supplementary Tables 1 and 2),

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were screened in symbiosis with *Medicago sativa* cv. Europe. These sets, which were assembled from a previously described signature-tagged mutant library (Pobigaylo et al. 2006), were, for the biggest part, nonredundant. Seventeen genes were represented by two or three different mutants in order to test whether they behaved similarly in the competition experiments. Furthermore, many mutants with a transposon insertion in genes known to be involved in symbiosis were included, as well as in genes that may play a role in symbiosis and in genes encoding hypothetical proteins.

In order to screen these large mutant sets in planta, an aeroponic plant cultivation system was used, which enabled an equivalent spreading of mutant suspension to all roots and guaranteed the formation of a high quantity of nodules. The composition of tags in the output pool (nodules) was compared with the composition of tags in the input pool (culture used to inoculate the plants) in order to identify mutants which were attenuated or over-represented in symbiosis. Four microarray hybridizations were carried out for each set. Three of these hybridizations were performed with targets synthesized from separate DNA isolations from the different aliquots of nodules. The fourth hybridization for each set was made using targets synthesized from a mixture of DNA from all three aliquots. In order to filter out possible false-positive clones, the results from three grouped hybridizations were compared with the data from the fourth pooled hybridization (Fig. 1). In all, 67 attenuated (30

from set 1 and 37 from set 2) (Table 1) and 29 over-represented (22 from set 1 and 7 from set 2) mutants were identified. This study focuses on the attenuated mutants identified in these experiments.

Most nodules in aeroponic conditions were occupied by a single mutant clone.

In order to determine how many mutants enter one nodule in aerobionics, nine nodules from each aeroponic tank were tested individually. The DNA from each of these nodules was used to perform separate target preparations and microarray hybridizations. The presence of a specific mutant in a nodule was detected based on high average spot intensity values for both tags. From 18 tested nodules, 15 were occupied by a single mutant clone. Two nodules contained two different mutant clones and one nodule contained three different mutant clones. Two of these nodules (set 1, nodules 7 and 9) contained the same mutant clone; all other nodules contained different mutant clones (Supplementary Table 3). Therefore, we conclude that most of the nodules in aeroponic conditions were occupied by a single mutant clone.

Reduced competitiveness was confirmed for a large proportion of the attenuated mutants.

To verify the attenuated phenotype observed in the STM experiments, 38 mutants that showed an attenuated phenotype

Table 1. Characteristics of mutants showing an attenuated phenotype under symbiotic conditions in both mutant sets

Mutant ID	Insertion in gene	Function	<i>m</i> value hybridization 4	Mean weighted value ^a	Mutant in nodules (%) ^b
2011mTn5STM.1.10.C09	SMa0244	Hypothetical protein	NA	-0.82	8.50
2011mTn5STM.2.09.D02 ^c	SMa0322	Hypothetical protein	-0.87	-0.80	53.15
2011mTn5STM.4.09.E06	SMa0414	Hypothetical protein	NA	-0.80	32.03
2011mTn5STM.1.05.D11	SMa0814	NifB FeMo cofactor biosynthesis protein	-1.06	-1.09	NT
2011mTn5STM.3.12.A01	SMa0819	FixB electron transfer flavoprotein α chain	NA	-0.87	NT
2011mTn5STM.2.07.E06	SMa0829	NifK nitrogenase Fe-Mo β chain	-1.10	-0.96	NT
2011mTn5STM.1.05.D09	SMa0840	NodD3 transcriptional regulator	NA	-0.93	NT
2011mTn5STM.2.08.D05	SMa0849	SyrM transcriptional regulator	-0.96	-0.78	NT
2011mTn5STM.3.06.G06	SMa0863	NodJ membrane transport protein	-1.03	-0.82	NT
2011mTn5STM.1.10.H04	SMa0873	NifN Nitrogenase Fe-Mo cofactor biosynthesis protein	NA	-0.97	NT
2011mTn5STM.1.11.A04	SMa1004	Hypothetical protein	-0.83	-0.86	23.97
2011mTn5STM.1.01.C04	SMa1229	FixL oxygen regulated histidine kinase	-1.11	-1.11	NT
2011mTn5STM.1.13.C08	SMa1229	FixL oxygen regulated histidine kinase	-1.08	-0.96	NT
2011mTn5STM.3.05.H07	SMa1229	FixL oxygen regulated histidine kinase	-1.09	-0.91	NT
2011mTn5STM.4.10.B08	SMa1742	Putative iron uptake protein	-0.72	-0.73	26.12
2011mTn5STM.1.08.G06	SMa1798	Kup2 Potassium uptake protein	-1.05	-0.94	20.85
2011mTn5STM.4.08.F03 ^c	SMa1913	Putative transport protein	-0.76	-0.86	77.73
2011mTn5STM.4.04.D06	SMb20227	Nutrient deprivation-induced protein A (NdiA1)	-0.77	-0.75	25.46
2011mTn5STM.4.04.D04 ^d	SMb20481	AsnO asparagine synthase, glutamine-hydrolyzing	-1.05	-1.10	18.04
2011mTn5STM.3.06.H03	SMb20509	Putative transcriptional regulator	-0.74	-0.92	28.25
2011mTn5STM.2.06.H09	SMb20612	C4-dicarboxylate transport sensor protein DctB	-0.83	-0.76	NT
2011mTn5STM.2.05.D11	SMb20615	Putative thiamine biosynthesis protein ThiC	-1.06	-0.92	0.34
2011mTn5STM.1.04.A09	SMb20616	ThiO putative thiamine biosynthesis oxidoreductase protein	-0.98	-0.88	1.89
2011mTn5STM.2.08.E08	SMb20757	Methylmalonyl-CoA mutase protein BhhA	-0.84	-0.87	24.35
2011mTn5STM.4.04.E11	SMb20942	UDP glucose 4-epimerase protein ExoB	-1.14	-1.07	NT
2011mTn5STM.2.05.G06	SMb20943	Acetyltransferase protein ExoZ	-1.12	-1.05	NT
2011mTn5STM.1.12.G08	SMb20948	ExoU glucosyltransferase	-1.05	-1.06	NT
2011mTn5STM.3.10.B09	SMb20957	Exopolysaccharide biosynthesis protein ExoA	-1.15	-1.18	NT
2011mTn5STM.1.01.B09	SMb20958	Exopolysaccharide biosynthesis protein ExoM	-1.08	-1.02	NT
2011mTn5STM.2.09.C09	SMb20959	Probable exopolysaccharide biosynthesis protein ExoO	-1.05	-1.08	NT
2011mTn5STM.1.07.B09	SMb20962	Probable phosphomethylpyrimidine kinase protein ThiD	-1.13	-1.05	4.96
2011mTn5STM.4.08.E12	SMb21158	Putative transcriptional regulator, probably of sugar phosphate metabolism protein	-1.13	-0.96	29.20
2011mTn5STM.2.12.A10	SMc00129	Sensor histidine kinase protein FeuQ	-0.99	-0.96	4.54
2011mTn5STM.1.11.A01	SMc00169	NAD-malic enzyme oxidoreductase protein Dme	-0.94	-0.99	NT
2011mTn5STM.2.05.C07	SMc00236	Probable indole-3-glycerol phosphate synthase TrpC	-0.99	-0.82	3.03

(continued on following page)

^a Mean weighted value, three hybridizations combined.

^b Average percentage of mutant in nodules in the individual test; NT = not tested.

^c False positives.

^d Clone has an unidentified secondary mutation responsible for the mutant phenotype.

and had no previously known fixation or nodulation defect were tested individually for symbiotic competitiveness in comparison with wild type-strain *S. meliloti* Rm2011, which was the parental strain of the STM mutant library (Pobigaylo et al. 2006). In this assay, *M. sativa* plants grown on agar plates were inoculated by a 1:1 mixture of mutant and wild-type cultures, and the ratio of the mutant to the wild type in the formed nodules was determined based on the neomycin resistance of the mutants. A severe defect in symbiotic competitiveness was confirmed for 23 mutants that displayed occupancy of nodules of less than 20%. Nine mutants displayed an occupancy of nodules between 20 and 30%, three mutants displayed an occupancy between 30 and 40%, and only three mutants were identified as definite false positives showing occupancy of nodules of more than 40% (Table 1).

Additionally, four mutants that showed nonsignificant mean_w (mean weighted) values in the STM experiment ranging between -0.01 and 0.39 were tested in the same way as the attenuated mutants (Supplementary Table 4). For these control clones, the mixtures of wild type and mutant used to inoculate the plants also were plated on medium containing neomycin and on medium without this antibiotic prior to inoculation. Control clone/wild type ratios prior to inoculation ranged between 49.48 and 55.80%, whereas the occupancy of nodules by the control clones ranged between 34.25 and 48.30%. Based on these nodule occupancy values, we decided to further study only those attenuated clones, which showed nodule occupancy of less than 20% in the individual competitiveness assay.

In all, 10 mutants were identified that showed symbiotic phenotypes other than reduced symbiotic competitiveness.

Each of the 38 mutants that displayed reduced competitiveness in the first screen also was tested individually for deficiencies in symbiosis. Only severe symbiotic defects, such as a non-nodulating (Nod⁻), infection deficient (Inf⁻) or nonfixing (Fix⁻) phenotype or a strong delay in nodulation were considered. The following mutants showed such symbiotic defects.

Mutant 2011mTn5STM.3.08.G1 carrying a transposon insertion in the *ccmC* (SMc03849) gene showed a Fix⁻ phenotype: the induced nodules were white and the plant shoots started to turn yellow 2.5 to 3 weeks after inoculation. In the nodulation kinetics test, this mutant induced a high quantity of small white nodules, which is usual for nonfixing *S. meliloti* mutants (Pauu et al. 1985) (Fig. 2A).

Mutant 2011mTn5STM.4.04.D04 with a transposon inserted into *asnO* (SMb20481) induced very few nodules; however, most of these nodules had a Fix⁺ phenotype (Fig. 2E). Clones 2011mTn5STM.3.10.F05 and 2011mTn5STM.3.05.A05 containing a transposon insertion in *ilvI* (SMc01431) and *ilvD2* (SMc04045), respectively, had an Inf⁻ and a partial Nod⁻ phenotype, inducing only a few pseudonodules on a fraction of plants (Fig. 2A).

Mutants 2011mTn5STM.2.05.D11, 2011mTn5STM.1.04.A09, and 2011mTn5STM.1.07.B09, carrying a transposon insertion in *thiC* (SMb20615), *thiO* (SMb20616), and *thiD* (SMb20962), respectively, showed delayed nodulation but, after

Table 1. Continued from preceding page

Mutant ID	Insertion in gene	Function	<i>m</i> value hybridization 4	Mean weighted value ^a	Mutant in nodules (%) ^b
2011mTn5STM.1.12.E10	SMc00349	GTP-binding membrane protein LepA	-0.97	-0.94	3.97
2011mTn5STM.4.02.C03	SMc00644	Hypothetical protein	-1.13	-1.08	NT
2011mTn5STM.4.03.B06	SMc00781	Methylmalonate-semialdehyde dehydrogenase MmsA (IolA)	-1.09	-1.03	2.94
2011mTn5STM.4.02.G03	SMc00828	Hypothetical protein	-0.87	-0.86	31.61
2011mTn5STM.3.07.B02	SMc00963	Biotin ABC transporter, permease component BioN	-1.09	-1.07	NT
2011mTn5STM.4.04.C12	SMc01053	Siroheme synthase protein CysG	-1.06	-1.06	0.45
2011mTn5STM.4.02.D11	SMc01139	RNA polymerase sigma-54 factor	-1.17	-1.03	NT
2011mTn5STM.4.05.E02	SMc01183	Putative LexA repressor regulator	-0.89	-0.86	16.67
2011mTn5STM.2.12.H01	SMc01219	Lipopolysaccharide core biosynthesis mannosyltransferase LpsB	-0.91	-0.84	NT
2011mTn5STM.3.10.F05	SMc01431	Probable acetolactate synthase isozyme III large subunit protein IlvI	-0.99	-0.83	0.04
2011mTn5STM.1.02.G07	SMc01877	Probable DNA repair protein RecN	-0.77	-0.73	21.70
2011mTn5STM.3.10.C03	SMc01929	Hypothetical protein	-0.70	-0.79	34.24
2011mTn5STM.2.10.D01	SMc01950	High-affinity branched-chain amino acid ABC transporter, permease protein LivM	-0.86	-0.81	15.31
2011mTn5STM.2.09.A02	SMc02109	Probable ATP-dependent CLP protease ATP-binding subunit protein ClpA	-0.96	-1.03	13.31
2011mTn5STM.4.10.A07	SMc02123	Hypothetical protein	-0.81	-0.77	27.16
2011mTn5STM.1.13.C06	SMc02143	Putative phosphate transport system permease ABC transporter protein PstA	-0.95	-0.95	11.45
2011mTn5STM.4.09.B12	SMc02144	Phosphate transport system permease ABC transporter protein PstC	-0.76	-0.93	4.06
2011mTn5STM.2.01.D04	SMc02165	Probable orotate phospho-ribosyltransferase protein PyrE	-0.96	-0.84	0.01
2011mTn5STM.4.02.E06	SMc02226	Hypothetical protein	-1.05	-0.97	1.24
2011mTn5STM.4.08.A01	SMc02274	Putative capsule polysaccharide export protein precursor RkpU	-0.75	-0.74	3.93
2011mTn5STM.1.10.H06	SMc02361	Cytochrome C-type biogenesis transmembrane protein CycH	-0.85	-0.90	NT
2011mTn5STM.3.06.F10	SMc02562	Phosphoenolpyruvate carboxykinase protein PckA	-1.04	-0.85	NT
2011mTn5STM.1.09.H12	SMc02767	N-5'-phosphoribosylanthranilate isomerase TrpF	-0.95	-0.94	0.00
2011mTn5STM.2.10.A06	SMc03181	pH adaptation potassium efflux system transmembrane protein PhaD1	-1.03	-0.92	NT
2011mTn5STM.3.08.G09 ^c	SMc03277	MFS-type transport protein	-0.93	-0.88	49.43
2011mTn5STM.4.09.F03	SMc03797	Homoserine O-succinyltransferase MetA	-1.20	-1.20	0.04
2011mTn5STM.3.08.G12	SMc03849	Cytochrome c-type biogenesis protein CcmC	-1.06	-0.98	7.93
2011mTn5STM.4.08.B05	SMc03900	β-Glucan export ATP-binding protein Ndva	-1.06	-1.02	NT
2011mTn5STM.3.05.A05	SMc04045	Probable dihydroxy-acid dehydratase protein IlvD2	-0.99	-0.85	0.03
2011mTn5STM.1.08.C11	SMc04346	IlvC ketol-acid reductoisomerase protein	-0.83	-0.78	NT
2011mTn5STM.1.03.D11	SMc04346	IlvC ketol-acid reductoisomerase protein	NA	-0.88	NT
2011mTn5STM.2.02.E07	SMc04405	3-Isopropylmalate dehydrogenase LeuB	-1.12	-0.94	NT

4 weeks, induced a higher quantity of nodules than the wild type (Fig. 2A and C). Because *thiC* is situated immediately upstream of *thiO*, only *thiC* and *thiD* mutants were studied further.

Mutants 2011mTn5STM.2.01.D04, 2011mTn5STM.4.04.C12, and 2011mTn5STM.4.09.F03 carrying a mTn5 insertion in *pyrE* (SMc02165), *cysG* (SMc01053), and *metA* (SMc03797), respectively, also displayed a delayed nodulation phenotype (Fig. 2C). The *metA* mutant induced pink effective nodules on all plants 3 weeks after inoculation. In contrast, *cysG* and *pyrE* mutants had a more severe symbiotic defect. Whereas, on most plants, nodules eventually turned pink, on some plants they stayed white even 4 weeks after inoculation. In the case of all mutants that displayed a delayed nodulation phenotype, the overall quantity of nodules formed on inoculated plants 24 days after inoculation exceeded those of the plants inoculated by the wild-type Rm2011 by 25 to 40%.

In order to confirm that the observed phenotypes originated from the mutation caused by the transposon insertion, these insertions were transferred to the wild type by general transduction or mutant phenotypes were complemented by a plasmid-borne wild-type copy of the targeted gene. For complementation, a wild-type copy of the mutated gene was cloned under the control of a constitutive *Escherichia coli lac* promoter into the low-copy vector pPHU231 (Hübner et al. 1991). Because the mTn5 transposon used for mutagenesis contains a terminator (Reeve et al. 1999), the mutations caused by insertion of this transposon have a polar effect on downstream genes in operon structures. Therefore, we expected successful complementation

only for mutations in genes which are not co-transcribed with downstream genes, or in cases where co-transcribed downstream genes have no effect on the symbiotic phenotype. Complementation or transfer of the mutant phenotype by transduction was evaluated by macroscopic inspection of the induced nodules and comparison of nodulation kinetics of the complemented or transduced strain to the wild type (Fig. 2).

Complementation clones of the mutants in *ccmC*, *ilvI*, *ilvD2*, and *thiD* showed a restored wild-type phenotype. Compared with the mutants, the complementation clones induced nodules on *M. sativa* at rates much more similar to those of wild-type Rm2011. Particularly, the complementation clone of the *ccmC* mutant had a Fix⁺ phenotype and induced fewer nodules than the original mutant. Interestingly, plants inoculated by the complementation clones of the *ilvI* and *ilvD2* mutants formed even more nodules at the early nodulation stages than plants inoculated by the wild type (Fig. 2B). This might have been caused by a higher rate of expression of *ilvI* and *ilvD2* directed by the constitutive *lac* promoter in the complementation clones than by the native promoter in the wild type. The complementation clone of the *thiD* mutant already reached wild-type nodulation rates 11 days after inoculation, compared with approximately 20 days after inoculation in the mutant, and induced the same overall quantity of nodules as the wild type.

The phenotypes of mutants in *metA*, *cysG*, *pyrE*, and *thiC* genes could not be complemented by the method described above. This may be explained by the polycistronic structure of operons containing these genes, where the transcription of downstream genes is influenced by the polar effect of the transposon insertion. However, transduction of these mutations into wild-type Rm2011 resulted in strains with the same phenotype as observed for the original transposon mutants (Fig. 2C and D). In contrast, the mutant phenotype of clone 2011mTn5STM.4.04.D04 carrying a transposon insertion in *asnO* (SMb20481) could be neither complemented nor transferred by transduction of the transposon insertion (Fig. 2E).

ilvI and *ilvD2* mutants do not cause formation of infection threads.

For the *ilvI* and *ilvD2* mutants that showed a partial Nod⁻ and Inf⁻ phenotype, fluorescent confocal microscopy was performed in order to determine whether these mutants cause root hair deformation and curling and infection thread formation. Microscopic images indicate that both mutants have a defect in infection. This corresponds to the phenotype observed in the plant assays. The *ilvD2* mutant induced root hair deformation (swellings and branchings) and, occasionally, root hair curling (Fig. 3D, E, and F). A more severe symbiotic defect of the *ilvI* mutant was observed. Root hair deformation was induced only in rare cases (Fig. 3B and C). Infection threads observed in plants invaded by wild-type Rm2011 (Fig. 3A) were not observed on plants inoculated by either the *ilvI* or the *ilvD2* mutant strain.

DISCUSSION

STM has been applied successfully to many pathogenic bacteria (Saenz and Dehio 2005) and several symbiotic or environmentally important organisms, such as *Xenorhabdus nematophila* (Heungens et al. 2002), *Burkholderia vietnamiensis* (O'Sullivan et al. 2007), *Desulfovibrio desulfuricans*, and *Shewanella oneidensis* (Groh et al. 2005). Here, for the first time, the STM technique was applied to study a symbiotic relationship between legume plants and nodulating bacteria. The establishment of a large signature-tagged transposon library (Pobigaylo et al. 2006) allowed generating and screening of sets that contain up to 412 differently tagged mutants. In this

Fig. 1. Comparison of mean weighted values from three grouped hybridizations (y axis) to the *m* values from the fourth pooled hybridization (x axis) for **A**, mutant set 1 and **B**, set 2. Mutants showing attenuated or strongly nodulating phenotypes are marked as crosses and mutants showing no difference to the input pool are denoted as dots.

work, 756 such mutants split into two sets were screened in planta in order to identify genes important for survival or competitiveness in symbiosis. Mutants, which were attenuated in symbiosis, are reviewed below.

Mutants with known symbiotic defects were detected as attenuated in the STM experiments.

As internal controls, mutants in genes with a known role in symbiosis were included in the sets of mutants which were tested in planta. In fact, 29 of 67 attenuated mutants were expected to be impaired in competitiveness, because symbiotic defects have been reported previously for such mutants in *S. meliloti* Rm2011 or closely related strains. This finding strongly supports the reliability of the STM approach in this study. The mutants reidentified as impaired in symbiotic competitiveness carried transposon insertions in the following genes.

Exopolysaccharide synthesis (*exo*) genes.

From nine mutants in *exo* genes, related to the biosynthesis of the symbiotically active exopolysaccharide succinoglycan, six were detected as attenuated. The attenuated mutants had

transposon insertions in *exoA*, *exoB*, *exoO*, *exoM*, *exoU*, and *exoZ*. *exoA*, *exoB*, *exoO*, *exoM*, and *exoU* mutants of *Sinorhizobium meliloti* Rm2011 do not produce succinoglycan and were shown to induce empty Fix⁻ nodules on *M. sativa* (Becker et al. 1993b,c; Buendia et al. 1991). An *exoZ* mutant of *S. meliloti* Rm1021 showed a reduced efficiency in the initiation and elongation of infection threads (Cheng and Walker 1998) which may explain the impaired competitiveness. The mean_w values of the *exoK*, *exoN2*, and *exoP2* mutants were below the threshold in the STM experiment and indicated a non-attenuated phenotype. An *S. meliloti* Rm2011 *exoK* mutant was shown to induce pink nodules at a normal rate on *M. sativa* (Becker et al. 1993a). *exoN2* and *exoP2* genes were not required for synthesis of succinoglycan (data not shown) and may be involved in synthesis of an as-yet-unknown polysaccharide.

Nod factor synthesis and transport (*nod*) genes.

Two *nod* mutants (*nodD3* and *nodJ*), which were included in the screened sets, were detected as attenuated. *S. meliloti* Rm1021 *nodD3* (Honma and Ausubel 1987) and *S. meliloti*

Fig. 2. Nodulation kinetics of **A** and **C**, mutants; **B**, respective complementation clones; and **D**, transductants. **E**, Comparison of mutant, complementation clone, and transductant for the *asnO* mutant. Results are given as the percentage of nodules induced by the respective clone setting the quantity of nodules induced by wild-type Rm2011 in the same experiment to 100%; * = complementation clones and ** = transductants.

102L4 *nodJ* (Barran et al. 2002) mutants showed a delayed nodulation phenotype on *M. sativa*. Interestingly, a *nodJ* mutation in *S. meliloti* RCR2011 had no significant effect on nodulation or infection thread formation (Roche et al. 1996). This indicates that the *nodJ* gene, whose product is involved in Nod factor transport, has a quite subtle influence on symbiosis.

The *nif* and *fix* genes.

The *nif* and *fix* genes are involved in nitrogen fixation. We expected mutants in these genes to be attenuated because many of them have a Fix^- phenotype. It previously was shown that such a Fix^- phenotype leads to premature degradation of bacteroids (Hirsch and Smith 1987). This would cause a reduced detection of such mutants in the output pool, because DNA from whole nodules was analyzed in the STM screen.

Four *fix* mutants were identified as attenuated: all three *fixL* mutants included in the sets (one *fixL* mutant in set 1 and two *fixL* mutants in set 2) and a *fixB* mutant. *S. meliloti* Rm2011 mutants in *fixL* (Batut et al. 1985; Vasse et al. 1990) and an *S. meliloti* Rm1021 mutant in *fixB* (Hirsch and Smith 1987) were shown to have a Fix^- phenotype. Moreover, all three *nif* mutants in the sets with transposon insertions in *nifB*, *nifK*, and *nifN* were attenuated in the STM experiment. *nifB* (Buikema et al. 1987; Hirsch and Smith 1987) and *nifK* (Ruvkun et al. 1982; Zimmerman et al. 1983) genes were proven to be required for nitrogen fixation in *S. meliloti* Rm1021, and the same was shown for *nifN* in Rm2011 (Aguilar et al. 1985, 1987).

Other genes.

Mutations in these genes were shown to cause diverse symbiotic defects in *S. meliloti*, ranging from Nod^- or Fix^- pheno-

types to reduced nitrogen fixation and reduced competitiveness phenotypes. Two *ilvC* mutants were present in the screened sets, and both were detected as attenuated. A mutation in the *ilvC* gene in *S. meliloti* Rm1021 was reported to result in a Nod^- phenotype (Aguilar and Grasso 1991; López et al. 2001). A *phaD1* mutant of *S. meliloti* AK631 (Putnoky et al. 1998) and an *ndvA* mutant of *S. meliloti* 102F34 (Dickstein et al. 1988; Stanfield et al. 1988) induced empty nodules devoid of bacteroids. *rpoN* (Ronson et al. 1987) and *dme* (Driscoll and Finan 1993) mutants of Rm1021 and a *cycH* mutant of *S. meliloti* Rm41 (Kereszt et al. 1995) showed Fix^- phenotypes. The *leuB* mutant was expected to be attenuated because it was shown that an *S. meliloti* Rm2011 *leuB* mutant was auxotrophic for leucine (Barsch et al. 2004). *S. meliloti* leucine auxotrophs induced ineffective nodules, and this phenotype could be compensated by adding leucine (Truchet et al. 1980). A *pckA* mutant of Rm1021 (Finan et al. 1991; Osteras et al. 1995) and a *dctB* mutant of Rm2011 (Engelke et al. 1987) were reported to elicit nodules with reduced nitrogen-fixing activity. An *syrM* mutation was shown to cause a delayed nodulation phenotype in Rm41 (Kondorosi et al. 1991). Mutation in *bioN* eliminated growth of the respective mutant of Rm1021 on *M. sativa* roots (Entcheva et al. 2002). Reduced growth on roots was shown to cause low competitiveness of such mutant strains (Guillen-Navarro et al. 2005). An *lpsB* mutant of Rm1021 had an altered lipopolysaccharide (LPS) structure and was compromised in infection thread formation and nitrogen fixation (Campbell et al. 2002, 2003). In Rm1021, mutation of SMC00644 resulted in a slightly altered LPS, which caused increased sensitivity to phages and deoxycholate, reduced nitrogenase activity, and impaired symbiotic competitiveness (Campbell et al. 2003).

Fig. 3. Confocal microscopy of the infection process by the pHC60-marked **A**, Rm2011 wild type; **B** and **C**, *ilvI* mutant; and **D**, **E**, and **F**, *ilvD2* mutant.

Reduced competitiveness phenotypes were identified for 23 novel mutants.

From 38 individually tested mutants, 23 were strongly attenuated in the individual competition assays with the wild type. These mutants, which had a defect in competitiveness but no other obvious nodulation or nitrogen fixation defect, carried a transposon insertion the following genes.

Gene *feuQ* (SMc00129) codes for a sensor histidine kinase, which is predicted to be a part of the two-component regulatory system FeuPQ. In *Rhizobium leguminosarum*, a mutation in *feuQ* led to the loss of the high-affinity iron uptake system, although siderophores were still produced (Yeoman et al. 1997). Interestingly, the *feuPQ* operon maps closely upstream of the cytochrome maturation operon *cycHJKL* in both *R. leguminosarum* (Yeoman et al. 1997) and *S. meliloti*. However, a mutation in *feuQ* did not affect cytochrome *c* function or expression of the *cycHJKL* operon in *R. leguminosarum* (Yeoman et al. 1997). Still, the expression of the *feuPQ* and *cycHJKL* clusters could be connected in some way, because the *R. leguminosarum* *cycK* mutant, similarly to the *feuQ* mutant, was deficient in high-affinity iron acquisition (Nadler et al. 1990; Yeoman et al. 1997). The defect of the *feuQ* transposon mutant in symbiotic competitiveness observed in this study probably originates from a disruption of the regulatory pathway controlled by the FeuPQ sensor system. Because the *S. meliloti* FeuQ has similarities to iron and copper ion sensor proteins, it might regulate metal ion transport similarly to FeuQ in *R. leguminosarum*.

Genes *pstA* (SMc02143) and *pstC* (SMc02144) encode components of the high-affinity phosphate transporter system PstSCAB (Yuan et al. 2006), which is one of three phosphate transport systems in *S. meliloti*. Two other systems are an ABC-type high-affinity P_i transport system, encoded by *phoCDET*, and a low-affinity P_i transport system, encoded by the *orfA-pit* genes (Voegelé et al. 1997). A *phoCDET* mutation in *S. meliloti* Rm1021 was shown to cause a Fix^- phenotype (Bardin et al. 1996). However, it was shown recently that strain Rm1021, in contrast to strain Rm2011 used here, carries a frameshift mutation in *pstC* (Krol and Becker 2004; Yuan et al. 2006). Correction of this mutation in the Rm1021 *phoCDET* mutant resulted in a Fix^+ phenotype on *M. sativa* (Yuan et al. 2006). Therefore, activity of only one of these high-affinity P_i transport systems is sufficient for an effective symbiosis.

pstA and *pstC* belong to the same gene cluster, where *pstC* is situated downstream of *pstS* and upstream of *pstA*, *pstB*, *phoU*, and *phoB*. It was shown that *pstSCAB* and *phoUB* are transcribed from a single promoter (Krol and Becker 2004; Yuan et al. 2006). Because the transposon mutations in *pstA* and *pstC* were polar, transcription of all downstream genes in the same operon was disrupted. This is particularly important in the case of *phoB* encoding a transcriptional regulator that is activated by the sensor kinase PhoR in conditions of low external phosphate concentration. PhoB regulates many cell processes, including phosphate transport (Bardin and Finan 1998; Bardin et al. 1996), exopolysaccharide biosynthesis (Rüberg et al. 1999), phosphonate degradation (Parker et al. 1999), acetyl phosphate metabolism (Summers et al. 1999), oxidative stress response, and iron homeostasis (Krol and Becker 2004; Yuan et al. 2006). The attenuated symbiotic competitiveness phenotype of the *pstA* and *pstC* transposon mutants may originate from a combinational effect of PstSCAB⁻ and PhoB⁻ phenotypes. However, it is unlikely that the polar *pst* mutants suffer from phosphate starvation, because phosphate transport still can be carried out by the OrfA-Pit phosphate transport system, which is active in *phoB* null mutants (Bardin and Finan 1998).

Gene *livM* (SMc01950) encodes the permease protein of a high-affinity amino acid ABC transporter that belongs to the

hydrophobic amino acid transporter (HAAT) family. *S. meliloti* *livM* shares 78% identity at the amino acid level to *braE* from *R. leguminosarum* bv. *trifolii*. In *R. leguminosarum*, amino acid uptake is performed by two broad-range amino acid ABC transporters, Aap and Bra (Hosie et al. 2002). The double *aap-bra* mutant was shown to retain nitrogenase activity at rates per bacteroid that were equal to or exceeded the wild-type rates; however, the plants nodulated by this mutant were severely nitrogen starved. It was proposed that Aap/Bra are involved in the efflux as well as influx of amino acids that drive amino acid cycling, which is crucial for the mutualistic symbiosis in *R. leguminosarum* (Lodwig et al. 2003; Prell and Poole 2006). A closer inspection of the *livM* function in *S. meliloti* is needed to investigate whether it may participate in amino acid transport relevant to symbiosis as in *R. leguminosarum* and to explain the attenuated phenotype of the *livM* mutant observed here.

Gene *lexA* (SMc01183) codes for a transcriptional repressor. LexA negatively regulates SOS genes under normal growth conditions by binding to a 20-bp consensus sequence (the SOS box). The RecA protein, stimulated by single-stranded DNA, interacts with LexA, causing an autocatalytic cleavage which disrupts the DNA-binding part of LexA, leading to derepression of the SOS regulon and, eventually, to DNA repair (Brent and Ptashne 1980). *S. meliloti* *recA* mutants exhibited an increased sensitivity to UV irradiation, showed a somewhat reduced growth rate compared with the wild-type strains (Selbitschka et al. 1991), and were slightly impaired in nodulation competition in environmental release experiments (Niemann et al. 1997; Selbitschka et al. 2006); however, the phenotype of rhizobial *lexA* mutants has not been studied yet. Constant derepression of the SOS regulon might reduce general fitness of the *lexA* mutant which, in turn, can cause a symbiotically attenuated phenotype.

Gene *lepA* (SMc00349) encodes a highly conserved membrane protein that belongs to the GTP-binding elongation factor family. Recently, it was demonstrated that LepA has the ability to back-translocate posttranslational ribosomes after a defective translocation event, thus giving EF-G a second chance to translocate the tRNAs correctly (Qin et al. 2006). Furthermore, an interaction between HolA (delta subunit of DNA polymerase III holoenzyme) and LepA was detected in *Bacillus subtilis*, suggesting that LepA might act to coordinate DNA replication and protein synthesis (Noirot-Gros et al. 2002). It is not clear, however, which role LepA can play in symbiosis or competitiveness.

Gene *clpA* (SMc02109) encodes the regulatory subunit of an ATP-dependent Clp protease. ClpA is a member of the Hsp100/Clp family of molecular chaperones. It associates with the ClpP serine-type protease protein, forming the active holoenzyme. Usually, Clp proteases are present at low levels during normal growth but are inducible during many types of stress. Their functions include degradation of damaged proteins that occur in stress conditions and control of diverse cell activities (Porankiewicz et al. 1999). *clpP* mutants of several pathogenic bacteria were more susceptible to several in vitro stresses and showed attenuated virulence in the STM screens in the host (Butler et al. 2006). Furthermore, in *Bradyrhizobium japonicum* USDA 110, a mutation in the *clpA* gene caused changes in exopolysaccharide (EPS) synthesis. The mutant strain produced approximately twofold higher levels of low molecular weight EPS compared with the wild-type strain USDA 110 (Louch and Miller 2001). It is not known if this connection between a *clpA* mutation and EPS synthesis exists in *S. meliloti* as well. Because EPSs are among the most important symbiotic determinants of *S. meliloti*, changes in their amount and molecular weight distribution can significantly in-

fluence symbiotic effectiveness (Frayse et al. 2003). On the other hand, the *clpA* mutant might be more vulnerable in stress conditions, which would lead to impaired survival during the infection process.

Genes *trpC* (SMc00236) and *trpF* (SMc02767) both are involved in the synthesis of tryptophan and encode an indole-3-glycerol phosphate synthase and an N-5'-phosphoribosylanthranilate isomerase, respectively. *S. meliloti trpC* and *trpF* mutants are tryptophan auxotrophs and accumulate anthranilate as an intermediate (Barsomian et al. 1992). *S. meliloti* mutants in *trpE(G)*, blocked at the first step of tryptophan synthesis, were shown to have a Fix⁻ phenotype, whereas *trpC* and *trpF* mutants induced pink nodules (Barsomian et al. 1992). Prasad and associates (2000) also showed that a leaky mutant in *S. meliloti* strain Rmd201, which has a mutation in *trpC*, *trpD*, or *trpF*, was partially effective in nitrogen fixation. However, no competitiveness phenotype was reported for these mutants until now. Interestingly, an *R. etli trpB* mutant, shown to be a tryptophan auxotroph, was unable to produce Nod factors in free-living conditions in the presence of the inducer naringenin unless tryptophan was added to the growth medium. The mutant clone formed fewer nodules than the wild type, and the nodules were white or pale pink (Taté et al. 1999).

A possible explanation for the attenuated symbiotic phenotype of *trpC* and *trpF* *S. meliloti* mutants is the fact that rhizobia use tryptophan to synthesize indole-3-acetic acid (IAA, auxin) (Kittell et al. 1989), a phytohormone that plays an important role in nodule formation. Plants as well as rhizobia can synthesize IAA (Woodward and Bartel 2005). However, it was shown that IAA-overproducing *S. meliloti* strains increased nodulation in *Medicago* spp. (Pii et al. 2007). The defect in tryptophan synthesis may cause a decrease in IAA synthesis and, therefore, be a symbiotic detriment.

Gene *rkpU* (SMc02274) codes for a protein related to export of capsular polysaccharides. Capsular polysaccharide contributes to the protection against abiotic factors, particularly against legume defense products during the infection process (Campbell et al. 1998; Roberts 1996). Furthermore, it was shown that capsular polysaccharides of *S. meliloti* are able to induce transcript accumulation of alfalfa genes encoding enzymes of the isoflavonoid biosynthetic pathways (Becquart-de-Kozak et al. 1997). The role of *rkpU* in symbiosis or its exact function in synthesis or export of the capsular polysaccharide has not yet been elucidated.

Gene *iolA* (*mmsA*, SMc00781) encodes a methylmalonate-semialdehyde dehydrogenase involved in myo-inositol catabolism. Inositol derivatives commonly are found in legume nodules (Scot and Egsgaard 1984; Streeter 1987). It was shown that an *iolA* mutant of *R. leguminosarum* occupied less than 5% of the nodules when co-inoculated with the wild type (Fry et al. 2001). The authors suggested that the nodulation advantage of the wild type was not the result of superior growth in the rhizosphere but was due to faster growth during the early stages of nodule development and infection thread formation. In *S. meliloti*, the ability to catabolize myo-inositol is important for the strains that are able to catabolize rhizopine (L-3-O-methyl-scylo-inosamine) (Galbraith et al. 1998), which is not the case for strain Rm2011 used here. Interestingly, interruption of the inositol site in *S. meliloti* PC was reported to increase alfalfa yield in field tests (Scupham et al. 1996); however, it is unclear what causes this beneficial effect. The attenuated symbiotic phenotype of the *iolA* mutant observed here indicates that the myo-inositol metabolism is important for competitiveness in non-rhizopine-catabolizing strains, similarly to the observations in *R. leguminosarum*.

Genes SMc02226 and SMA0244 encode conserved hypothetical proteins. SMA0244 encodes a protein containing an

FAD-binding domain, probably an FAD-dependent dehydrogenase. SMc02226 does not show significant homologies to known genes; however, its product was detected as a bacteroid-specific protein by Djordjevic and associates (2003). It is not clear yet what causes the attenuated symbiotic phenotype of these two mutants.

Symbiotic defects were identified for nine novel mutants.

Although the main goal of the STM experiment in planta was to identify genes important for symbiotic competitiveness, it also was of interest to test whether the attenuated mutants exhibit other symbiotic phenotypes. As a result, two Inf⁻ mutants, one Fix⁻ mutant, and six mutants with a delayed nodulation phenotype were identified.

ilvI (SMc01431) and *ilvD2* (SMc04045) mutants had an Inf⁻ phenotype. These mutants induced small, ineffective nodules on a minor fraction of plants but induced no infection threads. *ilvI* codes for the large subunit of an acetolactate synthase, which catalyzes the first step of isoleucine/valine biosynthesis, and *ilvD2* codes for a dihydroxy-acid dehydratase, which catalyzes the third step in the same pathway. Symbiotic phenotypes of *ilvI* and *ilvD2* mutants are strikingly similar to each other and to the previously described phenotype of *ilvC* mutants, obtained in different *S. meliloti* strains (Aguilar and Grasso 1991; López et al. 2001). *ilvC* encodes an acetohydroxyacid isomeroreductase, which catalyzes the second step in the isoleucine/valine synthesis pathway. It was shown that, in the *ilvC* mutant of *S. meliloti* Rm1021, the nodulation genes *nodABC* were not activated by luteolin (Aguilar and Grasso 1991). Because *S. meliloti* isoleucine/valine auxotrophic mutants displaying a wild-type nodulation phenotype could be isolated, it was suggested that the symbiotic defect was not caused by the isoleucine/valine auxotrophy (Aguilar and Grasso 1991). The fact that the *ilv* mutants have similar phenotypes indicates, however, that the reason for the symbiotic defect may be the disruption of a metabolic pathway in which the products of all three genes are involved.

The *ccmC* (SMc03849) mutant showed a Fix⁻ phenotype. *ccmC* (*cycZ*) codes for a cytochrome c-type biogenesis protein. This gene belongs to the *ccmABCDG* cluster, where *ccmA*, *ccmB*, *ccmC*, and *ccmD* encode components of the heme transporter and *ccmG* encodes a periplasmic thiol:disulfide oxidoreductase. Nitrogen fixation in *S. meliloti* depends on a respiratory chain, which consists of the cytochrome bc₁ complex and a cytochrome cbb3 terminal oxidase. Because the c-type cytochromes are essential components of this respiratory chain, the disruption of the c-type cytochrome maturation causes severe symbiotic defects (Delgado et al. 1998). Recently, a Fix⁻ phenotype of a *ccmC* transposon mutant was described by Yurgel and associates (2007). Capela and associates (2006) showed that a polar *ccmA* mutant had a Fix⁻ phenotype as well. The fact that complementation with the *ccmC* gene alone was successful indicates that *ccmD* and *ccmG*, located downstream of *ccmC*, are not essential for nitrogen fixation, or that their transcription is not completely disrupted by the terminator in the polar *ccmC* mutant.

pyrE (SMc02165) and *cysG* (SMc01053) mutants had a delayed nodulation phenotype and induced white nodules on some plants and pink nodules, or a mixture of pink and white nodules, on other plants. *pyrE* codes for an orotate phosphoribosyltransferase, involved in pyrimidine biosynthesis. *pyrE/pyrF* mutants of *S. meliloti* strain Rmd201 showed a Fix⁻ phenotype (Vineetha et al. 2001), but we did not observe this phenotype in Rm2011. We were unable to complement the symbiotic deficit of the *pyrE* mutant by the *pyrE* gene alone; however, transduction-mediated transfer of this mutation to the wild type resulted in the mutant phenotype. Therefore, it might

be possible that the delayed nodulation phenotype was caused by a polar effect on the downstream gene *frk*, which encodes a probable fructokinase, or by a combined *pyrE⁻ frk⁻* genotype. In *S. meliloti* L5-30, a fructokinase mutant was unable to fix nitrogen (Duncan 1981). However, it is not clear whether the polar Rm2011 *pyrE* mutant completely lacks fructokinase activity because the *frk* gene has paralogues in the sequenced genome of the closely related *S. meliloti* strain Rm1021 (e.g., SMb21374).

cysG encodes a siroheme synthase (uroporphyrinogen III methylase) which is required for siroheme synthesis. Siroheme is the prosthetic group of many nitrite and sulfite reductases that function in the conversion of the highly oxidized forms of nitrogen and sulfur to the fully reduced forms (NH_4^+ and S^{2-}). Unlike other *cys* genes, *cysG* is transcribed together with a nitrite reductase gene and not with genes related to cysteine synthesis. In *R. etli*, a *cysG* mutant was cysteine auxotrophic (Taté et al. 1997). Furthermore, it did not grow with nitrate as the sole nitrogen source and showed a poor competitiveness phenotype which could be almost completely restored by the addition of an organic sulfur source. The authors concluded that the impaired competitiveness probably originates from the cysteine auxotrophy of the *cysG* mutant. This might be the case also in *S. meliloti*, though it cannot be excluded that the *cysG* mutation has a more profound influence on symbiosis due to the involvement of the CysG protein in several important biochemical pathways.

metA (SMc03797), *thiC* (SMb20615), *thiO* (SMb20616), and *thiD* (SMb20962) mutants displayed delayed nodulation. *metA* codes for a homoserine *O*-succinyltransferase which catalyzes the first step in methionine biosynthesis in gram-negative bacteria. Mutations that cause methionine auxotrophy resulted in a Fix^- phenotype in *S. meliloti* 104A14 (Kerppola and Kahn 1988) and *R. fredii* HH303 (Kim et al. 1988). Unfortunately, in these studies, the genes which were disrupted in the auxotrophic mutants were not deduced. On the other hand, inactivation of *metH* encoding methionine synthase in *S. meliloti* 102F34 did not cause methionine auxotrophy and resulted in an attenuated competitiveness phenotype of mutants, but not in a Fix^- phenotype (Barra et al. 2006). Because the *metA* mutant studied here did not have a Fix^- phenotype and could grow on minimal medium (data not shown), it is not strictly auxotrophic for methionine. The *galM* gene encoding an aldose 1-epimerase protein involved in galactose metabolism maps downstream of *metA*. It has not been elucidated yet whether *galM* plays any role in symbiosis. Therefore, the attenuated phenotype of the *metA* mutant may be caused by the mutation in the *metA* gene, by the polar effect on the *galM* gene, or by the combination of both.

thiD encodes a probable phosphomethylpyrimidine kinase, *thiC* encodes a thiamine biosynthesis protein of the ThiC family, and *thiO* encodes a putative oxidoreductase protein. These genes all are involved in thiamine synthesis and map to pSymB, where *thiC* and *thiO* belong to the same cluster, with *thiC* being situated upstream of *thiO*. *S. meliloti* is able to synthesize thiamine, but colonization of roots is limited by the availability of this vitamin (Streit et al. 1996). On the other hand, it was shown that, in an *R. etli* strain constitutively expressing the *thiCOGE* cluster, the *fixNOQP* operon was constitutively expressed (Miranda-Rios et al. 1997). This strain also showed an increased capacity to fix nitrogen during symbiosis (Soberon et al. 1990). It was suggested that a metabolite related to the purine-thiamine biosynthetic pathway, probably 5-amino-4-imidazolecarboxamide (AICAR), is a negative effector for expression of *fixNOQP*. The constitutive expression of *thiC* could lower the concentration of several metabolites of the purine biosynthetic pathway, particularly AICAR, and, as a

result, block the negative regulation of *fixNOQP* (Miranda-Rios et al. 1997). Therefore, mutations in thiamine synthesis genes could have an effect on symbiotic competitiveness through a deficit in thiamine availability or through downregulation of nitrogen-fixation genes.

Clone 2011mTn5STM.4.04.D04 displayed a severe defect in nodulation efficiency. When the plants were inoculated by this mutant only, it induced nodules at less than 5% of the wild-type nodulation rate. Interestingly, when the plants were inoculated with a balanced mixture of wild-type Rm2011 and 2011mTn5STM.4.04.D04, the average percentage of the mutant in nodules reached 18%, indicating that the presence of the wild-type Rm2011 might facilitate infection of plants by this mutant. The transposon insertion in the clone 2011mTn5STM.4.04.D04 mapped to the *asnO* (SMb20481) gene, which codes for a protein homologous to glutamine-dependent asparagine synthetases. However, the symbiotic defect could not be complemented by the *asnO* gene, and transfer of the transposon mutation by transduction to the wild type did not cause the described nodulation defect in the transductants (Fig. 2E). The phenotype of this mutant also did not match the phenotype of an *asnO* mutant of *S. meliloti* GMI211 reported by Berges and associates (2001). Therefore, we conclude that the phenotype of this mutant originated from a different (secondary) mutation and not from the transposon insertion in the *asnO* gene.

Concluding remarks.

The present study shows that STM is a powerful and straightforward tool to study bacteria-plant symbiosis. The fact that many mutants that possessed a known symbiotic defect could be detected in the STM experiment supports the reliability of our detection system. Importantly, the mutants that had a defect at late stages of symbiosis, such as *fix* or *nif* mutants, or mutants with only a slight symbiotic deficiency, such as *lpsB* or *dctB* mutants, also could be detected as poorly competitive by this method. The sensitivity of the STM approach in this study of symbiotic competitiveness originates from the fact that the representation of each tagged mutant in the output pool DNA isolated from the nodules results from the competition that starts already at the stage of colonization of the plant roots and continues even in the mature nodules. Furthermore, it should be taken into account that only a small part of initiated infections result in the formation of nodules (Vance 1983). This, together with the high number of analyzed nodules and the fact that a part of the nodules is occupied by more than one mutant clone, results in a high statistical significance of the results of the STM screen. Furthermore, the representation of a particular mutant in the output pool depends not only on the number of nodules occupied by this mutant but also on the size of the nodules and the density of the bacterial population in each nodule. The latter is dependent on how many nodule cells are infected, how well they are infected, and how stable the infection is. All these factors are influenced by the fitness of the clones that occupy the nodules and are reflected in the composition of the output pool.

Apart from mutations in genes with known or probable functions deduced from sequence similarities, mutations in genes encoding hypothetical proteins showed reduced competitiveness in the STM screen. Furthermore, the attenuated phenotypes detected in the aeroponic cultivation system with a mixture of many mutants could, for the most part, be confirmed in a different assay, growing plants on solid plant medium and in competition with the wild type only. This indicates robustness of the phenotypes observed in the STM screen. On the other hand, it is difficult to speculate about the function of the genes disrupted in the attenuated mutants at the moment. We do not know if the attenuated phenotypes of these mutants originate

from disruptions in specific plant–microbe interaction pathways or simply reflect general reductions in growth rate and ability to adapt to stress conditions which could be important as well under many other conditions unrelated to symbiosis. Therefore, further investigation of the identified genes is necessary to elucidate their role in symbiosis and competitiveness.

In this study, only a small part of the library of signature-tagged mutants was screened. The screening of the rest of the library certainly will reveal many new mutants with reduced competitiveness. The genes identified in this study as important for symbiotic competitiveness open up venues for further research.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids are listed in the Table 2. Mutants containing different tags were mixed in roughly equal quantities and the mixtures were stored as a glycerol stock at -20°C . *E. coli* strains were grown at 37°C in Luria-Bertani medium (Sambrook et al. 1989). *S. meliloti* cells were grown at 30°C in tryptone-yeast (TY) complex medium (Beringer 1974) and in $2\times$ TY medium (10 g of tryptone, 6 g of yeast extract, and 0.4 g of CaCl_2). When required, the media were supplemented with tetracycline (5 $\mu\text{g/ml}$) for *E. coli* and with neomycin (120 $\mu\text{g/ml}$), tetracycline (8 $\mu\text{g/ml}$), nalidixic acid (10 $\mu\text{g/ml}$), or a combination of the three for *S. meliloti*.

Screening of signature-tagged mutants in planta.

Plants were grown as described by Gallusci and associates (1991). Seed of *M. sativa* cv. Europe were sterilized and cultivated for 8 days on solid plant medium plates and then transferred to the aeroponic tanks. After 10 days of aeroponic cultivation in the presence of nitrogen, the medium was replaced with fresh medium lacking ammonium nitrate. After two more days, plants were inoculated with 17 ml of *S. meliloti* mutant mixed culture (freshly grown in TY to an optical density at 600 nm $[\text{OD}_{600}] = 0.3$, washed twice in plant medium) to promote nodulation. A part of each mixed-mutant culture was stored separately and used as the reference (input pool). Nodules

were harvested 24 days after inoculation and frozen in liquid nitrogen. For each set, a separate aeroponic tank with approximately 60 *M. sativa* plants was run. In all, 18 and 16 ml of nodules (approximately 1,500 to 2,000 nodules) were collected for set 1 and set 2, respectively. Nodules were stored in nine aliquots at -80°C (five aliquots for set 1 and four aliquots for set 2). Prior to DNA isolation, nodules were washed with 70% ethanol and rinsed three to four times with sterile water. Total DNA from nodules (output pool) was isolated using the DNeasy plant mini kit from Qiagen (Hilden, Germany). DNA isolations were performed for three aliquots of nodules per mutant set (1.5 to 2.5 g wet weight per aliquot), where a separate DNA isolation was made for each aliquot. DNA from the input pool cultures was isolated using the NucleoSpin Tissue kit from Macherey-Nagel (Düren, Germany). For determination of the quantity of mutants in single nodules, individual nodules from the aeroponic tanks were rinsed as above, crushed in 700 μl of sterile water, and boiled for 3 min. Then, 5 μl of the suspension was used as a template for the PCR reaction.

Microarray hybridization and statistical analysis.

The tags were amplified applying Cy3- or Cy5-labeled primers (in the case of the output or input pools, respectively) (21 cycles), purified, and hybridized to the tag microarray, carrying at least four replicates for each tag. Production of microarrays, hybridization, scanning, microarray image processing, and data preprocessing were performed as described previously (Pobigaylo et al. 2006). The $\log_2$ of the ratio of intensities (m_i value) was calculated for each spot using the formula $m_i = \log_2(R_i/G_i)$. $R_i = I_{\text{ch1}_i} - B_{\text{gch1}_i}$ and $G_i = I_{\text{ch2}_i} - B_{\text{gch2}_i}$ where I_{ch1_i} and I_{ch2_i} are intensities of spots in channel 1 and channel 2, respectively, B_{gch1_i} and B_{gch2_i} are background values of spots in channel 1 and channel 2, respectively. The normalization method used in this experiment was a modification of the global median normalization. A bias toward low m values was expected; therefore, 15% of the lowest m values were excluded from the pool of data used to calculate the median. At the next step of statistical analysis, the mean_w values were calculated for each mutant, grouping the data from three hybridizations that represented three aliquots of

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Sinorhizobium meliloti</i>		
Rm2011	Wild type, streptomycin-resistant	J. Dénarié, France
<i>Escherichia coli</i>		
DH5 α	<i>F</i> supE44 Δ lacU169 (ϕ 80lacZAM15) <i>hsdR17 recA1 endA17 gyrA96 thi-1 relA1</i>	Sambrook et al. (1989)
S17-1	MM294, RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al. (1983)
Plasmids		
pHC60	Stable <i>IncP</i> plasmid that constitutively expresses green fluorescent protein	Cheng and Walker (1998)
pPHU231	pRK290 derivative, broad-host-range vector	Hubner et al. (1991)
pCTF3	pPHU231 containing a 0.8 kb <i>KpnI-XbaI</i> fragment with the <i>ccmC</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTF6	pPHU231 containing a 0.8 kb <i>KpnI-XbaI</i> fragment with the <i>thiD</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTF8	pPHU231 containing a 1.85 kb <i>KpnI-XbaI</i> fragment with the <i>ilvD2</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTF10	pPHU231 containing a 1.8 kb <i>EcoRI-XbaI</i> fragment with the <i>ilvD2</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTN1	pPHU231 containing a 1.8 kb <i>SacI-XbaI</i> fragment with the <i>asnO</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTN2	pPHU231 containing a 1 kb <i>KpnI-XbaI</i> fragment with the <i>metA</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTN4	pPHU231 containing a 1.5 kb <i>KpnI-XbaI</i> fragment with the <i>cysG</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTN9	pPHU231 containing a 1.85 kb <i>BamHI-SphI</i> fragment with the <i>thiC</i> gene under control of <i>E. coli lac</i> promoter	This work
pCTN11	pPHU231 containing a 0.7 kb <i>KpnI-XbaI</i> fragment with the <i>pyrE</i> gene under control of <i>E. coli lac</i> promoter	This work
Phage		
Φ M12	<i>S. meliloti</i> transducing phage	Finan et al. (1984)

nodules. Mutants were regarded as attenuated or over-represented if P value (t) < 0.01 and mean_w < -0.7 or > 0.7, respectively. As a result, 83 mutants were found to be attenuated (37 in set 1 and 46 in set 2) and 50 mutants were found to be highly represented in the sets under symbiotic conditions (30 in set 1 and 20 in set 2). The mean_w values from three grouped hybridizations were compared with the m values from the fourth pooled hybridization and the mutants were filtered out whose absolute m values were lower than 0.7 in the fourth hybridization.

Individual competition assays and kinetics of nodule formation.

In the individual competition assays, 2-day-old *M. sativa* seedlings were placed on agar plates with plant minimal medium (Broughton and Dilworth 1971) and inoculated with a 1:1 mixture of wild-type Rm2011 and mutant (2×10^7 bacterial cells/plate). Prior to the inoculation, both wild-type and mutant strains were grown in the same medium containing nalidixic acid. Cell numbers were deduced from OD₆₀₀ measurements at the same growth phase. After 4 to 5 weeks, the nodules were harvested and surface sterilized with 70% ethanol for 1 min, rinsed three to four times with sterile water, and crushed in 1 ml 0.85% NaCl. To determine the relative proportions of mutant bacteria in the nodules, 100 μ l of crushed nodule suspension and 100 μ l of 1:100 dilution of the nodule suspension were replica plated onto medium containing nalidixic acid and onto medium containing nalidixic acid and neomycin. For each mutant, 25 to 30 nodules from 15 to 18 plants were analyzed, and the mean value of the percentage of the mutant in each nodule was calculated.

To study the kinetics of nodule formation, *M. sativa* plants, grown as described above were inoculated with 2×10^7 bacterial cells/plate of the respective mutant, the complementation clone, the transductant with transferred mutation, or the wild type. For each clone and the wild type, at least 90 plants were monitored during 24 days after inoculation.

Confocal microscopy.

Mutant clones and the wild type were transformed with plasmid pHc60 that constitutively expresses *gfp*. *M. sativa* plants, grown on agar plates as described above, were inoculated by fluorescent mutant clones and inspected 7 days after inoculation. Confocal microscopy was performed using the laser-scanning unit TCS SP2 mounted to a DM RE-7 SDK microscope (both from Leica Microsystems, Wetzlar, Germany) equipped with a $\times 20$ 0.7 HC PLAPO CS oil immersion objective. Green fluorescent protein was excited at 488 nm and emission was detected between 500 and 600 nm.

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