Expression of the Bacterial Catalase Genes During *Sinorhizobium meliloti*–*Medicago sativa* Symbiosis and Their Crucial Role During the Infection Process

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Sinorhizobium meliloti possesses three distinct catalases to cope with oxidative stress: two monofunctional catalases (KatA and KatC) and one bifunctional catalase-peroxydase (KatB). The *katB* gene is constitutively expressed during growth in batch culture and is not induced under oxidative stress conditions. In contrast, the expression of *katA* and *katC* genes is mainly regulated at the transcription level in these conditions. A differential expression of *kat* genes was observed during the development of the nodule. A high expression of *katA* gene was detected in bacteroids, suggesting that the nitrogen-fixation process induces a strong oxidative stress. In contrast, bacteria express *katB* and *katC* genes and not the H_2O_2 -inducible *katA* gene in infection threads despite the detection of H_2O_2 around the bacteria. A *katB katC* double mutant nodulated poorly and displayed abnormal infection. After nonefficient release into plant cells, bacteria failed to differentiate into bacteroids and rapidly underwent senescence. Our results indicate that these two catalases are essential for the establishment of the symbiosis. They also suggest that the bacteria are in a nonexponential growth phase in infection threads and corroborate previous studies on the growth rate of bacteria inside the plant.

Sinorhizobium meliloti is an aerobic gram-negative bacterium from the rhizosphere able to infect roots of *Medicago* species and develop a symbiosis. During this process, a sophisticated exchange of recognition signals occurs between both partners (Long 1996). In response to flavonoids secreted by legume roots, the microsymbionts produce nodulation factors, which are essential for the formation of a new organ, the root nodule (Lerouge et al. 1990; Stougaard 2000). Inside the plant cell, bacteria differentiate into their symbiotic forms, the bacteroids that are able to reduce atmospheric nitrogen to ammonia. The key enzyme of nitrogen fixation, nitrogenase, is rapidly and irreversibly inactivated by oxygen. To avoid this inactivation, a diffusion barrier in the cortex of nodules limits permeability to oxygen, which is delivered to bacteroids by leghemoglobin, a plant oxygen carrier. Despite this efficient system to reduce the diffusion of an excess of oxygen to the bacteroid, a high rate of respiration is necessary to supply the energy required by the nitrogen reduction process. This leads to the production of reactive oxygen species such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2). In addition, an oxidative burst including an accumulation of O_2^- and H_2O_2 occurs

during the early stages of the symbiotic interaction, especially in the infection threads (Santos et al. 2001).

The response of both free-living bacteria and bacteroids to the toxic form of oxygen is an important factor for nodulation and nitrogen fixation. The dismutation of O_2^- to H_2O_2 and O_2 is catalyzed by an atypical superoxide dismutase SodA in *S. meliloti* (Santos et al. 1999). Disruption of the *sodA* gene affected the symbiotic properties of *S. meliloti* in alfalfa. Most mutant bacteria nodulated poorly and did not reach the differentiation stage of nitrogen-fixing bacteroid (Santos et al. 2000). As other root nodule bacteria, *S. meliloti* free-living cells had higher susceptibility to H_2O_2 than most of the other genera of aerobic or facultative anaerobic bacteria (Hérouart et al. 1996; Ohwada et al. 1999). Analysis of H_2O_2 -scavenging enzymes on native gels revealed that, in *S. meliloti*, the dismutation of H_2O_2 into O_2 and H_2O occurs through the action of three different catalases, two monofunctional catalases (KatA and KatC) and one bifunctional catalase-peroxidase (KatB) (Hérouart et al. 1996). A switch of catalase activity pattern occurred during bacterial growth in minimum medium (Sigaud et al. 1999). KatA was mainly detectable during the exponential phase while, during the stationary phase, KatC activity increased as KatA activity gradually decreased. KatB activity was detected throughout the growth of bacteria in minimum medium and represented around 90% of the total catalase activity, suggesting an important role of this bifunctional catalase in free-living bacteria. Analysis of *S. meliloti* catalase profiles after several treatments that are known to produce an oxidative stress has shown an increase in KatA activity in response to exogenous H_2O_2 . In contrast, an induction of *katC* gene was only observed after bacteria had been exposed to heat stress, salt stress, or ethanol. Analysis of other root nodule bacteria such as *Bradyrhizobium japonicum*, *S. fredii*, *Rhizobium leguminosarum* bv. *trifolii*, and *R. leguminosarum* bv. *Phaseoli*, on native gel also revealed the presence of three catalases. The catalase activity of these bacteria profiles also varied either in the presence of H_2O_2 in the environment (Ohwada et al. 1999), during growth, or both, suggesting a cell-density regulation of catalase (Crockford et al. 1995). However, the role of each catalase in the response of bacteria to oxidative stress and especially during the symbiotic interaction is still unknown. In *S. meliloti*, single *katA* or *katC* mutants showed similar nodulation and nitrogen fixation capacities as compared with a wild-type strain (Sigaud et al. 1999). In contrast, a dramatic decrease of nitrogen fixation capacity in a *katA katC* double mutant was already observed (Sigaud et al. 1999). In this paper, the expression of the three catalase genes is analyzed in free-living cells and during the establishment and the maintenance of nitrogen-fixing nodules.

Moreover, the analyses of nodules incited by double catalase mutants are presented at structural and ultrastructural levels.

RESULTS

Cloning and analysis of the *katB* gene in *S. meliloti*.

Three catalase genes have been predicted, based on the analysis of the complete genome of *S. meliloti* strain Rm1021 (Galibert et al. 2001). The sequence of the megaplasmid pSymA (Barnett et al. 2001) reveals the presence of an open reading frame showing high homology with other bacterial catalase-peroxidase, such as *Mesorhizobium loti* (69% identity) and *Escherichia coli* HPI catalase (58% identity). However, before the publication of the Rm1021 genome sequence, we amplified a polymerase chain reaction (PCR) fragment (*SmkatB*) using a nested-PCR strategy with three degenerated primers that were deduced from a consensus sequence of five bifunctional catalase-peroxidases as described below. *SmkatB* was used as probe to screen a genomic library and to localize *katB* on a 3-kb *SmaI*-*ApaI* DNA fragment (subclone pBSKB1). Alignment of the deduced amino acid sequences of *katB* and the published sequence (Sma2379) revealed one punctual mutation (T → G) resulting in one substitution at amino acid level (Met364 → Arg364). The Met364 is conserved in all bacterial catalase peroxidases published so far. Specific primers were synthesized to amplify this genomic region, using Rm1021 DNA as template (discussed below). The amplified fragment was purified and was cloned in the pGEM-T vector. The sequencing of four independent recombinant plasmids indicates that this punctual mutation is present in the pSymB of strain Rm1021. Analysis of Rm1021 cell extracts on native gel using the DAB-negative coloration of catalase profiles showed that the achromatic band corresponding to the KatB activity was still present despite the Met364 substitution (Fig. 1B). However, higher KatA and lower KatB activities were observed on native gel for strain Rm1021 compared with GMI211 (Fig. 1A) and Rm5000 (Sigaud et al. 1999). The sequence of the region Sma2379 can be noted as *katB*.

Phenotypic of *katB* mutant in free-living conditions.

To evaluate the effect of *katB* disruption on free-living phenotype, a transcriptional *katB::lacZ* fusion was constructed by insertion of a promoterless *lacZ* cassette into the *katB* coding region (discussed below). This fusion was transferred into strain GMI211 (LacZ⁻) by conjugation, and the disruption of *katB* was confirmed by Southern analysis of the recombinant mutant strain (GKBZ01). The total catalase activity in GKBZ01 (14.2 ± 4.3 U per mg of proteins) during the exponential growth phase was 2.5-fold less than in control strain

GMI211 (36 ± 6.7 U per mg of proteins). In contrast, no significant difference in total catalase activity was detected in either strain when bacterial extracts were prepared from bacteria in stationary phase (20.6 ± 7.9 and 17 ± 7.2 U per mg of proteins, respectively). Analysis on native gel of catalase profiles of cell extracts of GMI211 and GKBZ01 showed that KatB activity was present in GMI211 (I) and absent in GKBZ01 (II), confirming that this gene effectively encodes KatB (Fig. 1A). Introduction of a stable plasmid containing *katB* into GKBZ01 restored the KatB activity (data not shown). Growth curves of these two strains on either LB-MC or minimum medium were not significantly different (data not shown). The deficiency of KatB was not compensated for by the induction of either KatA or KatC or both monofunctional catalases in midexponential phase (Fig. 1A; 24 h). In contrast, in stationary phase (Fig. 1A; 72 h), KatA activity was still detected in GKBZ01 and not in GMI211 despite the induction of KatC in both strains, indicating a deregulation of the switch-off of KatA in the *katB* mutant strain and thus providing an explanation for our data on total catalase activities.

Growth and catalase gene expression.

To complete our knowledge on the expression of each catalase gene during growth, two new recombinant strains harboring promoter-*lacZ* fusions were constructed. The transcriptional *katC::lacZ* fusion described previously (Sigaud et al. 1999) was introduced in the GMI211 LacZ⁻ strain using triparental mating, resulting in the recombinant GKZ01 strain. In parallel, a transcriptional *katA::lacZ* fusion was constructed and cloned in the suicide plasmid pSUP202 in order to obtain the recombinant GKAZ01 strain. No difference in growth on LB-MC medium was observed between the three *kat::lacZ* recombinant strains and the GMI211 control strain (data not shown). β -galactosidase activities were determined during their growth. A fourfold increase of β -galactosidase activity was observed in the stationary phase of GKZ01 confirming that the growth-dependent KatC regulation is mainly transcriptional in *S. meliloti*. No significant modification of β -galactosidase activity was detected in GKBZ01, indicating that KatB could be mainly considered as a housekeeping bifunctional catalase-peroxidase enzyme (data not shown). In contrast, a threefold greater β -galactosidase activity was detected during the early exponential phase in GKAZ01 as compared with the very low basic activity during stationary phase (Fig. 2). These data are correlated with the KatA catalase pattern observed in GMI211 on native gel (Fig. 1A), indicating that *katA* is mainly regulated at a transcriptional level during growth in batch culture.

Oxidative stress and catalase gene expression.

Previous analysis of a recombinant Rm5000 strain harboring a *katC::lacZ* fusion revealed that *katC* expression is induced by some treatments that are known to induce an oxidative stress, such as heat stress (37°C), salt stress (NaCl), ethanol, and paraquat (a superoxide-generating compound). On the other hand, no induction of *katC* by exogenous H₂O₂ was observed (Sigaud et al. 1999). A similar expression pattern was observed in GKZ01 when treatments were applied to bacteria in exponential phase (data not shown). No induction of *katB* was detected in GKBZ01 following any of the treatments tested in this paper. In contrast, when exogenous H₂O₂ (1 mM) was added to the growth medium, a sixfold increase of β -galactosidase activity in GKAZ01 was measured after 1 h (430 ± 23 Miller units) as compared with nontreated bacteria (70 ± 4 Miller units). It is interesting to point out that no induction of any *kat* gene expression was observed when the oxidative treatments were applied to bacteria in stationary growth phase (data not shown).

Fig. 1. Catalase activity patterns of *Sinorhizobium meliloti* strains. Stationary cultures were inoculated in fresh LB-MC medium to an initial optical density at 600 nm of 0.01. Cell extracts were prepared from GMI211 (I), the *katB* mutant GKBZ01 (II), and Rm1021 (III) strains after 24 h (midexponential phase) and 96 h (stationary phase). Proteins (30 μ g) were submitted to electrophoresis on a native polyacrylamide gel (7%) that had been stained for catalase activity. The positions of KatA, KatB, and KatC are indicated according to Sigaud and associates (1999).

Nodule development and catalase gene expressions.

To investigate the effect of *katB* disruption on nodulation and fixation capacities of the bacteria, alfalfa seedlings were infected with the null *katB* mutant GKBZ01 and with GMI211 as control. No significant difference was observed five weeks after infection: 98% of inoculated plants produced nodules with an average of 10 nodules per plant and an acetylene reduction activity of 53 ± 5 nmole C_2H_4 per min per mg of fresh weight (FW).

To analyze the expression of each catalase gene during the development of the root nodule, alfalfa seedlings were independently infected by the three strains harboring one transcriptional *kat::lacZ* fusion (GKAZ01, GKBZ01, and GKCZ01). Strain GMI211 was used as control. Analysis of total β -galactosidase activity in 6-week-old nodule extracts showed that *kata* was highly expressed ($3,572 \pm 182$ Miller units per μ g of proteins) compared with *katB* and *katC* (545 ± 101 and 262 ± 77 Miller units per μ g of proteins, respectively). The histochemical detection of β -galactosidase activity was performed using X-gal as substrate (Fig. 3). Three days after infection, analysis of root hairs revealed a blue staining inside infection threads only with strains GKBZ01 and GKCZ01 (Fig. 3B and

C). During the first steps of root infection by *S. meliloti*, an accumulation of H_2O_2 has been detected around bacteria inside some infection threads (Santos et al. 2001). However, analysis of plants infected by GKAZ01 revealed no expression of the *katA::lacZ* fusion inside infection threads (Fig. 3A). Moreover, treatment of roots with exogenous H_2O_2 at several concentrations (0.1 mM, 1 mM, and 10 mM) for 1 h before histochemical staining with X-gal had no effect on *katA* expression inside infection threads (data not shown). To complete our investigation on the expression of *kat* genes, 7-day-old and 6-week-old nodules were analyzed (Fig. 3D through F and 3G through I, respectively). As expected, no X-gal staining was observed in the meristematic zone of root nodules (I). The *katB::lacZ* fusion was expressed in several morphological zones: infection (II), fixation (III), and senescent (IV) zones of young and mature nodules (Fig. 3E and H, respectively). In contrast, the *katA::lacZ* was strongly expressed only in nitrogen fixing bacteroids (Fig. 3G). This result is consistent with our previous analysis of catalase activity pattern in bacteroids. A higher catalase activity (sevenfold) has been measured in bacteroids extracted from 5-week-old nodules than in free-living bacteria, and KatA is the major isoform detectable on native gel in bacteroid protein extract (Sigaud et al.

Fig. 2. Growth stage induction of *katA* gene. Stationary cultures of GKAZ01 (*katA::lacZ* transcriptional fusion) were inoculated in fresh LB-MC medium to an initial optical density at 600 nm of 0.05. At the indicated times, samples were taken to assay β -galactosidase activity. Values represent averages of three assays. Error bars show standard deviation.

1999). Finally, the *katC::lacZ* fusion was expressed in infection zone II and in the interzone II-III of young nodules (Fig. 3F) while, in mature nodules, a blue staining was observed in bacteria of infection zone II and in some bacteria at the extreme limit of zone IV (Fig. 3I).

Symbiotic phenotype of a *katB katC* double mutant.

During the first steps of root infection by *S. meliloti*, it appears that both *katB* and *katC* are expressed in the infection threads. Single null mutation of *katB* or *katC* had little effect on bacteria growth, root nodulation, or nitrogen fixation. To in-

Fig. 3. Histochemical localization of bacterial *kat* genes expression during *Sinorhizobium meliloti*–*Medicago sativa* nodule development. β-galactosidase activity was detected using X-Gal as substrate for **A, D, and G**, *katA::lacZ*; **B, E, and H**, *katB::lacZ*; and **C, F, and I**, *katC::lacZ* fusions in hair roots four days after infection (**A through C**); in 7-day-old nodules (**D through F**); and in 6-week-old nodules (**G through I**). β-galactosidase staining of a 6-week-old nodule infected by **J**, control strain GM1211. Arrows indicate infection threads. Spatial development zones (I, II, III, or IV) are indicated on nodule cross sections. Scale bars = 50 μm (**A through C**), 100 μm (**D through F**), and 200 μm (**G through J**).

investigate the combined role of these two catalases, a double mutant strain *katB katC* (named MK5004) was constructed by conjugation, using the *katC* mutant as the recipient strain as described below. Disruption of the *katB* gene was confirmed by Southern analysis of the double mutant (data not shown). In free-living conditions, the growth of the MK5004 strain was slightly affected in LB-MC; doubling time was about 1.4-fold longer, but maximal growth was the same after 50 h (data not shown). The total catalase activity of MK5004 (7.8 ± 2.4 U per mg of proteins) in exponential phase was threefold lower than in control strain Rm5000 (24.2 ± 5.6 U per mg of proteins) and analysis on native gel confirmed the absence of KatB and KatC activities (data not shown).

Only 45% of plants infected with MK5004 produced nodules. These nodules were white and 75% of them were small and spherical. In contrast, a high efficiency of nodulation (70 to 90%) was observed with the wild-type strain Rm5000 and the single *katB* or *katC* mutants. Moreover, the average number of nodules per nodulated plant decreased from 2.75 (Rm5000) to 1.76 (MK5004). Acetylene reduction activity was determined in nodulated *M. sativa* 5 weeks after bacterial infection. A drastic decrease in C_2H_2 reduction was observed for the *katB katC* double mutant (3.3 ± 0.9 nmole C_2H_4 per min per mg of FW) as compared with strain Rm5000 (65.8 ± 24.5 nmole C_2H_4 per min per mg of FW). Moreover, this decrease is far greater than that observed for the *katA katC* double mutant (Sigaud et al. 1999).

Bacteroid differentiation in nodules from *katB katC* and *katA katC* double mutants.

Alfalfa nodules from plants infected with MK5003 (*katA⁻ katC⁻*), MK5004 (*katB⁻ katC⁻*) and Rm5000 (wt) were studied at structural and ultrastructural levels by optical and electronic microscopy. In nodules obtained after infection with Rm5000, the five typical steps in bacteroid development (type 1 to 5) described by Vasse and colleagues (1990) were observed. The meristematic zone I is bacteria-free. Bacteria released from infection threads in plant cells are surrounded by a peribacteroid membrane in the distal infection zone II (bacteroid of type 1). Bacteroids elongated without dividing in the proximal infection zone II (bacteroid of type 2). In interzone II-III, bacteroids stopped elongating and displayed cytoplasmic heterogeneity (bacteroid of type 3). They showed a higher cytoplasmic heterogeneity and differentiated into a type 4 nitrogen-fixing bacteroid in infected cells of zone III. (Fig. 4A and B). Both symbionts degenerated in the senescent zone IV. Undifferentiated bacteria reinfected plant cells but did not differentiate into bacteroids in the saprophytic zone V (Timmers et al. 2000). The differentiation of bacteroids was blocked at different steps in nodules infected by MK5003 or MK5004.

When compared with control Rm5000 nodules, a larger interzone II-III containing typical amyloplast-rich cells (Fig. 4C) was observed in MK5003 nodules. No clear zone III was observed, and the senescent zone IV appeared to be adjacent to interzone II-III. Ultrastructural studies showed that, in most plant cells, bacteria were correctly released from infection threads and they differentiated into type 2 and type 3 bacteroids (Fig. 4D). However, bacteroids were more sparsely distributed in MK5003-infected cells than in Rm5000-infected cells, and some bacteroids were irregular in shape (Fig. 4D). In a few cells, bacteroids of type 4 were observed confirming the fix⁺ phenotype of the MK5003 strain (Sigaud et al. 1999). Moreover, in some infected cells of the interzone II-III, some bacteroids of type 1 directly underwent senescence and contained high amounts of polyhydroxybutyrate (Fig. 4E).

In MK5004 nodules, all plant cells were free of bacteroids despite the presence of many infection threads (Fig. 4F). A large number of amyloplasts were observed (Fig. 4G). Bacteria

were released from infection threads without peribacteroid membrane and directly underwent senescence accumulating high amount of polyhydroxybutyrate (Fig. 4H).

DISCUSSION

Production of reactive oxygen species appears to be a general response of plants to biotic and abiotic stress (Dat et al. 2000). In the case of alfalfa-*Sinorhizobium meliloti* symbiosis, superoxide radicals and hydrogen peroxide have been detected, especially in some infection threads (Santos et al. 2001) but never inside bacteria and bacteroids during the development of the root nodule. This suggests the presence of a very efficient bacterial H_2O_2 -scavenging defense.

Unlike *E. coli* and *Botrytis subtilis*, which have been shown to contain two catalase isozymes, *S. meliloti* clearly expresses three genes encoding one bifunctional catalase-peroxidase KatB and two monofunctional catalases KatA and KatC. The *katB* gene was cloned using a nested-PCR strategy followed by a genomic bank screening. This gene encodes a protein that shows clear homology to the group II of bacterial catalase-peroxidase (Loewen et al. 2000). Analysis using a recombinant strain harboring a *katB::lacZ* fusion showed no significant variation of *katB* gene expression during growth of bacteria in rich medium. The noninducibility of *katB* in bacteria submitted to oxidative stress conditions tested in this paper is another major argument in support of the idea that KatB can be considered as a housekeeping bifunctional catalase-peroxidase enzyme in *S. meliloti*. A lower KatB activity is detectable in Rm1021 as compared with Rm5000 and GMI211 strains. It will be interesting to study whether there is a relationship between this phenotype and the substitution of the highly conserved Met364 by an arginine in the Rm1021 catalase-peroxidase sequence. Histochemical detection of β -galactosidase activity during nodule development revealed that *katB* is constitutively expressed in bacteria in infection threads, during infection of plant cells in zone II, in nitrogen-fixing bacteroids of zone III, and during senescence of the nodule. However, KatB does not seem to be essential to the symbiotic process, since efficient nitrogen-fixing nodules developed on roots of plants infected with the null *katB* mutant. A large divergence in the structure of the *katB* allele is observed between *S. meliloti* and *M. loti*, the two rhizobacteria for which the complete genome is available. In *M. loti*, the *katB*-homologous gene is localized on the chromosome and divergently arranged from an *oxyR*-like gene, which is known to encode the peroxide-sensing transcription factor essential for the induction of *katG* encoding the HPI bifunctional catalase-peroxidase in Enterobacteria (Zheng and Storz 2000). In the *S. meliloti* genome, only one *oxyR*-like gene is present and is localized on the chromosome divergently upstream of *katA* and not upstream of *katB*. This could explain why only *katA* is inducible by exogenous H_2O_2 in *S. meliloti*.

The *katA* transcription is largely induced during exponential phase. This expression pattern in free-living cells is similar to that of *katG* in *E. coli*, suggesting that a significant amount of the homeostatic control of intracellular H_2O_2 concentration during exponential growth seems to be accomplished by the transcriptional regulation of *katA* in *S. meliloti*. *KatA* is also the major catalase gene expressed in bacteroids, indicating that the nitrogen-fixation process induces a strong oxidative stress, probably due to the high rate of bacteroid respiration and to the stringent conditions required to reduce nitrogen. However, we cannot exclude the possibility that the protective role of *katA* activation in bacteroids is a population-wide phenomenon rather than one restricted to cells occasionally undergoing more intense oxidative stress, as has been demonstrated for OxyR activation in *E. coli* (Gonzalez-Flecha and Demple 1997). Two

potential NifA and OxyR binding sites are present upstream of *kata*, suggesting that *kata* could be regulated at least by these two regulators. Unexpectedly, no detection of the expression of *kata::lacZ* fusion was observed in bacteria inside the infection

threads, despite the detection of H_2O_2 all around the bacteria in some infection threads (Santos et al. 2001). This could suggest that the accumulation of the H_2O_2 levels in infection threads is not sufficient to induce the transcription of *kata*. However, fol-

Fig. 4. Structure and ultrastructure of 5-week-old alfalfa nodules. Nodules were incited by **A and B**, Rm5000; **C, D, and E**, MK5003; and **F, G, and H**, MK5004 bacteria strains. Studies were performed at structural (**A, C, and F**) and ultrastructural (**B, D, E, G, and H**) levels. a = amyloplast; cw = cell wall; it = infection thread; and v = vacuole. Arrows indicate peribacteroid membranes and * indicate polyhydroxybutyrate white granules (**E** and **H**). Scale bars = 20 μ m (**A, C, and F**) and 1 μ m (**B, D, E, G, and H**).

lowing treatment of root hairs showing infection threads with different concentrations of exogenous H₂O₂, no induction of *katA* was observed that is comparable to our results on free-living bacteria in stationary growth. On the other hand, our analysis using *katC::lacZ* fusion showed that *katC* is expressed not only in late exponential and stationary phases in batch culture but also in all infection threads. Moreover, the analysis of the bacterial growth rate inside the infection thread using a *S. meliloti* strain that constitutively expresses a variant of green fluorescent protein indicated a bacterial doubling time of about 11 h, which may reflect slower, or nonexponential, growth (Gage et al. 1996). All these results indicate that bacteria inside the infection thread are in a similar physiological state to free cells in stationary phase. The limitation of the bacterial growth in the infection threads could be due to peroxide-driven cross-linking of root nodule extensins as recently proposed (Rathbun et al. 2002; Wisniewski et al. 2000). Finally, *katC* is also expressed in vegetative bacteria released in the cells of the highly senescent zone IV of mature 6-week-old nodules, which has been recently designated as an additional zone V (Timmers et al. 2000). Moreover, the absence of *katA* expression in zone V correlated with the fact that intracellular Rhizobia in zone V do not appear to fix nitrogen (Timmers et al. 2000).

Our results show that the catalase activity profiles during growth of free-living wild-type bacteria and after differentiation into bacteroids are correlated with the expression of the reporter gene *lacZ* in each *kat-lacZ* strain, suggesting that all catalases are mainly regulated at the transcriptional level in *S. meliloti*. However, it can not be excluded that the disruption of each *kat* gene, by insertion of promoterless *lacZ* gene, could lead to an artificial over-induction of the reporter gene by increasing oxidative stress in recombinant strains.

Does a relationship exist between the in planta expression profiles of each *kat* gene and the phenotype of recombinant *kat* mutants? All simple *kat* null mutants in different genetic backgrounds show a nod⁺ and fix⁺ phenotype. This could be due to the fact that, in each different region of nodules, at least two *kat* genes are expressed. In contrast, the disruption of two *kat* genes induces numerous problems during the nodulation or nitrogen fixation process, or both. Only 75 and 45% of the plants inoculated with the *katA katC* (MK5003) and *katB katC* (MK5004) double mutant strains, respectively, showed a nodulating phenotype. A decrease in the nitrogen fixation capacity was observed for MK5003-nodulated plants (Sigaud et al. 1999). This phenotype is probably due to a very bad differentiation of bacteroids into a type 4 nitrogen-fixing form in infected cells. A more drastic decrease in nitrogen fixation capacity was observed for the MK5004 mutant since the bacteria are not correctly released into plant cells, showed no peribacteroid membrane, and did not differentiate into bacteroids, indicating that the expression of *katB* and *katC* genes are essential for an efficient infection of plant cells. Compared with many mutants already described (Oke and Long 1999), the *katB katC* double mutant shows some similarities with the *BacA* mutant strain (Glazebrook et al. 1993). As yet, the effects of the disruption of *katA* and *katB* can not be analyzed, since the construction of this double mutant by transduction or conjugation has failed so far. This could indicate that both genes are probably essential for survival of bacteria on LB plates, since only these two *kat* genes are expressed during the exponential growth phase.

All these results indicate that sophisticated regulation of the *kat* genes occurs not only in batch culture, as for many other bacteria, but also during the establishment and the maintenance of functional symbiotic root nodules in alfalfa. Our studies on

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	References
Strains		
<i>Sinorhizobium meliloti</i>		
RCR2011	SU47, wild type, Nod ⁺ Fix ⁺	Rosenberg et al. 1981
Rm1021	Derivative of RCR2011, Sm ^r	S. Long
Rm5000	SU47, <i>rif</i> ^r -5	Finan et al. 1984
GMI211	Derivative of RCR2011, <i>lac</i> , Sm ^r	Niel et al. 1977
GKAZ01	Same as GMI211 but with <i>pkatA-lacZ</i> fusion	This study
GKBZ01	Same as GMI211 but with <i>pkatB-lacZ</i> fusion	This study
GKCZ01	Same as GMI211 but with <i>pkatC-lacZ</i> fusion	This study
MK5002	Same as Rm5000 but with <i>katC::Ω</i> Sm ^r /Sp ^r	Sigaud et al. 1999
MK5003	Same as MK5002 but with <i>katA::Tn5</i>	Sigaud et al. 1999
MK5004	Same as MK5002 but with <i>pkatB-lacZ</i> fusion	This study
<i>Escherichia coli</i>		
DH5α	<i>F supE44 ΔlacU169(Φ80dlacZDM15) hsdR17(r_K⁻m_K⁺) recA1 endA1 gyrA96 thi-1 relA1</i>	Bethesda Research Laboratories
MT607	<i>pro-82 thi-1 hsdR17 supE44 recA56</i>	Finan et al. 1986
MT616	MT607 (pRK600)	Finan et al. 1986
Plasmids		
pLAFR1	IncP1 cosmid cloning vector Tc ^r	Friedman et al. 1982
pRK600	ColE1 replicon with RK2 transfer region Cm ^r	Finan et al. 1986
pBluescriptKS(+)	Derivative of pUC19 with fl(+) <i>ori</i> R, Ap ^r	Stratagene, La Jolla, CA. U.S.A.
pGEM-T	T vector with fl <i>ori</i> R, Ap ^r	Promega Corp.
pSUP202	ColE1, Mob ⁺ , Tc ^r Ap ^r Cm ^r	Simon et al. 1983
pKOK5	Ap ^r Km ^r , pSUP202 derivative; source of <i>lacZ</i> -Km ^r	Kokotek and Lotz 1989
pLRK3	pLAFR1, <i>S. meliloti</i> cosmid with <i>katB</i>	This study
pLRK4	pLAFR1, <i>S. meliloti</i> cosmid with <i>katB</i>	This study
pBSKB1	pBluescript, 3.0-kb <i>Sma</i> I- <i>Apa</i> I fragment with <i>katB</i>	This study
pBSKB2	pBluescript, 2.4-kb <i>Eco</i> RV- <i>Apa</i> I fragment with <i>katB</i>	This study
pGEMKB	pGEM-T, with 581-bp PCR-amplified fragment of <i>katB</i>	This study
pBSKBZ	Same as pBSKB1 but with <i>katB::lacZ</i>	This study
pSUPKBZ	pSUP202, <i>Bam</i> HI- <i>Bam</i> HI fragment of pBSKBZ	This study
Complementation <i>katB</i> -		
pBSKA1-1	pBluescript, 1.4-kb <i>Eco</i> RI- <i>Apa</i> I fragment with partial <i>katA</i>	Hérouart et al. 1996
pBSKAZ	Same as pBSKA1-1 but with <i>katA::lacZ</i>	This study
pSUPKAZ	pSUP202, <i>Pst</i> I- <i>Pst</i> I fragment of pBSKAZ	This study

^a Abbreviations: Tc, tetracycline; Sm, streptomycin; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; and Sp, spectinomycin.

the *katB katC* double mutant strain confirm the critical protective role of these two catalases during the infection process. We further propose that these genes could also be used as markers to evaluate the physiological response of bacteroids in nodulated plants submitted to different environmental conditions leading to oxidative stress.

MATERIALS AND METHODS

Bacteria strains and growth conditions.

The bacterial strains and plasmids used are listed in Table 1. *Sinorhizobium meliloti* was grown in LB-MC medium as described previously (Hérouart et al. 1996). Antibiotics are added as required at the following concentration ($\mu\text{g per ml}$): ampicillin (100), chloramphenicol (30), kanamycin (50), neomycin (200), spectinomycin (100), streptomycin (100), and tetracycline (10).

General molecular techniques.

The molecular cloning techniques, gel electrophoresis, and small-scale preparation of total DNA from *S. meliloti* were essentially performed according to Ausubel and associates (1993). Plasmid DNA and DNA fragments were purified by using Qiagen kit and Quiaex kit, respectively (Qiagen, Courtaboeuf, France). Sequence alignments and analysis were performed using bioinformatics tools of NCBI and INFOBIOGEN servers.

Cloning of the *S. meliloti katB*.

To perform nested-PCR, three different homology boxes were chosen in the consensus sequence: peptide 1 (IYVNP-EG), peptide 2 (MNDEETVA), and peptide 3 (HRDMGP). The three degenerated oligonucleotides *katB1* (5'ATHTAYGTNAA-YCCNGARGG), *katB2* (5'GCNRCNGTYTCNWCRTCTRY-CAT), and *katB3* (5'GGNCCCATRTCNCKRTG) corresponding respectively to peptides 1 (IYVNP-EG), 2 (MNDEETVA), and 3 (HRDMGP) were synthesized. A first PCR with *katB1* and *katB3* primers was performed on *S. meliloti* total DNA using the protocol previously described (Hérouart et al. 1996). The size of the amplified fragment was 581 bp, which corresponded to the predicted size from the sequence alignment. Using this fragment as a template, a second PCR using *katB1* and *katB2* produced a 108-bp fragment, as expected. The PCR-amplified 581-bp fragment (*SmkatB*) was subcloned into pGEM-T vector, sequenced, and ^{32}P -labeled with the prime-a-gene labeling system (Promega, Charbonnières, France). Screening of 3,000 colonies of the genomic cosmid library was performed as described previously (Hérouart et al. 1996). Two positive clones (pLRK3 and pLRK4) carried an insert of 22 kb exhibiting the same restriction map. Southern blotting and deletion analysis of the positive cosmid pLRK3 allowed the localization of *katB* on a 3-kb *SmaI-ApaI* DNA fragment (subclone pBSKB1).

Construction of a *katB* mutant strain (GKBZ01).

Plasmid pBSKB2 carries the 2.4-kb *EcoRI-ApaI* fragment purified from pBSKB1 and subcloned into pBluescript KS (+). The coding region of *katB* was disrupted by insertion of a promoterless *lacZ-Km^r* cartridge, allowing also the construction of a *katB-lacZ* fusion. A *BamHI-BamHI* fragment containing the cartridge was purified from plasmid pKOK5 and inserted in the *BglII* site of pBSKB2, giving the pBSKBZ plasmid. Using the two *BamHI* sites, which were present in the polylinker of the vector and in the *katB* gene, the *katB-lacZ* fusion was cloned at the unique *BamHI* of the vector pSUP202, resulting in the pSUPKBZ plasmid. The recombinant suicide plasmid was transferred to the GMI211 recipient strain by triparental mating

using the MT616 *E. coli* strain as a helper, as described previously (Glazebrook and Walker 1991). Simple recombinant clones were selected on LB-MC medium containing streptomycin, neomycin, and X-gal. Recombination at the correct location was checked by Southern hybridizations using the *EcoRI-BamHI* fragment of the *katB* gene as a probe. One *S. meliloti* clone, noted GKBZ01, was selected for further studies.

Construction of the *katA::lacZ* fusion (strain GKAZ01).

To construct a transcriptional *lacZ* fusion to the *katA* promoter, the plasmid pKOK5 DNA was digested with *PstI* and the *lacZ-Km^r* cartridge was inserted in the *NsiI* site of pBSKA1-1, which has been shown to carry partial *katA* (Hérouart et al. 1996). The *PstI-PstI* fragment of the recombinant plasmid harboring the correct *katA::lacZ* orientation was inserted in the unique *PstI* site of pSUP202. After triparental conjugation using GMI211 as recipient, simple recombinant clones were selected on LB-MC medium containing tetracycline, neomycin, and X-Gal. After Southern checking of a correct recombination, one *S. meliloti* clone, noted GKAZ01, was selected for further studies.

Construction of the *katB katC* double mutant (strain MK5004).

To construct the *katB katC* double mutant, the suicide plasmid pSUPKBZ was transferred to the *katC* mutant MK5002 recipient strain (Sigaud et al. 1999) by triparental mating using the MT616 *E. coli* strain as a helper, as described previously (Glazebrook and Walker 1991). Simple recombinant clones were selected on LB-MC medium containing streptomycin, spectinomycin, and neomycin. Recombination was verified by Southern hybridizations with the *EcoRI-BamHI* fragment of the *katB* gene as probe, and one *S. meliloti* clone, noted MK5004, was selected for further studies.

Plant nodulation assays.

Medicago sativa L. var. Europe (alfalfa) was used as host plant to test nodulation and nitrogen fixation of *S. meliloti* strains. Surface-sterilized germinating seedlings were grown in test tubes and were inoculated with the appropriate strain by using the protocol described previously (Hérouart et al. 1996). Nitrogenase activity was determined by acetylene reduction using a gas chromatograph as described by Rigaud (1976).

Enzyme activity assays and oxidative stress conditions.

Bacterial crude extracts were prepared from free-living bacteria by sonication in a phosphate buffer (Hérouart et al. 1996). Total catalase activity was measured spectrophotometrically by following the decomposition of H_2O_2 at 240 nm (Jones 1982). Catalase was visualized on nondenaturing polyacrylamide gels by inhibition of diaminobenzidine oxidation, as previously described (Clare et al. 1984; Hérouart et al. 1996). Protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford 1976). β -galactosidase activity in crude extracts of bacteria and nodules was carried out using o-nitrophenyl- β -D-galactoside as the substrate, according to Miller's method (Miller 1972). Histochemical detection of β -galactosidase activity in entire roots and in cross sections was performed as described previously (Boivin et al. 1990). Sections of nodules (150 μM) were cut with a vibratome 1000 Plus (Labonord, Templemars, France) and were mounted on slides for observation and photography with an Olympus microscope. To evaluate the effect of different oxidative stresses on the regulation of catalase gene expression in GKAZ01, GKBZ01, and GKCZ01 strains, bacteria have been inoculated at optical density at 600 nm (OD_{600}) = 0.05 in LB-MC medium, grown overnight until OD_{600} = 0.4,

and submitted for 1 h to heat stress, salt stress, ethanol, paraquat, or H₂O₂. Values were obtained by using triplicate samples.

Electron microscopy studies.

For electron microscopy, nodules were harvested 5 weeks after inoculation. At least five nodules from different plants were fixed, post-fixed, dehydrated, and embedded, as described previously (Santos et al. 2000). Sections were performed with a diamond knife in a Reichert Ultracut Ultramicrotome (Leica, Rueil-Malmaison, France). After staining with uranyl acetate and Reynold's lead citrate, ultrathin sections were examined in a transmission electron microscope (CM12; Philips, Cambridge, U.K.).

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