The Response to Nitric Oxide of the Nitrogen-Fixing Symbiont *Sinorhizobium meliloti*

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Nitric oxide (NO) is crucial in animal– and plant–pathogen interactions, during which it participates in host defense response and resistance. Indications for the presence of NO during the symbiotic interaction between the model legume *Medicago truncatula* and its symbiont *Sinorhizobium meliloti* have been reported but the role of NO in symbiosis is far from being elucidated. Our objective was to understand the role or roles played by NO in symbiosis. As a first step toward this goal, we analyzed the bacterial response to NO in culture, using a transcriptomic approach. We identified approximately 100 bacterial genes whose expression is upregulated in the presence of NO. Surprisingly, most of these genes are regulated by the two-component system FixLJ, known to control the majority of rhizobial genes expressed in planta in mature nodules, or the NO-dedicated regulator NnrR. Among the genes responding to NO is *hmp*, encoding a putative flavohemoglobin. We report that an *hmp* mutant displays a higher sensitivity toward NO in culture and leads to a reduced nitrogen fixation efficiency in planta. Because flavohemoglobins are known to detoxify NO in numerous bacterial species, this result is the first indication of the importance of the bacterial NO response in symbiosis.

Nitric oxide (NO) is a water-soluble, free-radical reactive gas known to play a number of different roles in biological systems. NO plays a key role in intracellular signaling but is also potentially toxic due to its reaction with a variety of cellular targets, especially thiols and transition metal centers in proteins. It is also part of the antibacterial arsenal of mammalian cells. Thus, the inducible NO-synthase of macrophages generates toxic amounts of NO from arginine, in an attempt to kill off invading microorganisms. To counteract this hazard, bacteria have evolved a number of defense systems that make them capable of protecting themselves against NO stress. Bacterial responses to NO have been studied in model species *Escherichia coli* (Justino et al. 2005) and *Bacillus subtilis* (Moore et al. 2004), human pathogens *Mycobacterium tuberculosis* (Ohno et al. 2003), *Salmonella typhimurium* (Bang et al. 2006), and others (Elvers et al. 2004; Richardson et al. 2006), and to a lesser extent, in phytopathogenic bacteria (Boccaro et al. 2005; Mur et al. 2006). It has become apparent that multiple regulatory systems mediate the response to NO exposure,

some of them NO unspecific (SoxR, OxyR, Fur, and Fnr), while others could be dedicated NO sensors (NorR and probably NsrR) (Spiro 2007, 2008).

Different mechanisms are used by bacteria to counteract NO stress. One of the most frequent is the degradation of NO by a flavohemoglobin with nitrate as end product (Gardner et al. 1998; Gardner 2005; Angelo et al. 2008). Flavohemoglobins have been shown to protect against NO both anaerobically and aerobically (Justino et al. 2005), although NO consumption is more efficient in the presence of oxygen. Flavohemoglobins are important for the virulence of many bacteria such as *E. coli* (Stevanin et al. 2007), *Staphylococcus aureus* (Richardson et al. 2006), and *S. typhimurium* (Bang et al. 2006). Flavohemoglobin-like proteins have also been identified in numerous plant pathogens and, although little is known about their roles in these bacteria, it has been shown, in *Erwinia chrysanthemi*, that *hmpX* is a NO-related pathogenicity determinant (Favey et al. 1995). HmpX consumes NO and prevents inhibition by NO of cell respiration, indicating a role in protection from nitrosative stress. It was shown more recently that *hmpX* affects the plant hypersensitive response by intercepting NO produced by the host (Boccaro et al. 2005). Other systems of NO detoxification can play a role in NO response and be determinants of virulence. *Neisseria meningitidis* *norB* (Stevanin et al. 2005) and *Brucella suis* *norD* (Loisel-Meyer et al. 2006) are both involved in NO degradation. *N. meningitidis* *cycP* encodes cytochrome *c'*, a single heme-containing molecule, which binds and detoxifies NO in *Rhodobacter capsulatus* (Cross et al. 2000).

NO may also play a role in symbiotic interactions. During colonization of the squid light organ by *Vibrio fischeri*, NO is synthesized by the host tissues and may help to exclude non-symbiotic bacterial species (Davidson et al. 2004). The interaction between legume plants and some soil bacteria, collectively called rhizobia, leads to the establishment of symbiotic relationships, characterized by the formation of new differentiated root organs called nodules which provide a niche for bacterial nitrogen fixation. Several indications of the occurrence of NO during legume–rhizobia interactions have been reported. For example, NO bound to Lb as nitrosylleghaemoglobin (LbNO) was detected in nodules of soybean infected by *Bradyrhizobium japonicum* (Mathieu et al. 1998; Meakin et al. 2007), and NO was transiently observed in *Lotus japonicus* and *Medicago sativa* roots 4 h after infection by their cognate symbiont (*Mesorhizobium loti* and *Sinorhizobium meliloti*, respectively) (Nagata et al. 2008; Shimoda et al. 2005). Interestingly, class-I hemoglobin Hb1 is able to complex with NO and is present on the site of NO production, suggesting that modulation of NO levels and expression of Hb1 are involved in the establishment of the symbiosis (Nagata et al. 2008). Overexpression of Hb1

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and one supplementary table are published online.

enhances symbiotic nitrogen fixation activity, probably by removing NO as an inhibitor of nitrogenase (Shimoda et al. 2009). Moreover, Baudouin and colleagues clearly showed the presence of NO in functional nodules of *Medicago truncatula* infected by *S. meliloti* (Baudouin et al. 2006). NO was localized in infected cells of the nodule fixation zone. Pii and co-workers (2007) confirmed this observation and extended it to *M. sativa*. However, the role, if any, of this molecule in the symbiotic interaction remains unknown.

In contrast to all that has been carried out in other bacterial species, the response of rhizobia to the presence of NO has been poorly documented. In *B. japonicum*, only a few genes are known to be induced by the presence of NO. These genes are involved in the denitrification process, which is the complete reduction of nitrate or nitrite to N₂ with NO as intermediate (Bedmar et al. 2005). These genes were shown to be controlled by NnrR, whose expression is under the control of the FixLJ-FixK₂ signaling pathway (Mesa et al. 2003). The role in symbiosis of one of these genes, encoding the NO reductase, has been studied by analyzing the ability of a *norC* mutant to consume NO. The *norC* nodule bacteria were proficient in the ability to consume NO and the mutation had no effect on NO level, suggesting that other systems may be involved in NO detoxification (Meakin et al. 2006). The NO response of *S. meliloti* has been even less characterized than that of *B. japonicum*. A conserved operon has been shown to be induced by the presence of NO (de Bruijn et al. 2006). This operon encodes putative proteases, a possible sterol-binding protein, and a protein of unknown function. However, mutants in this operon displayed a wild-type phenotype in planta, indicating that these genes do not have an essential role in symbiosis.

How rhizobia cope with the presence of NO in nodules and what role is played by the bacterial NO response in the interaction with the host plant are questions far from being solved. As a first step to answer these questions, we report in this study on the identification of *S. meliloti* genes upregulated in the presence of NO and on regulators involved in the transcriptional activation of these genes. One of the genes (*hmp*) belonging to the NO stimulon encodes a flavohemoglobin, and *hmp* mutants displayed a deficiency in nitrogen fixation, suggesting that flavohemoglobin and NO have a role during the interaction between *S. meliloti* and alfalfa or the model plant *M. truncatula*.

RESULTS

Optimal conditions for transcriptomic studies in the presence of NO.

The global assessment of genes induced by NO was performed by means of a transcriptomic approach. Preliminary experiments were completed to determine the optimal NO concentration and length of exposure at which the subsequent experiments would be carried out. Spermine NONOate (SpNN) was used as a NO donor. Various concentrations of SpNN ranging from 0 to 500 μ M were tested for cell growth. The results are presented in Figure 1A. For the lowest concentration tested (10 μ M), no visible effect on growth of the wild-type strain (Rm1021) was observed whereas a modest slow down was detected at 25 μ M. At concentrations of 50 μ M and above, a transient growth arrest was observed whose length increased with the concentration of NO donor in the medium.

Two genes (*smal289* and *smal294*) were already known to be induced by NO (de Bruijn et al. 2006). In order to monitor their expression, we constructed genomic β -glucuronidase (*gus*) transcriptional fusions to each of these genes by insertion of a suicide plasmid (discussed below). The *gus* activity was measured at the highest NO donor concentration (25 μ M) which did not display a transient growth arrest. Both fusions

were induced, with an optimum reached when cells were exposed to NO for 1.5 h (Fig. 1B).

Hence, on the basis of these indications, a first set of preliminary microarrays was set up where the transcriptome of the wild-type strain in the presence of NO donor (25 μ M for 0.5 or 1.5 h) was compared with that of the same strain without NO donor. Genes were considered as induced when a threefold increase of gene expression was obtained in the presence of NO donor compared with the control condition. Using this criterion, similar numbers of genes were induced in the presence of 25 μ M SpNN for 30 min or for 1.5 h. Among the genes induced after 1.5 h of incubation, we found the sigma factor-encoding gene *rpoE2* as well as many genes whose expression is dependent

Fig. 1. Spermine NONOate (SpNN) (25 μ M) has a limited effect on cell growth and is sufficient to induce a transcriptional nitric oxide (NO) response. **A.** *Sinorhizobium meliloti* Rm1021 cells were cultured exponentially without or with various concentrations of SpNN as indicated. Cell growth was measured (optical density at 600 nm [OD₆₀₀]) every 2 h after addition of the compound. **B.** *S. meliloti* strains CBT731 or CBT732, carrying a genomic β -glucuronidase (*gus*) fusion to either *smal289* (squares) or *smal294* (circles), respectively, were cultured exponentially without (open symbols) or with (black symbols) SpNN (25 μ M). At different times after addition of the molecule, aliquots of the cultures were collected and tested for *gus* activity. The figure shows the results of a typical experiment (for each time point, $n = 1$) that was reproduced three times with similar results.

upon this factor (data not shown). RpoE2 was recently described as a sigma factor involved in the general stress response of *S. meliloti* (Sauviac et al. 2007) and its apparent activation could reflect a stress response rather than a specific NO response. In contrast, *rpoE2* was not induced after 30 min of incubation with SpNN. This was confirmed by real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (data not

shown). Therefore, incubation for 30 min in the presence of 25 μ M SpNN was chosen for further experiments.

NO upregulates approximately 100 genes in *S. meliloti* free-living cells.

We compared the transcript levels in the wild-type strain grown without or with 25 μ M SpNN for 30 min. We consid-

Table 1. Nitric oxide (NO) upregulated genes as identified with microarrays^a

Gene ID ^b	Gene description	Wild type (wt)		<i>fixJ</i>		Ratio
		FC	<i>P</i>	FC	<i>P</i>	wt/ <i>fixJ</i>
FixJ-dependent						
SMA0633	Hypothetical protein	4.1	0.01	1.2	0.58	3.3
SMA0657	Cytochrome c binding protein; probable amino term	3.2	0.01	1.6	0.15	2.0
SMA0669	Hypothetical transmembrane protein; HlyD-family	5.6	0.01	1.1	–	5.0
SMA0762	FixK2 transcription regulator	23.0	0.01	2.0	0.52	11.5
SMA0763	Hypothetical protein	10.0	0.04	2.4	0.14	4.3
SMA0767	FixQ2 cbb3-type cytochrome oxidase	6.9	0.04	1.2	0.60	5.8
SMA0815	NifA transcriptional activator	3.9	0.03	1.5	0.23	2.5
SMA1082	Hypothetical protein	11.0	0.01	2.8	0.01	4.1
SMA1084	Probable phosphoketolase	4.5	0.01	2.2	0.11	2.1
SMA1091	Hypothetical protein	4.1	<0.01	1.8	0.01	2.3
SMA1093	Hypothetical protein	3.7	0.02	1.6	0.06	2.3
SMA1095	Conserved hypothetical protein	3.3	0.02	1.3	0.41	2.5
SMA1101	Hypothetical protein	4.4	0.02	2.2	0.01	2.0
SMA1103	Adenylate/guanylate cyclase	5.1	0.01	2.2	0.01	2.3
SMA1120	ABC transporter; ATP binding protein. hypothetical	6.4	<0.01	2.7	<0.01	2.4
SMA1126	Putative protease; transmembrane protein	6.7	0.01	1.9	0.08	3.5
SMA1128	DegP4 protease like protein	4.5	0.01	1.5	0.19	3.0
SMA1131	Metallo-β-lactamase superfamily protein	3.6	<0.01	1.7	0.07	2.1
SMA1138	Truncated response regulator	3.4	0.01	1.5	0.05	2.3
SMA1146	Conserved hypothetical protein	5.7	0.01	2.7	<0.01	2.1
SMA1147	Conserved hypothetical protein	25.0	<0.01	4.6	<0.01	5.4
SMA1149	Conserved hypothetical protein	9.3	0.02	2.0	0.04	4.6
SMA1153	Hypothetical protein	5.9	0.01	1.9	0.26	3.1
SMA1154	Hypothetical protein	18.0	0.01	2.2	<0.01	8.1
SMA1155	Cation transport P-type ATPase. hypothetical	3.9	0.04	1.4	0.17	2.7
SMA1156	Alcohol dehydrogenase. Zn-dependent class III	8.2	0.03	2.5	0.06	3.3
SMA1158	Conserved hypothetical protein	8.9	0.04	2.5	0.01	3.6
SMA1170	Putative CycB2 cytochrome c552	10.0	<0.01	4.5	0.04	2.3
SMA1176	Hypothetical protein	3.8	<0.01	1.6	0.10	2.4
SMA1184	NosF copper ABC transporter	3.8	<0.01	1.2	0.39	3.1
SMA1185	NosY nitrous oxide metabolic protein	5.5	0.01	2.2	0.17	2.5
SMA1191	hmp-putative flavohemoglobin like protein	3.9	0.05	1.8	0.29	2.3
SMA1194	nnrS-Putative haem and copper membrane protein	4.4	0.01	2.1	0.10	2.1
SMA1201	Hypothetical protein	8.5	<0.01	3.8	0.01	2.2
SMA1209	FixI1 copper transport ATPase	4.9	<0.01	1.9	0.04	2.7
SMA1211	FixG Iron sulfur membrane protein	11.0	0.01	1.8	0.17	6.2
SMA1213	FixP1 Di-heme cytochrome c	5.6	0.02	1.2	0.38	4.6
SMA1214	FixQ1 cbb3-type cytochrome oxidase	7.0	0.04	1.5	0.03	4.5
SMA1220	FixN1 Heme b/copper cyt-c oxidase subunit	9.5	0.02	3.2	0.09	3.0
SMA1223	FixM flavoprotein oxidoreductase	12.0	0.03	1.2	0.90	10.2
SMA1225	FixK1 transcriptional activator	22.0	0.01	1.7	0.10	12.9
SMA1226	FixT1 inhibitor of FixL autophosphorylation	5.2	<0.01	1.1	0.61	4.6
SMA1231	Conserved hypothetical protein	3.9	0.04	1.4	0.59	2.8
SMA1276*	NorC nitric oxide reductase	14.0	<0.01	6.1	<0.01	2.3
SMA1283	NnrU-like transmembrane protein	16.0	<0.01	7.2	0.02	2.2
SMA1292	Peptidase	7.7	<0.01	3.8	<0.01	2.0
SMA1294*	Hypothetical protein	4.8	0.01	2.3	0.14	2.1
SMA1296	adhA1-Alcohol dehydrogenase; Zn-dep-class III	14.0	<0.01	6.4	0.01	2.2
SMb20139	Conserved hypothetical transmembrane protein	15.0	0.02	1.3	0.40	11.0
SMb20186	gfa-Glutathione-dep formaldehyde-activating enz	6.7	0.04	1.8	0.36	3.8
SMb20433	eutC-Probable ornithine cyclodeaminase	4.3	<0.01	2.1	0.01	2.1
SMc03253	L-proline cis-4-hydroxylase	34.0	0.01	1.9	0.01	18.2
SMc03254	fixT3_putative antikinase protein	5.3	0.03	1.1	0.59	4.6
FixJ-independent						
SMA0625	Hypothetical protein	5.2	0.01	3.8	0.01	1.4
SMA1086	Conserved hypothetical protein	3.2	0.01	1.7	0.19	1.9
SMA1087	Cation transport ATPase	3.2	<0.01	1.8	0.05	1.8
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^a FC: fold change values of a comparison of *Sinorhizobium meliloti* cells grown with (25 μ M) versus without NO donor; P = P value.

^b FixJ-dependent and -independent genes in the presence of NO, and Stress = stress-related genes. Gene ID in bold: genes known as FixJ-dependent in microaerobic conditions. Genes with an asterisk (*) were validated by quantitative reverse-transcription polymerase chain reaction as NnrR dependent.

ered as differentially expressed genes only those showing an M value ($\log_2$ ratio of intensities) $>$ or <1.58 (fold change of three) and P value ≤ 0.05 according to t statistics. Approximately 130 genes were downregulated in the presence of SpNN, many of them (approximately 50) encoding ribosomal proteins and translation factors. This indicates an effect of the

molecule, even moderate, on cell biosynthetic activity. In this study, only genes induced by NO were considered. Approximately 120 genes were upregulated by NO. In an attempt to eliminate those for which the response might not be specific to NO, we compared them with a list of genes known to respond to different stresses (carbon or nitrogen starvation, heat shock,

Table 1. (continued from preceding page)

Gene ID ^b	Gene description	Wild type (wt)		<i>fixJ</i>		Ratio
		FC	<i>P</i>	FC	<i>P</i>	wt/ <i>fixJ</i>
SMa1132	Hypothetical protein	3.9	0.02	4.2	0.06	0.9
SMa1134	Conserved hypothetical protein	4.8	<0.01	2.9	<0.01	1.7
SMa1169	Hypothetical protein	3.5	0.02	1.8	0.17	1.9
SMa1179	NosR Regulatory protein for N2O reductase	3.7	<0.01	2.2	0.06	1.7
SMa1182	NosZ N2O reductase	22.0	<0.01	11.9	<0.01	1.8
SMa1183	NosD periplasmic copper-binding precursor	11.0	<0.01	6.4	0.03	1.8
SMa1195*	Conserved hypothetical protein	7.1	0.01	3.9	0.01	1.8
SMa1207	FixK-like regulatory protein	16.0	<0.01	10.2	<0.01	1.6
SMa1233	NapB periplasmic nitrate reductase	3.8	0.02	3.7	0.11	1.0
SMa1240	NapF Fd component periplasmic nitrate reductase	3.6	0.02	2.3	0.12	1.5
SMa1243	AzuI pseudoazurin (blue copper protein)	44.0	<0.01	42.0	<0.01	1.1
SMa1247	NirV periplasmic nitrate reductase	4.8	0.01	2.5	0.05	1.9
SMa1250*	Putative NirK Cu-nitrite reductase	3.9	0.03	2.2	0.08	1.8
SMa1255	Conserved hypothetical protein	3.2	<0.01	2.0	0.07	1.6
SMa1256	Conserved hypothetical protein	4.2	0.01	2.5	0.04	1.7
SMa1259*	Conserved hypothetical protein	9.3	<0.01	8.1	<0.01	1.1
SMa1266	HemN coproporphyrinogen III oxidase	16.0	<0.01	8.4	<0.01	1.9
SMa1273	NorB nitric oxide reductase	9.5	<0.01	6.9	<0.01	1.4
SMa1279	NorE protein involved in nitric oxide reduction	3.1	<0.01	2.0	0.02	1.5
SMa1285	Probable decarboxylase	4.2	0.01	3.0	0.05	1.4
SMa1288*	Carboxy-lyase	3.1	<0.01	1.8	0.11	1.7
SMa1291	Conserved hypothetical protein	16.0	<0.01	10.3	<0.01	1.5
SMa1521	Conserved hypothetical protein	3.0	0.02	2.8	0.01	1.1
SMb20663	Conserved hypothetical protein	3.1	<0.01	2.4	0.01	1.3
SMb20664	Putative transcriptional regulator; GntR family protein	4.2	<0.01	3.1	0.01	1.4
SMb21011	xdhA2_probable xanthine dehydrogenase protein	3.5	0.02	4.2	0.01	0.8
SMb21212	Putative protein	3.6	0.01	5.1	0.01	0.7
SMb21284	Putative polysaccharide deacetylase	3.8	0.01	3.3	0.01	1.2
SMc00147	Conserved hypothetical protein	3.0	0.01	6.4	0.01	0.5
SMc00167	Conserved hypothetical signal peptide protein	3.1	0.02	3.7	0.01	0.8
SMc00276	Conserved hypothetical protein	3.6	0.01	3.7	0.04	1.0
SMc00277	Conserved hypothetical protein	8.2	0.02	9.0	<0.01	0.9
SMc00316	Oxidoreductase UbiH/COQ6 family	4.2	<0.01	4.7	0.01	0.9
SMc00317	Putative transporter	4.2	0.01	3.7	<0.01	1.1
SMc00338	Conserved hypothetical protein	4.5	<0.01	5.2	0.01	0.9
SMc00470	Hypothetical transmembrane protein	4.4	0.02	5.0	0.01	0.9
SMc00507	Hypothetical protein	3.9	0.05	4.1	0.02	0.9
SMc00998	Conserved hypothetical signal peptide protein	3.5	<0.01	3.6	<0.01	1.0
SMc01022	Putative cytochrome b transmembrane protein	4.8	<0.01	5.4	<0.01	0.9
SMc01843	metF-Prob. 5.10-methylenetetrahydrofolate reductase	3.1	0.05	2.4	0.01	1.3
SMc02021	Putative organic acid ABC transporter	3.0	0.05	2.8	0.01	1.1
SMc02239	Conserved hypothetical protein	3.3	0.04	3.0	0.09	1.1
SMc02255	qxtA_putative quinol oxidase subunit	3.3	<0.01	3.3	0.01	1.0
SMc03834	pcbD_putative pterin-4- α carbinolamine dehydratase	3.7	0.02	5.8	0.01	0.6
SMc03835	ligT_putative 2'-5' RNA ligase protein	3.6	0.01	4.5	0.01	0.8
SMc04150	Conserved hypothetical protein	4.0	0.02	2.5	<0.01	1.6
SMc04164	Hypothetical protein	3.4	0.02	5.1	0.03	0.7
Stress						
SMa0615	FixO3 cytochrome-c oxidase subunit	5.5	0.01	4.5	0.01	1.2
SMa1104	Hypothetical protein fragment	4.5	<0.01	3.7	0.01	1.2
SMa1450	Thiolase	3.6	0.04	4.2	<0.01	0.8
SMa1720	Putative Lysr-R family transcriptional regulator	3.1	0.01	3.3	<0.01	0.9
SMb20331	Hypothetical membrane spanning protein	3.5	0.05	3.8	0.01	0.9
SMb21456	Conserved hypothetical protein	5.4	<0.01	6.2	<0.01	0.9
SMc00137	Conserved hypothetical protein	4.1	0.01	4.4	<0.01	0.9
SMc00506	Hypothetical transmembrane protein	4.2	0.01	4.3	<0.01	1.0
SMc00885	Hypothetical transmembrane signal peptide protein	3.8	0.03	1.8	0.28	2.1
SMc01169	Ald-probable alanine deshydrogenase oxidoreductase	14.8	<0.01	20.1	<0.01	0.7
SMc01267	Conserved hypothetical protein	4.0	<0.01	2.2	0.15	1.8
SMc01815	Dihydropyrimidine dehydrogenase family protein	11.4	<0.01	8.4	<0.01	1.4
SMc02227	fadB putative fatty oxidation complex α subunit	4.0	0.01	6.6	<0.01	0.6
SMc02228	fadA putative acetyl CoA acetyltransferase	7.1	0.02	8.0	0.02	0.9
SMc03780	Conserved hypothetical protein	3.5	<0.01	3.9	0.01	0.9
SMc03999	Hypothetical protein	3.6	0.02	3.8	0.01	0.9

or NaCl) (Dominguez-Ferreras et al. 2006; Sauviac et al. 2007). Thus, we eliminated 16 genes which were induced under at least two other stress conditions (Table 1). Because SpNN generates NO and spermine in the culture, a control microarray experiment was performed to compare the transcriptomes of cells grown without or with 25 μ M spermine. No genes were differentially expressed in these conditions, indicating that NO was really responsible for the upregulation of genes mentioned above. Finally, the NO stimulon comprised 103 genes, which are listed in Table 1. Based on predictions of operon structure, approximately 30 genes may have been missed in our analysis; 10 of these were present in our data set but did not meet cut-offs for fold change or *P* value. Consequently, the NO stimulon could be composed of up to 130 genes. Interestingly, genes already known to be induced by NO (*sma1291-sma1294*) (de Bruijn et al. 2006) were detected in this study, confirming the validity of the approach used.

FixLJ and NnrR are major regulators of the NO response in *S. meliloti*.

Strikingly, we observed a remarkable overlap between the microoxia and NO stimulons because 70% of the genes upregulated in the presence of NO had been previously observed as activated in microaerobic conditions (Bobik et al. 2006). Because the two-component system FixLJ is the major regulator operating in microaerobic conditions in *S. meliloti*, we suspected that it could be involved in the NO response. Hence, we set up a microarray experiment where we compared the transcriptomes

of *fixJ* mutant cells grown in the presence and absence of SpNN (25 μ M). We considered genes for which the induction level in the presence of NO was reduced at least twofold in the mutant compared with the wild-type strain to be FixJ dependent (Table 1, column 7). Using this criterion, 53 genes were found dependent upon FixJ (Table 1, upper part). Most of them (*n* = 46) were already known to be FixJ targets in microaerobic or symbiotic conditions (Bobik et al. 2006). Direct targets of FixJ (*fixK₁*, *fixK₂*, *nifA*, and *smc03253*) were found in this study, and these results were confirmed by qRT-PCR analysis because we measured a gene induction of 700- and 15-fold for *fixK* and *nifA*, respectively, in the presence of SpNN compared with the control without NO donor. This activation was dependent upon FixJ because it was abolished in a *fixJ* mutant in the presence of NO (data not shown). All other FixJ targets had been identified previously as controlled by the intermediate transcriptional regulator FixK and, indeed, most of them display a FixK-binding sequence in their upstream regions (Bobik et al. 2006).

In the response to microoxia, FixJ functions in partnership with its cognate sensor histidine protein kinase FixL. In order to determine whether FixL is also involved in NO sensing, a transcriptomic analysis was performed to compare a *fixL* to a *fixJ* mutant in the presence of SpNN (25 μ M). No genes were differentially expressed, suggesting that FixL is the kinase responding to NO (data not shown). Therefore, these results show that the two-component system FixLJ is a major regulator of the NO response because it controls, either directly or via FixK, at least half of the NO-induced genes.

Among the 50 genes found to be FixJ independent in the presence of NO (Table 1, lower part), as many as 19 were known to be upregulated under microaerobic conditions in a FixJ-dependent manner (via FixK) (Table 1, lower part, gene ID in bold) (Bobik et al. 2006). Of these 19 genes, 13 could be partly FixJ dependent because the ratio *wt/fixJ* was found between 1.5 and 2. Among those were genes involved in the denitrification process (*nap*, *nir*, *nor*, and *nos*). Some of these genes are known to be regulated by the NO-dedicated regulator NnrR in *R. sphaeroides* (Laratta and Shapleigh 2003) and *B. japonicum* (Mesa et al. 2003). To test whether these genes are regulated by NnrR in *S. meliloti*, we analyzed the transcriptomes of *nnrR* mutant cells in the presence or absence of SpNN. In all,

Table 2. Quantitative reverse-transcription polymerase chain reaction validated NnrR-dependent genes

Gene	Fold change $\pm$ NO ($\pm$ SD) ^a	
	Wild type	<i>nnrR</i>
<i>sma1195</i>	399 ($\pm$ 165)	56 ($\pm$ 34)
<i>sma1250 (nirK)</i>	914 ($\pm$ 384)	73 ($\pm$ 39)
<i>sma1252 (nnrS)</i>	103 ($\pm$ 24)	13 ($\pm$ 7)
<i>sma1259</i>	856 ($\pm$ 456)	205 ($\pm$ 230)
<i>sma1276 (norC)</i>	4,192 ($\pm$ 557)	9 ($\pm$ 6)
<i>sma1288</i>	102 ($\pm$ 50)	7 ($\pm$ 6)
<i>sma1289</i>	2,292 ($\pm$ 976)	19 ($\pm$ 12)

^a NO = nitric oxide and SD = standard deviation.

Gene	Putative regulator-binding sequence (distance to start codon in bp)	Putative operon
<i>sma1195</i>	CTG TTTG CGCAAGCGCAAAGAC (121)	
<i>sma1250 (nirK)</i>	CTG TTTG TTCACCTCAAAGAG (70)	
<i>sma1252 (nnrS)</i>	GTC TTTG CCAATTGTCAAAGAA (52)	
<i>sma1261</i>	TTG TTTG CTCCCGTCAAAGTG (64)	
<i>sma1276 (norC)</i>	TTG TTTG CCGAAACGCAAAGAA (125)	
<i>sma1288*</i>	CAAT TTTG CGCTGGAGCAAAGAC (87)	
<i>sma1289*</i>	GTC TTTG CTCCAGCGCAAATTG (68)	

Fig. 2. Putative NnrR-binding sequences in front of NnrR-dependent genes. The first gene of each of the quantitative reverse-transcription polymerase chain reaction-validated NnrR-dependent transcription units is indicated in the first column. Asterisks indicate that the boxes are identical but read in opposite directions. Putative NnrR-binding-sequences are indicated in the second column. The distance to the start codon is indicated from the last nucleotide of the sequence shown in the column. When the transcription unit comprises more than one gene, it is shown in the last column (not to scale), and the genes found to be nitric oxide (NO)-dependent in the present study are indicated in gray.

22 genes covering 14 transcription units were NnrR dependent (data not shown). However, when assayed by qRT-PCR, only six could be clearly validated (i.e., *smal195*, *nirK*, *smal259*, *norC*, *1288*, and *smal289*) (Table 2).

Four of these transcription units had been predicted in a previous bioinformatic study (Rodionov et al. 2005), on the basis of the presence of a putative NnrR-binding sequence upstream of these genes (cTTGcgNNNNcgCAAag). In this analysis, *smal252* (*nnrS*) also displayed this consensus sequence but was found to be NO induced in our transcriptomic analysis. By using qRT-PCR, we showed that *smal252* was induced (100-fold) in the presence of NO in an NnrR-dependent manner (Table 2). Alignment of promoter regions of the seven identified NnrR-dependent transcription units revealed the presence of the sequence TTTGN₈CAAA in all of them. Therefore, we conclude that the NnrR regulon contains at least seven transcription units and that the DNA motif TTTGN₈CAAA is the likely sequence recognized by NnrR in *S. meliloti* (Fig. 2).

Among the genes that we failed to validate as FixJ or NnrR dependent, some were shown to be FixLJ/FixK dependent in microaerobic conditions (Table 1, lower part, gene ID in bold) and displayed a FixK-binding recognition sequence TTGAN₆T/GCAA in their promoter region (Bobik et al. 2006; Foussard et al. 1997). Interestingly, both FixK and NnrR are regulators of the FNR/CRP family, and their DNA-binding sites display sequence similarities and might overlap (i.e., TTTGAN₆T/GCAAA). Thus, some *S. meliloti* genes may be regulated by both FixK and NnrR. Although our data show that either FixLJ/FixK or NnrR regulates most genes induced by NO exposure, it is difficult to assign some of these genes to a specific regulon.

The putative flavohemoglobin encoded by the *hmp* gene plays a role in the bacterial resistance to NO but also in symbiosis.

Among the genes upregulated in the presence of NO, one (*hmp*) encodes a homologue of the flavohemoglobins characterized in a wide variety of eubacteria (Lira-Ruan et al. 2003) and known for its role in host-microbe interactions (Boccara et al. 2005; Bang et al. 2006; Stevanin et al. 2007). The induction of *hmp* in cells grown in the presence of NO was verified by qRT-PCR, and by measuring the activity of a genomic *hmp-gus* transcriptional fusion (Supplementary Fig. S1). In addition, expression of *hmp* was also detected in nodules.

In order to test whether the *S. meliloti* homologue of Hmp has a biological function in the NO response, we constructed two kinds of mutants and tested their sensitivity to NO in culture. The first mutant (*hmp*) was a null mutant constructed by integration of a suicide plasmid in the *hmp* gene. The second mutant (*hmp++*) overexpressed the flavohemoglobin from plasmid pBBR1-MCS5. qRT-PCR analysis showed that *S. meliloti* cells carrying this plasmid display approximately 1,000 times as much *hmp* transcripts as the wild-type cells (data not shown). The sensitivity of these mutants to the NO donor SpNN was compared with that of the wild-type strain. As already described above (Fig. 1), addition of SpNN to wild-type (Rm1021) cells led to a transient arrest of cell growth, whose duration was dependent on SpNN concentration (Fig. 3A). We observed that this growth arrest was approximately 90 min longer for the *hmp* mutant (CBT733) than for the wild strain (Fig. 3A; Supplementary Fig. S2). Similar results were obtained with an *hmp::mTn5* mutant strain derived from Rm2011 (GMI1549; data not shown), another *S. meliloti* strain closely related to Rm1021.

The growth of the wild-type strain Rm1021 carrying the empty plasmid pBBR1-MCS5 was completely arrested in cul-

ture containing 200 μ M SpNN (Fig. 3B). This result was also observed in the wild-type strain without plasmid (Fig. 1). In contrast, the *hmp++* strain was resistant to at least 200 μ M SpNN (Fig. 3B). Comparable results were obtained in the *S. meliloti* Rm2011 background. From all these experiments, we conclude that Hmp plays a role in the resistance to NO, presumably by detoxifying it.

The symbiotic properties of *hmp* null mutants in either Rm1021 or Rm2011 strain backgrounds were tested on *M. sativa* or *M. truncatula*, respectively. Both mutants displayed a Nod⁺ Fix⁺ phenotype. However, at 38 days postinoculation, the shoot dry weight and nitrogen fixation efficiency were reduced by as much as 30 and 56%, respectively, in *M. truncatula* infected with the *hmp* mutant (Rm2011 background) compared with the wild type (Fig. 4). Comparable results were obtained with *S. meliloti* (background Rm1021) and *M. sativa*, although the percentage of reduction was slightly lower in this case. Therefore, these data showed that Hmp could play a role, to some extent, in symbiosis.

Fig. 3. Hmp is involved in nitric oxide (NO) detoxification in *Sinorhizobium meliloti*. **A**, *S. meliloti* wt cells (black symbols) or *hmp* mutant cells (CBT733; open symbols) were cultured exponentially in the absence (squares) or presence of 50 μ M (circles) or 75 μ M (triangles) Spermine NONOate (SpNN). Cell density (optical density at 600 nm [OD₆₀₀]) was monitored over time. The figure shows the results of a typical experiment (for each time point, $n = 1$) reproduced at least three times. **B**, *S. meliloti* wild type (Rm1021) containing either the empty plasmid pBBR1-MCS5 (black symbols) or pBBR1-*hmp* (open symbols) was cultured without (squares) or with (diamonds) 200 μ M SpNN. Cell density (OD₆₀₀) was measured at the indicated times. The figure shows the results of a typical experiment ($n = 1$) reproduced three times with pBBR-*hmp* or twice for the empty vector.

Fig. 4. *Hmp* is required for efficient nitrogen fixation of *Sinorhizobium meliloti* in *Medicago truncatula* nodules. *M. truncatula* plantlets were inoculated with *S. meliloti* wild type (WT) (Rm2011) or *hmp* mutant cells (GMI11549). Negative controls were performed where H₂O was added on the plantlets. At 38 days after inoculation, plants were tested for **A**, dry weight of the aerial parts of the plants or **B**, nitrogen fixation efficiency, using an acetylene reduction assay. Values are means $\pm$ standard deviation of data obtained from 10 plants for each strain and five control plants (H₂O).

DISCUSSION

NO produced during infection plays a crucial role in host defense mechanisms. Thus, pathogens evolved mechanisms to detect NO and induce an adaptive response to this molecule in order to survive phagocyte killing. NO may also have a role in symbiotic interactions, because NO is produced in plant root nodules of legumes such as *M. truncatula* (Baudouin et al. 2006) or *M. sativa* (Pii et al. 2007). We can speculate that the role of NO in symbiosis with nitrogen-fixing bacteria would be different from that known in pathogenic interactions; however, the function or functions of NO are not identified yet. Although the response to nitrosative stress is well documented in many bacteria, data concerning the response of rhizobia to the presence of NO were very limited prior to this study. Our transcriptome analyses show that approximately 100 genes are upregulated by NO in *S. meliloti* grown in culture. If we consider the genes which could have been missed in putative operations, the NO stimulon of *S. meliloti* could comprise up to 130 genes.

The first striking observation of this study was the remarkable overlap between the NO and microaerobic stimulons, because at least 70% of the genes upregulated by NO had been described before as being induced under microaerobic conditions (Bobik et al. 2006). We rule out the possibility that SpNN itself could have triggered a microaerobic environment because it was shown before that the oxygen tension was identical in a culture containing or not containing this molecule (Ohno et al. 2003). We showed that most of the genes common to the NO and microoxia stimulons are regulated by the two-component system FixLJ (Fig. 5). The NO stimulon includes all the direct targets of FixLJ (i.e., *nifA*, *fixK*, and *smc03253*), confirming that NO acts at the FixLJ level. In *S. meliloti*, FixJ is a major regulator because it controls two-thirds of the genes induced by microaerobiosis and the majority of rhizobial genes expressed in planta in mature nodules (Bobik et al. 2006). The FixJ cognate sensor histidine kinase FixL possesses a heme-binding domain, and the oxygen-bound form (dissociation

Fig. 5. Regulation of nitric oxide (NO)-induced genes: a schematic model. Number of targets indicated in the boxes is underestimated due to the genes present in the same transcription unit but not found in our study; * indicates genes identified following quantitative reverse-transcription polymerase chain reaction analysis.

constant 50 to 140 μM) is the off-state whereas the unliganded form is the active kinase (on-state). It has already been known that FixL could bind ligands other than O_2 such as NO and CO. FixL affinity was shown to be higher for NO than for O_2 ; however, oxygen was the only compound able to abolish its kinase activity (>200-fold inhibition) (Gilles-Gonzalez et al. 2008). Therefore, the on-state of the kinase in the presence of NO could be due to the higher affinity of FixL for NO compared with oxygen. Whether NO signaling via FixLJ has a biological significance in either free-living cells or symbiosis remains to be established. In the soil, *S. meliloti* is submitted to microoxic conditions and also to nitrogen oxides generated by either denitrifying microorganisms or nitrate supplements. Low oxygen concentrations encountered by *S. meliloti* during symbiosis trigger FixLJ-mediated gene expression (Reyrat et al. 1993). However, NO is present in nodules and may serve as an additional signal to activate the FixLJ regulon. The concentration of NO in nodules has not been determined precisely but is estimated to be in the micromolar range (R. Brouquisse, *personal communication*). Heurlier and colleagues (2008) measured the concentration of NO released by SpNN in the medium. Based on their results, we calculated that NO is in the micromolar range in our experiments (i.e., of the same order of magnitude as that estimated in nodules, where it could therefore be considered as a putative signal for FixL). The availability of plant mutants deficient in NO synthesis could help settle the question of whether NO is a symbiotic signal of the FixLJ signaling pathway; unfortunately, the enzymatic sources of NO in plants are not fully characterized yet (Besson-Bard et al. 2008). In other bacterial species, two-component systems have also been described to respond to NO (Spiro 2007). In *Mycobacterium tuberculosis*, a significant overlap between the transcriptional responses to NO and hypoxia has been described because, of the 36 genes determined to be NO inducible, 27 were also induced under microaerobic conditions. *M. tuberculosis* DosR is a response regulator that controls the expression of at least 50 genes in response to anoxia. DosR can be phosphorylated by two histidine kinases, DosS and DosT, both of which respond to low oxygen concentration (Roberts et al. 2004). There is good evidence to suggest that the DosRST system is also involved in mediating the response to NO, given that most DosR-regulated genes are induced in activated macrophages in an NO-synthase-dependent manner (Ohno et al. 2003). It has been proposed that NO present at a high concentration during the inflammatory response displaces oxygen from the haem group but is a poor inhibitor of the histidine kinase activity, thus explaining how NO can induce the Dos regulon (Sousa et al. 2007). We reached a similar conclusion with FixLJ in *S. meliloti*.

We confirmed that NnrR, a member of FNR/CRP family, is also involved in the NO response of *S. meliloti*. NnrR is a dedicated NO sensor, first identified as activating gene transcription in response to NO in a denitrifying strain of *R. sphaeroides* (Tosques et al. 1996). This protein controls the transcription of *nor* genes, which encode the NO reductase. Surprisingly, the *nirKV* operon encoding the nitrite reductase responsible for the formation of NO is also a target for NnrR regulation. This coordinated regulation is common in denitrifiers and prevents the toxic accumulation of NO. Rodionov and colleagues (2005) found orthologues of NnrR in six α -proteobacteria, all of which also possess the *nir* and *nor* genes involved in denitrification. *S. meliloti* NnrR was shown previously to regulate, in the presence of NO, an operon (*smal289-smal294*) whose function is yet unknown (de Bruijn et al. 2006). In this study, we confirmed this finding and we identified seven transcription units regulated by NnrR (Table 2). All are located on the symbiotic plasmid pSymA and display a consensus NnrR box

in their promoter region (TTTGN₈CAAA). Our work agrees with the bioinformatic predictions of Rodionov, who identified eight candidate NnrR targets in *S. meliloti* (Rodionov et al. 2005; Bobik et al. 2006). Our analyses recovered five of these transcription units (*smal195*, *nirK*, *nnrS*, *norC*, and *smal289*) and found two new ones (*smal259* and *smal288*).

For a subset of NO-induced genes, the issue remains whether these genes are controlled by FixK or NnrR in the presence of NO. Both regulators belong to the FNR/CRP family, and their DNA-binding sites display high sequence similarities and sometimes overlap. Hence, we propose that at least some of these genes could be regulated alternately by either FixK or NnrR in the presence of NO, which renders difficult their classification in one regulon or the other.

Another important question is whether or not both regulators FixK and NnrR are on the same signaling pathways. In *B. japonicum*, a model has been described by Mesa and coworkers where *nnrR* is placed in the FixLJ-FixK₂ cascade, downstream of the FixK₂ regulator. The expression of *nnrR* in the wild-type strain is quite low, rendering its expression in the *fixK* mutant even more difficult to analyze. Also, the FixK box present in the promoter region of *nnrR* is poorly conserved (Mesa et al. 2003). However, a recent transcriptome analysis confirmed that expression of *nnrR* is regulated by FixJ and FixK₂ under microaerobic conditions, although this does not seem to be the case in planta (Mesa et al. 2008). This model is unlikely to be applicable to *S. meliloti* because (i) *nnrR* was not found as a FixK target in a previous transcriptomic study (Bobik et al. 2006), (ii) there is no FixK-box in the promoter region of *nnrR* in *S. meliloti*, and (iii) we could find genes regulated by NnrR and not by FixJ/FixK in the presence of NO (i.e., *smal289*) (this work and data not shown). On the whole, these data suggest that FixK and NnrR are part of two different signaling pathways, both being responsive to NO.

There are approximately 25 genes for which regulators remain to be found. Various regulators involved in NO response have been described in other bacteria (Spiro 2007); at least two of these regulators (i.e., SoxR and OxyR) are encoded by the genome of *S. meliloti* and could be responsible for NO upregulation of some of these genes. Alternatively, because NO poorly induces expression of most of these, they may not be genuine NO-induced genes. Most of these genes encode hypothetical or poorly characterized proteins.

In terms of physiological response to NO in *S. meliloti*, *hmp*, encoding a flavohemoglobin, is a relevant gene. Flavohemoglobins participate in NO detoxification in many organisms and are often pathogenicity determinants. We showed that, in *S. meliloti*, *hmp* is clearly involved in NO response because null mutants in this gene displayed a higher sensitivity to NO than the wild type whereas overexpression of Hmp enhanced NO resistance. We further showed that this gene plays a role in symbiosis because *hmp* null mutants displayed lower nitrogen fixation efficiency than the wild type. This could be the consequence of NO accumulation in the mutant cells and negative effects of this accumulation on the function of proteins such as the nitrogenase, that has been shown to be sensitive to NO (Trinchant and Rigaud 1982). Hmp converts NO to nitrate or nitrous oxide in aerobic or anaerobic conditions, respectively (Justino et al. 2005). Because microaerobic conditions prevail inside nodules, detoxification of NO by Hmp could lead to the formation of either nitrate or nitrous oxide, which may both enter the denitrification pathway.

Gene *smal195* is similar to *Escherichia coli* *ytfE*. The expression of this gene in *E. coli* is increased upon exposure to NO and it has been shown to be critical for repair of iron clusters damaged by oxidative or nitrosative stress conditions (Justino et al. 2005, 2007; Overton et al. 2008). These repair-

of-iron-center family proteins are encoded in many pathogen genomes; one has been shown to be essential for *Hemophilus influenzae* pathogenicity (Harrington et al. 2009). Among the genes induced by NO are those involved in denitrification and in functions related to this process, such as *azul* (encoding a pseudoazurin), *hemN* (encoding a coproporphyrinogen III oxidase), *nnrU* (encoding a putative regulatory protein), and *smal252* encoding a member of the NnrS family. NnrS is a putative heme-copper protein and a member of the short-chain dehydrogenase family. This protein has been identified in *R. sphaeroides* and its role, although not studied in details, could be in the denitrification process (Bartnikas et al. 2002). Denitrification is a widespread respiratory pathway in bacteria in which nitrogen oxides are used as electron acceptors in the absence of oxygen, which may explain why most of the denitrification genes are expressed in microaerobic conditions (albeit at a lower level than in the presence of NO for nitrite reductase-*nir* and NO reductase-*nor*). The role of these genes in symbiosis is not known, although denitrification activity has been detected in *S. meliloti* bacteroids (O'Hara et al. 1983). More recently, bacteroidal nitrate and nitrite reductase activities were also detected in soya bean nodules treated with low concentrations of nitrates (Mesa et al. 2004; Meakin et al. 2007). There is evidence of a role of denitrification genes in the successful interaction of bacteria with their host (Baek et al. 2004; Philippot 2005). Denitrification may serve an energetic function in bacteroids but also as a protection against NO during infection. Indeed, we observed that a *norB* mutant (encoding NO reductase) is more sensitive to NO than is the wild type in culture (data not shown). Denitrification could protect the nitrogenase against inhibition by nitrate or NO (Lucinski et al. 2002). However, data obtained in *B. japonicum* indicate that *nor* gene products are not the only ones responsible for NO degradation in soya bean nodules and that another detoxification system must exist. It could be a flavohemoglobin because *B. japonicum* possesses a *hmp* orthologue in its genome (Meakin et al. 2006).

It was recently described that nitrate reductase, product of the *nap* genes (but probably not nitrite reductase-*nirK*), contributes to the formation of NO in soya-bean nodules as measured by the formation of leghemoglobin-NO (Meakin et al. 2007). Hence, bacteria could contribute to the formation of NO in the nodules. A role for the bacterial NO production has been shown recently in macrophages infected with *B. anthracis*

spores, where NO antagonizes the antimicrobial action of the oxidative burst generated by macrophages (Shatalin et al. 2008). A similar role could be conceived in the symbiotic interaction where an oxidative burst occurs in alfalfa early after infection with symbiotic bacteria.

In conclusion, the flavohemoglobin Hmp has emerged as the most significant NO-detoxifying protein in many diverse microorganisms, particularly pathogenic bacteria, and we show here that it could also serve this role in the symbiotic interaction between *S. meliloti* and alfalfa or the model plant legume *Medicago truncatula*. Other bacterial genes upregulated by NO such as those encoding NO reductase (*nor*) could be involved in NO detoxifying, perhaps together with plant hemoglobins, which participate in limiting NO toxicity inside nodules. However, the functions of many genes implicated in the NO response are still unknown and their implication in the establishment or maintenance of symbiosis could prove to be more subtle than the sole detoxification function.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The strains and plasmids used in this study are listed in Table 3. *S. meliloti* strains were grown in aerobic conditions at 28°C in Vincent minimal medium (VMM): 5.74 mM K₂HPO₄, 7.35 mM KH₂PO₄, 1 mM MgSO₄, 18.7 mM NH₄Cl, 10 mM Na₂ succinate, 456 µM CaCl₂, 35 µM FeCl₃, 4 µM biotine, 48.5 µM H₃BO₃, 10 µM MnSO₄, 1 µM ZnSO₄, 0.5 µM CuSO₄, 0.27 µM CoCl₂, and 0.5 µM NaMoO₄.

Strain constructions were performed in Luria-Bertani medium supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄ (LBMC). Antibiotics were added at the following concentrations: streptomycin (Sm) at 100 to 200 µg ml⁻¹, tetracycline (Tc) at 10 µg ml⁻¹, gentamicin (Gm) at 40 µg ml⁻¹, and neomycin (Nm) at 100 µg ml⁻¹.

The NO donor SpNN was purchased from Cayman Chemicals Coger (CAY-82150-M100; Paris). A 100-mM stock solution was prepared, just before use, in Na-phosphate buffer (0.1 M), pH 6.9, and then diluted in the cell culture medium at the appropriate concentrations.

Strain and plasmid constructions.

All plasmid constructions were performed in the *E. coli* DH5α strain. The DNA sequences of oligonucleotide primers

Table 3. Strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
<i>Sinorhizobium meliloti</i>		
Rm1021	Wild-type strain, SU-47 derived, Sm ^r	Meade et al. 1982
Rm2011	Wild-type strain, SU-47 derived, Sm ^r	Pobigaylo et al. 2008
EK101	Rm1021 derivative, <i>fixJ</i> ::Tn5	Ernö Kiss
EK104	Rm1021 derivative, <i>fixL</i> ::Tn5	Ernö Kiss
GMI11548	Rm1021 <i>smal207</i> ::pK19	Anke Becker
GMI11549	Rm2011 <i>hmp</i> ::mTn5-STM-2.12.A01	Anke Becker
CBT515	Rm1021 <i>nnrR</i> ::pVO155	Silvia Rosbach
CBT731	Rm1021 <i>smal289</i> ::pVO155	This work
CBT732	Rm1021 <i>smal294</i> ::pVO155	This work
CBT733	Rm1021 <i>hmp</i> ::pVO155	This work
<i>Escherichia coli</i>		
DH5α	<i>supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook et al. 1989
Plasmids		
pRK2013	Helper plasmid for triparental matings	Figurski and Helinski 1979
pGEM-T	Cloning vector, Amp ^r	Promega
pBBR1MCS-5	Cloning vector, Gm ^r derivative of pBBR1	Kovach et al. 1995
pVO155	Integrational plasmid with promoterless <i>uidA</i> gene, Kan ^r	Oke and Long 1999
pGEM-hmp	pGEM-T + <i>hmp</i>	This work
pBBR-hmp	pBBR1MCS-5 + <i>hmp</i>	This work

^a Sm^r, Amp^r, Gm^r, and Kan^r = resistant to streptomycin, ampicillin, gentamicin, and kanamycin, respectively.

used for PCR amplifications are shown in Supplementary Table S1.

For constructing plasmid pBBR-hmp, a DNA fragment containing the *hmp* gene with 100 nucleotides upstream of ATG was generated by PCR (1,319 bp) using primers *sma1191exp*, genomic DNA of the strain Rm1021 as template, and ligated in pGEM-T plasmid, yielding pGEM-hmp. The cloned *S. meliloti* DNA region was verified by DNA sequencing. This plasmid was digested with *Bam*HI-*Xba*I and ligated into *Bam*HI-*Xba*I-digested pBBR1MCS-5, yielding pBBR-hmp. These plasmids were introduced in *S. meliloti* by triparental mating using pRK2013 as helper.

To construct *S. meliloti* null-mutant strains, DNA fragments of *sma1191*, *sma1289*, and *sma1294* were produced by PCR. PCR products were digested with *Bam*HI and *Xba*I and ligated into the *Bam*HI-*Xba*I digested pVO155 plasmid. The resulting plasmid constructs were checked by sequencing and introduced into *S. meliloti* Rm1021 by triparental mating. Transconjugants were selected three times on LBMC plates supplemented with Sm and Nm. A single colony was selected and the correct integration of the plasmid was confirmed by PCR.

Preparation of samples for microarrays and qRT-PCR.

For microarray and qRT-PCR analyses, strains were grown exponentially at 28°C in 25 ml of VMM. At an optical density at 600 nm (OD_{600}) between 0.15 and 0.25, spermine or SpNN was added at the appropriate concentration. The cultures were incubated at 28°C for 30 min; then, cells were collected by filtration and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. RNA was prepared from the collected samples as previously described (Bobik et al. 2006), followed by DNaseI treatment (Qiagen clean-up procedure [Qiagen, Hilden, Germany]).

Labeling of hybridization probes, microarray hybridizations, and analyses.

Cy3- and Cy5-labeled cDNAs were prepared from 20 µg of RNA according to Bobik and associates (2006). For each experiment, RNA preparations from at least three independent cultures were used. Sm14kOLI microarrays were purchased from A. Becker (University of Bielefeld, Germany). They consisted of glass slides carrying mainly 70-mer oligonucleotides representative of the 6,208 predicted open reading frames of *S. meliloti* spotted in duplicate, as well as 50- to 70-mer oligonucleotides representative of intergenic regions, and a number of control spots. Hybridizations were performed as described previously (Bobik et al. 2006). Data were acquired on a GenePix 4000 scanner (Axon Instruments, Sunnyvale, CA, U.S.A.) and quantification of mean signal intensities for each spot was performed using GenePix Pro 3.0 (Axon Instruments). Data analyses were carried out using Genesight (version 3.5; Bio-discovery Inc., El Segundo, CA, U.S.A.) with normalization by median of the signal and statistical *t* test. The whole set of data is available at ArrayExpress (accession E-MEXP-2515).

qRT-PCR analyses.

RT was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and random hexamers as primers. RNA samples isolated from at least three independent cultures were tested for each condition. Real-time PCR tests were run on a LightCycler system (Roche, Mannheim, Germany) using the FastStart DNA Master SYBRGreen I kit (Roche) according to the manufacturer's instructions. 16S rRNA was used as a reference for normalization. In these experiments, we observed variability between biological repeats. It may be due to the degradation kinetic of SpNN (particularly dependent upon pH and temperature of the medium), which

may vary slightly from one culture to another and, hence, impact the level of NO-induced gene expression. Also, SpNN is not stable in solution and, hence, must be prepared just before each experiment by weighing very small amounts of the molecule, which may be another source of variability.

Plant assays.

Seeds of *M. sativa* cv. Europe or *M. truncatula* cv. Jemalong A17 were surface sterilized, germinated on agar plates, and allowed to grow on nitrogen-free Fahreus medium in test tubes during three (*M. sativa*) or two (*M. truncatula*) days (Barker et al. 2006). Plants were inoculated (*S. meliloti* strains at 100 µl/plant resuspended in sterile water at $OD_{600} = 0.001$). Series of 10 plants were inoculated with either the wild type or the different mutants, and series of five plants were inoculated with 100 µl of water as controls. Plants were grown in a culture room (22°C) with day and night cycles of 16 and 8 h, respectively. Dry weights of the plant shoots were measured 38 days after inoculation.

Nitrogenase activity was determined by the acetylene reduction assay (Hardy et al. 1968). Nitrogen fixation was assayed using plants grown in test tubes fitted with rubber stoppers. Air (6 ml) was removed from the tubes and the same amount of acetylene was added. Gas samples (200 µl) were taken after 2.5 h and analyzed for ethylene and acetylene in a gas chromatograph equipped with a hydrogen flame ionization detector (Intersmat IGC 120FL). For each plant, the amount of ethylene produced is assessed by measuring the height of the ethylene peak on the chromatogram.

Gus activity assay.

S. meliloti cells were grown to exponential phase at 28°C in VMM. At $OD_{600} = 0.2$, SpNN was added and the cultures were incubated at 28°C for the indicated times. Aliquots of 300 or 1,000 µl were centrifuged and resuspended in 300 µl of sterile water. Gus activity was assayed on the samples as referenced by Blanvillain and associates (2007).

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