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Cloning and Characterization of the *katA* Gene of *Rhizobium meliloti* Encoding a Hydrogen Peroxide-Inducible Catalase

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To investigate the involvement of bacterial catalases of the symbiotic gram-negative bacterium *Rhizobium meliloti* in the development of *Medicago-Rhizobium* functional nodules, we cloned a putative *kat* gene by screening a cosmid library with a catalase-specific DNA probe amplified by PCR from the *R. meliloti* genome. Nucleotide sequence analysis of a 1.8-kb DNA fragment revealed an open reading frame, called *katA*, encoding a peptide of 562 amino acid residues with a calculated molecular mass of 62.9 kDa. The predicted amino acid sequence showed a high homology with the primary structure of monofunctional catalases from eucaryotes and procaryotes. The *katA* gene was localized on the chromosome, and the *katA* gene product was essentially found in the periplasmic space. A *katA*::Tn*5* mutant was obtained and showed a drastic sensitivity to hydrogen peroxide, indicating an essential protective role of KatA. However, neither Nod nor Fix phenotypes were impaired in the mutant, suggesting that KatA is not essential for nodulation and establishment of nitrogen fixation. Exposure to a sublethal concentration of H_2O_2 enhanced KatA activity (100-fold) and also increased survival to subsequent H_2O_2 exposure at higher concentrations. No protection is observed in *katA*::Tn*5*, indicating that KatA is the major component of an adaptive response.

Reactive oxygen species such as superoxide radical, hydrogen peroxide, and hydroxyl radical naturally arise during normal metabolism in aerobically growing cells as a result of the incomplete reduction of molecular oxygen. These species can damage lipids, proteins and DNA and are probably involved in some degenerative processes in living cells (23). In this framework, there is increasing evidence that oxygen-derived species play an important role in *Rhizobium*-legume symbiosis, at least during the senescence period. This symbiotic association leads to the formation of nodules where a high degree of interaction between the host cell and the microbial symbiont is found (35). The symbiosis is mainly characterized by its ability to fix atmospheric nitrogen, and the key enzyme of this process, the nitrogenase located in the microsymbiont, is rapidly and irreversibly inactivated by oxygen; the possible role of oxygen-derived species in this inactivation is still an open question (40). Nodules have a high potential to produce damaging oxygen-derived species such as hydrogen peroxide because of the strong reducing conditions required for nitrogen fixation and the action of several proteins, including ferredoxin, uricase, hydrogenase, and leghemoglobin (12). This hemoprotein, present in large amounts in legume nodules, is itself subject to an autoxidation process, generating superoxide anion and hydrogen peroxide (41). Furthermore, hydrogen peroxide has been shown to react with leghemoglobin to generate further damaging species (13), and significant amounts of hydroxyl radicals have been found in senescing nodules (3). This can be related to the high concentration of catalytic iron, which is able to catalyze the hydroxyl radical formation from hydrogen peroxide via the Fenton reaction in the cytosol of these nodules (3). Thus, hydrogen peroxide removal appears essential in functioning nodules. Dalton et al. (12) showed that an efficient ascorbate-glutathione cycle, which can adjust to various physiological conditions, is present in the host cell cytosol and that there is a key link between nitrogen fixation and this detoxification process (11).

Another possible process of hydrogen peroxide detoxification can be provided by catalase, which catalyzes the conversion of hydrogen peroxide into water and oxygen. Catalases occur in both the plant cytosol and microsymbiont (20) and appear to represent a well-conserved group of proteins (53) in spite of the existence of variant forms. For example, *Escherichia coli*, the only gram-negative bacterium whose catalase has been studied in depth, produces two species of hydroperoxidases (HPI and HPII) whose synthesis is genetically controlled by at least four noncontiguous loci (*katE*, *rpoS*, *katG*, and *oxyR*). HPI, encoded by *katG* (49), is a bifunctional enzyme, having both catalase and peroxidase activities which are dependent on the oxidative stress response (*oxyR*) regulon (8). HPII, encoded by *katE* (54), possesses only catalase activity and is maximally synthesized as cells enter the stationary phase of growth (34) and undergo osmotic changes (27). The expression of HPII requires a functional *rpoS* (*katF*) gene (for a review, see reference 33) which has been shown to also be a regulator of *katG* transcription in an *oxyR*-independent manner (26).

Little is known about the catalases in the family *Rhizobiaceae*. It has recently been reported that *Rhizobium leguminosarum* bv. phaseoli responds to H_2O_2-mediated oxidative stress by a modest induction of catalase activity (10). Catalase showed a cell density-dependent regulation controlled by the accumulation of extracellular components in the growth medium (10). Moreover, the catalase activity of a *Rhizobium japonicum* strain collected from effective soybean nodules was greater than that in a strain from the ineffective root structures (20).

In this paper, we describe the characterization of a chromo-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
R. meliloti		
RCR2011	SU47, wild type	J. Dénarié
Rm5000	*rif-5*	16
MK5001	Same as Rm5000 but with *katA*::Tn*5*	This work
A. tumefaciens		
GMI9023	C58 cured of pAtC58 and pTiC58	44
At125	GMI9023 pRmeSU47b	17
At128	GMI9023 pRmeSU47a	17
B. japonicum		
USDA110	*B. japonicum*, wild type	31
E. coli		
XL1-Blue MRF′ Kan	Δ(*mcrA*) *183*Δ(*mcrCB-hsdSMR-mrr*) *173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F′*proAB lacI*^{q} *Z*Δ M15 Tn*5* (Kan^r)]	Stratagene
DH5α	F$^-$ *supE44* Δ*lacU169*(ϕ80*dlacZ*ΔM15) *hsdR17*($r_K^-m_K^+$) *recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
HB101	F′ *hsdS20*($r_B^-m_B^-$ *recA13*(Sm) *ara14 proA2 lacY1 galK2 leuB6 xyl5 mtl1 supE44 rpsL20*	Bethesda Research Laboratories
MT607	*pro-82 thi-1 hsdR17 supE44 recA56*	17
MT609	*thyA36 polA1* Sp^r, recipient for Tn*5* mutagenesis of IncP1 plasmids	51
MT614	MT607ΩTn*5*	56
MT616	MT607(pRK600)	17
Plasmids		
pLAFR1	IncP1 cosmid cloning vector Tc^r	21
pRK600	ColE1 replicon with RK2 transfer region Cm^r	17
pPH1JI	IncP1 Gm^r Sp^r Cm^r	4
pBluescriptKS(+)	Derivative of pUC19 with f1(+)*oriR*, Ap^r	Stratagene
pPCR-ScriptSK(+)	Derivative of pBluescriptSK(+) with SrfI site, Ap^r	Stratagene
pLRK1	pLAFR1, *R. meliloti* cosmid clone with *katA*	This work
pLRK13	Same as pLRK1 but with *katA*::Tn*5*	This work
pLRK14	Same as pLRK1 but with *katA*::Tn*5*	This work
pLRK16	Same as pLRK1 but with *katA*::Tn*5*	This work
pBSKA1-1	pBluescript, 1.4-kb *Eco*RI-*Apa*I fragment with partial *katA*	This work
pBSKA1-2	pBluescript, 442-bp *Pst*I-*Apa*I fragment with partial *katA*	This work

[a] Abbreviations: Tc, tetracycline; Sm, streptomycin; Ap, ampicillin; Kan, kanamycin; Cm, chloramphenicol; Gm, gentamicin; Sp, spectinomycin.

somal *katA* gene from *R. meliloti*, encoding the monofunctional hydrogen peroxide-inducible catalase KatA. The sensitivity to H_2O_2 and the ability to establish a functional symbiosis for a *katA*::Tn*5* mutant are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and transposon. Bacterial strains, plasmids, and the transposon used in this study are listed in Table 1.

Media, antibiotics, and growth conditions. *E. coli* was maintained and grown at 37°C in Luria Bertani (LB) medium (yeast extract, 5 g/liter; tryptone, 10 g/liter; NaCl, 10 g/liter). *R. meliloti* was grown in LB-MC, which is LB medium containing 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$. Thymine (60 μg/ml), ampicillin (100 μg/ml), rifampin (20 μg/ml), spectinomycin (100 μg/ml), tetracycline (10 μg/ml), gentamicin (70 μg/ml), chloramphenicol (30 μg/ml), neomycin (200 μg/ml), and kanamycin (50 μg/ml) were added to the medium as needed.

DNA preparation, screening of library, and sequencing. Total DNA from *Rhizobium meliloti* was isolated according to the method of Ausubel et al. (1). Restriction endonuclease digestions and analyses, ligations, transformations in DH5α, plasmid DNA isolations, and Southern transfer were performed by standard methods (46). Probes were ^{32}P labeled with the Prime-a-gene labeling system (Promega, France). Screening of the genomic library, constructed in pLAFR1 (21), was essentially performed according to standard procedures (1). A filter containing 2×10^3 bacterial clones was hybridized overnight with the probe in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.05% sodium pyrophosphate at 60°C. Subsequently, unbound probe was removed by washing the filters four times for 30 min in 6× SSC–0.5% sodium dodecyl sulfate at 60°C. The filter was autoradiographed on Kodak XAR-5 film with intensifying screens. Southern analysis of genomic DNA was performed with similar hybridization and washing conditions. The nucleotide sequences of both strands of the *katA* coding region were determined by the dideoxy chain termination method (47) according to the U.S. Biochemicals protocol for the Sequenase 2.0 enzyme with α-^{35}S-dATP (ICN, Orsay, France). Sequence data were analyzed with the University of Wisconsin Genetics Computer Group program library (15).

Amplification of DNA by PCR. PCR mixtures contained 100 pmol of each primer; 300 ng of genomic DNA or 100 ng of amplified DNA; 200 mM (each) dATP, dTTP, dGTP, and dCTP; 1× *Taq* polymerase buffer (Appligen, Illkirch, France); and 1 U of *Taq* DNA polymerase (Appligen) in a final volume of 100 μl. Samples were overlaid with mineral oil. Reaction mixtures were cycled automatically with a PHC-3 Thermal Cycler (Techne-Cambridge Ltd., Cambridge, United Kingdom) through temperature-time cycles as follows: denaturation, 95°C for 1 min; annealing, 42°C for 1 min; extension, 72°C for 1 min. Denaturation time of the first cycle was prolonged to 4 min to ensure a single-stranded template for the PCR, and the final extension time was increased to 5 min to ensure completion of strand synthesis. Ten microliters of reaction mixture was analyzed by electrophoresis on 1.5% agarose gels. PCR-amplified DNA was excised and recovered by using the GeneClean II kit (Bio 101, Inc., La Jolla, Calif.).

Transposon mutagenesis of the *R. meliloti katA* locus. For Tn*5* insertion in the *katA* coding region, the MT614 *E. coli* strain carrying the Tn*5* transposon on the chromosome was transformed with the plasmid pLRK1. Recombinant plasmids were transferred to the MT609 *E. coli* recipient strain by triparental mating, using the MT616 *E. coli* strain as a helper according to the protocol previously described (22). The recipient strains containing the recombinant plasmid were selected by growth on medium containing tetracycline, spectinomycin, and thymine. Plasmid DNA was prepared from 48 different clones. Restriction analyses of plasmid DNA revealed that three different cosmids (pLRK13, pLRK14, and pLRK16) carried an insertion of the Tn*5* transposon in the 1.4-kb *Apa*I-*Eco*RI fragment corresponding to the coding region. For genomic recombination, these three tetracycline-resistant IncP1 group plasmids were introduced into the rifampin-resistant SU47 derivative Rm5000 by triparental mating, using the MT609 *E. coli* strain as a helper. The plasmid incompatibility technique (45) was then used to detect strains in which the insertion had recombined from the plasmid to the *R. meliloti* genome. Recombinants carrying Tn*5* on the *katA* gene (MK5001) were selected for the gentamicin- and spectinomycin-resistant plasmid pH1JI (4).

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Bacterial extract preparation and biochemical assays. Cell crude extracts were prepared from saturated cultures (100 ml) harvested by centrifugation at 7,000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 1 ml of ice-cold phosphate buffer (50 mM KH_2PO_4, 0.1 mM EDTA) and sonicated on ice (six times during 30 s, one pulse per s). The sonicated extract was then clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. Isolation of periplasmic proteins from saturated cultures was performed by using a cold osmotic shock as described previously (32). Protein concentration was determined according to the method of Bradford (6). The amount of malate dehydrogenase, a cytoplasmic enzyme, was measured by the production of NADH monitored at 340 nm in an assay mixture containing 2.5 mM NAD^+, 85 mM L-malate (pH 7.5), and 100 mM glycine (pH 10). One unit of malate dehydrogenase is the amount of enzyme which produces 1 nmol of NADH per min at 25°C. Catalase activity was measured spectrophotometrically by following the decomposition of H_2O_2 at 240 nm, using an extinction value of 43 M^{-1} cm^{-1} (28). One unit of catalase is the amount of enzyme which decomposes 1 μmol of H_2O_2 per min at 25°C. Bacterial extracts were loaded on 8% nondenaturing polyacrylamide gels (Mini-cuve; Bio-Rad) and submitted to electrophoresis at 100 V for 3 h. Catalase and peroxidase activities in polyacrylamide gels were visualized via inhibition of diaminobenzidine oxidation by H_2O_2 in the presence or in the absence of horseradish peroxidase-H_2O_2, respectively (9).

H_2O_2 sensitivity assay and H_2O_2 induction treatment. Cells grown to an optical density at 600 nm of 0.2 in LB-MC medium with shaking at 30°C were challenged with H_2O_2. Aliquots were withdrawn at various times during 6 h, and survival was assessed by plating onto LB plates. Colonies were counted after 3 days of incubation at 30°C. H_2O_2 pretreatments and H_2O_2 induction experiments were performed using a protocol adapted from that of Brown et al. (7); 200 μM H_2O_2 was added five times at 12-min intervals to the exponential culture (1 mM, final concentration).

Nodulation and nitrogen fixation assays. *Medicago sativa* was used as the host plant to test the nodulation and fixation of *R. meliloti* strains. Plants were grown in sterile tubes (2 plantlets per tube) containing 20 ml of an N-free nutrient medium with 0.8% agarose prepared as a slope. The plants were inoculated twice, 1 and 2 weeks after germination, with the appropriate *R. meliloti* strains. The plants were visually screened for nodule formation by observing the root system 4 weeks after the second *Rhizobium* inoculation. Phenotypes of bacteria recovered from nodules were checked on the appropriate media. N_2 fixation activity was determined by C_2H_2 reduction, using a gas chromatograph (ATI-Unicam, model 610) equipped with a column of Porapak T (80/100 mesh) as described by Rigaud (42). Incubations of nodulated plantlets were made, in four replicates, at 25°C, in 80-ml rubber-capped tubes containing O_2 (20 kPa) and C_2H_2 (10 kPa) in argon.

Nucleotide sequence accession number. The DNA sequence of the *R. meliloti katA* gene has been submitted to GenBank and assigned accession number U59271.

FIG. 1. Multiple alignment of the predicted amino acid sequence of an *R. meliloti* PCR fragment amplified between *kat 1* and *kat 3* (rmkatA) with sequences of bovine liver catalase (blc; GenBank accession no. P00432) and procaryotic catalases from the following organisms: *Rhizobium sp.* strain SNU003 (rsp; GenBank accession no. U56239), *Bacillus subtilis* (bsub; GenBank accession no. M80796), *B. abortus* (bruab; GenBank accession no. U11439), *Bacteroïdes fragilis* (bfra; GenBank accession no. U18676), *H. influenzae* (catalase *hktE*) (hiu; GenBank accession no. U02682), *B. pertussis* (borper; GenBank accession no. U07800), *E. coli* (hyperoxidase II) (ecolihpii; GenBank accession no. M55161), and *P. syringae* (*catF*) (psesyr; GenBank accession no. U03465). Identities between the published sequences are shaded in grey. The consensus sequence has been determined, and gaps (dots) have been introduced in protein sequences to optimize the match, using the Genetics Computer Group program. Identities between rmkatA and the consensus sequence are boxed. Amino acids chosen for the synthesis of PCR primers are underlined. The active-site residues (His-74, Ser-113, and Asn-147) and the distal heme site ligands (Val-73, His-74, Asn-147, and Phe-152) of blc are indicated by asterisks and solid circles, respectively.

RESULTS

Cloning of a *kat* gene in *R. meliloti* by nested PCR. The three-dimensional structures of bovine liver catalase (39), *Penicillium vitale* catalase (50), and *Micrococcus lysodeikticus* catalase (38) have been solved and reveal four distinct domains: the N-terminal extended arm, the β-barrel core, the wrapping domain, and the C-terminal external α-helices (18, 19). Alignment of the amino acid sequences of several catalase sequences from different species revealed homology boxes particularly in the C-terminal portion of the extended arm and the N-terminal region of the barrel core (Fig. 1). This permitted the identification of a catalase consensus sequence. A similar high degree of amino acid homology was found during a comparative study of 20 different catalase proteins (53). In order to isolate a *kat* gene from *R. meliloti* using a PCR strategy, three degenerate oligonucleotides, 5′CCIGARMGIGTIGTICA YGC (*kat 1*), 5′ARRTTYTAYACIGARGARGG (*kat 2*), and 5′GTIGGIAAYAAYACICCIGTITTYTT (*kat 3*) derived from amino acids 69 to 75 (PERVVHA), 134 to 140 (KFYT EEG), and 145 to 154 (VGNNTPVFF) of the consensus sequence (Fig. 1), were synthesized and used as primers to amplify DNA from the *R. meliloti* genome. The first PCR with *kat 1* and *kat 3* primers produced a fragment of approximately 250 bp, which corresponded to the expected size from the amplified region of a *kat* gene. Using this 250-bp fragment as a DNA template, a second PCR with *kat 1* and *kat 2* primers produced a fragment of 220 bp as expected. The PCR-amplified 250-bp fragments were ligated into pPCR-ScriptSK(+) and transformed into the *E. coli* XL1-Blue MRF′ Kan strain. Sequencing of the inserts from four clones showed that the 246-bp fragment contained an open reading frame encoding part of a protein which is 70% identical to the catalase consensus sequence (Fig. 1). The insert from one of the plasmids was then radiolabeled and used as a hybridization probe to screen a genomic DNA library of *R. meliloti* (21).

Nucleotide and predicted amino acid sequence analysis of the *katA* gene. Analyses of three positive clones carrying a DNA insert of 22 kb revealed the same restriction map; one of those clones, called pLRK1, was used for further studies. Sequencing and analysis of a 1,944-bp DNA fragment carried by pLRK1 identified one major open reading frame of 562 residues with an ATG start codon corresponding to a protein with an M_r of 62,973 and a pI of 7.0 (Fig. 2). An inverted repeat sequence is present downstream of the stop codon and might serve as a transcription terminator. The ATG was preceded by a potential ribosome binding site located 10 bp upstream. The G+C content (63%) of the putative *katA* gene is similar to that of other genes from *R. meliloti*. A search of the current DNA and protein in databases with the BLAST algorithm (Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, Calif.) revealed significant similarities to large regions of numerous procaryotic and eucaryotic catalases. The highest amino acid identity was found with *Brucella abortus* catalase (71% identity), and paradoxically, catalase sequences of *R. meliloti* and *Rhizobium* sp. strain SNU003 showed only 44% identity. The predicted *katA* gene product also showed high identity to catalases from several

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PstI
5'CTGCAGATCTGCGCCATATTTGCCATAAAA CCGCAATATACGGCACCGATCATAACATCA GAAATCATTACAGCGCGTTACAACTGGATC TTTCAACGCTTCGGCCGGGTCGCCTGAAGC   120
                                SD
ATGAACGCAAAACCGGCGCGCACCACTGCT TCATTGAAACCGTGAGCATCCAAAAAATGC CGCCGCCCCTTCCGGAGCGGCGGCATTTGA TTTGCGCATGTGTAGGAACCTACTGCGGCT   240
                                                          M P   P P L P E R R H L I   C A C V G T Y C G C

GCGGTTCGAACCCGCCTTCAGGCTCTTCGC CCTTCGTGCCGGCTTCCTTCTTCGTACCGG CCGAAGGCACTGCGGAGCCGCGATGCGGCG GTGTATCGTCCCCAAGATCGCGCGCCGGCT   360
23  G S N P P S G S S P   F V P A S F F V P A   E G T A E P R C G G   V S S P R S R A G F
                                                                  EcoRI
TCTCGCCGCGGATCAGAGCCTTGATCTCGT CGCCGGTCAGCGTCTCGTATTCGAGCAAGC CTTCCGCGAGAGCCACGAATTCGCTGACGG CAGGCCCGCGTGGCGGCATCATGCTGCAGG   480
63  S P R I R A L I S S   P V S V S Y S S K P   S A R A T N S L T A   G P R G G I M L Q D

ATTATCAGCTCATCGAGAAGCTGGCGCACC AGAACCGCGAGCGCATTCCGGAACGCGTCG TCCATGCCAAGGGCTGGGGTGCTTTCGGCT CGCTGAAGATCACCGGCGACATCTCGCAAT   600
103 Y Q L I E K L A H Q   N R E R I P E R V V   H A K G W G A F G S   L K I T G D I S Q Y
                                                                  SacII
ACACCAGGGCGAAGTGTCTCCAGCCCGGCG CGGAAACGCCGATGCTGGCCCGCTTCTCGA CCGTTGCCGGCGAACAGGGCGCCGCCGACC ACGAGCGCGACGTCCGCGGTTTCGCGCTGA   720
143 T R A K C L Q P G A   E T P M L A R F S T   V A G E Q G A A D H   E R D V R G F A L K

AATTCTACACCGATGAAGGCAACTGGGACC TCGTCGGCAACAATACTCCGGTCTTCTTCA TTCGCGATCCCTACAAGTTCCCCGACTTCA TCCACACGCAGAAGCGTCATCCGAAGACCA   840
183 F Y T D E G N W D L   V G N N T P V F F I   R D P Y K F P D F I   H T Q K R H P K T N
                                                                  SacII
ATCTCCGTTCCGCCACTGCCATGTGGGACT ACTGGTCCCTGTCGCCGGAAAGCCTGCATC AGGTGACGATCCTGATGTCCGACCGCGGCC TCCCGCAGACGCCGATGCATATGAACGGCT   960
223 L R S A T A M W D Y   W S L S P E S L H Q   V T I L M S D R G L   P Q T P M H M N G Y

ACGGCTCGCACACCTATTCCTTCTGGAACG ACGCGGGCGAACGCTATTGGGTCAAGTTCC ACTTCAAGACCCAGCAGGGCCATAAGTTCT TCACCAACGAGGAAGGCGAGGCGGTCATCG  1080
263 G S H T Y S F W N D   A G E R Y W V K F H   F K T Q Q G H K F F   T N E E G E A V I G
                                                                  SacI
GCAAGACCCGCGAGGGCTATCAGGAGTCAC TGTTCTACGCCATCGAGAACGGCGAGTTCC CGCGCTGGACGGTGCAGGTGCAGATCATGC CAGAGCTCGACGTCGAGAAGACGCCCTACA  1200
303 K T R E G Y Q E S L   F Y A I E N G E F P   R W T V Q V Q I M P   E L D V E K T P Y N

ACCCGTTCGACCTGACCAAGGTCTGGCCGC ATGCCGACTACCCGCCGGTCGAGATCGGCG TGCTGGAGCTGAACCGCAACCCGGAAAACT ATTTTGCCGAGATCGAGAACGCAGCCTTCT  1320
343 P F D L T K V W P H   A D Y P P V E I G V   L E L N R N P E N Y   F A E I E N A A F S

CGCCCTCCAATATCGTGCCCGGCATCGGCT TCTCGCCGGACAAGGTGCTGCAGGCCCGCA TCTTCTCCTATGCGGACGCCCACCGCTACC GCCTCGGCACGCATTACGAGCACATCCCGG  1440
383 P S N I V P G I G F   S P D K V L Q A R I   F S Y A D A H R Y R   L G T H Y E H I P V

TCAACCAGCCGCGCTGCCCGGTTCACCACT ATCATCGCGATGGTCAGATGAACACCTATG GCGGCATTCGCACCGGCAATCCGGACGCCT ATTACGAGCCCAACTCCTTCAATGGCCCGG  1560
423 N Q P R C P V H H Y   H R D G Q M N T Y G   G I R T G N P D A Y   Y E P N S F N G P A

CCGAACAGCCGCTGGCCAAGGAACCGCCGC TCAGAATAGACGGCGACATGTCCCGCTACG ACCACCGCGTCGGCAATGACGACTACGTCC AGGTGCGGGCGCTGTTCGGCCTCTTCGACG  1680
463 E Q P L A K E P P L   R I D G D M S R Y D   H R V G N D D Y V Q   V R A L F G L F D E
                                                SmaI
AAGGGCAGAAGTCGCGCCTCTTCTCCAACA TCGCGGCCGCCATGGGCGGCGTCCCGGGCG AGATCATCGAGCGGCAGCTCATTCATTTCG CCCGTGTCCACCCGGAATACGAAGCGGGCG  1800
503 G Q K S R L F S N I   A A A M G G V P G E   I I E R Q L I H F A   R V H P E Y E A G V
          * * *                                                   ApaI
TCCGCCAGGCACTGAAGACCGCCCACGGCT ACGAGGCGGACACGATCAGCACGGCGGCCG AGTAATCAGGGCCCAATTCCCGACAAGGGC GAAGCCACGCCTTCAGGAAGGCGTGGCCT  1920
543 R Q A L K T A H G Y   E A D T I S T A A E   OC

TCGTCAATTGGAGGAGCCGTTGTT -3'
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FIG. 2. Nucleotide sequence of the 1,944-bp DNA fragment containing the *R. meliloti katA* gene. The deduced amino acid sequence of the *katA* gene product is presented underneath the DNA sequence. The putative Shine-Dalgarno (SD) sequence, the potential terminator hairpin, and the restriction enzyme sites used in this study are underlined. Numbers on the left indicate amino acid positions; those on the right indicate nucleotide positions. The putative histidine active-site residues (His-123, Ser-161, and Asn-195) and the proximal (Pro-383, Arg-401, and Tyr-405) and distal (Val-122, His-123, Asn-195, Phe-200, and Phe-208) heme site ligands are indicated by solid circles. The putative SKL sequence is indicated by asterisks.

other bacteria such as *Bordetella pertussis* (51% identity), *Haemophilus influenzae* (50% identity), and *E. coli* HPII (34% identity) as well as to eucaryotic catalases such as bovine liver catalase (43% identity), human catalase (36% identity), and catalases from *Nicotiana tabacum* (34% identity) and *Arabidopsis thaliana* (32% identity).

Location of the *katA* gene on the *R. meliloti* chromosome. The genome of *R. meliloti* 1021, like those of many *R. meliloti* strains, exhibits three replicons: the chromosome, 3.4 Mb, and two megaplasmids, pSym-b (pRmSU47b), 1.7 Mb, and pSym-a (pRmSU47a), 1.4 Mb (25). In order to determine on which replicon the *katA* gene is located, each plasmid was mobilized into *Agrobacterium tumefaciens* (17), and total DNA from resulting hybrid strains was tested by Southern analysis using as a radiolabeled probe the 1.4-kb *Apa*I-*Eco*RI fragment corresponding to the *katA* coding region (Fig. 3). No significant hybridization of the probe was detected in hybrid strains, indicating that the *katA* gene is located on the chromosome of *R. meliloti* and not on one of the Sym plasmids. Under these stringent conditions, no cross-hybridization was observed with *E. coli*, *A. tumefaciens*, or *Bradyrhizobium japonicum* DNA. However, low hybridization of the probe with a 14-kb *Apa*I-

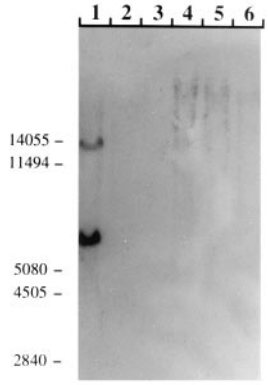

FIG. 3. Chromosomal location of the *R. meliloti katA* gene by Southern analysis. Total DNA of different strains was digested with *Apa*I, transferred onto a nylon membrane after electrophoresis, and probed with the radiolabeled 1.4-kb *Eco*RI-*Apa*I fragment from pBSKA1-1. Lane 1, *R. meliloti* RCR2011; lane 2, *B. japonicum* USDA110; lane 3, *E. coli* HB101; lane 4, *A. tumefaciens* GMI9023; lane 5, *A. tumefaciens* At125; lane 6, *A. tumefaciens* At128 (see Table 1 for relevant characteristics). Numbers on the left indicate sizes of selected lambda markers (in base pairs).

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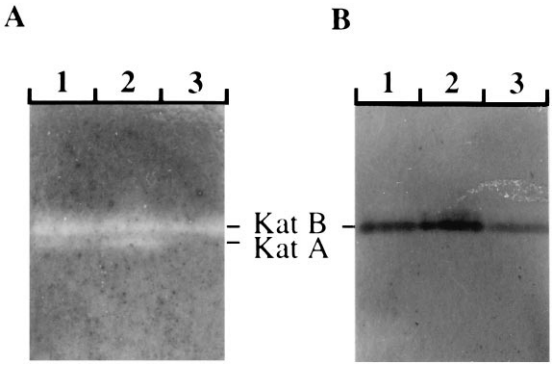

FIG. 4. Effect of a *katA* mutation on the pattern of catalase and peroxidase activities. Catalase activity (A) and peroxidase activity (B) were detected in samples (25 μg of protein) after electrophoresis on native polyacrylamide gels. Lane 1, RCR2011; lane 2, Rm5000; lane 3, MK5001.

*Apa*I fragment of the *R. meliloti* chromosome DNA was detected, suggesting the presence of a second catalase gene homologous to the *katA* gene. These results were confirmed by Southern analysis of *Eco*RI-digested DNA, showing also a pattern of two bands for chromosomal DNA of *R. meliloti* (data not shown).

Transposon mutagenesis of the *katA* gene shows that this gene encodes a catalase. Recombinant Rm5000 strain carrying the Tn*5* transposon on the *katA* gene (MK5001) was constructed as described in Materials and Methods, and the insertion of Tn*5* in the coding region of *katA* was confirmed by Southern analysis of the MK5001 genomic DNA (data not shown). To determine whether the mutation of the *katA* gene resulted in the loss of catalase activity associated with the *katA* gene product, protein extracts of stationary-phase cells from RCR2011 and Rm5000 wild-type strains and MK5001 *katA*::Tn*5* mutant were analyzed for catalase and peroxidase activities on a native polyacrylamide gel (Fig. 4). The enzyme assays revealed that *R. meliloti* showed at least two catalases (KatA and KatB). The lower band (KatA) was only present in wild-type strains, and this corresponds to the *katA* gene product. There was no corresponding peroxidase activity, indicating that KatA is a monofunctional catalase hydroperoxidase enzyme, possessing only catalase activity. In contrast, a peroxidase activity was detected at the level of the second catalase KatB, suggesting a bifunctional hydroperoxidase (Fig. 4B).

Sensitivity of the *katA* mutant to H_2O_2. To assess the contribution of the catalase encoded by *katA* to the H_2O_2 resistance of *R. meliloti*, the survival to various concentrations of H_2O_2 of wild-type Rm5000 was compared with that of mutant MK5001. Up to 2 mM H_2O_2, growth of both strains occurred at a rate similar to that of the untreated culture (data not shown). In the presence of 3 mM H_2O_2, an adaptation period of 3 h was required for the wild-type cells to acquire a survival rate similar to that of the untreated cells (Fig. 5A). In contrast, this characteristic V-shaped survival curve was not observed for the *katA* cells. Moreover, in the presence of 4 mM H_2O_2, mutant MK5001 was rapidly killed, whereas survival of wild-type cells was only slightly reduced (Fig. 5B; 40% wild-type survival versus 0.7% *katA* survival after a 2-h challenge). This implies that the catalase encoded by *katA* is essential for resistance to exogenous H_2O_2.

Induction of KatA and adaptation to H_2O_2. To further investigate the role of KatA in the resistance of *R. meliloti* to H_2O_2, *R. meliloti* strains were exposed to a sublethal dose of H_2O_2 (final concentration, 1 mM). The intensity of the total catalase activity was considerably increased in wild-type Rm5000 (100-fold), whereas no induction by H_2O_2 was observed in mutant MK5001 (Table 2). Analysis of the catalase activity on native gels showed a similar pattern for MK5001 with and without H_2O_2, indicating that the second hydroperoxidase (KatB) is not H_2O_2 inducible (data not shown). Moreover, H_2O_2 pretreatment of wild-type cells with this sublethal concentration (1 mM) allowed a total recovery of survival at 4 mM H_2O_2 (Fig. 6). This acquired resistance to 4 mM H_2O_2 can be blocked in the presence of the protein synthesis inhibitor chloramphenicol, indicating that de novo KatA synthesis is required for adaptation of *R. meliloti* to H_2O_2 (Fig. 6). In contrast, no enhanced protection was observed for mutant MK5001 after pretreatment with 1 mM H_2O_2 (Fig. 6), indicating that KatA is the major component of an adaptive response.

Periplasmic location of KatA. To test the localization of the H_2O_2-induced KatA, periplasmic extracts of Rm5000 with and without H_2O_2 treatment were prepared by the cold osmotic shock method, which is known to preserve the plasmic mem-

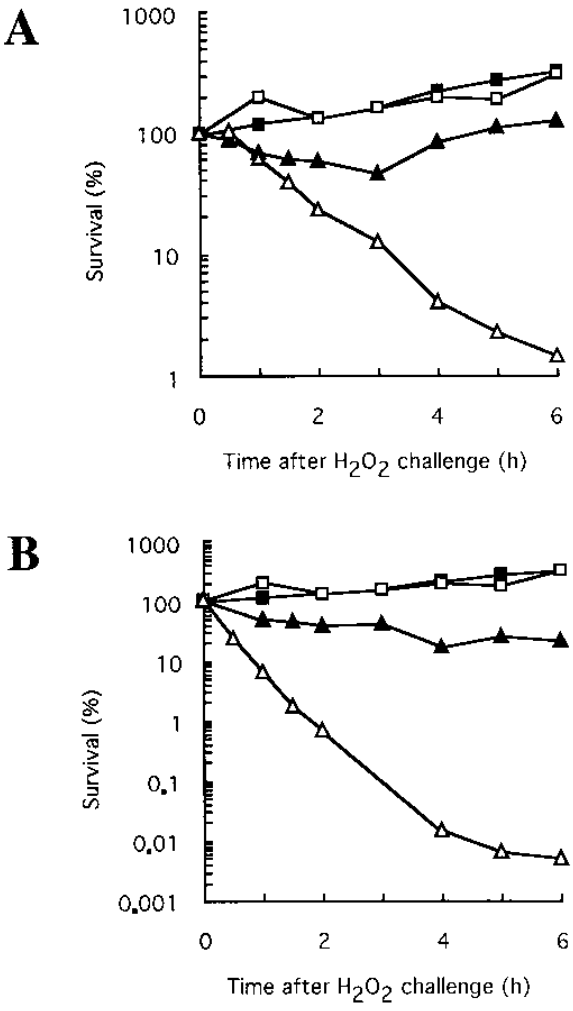

FIG. 5. Sensitivity of wild-type (■ and ▲) and *katA* mutant (□ and △) strains to H_2O_2. Cells were grown exponentially in LB-MC medium (4 ml) to an optical density at 600 nm of 0.2, which represents 100% survival. Cells were challenged with 3 mM H_2O_2 (A) and 4 mM H_2O_2 (B). ■ and □, control cells without H_2O_2; ▲ and △, H_2O_2-treated cells. Samples were taken periodically, diluted, and plated onto LB medium to determine cell viability. Datum points are the means of duplicates from representative experiments.

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TABLE 2. Effect of H_2O_2 on catalase activity in wild-type (Rm5000) and *katA*::Tn*5* mutant (MK5001) strains

R. meliloti strain	Crude extract[a]		Periplasm	
	Catalase activity	MDH[b]	Catalase activity	MDH
Rm5000	10.4 ± 0.5	678 ± 18	1.3 ± 0.2	ND[c]
Rm5000 + H_2O_2	1,178.8 ± 13.4	819 ± 11	122.8 ± 10.5	ND
MK5001	9.6 ± 0.4	1,077 ± 13	0.5 ± 0.1	ND
MK 5001 + H_2O_2	13.3 ± 0.8	1,173 ± 19	0.3 ± 0.1	ND

[a] Values were obtained by using triplicate samples from two independent experiments and are given in units per milligram of protein.
[b] MDH, malate dehydrogenase.
[c] ND, not detected.

brane integrity but allows only low release of the periplasmic proteins (only 1.2% of the periplasmic cyclic phosphodiesterase) and particularly those with a high molecular weight (32). In both extracts, the absolute absence of malate dehydrogenase activity (a cytoplasmic marker enzyme) suggested that there was no contamination by cytoplasmic proteins. Catalase activity was always detected in periplasm extracts of Rm5000, and there was a 100-fold increase of catalase activity in the periplasm after H_2O_2 treatment (Table 2). Analysis of extracts for catalase activity on nondenaturing gels suggested that KatA and KatB are located in the periplasm (Fig. 7, lane 2) and confirmed the induction of KatA by H_2O_2 (Fig. 7, lane 3).

The *katA* mutant has a Nod$^+$ Fix$^+$ phenotype. To test the capacity of the *katA*::Tn*5* mutant to nodulate *M. sativa* host plants, plants were inoculated with the MK5001 strain and the wild-type Rm5000 strain as a control. All the plants inoculated with MK5001 showed a nodulating phenotype (Nod$^+$). The number of nodules was comparable for plants nodulated with the MK5001 strain and those nodulated with the Rm5000 strain (data not shown). To study the nitrogen fixation of nodulated plants, acetylene reduction activity was assayed

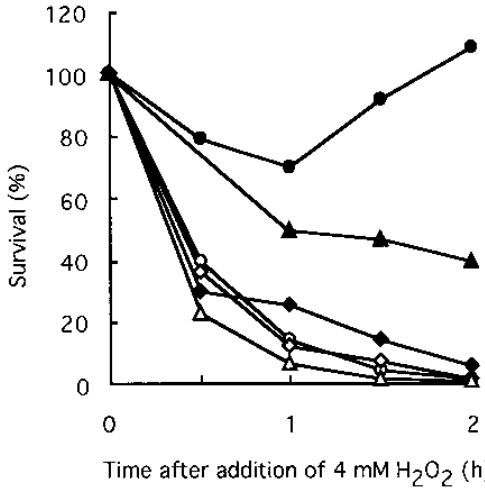

FIG. 6. Adaptation of wild-type (▲, ◆, and ●) and *katA* mutant (△, ◇, and ○) strains to H_2O_2. Cells were grown as described in the legend to Fig. 5 and then treated with 5× 200 μM H_2O_2 for 1 h in the absence and in the presence of chloramphenicol (100 μg/ml). After pretreatment, the cells were harvested by centrifugation at 3,000 × *g* for 2 min at room temperature. The cell pellet was resuspended in 4 ml of LB-MC medium containing 4 mM H_2O_2. Samples were taken periodically, diluted, and plated onto LB medium to determine cell viability. ▲ and △, no pretreatment; ● and ○, pretreated in the absence of chloramphenicol; ◆ and ◇, pretreated in the presence of chloramphenicol. Datum points are the means of duplicates from representative experiments.

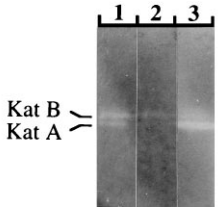

FIG. 7. Induction of KatA by H_2O_2. Strain Rm5000 protein (25 μg) was loaded on a native polyacrylamide gel and stained for catalase activity. Lane 1, crude extract; lane 2, periplasmic proteins; lane 3, periplasmic proteins from cells pretreated with 5× 200 μM H_2O_2 for 1 h.

for *M. sativa* nodulated with the MK5001 or Rm5000 strain. The level of C_2H_2 reduction observed for MK5001-nodulated plants was not significantly different from that of Rm5000-nodulated plants during the first 6 weeks of fixation (Fig. 8). This result shows that the *katA*::Tn*5* mutant strain has also a fixing phenotype (Fix$^+$).

DISCUSSION

With the aim of determining the role of catalases in the establishment and/or the maintenance of *Medicago-Rhizobium* functional nodules, we first cloned the *R. meliloti katA* gene, which encodes an H_2O_2-inducible catalase KatA. The *katA* gene encodes a protein of 562 amino acids, and multiple amino acid sequence alignment showed a strong relationship between KatA and the group of catalases including the *E. coli* monofunctional HPII catalase which has been localized in the cytoplasm (24). In contrast, no similarity to bacterial catalase-peroxidase-type enzymes such as periplasmic *E. coli* HPI was found, which is consistent with the absence of peroxidase activity of KatA. Since the HPII-type catalases appear to represent a well-conserved group of proteins, they were used for phylogenetic reconstruction by Von Ossowski et al. in 1993 (53). Indeed, the KatA amino acid sequence shows high identity with the animal pathogenic bacterium *B. abortus*, which has recently been shown to be a member of the phylogenetic *Rhizobiaceae* group close to *R. meliloti* on the basis of 16S rRNA

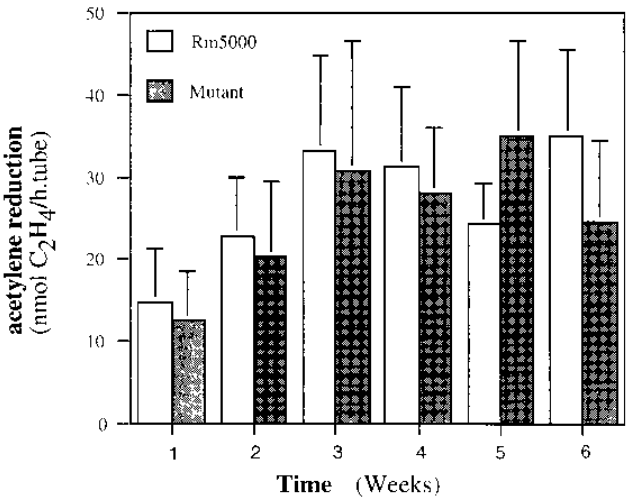

FIG. 8. Time course of C_2H_2 reduction activity. C_2H_2 reduction activity per tube containing two plants was measured at weekly intervals after the second inoculation by the MK5001 mutant strain or the wild-type Rm5000 strain. Values are means ± standard errors of the means (n = 4). Experimental data were assessed for statistical significance by means of the Student t test.

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sequence data (55). Surprisingly, the KatA sequence showed lower amino acid identity with the SNU003 *Rhizobium* sp. catalase (44% identity) than with the scotobacteria *B. pertussis* and *H. influenzae* (50% identity). Our results are consistent with the recent data of Mayfield and Duvall (36) showing that phylogenic trees based on nine procaryotic and five eucaryotic catalase sequences demonstrate no relationship to phylogenies based on ribosomal DNA gene sequences. Thus, there is no obvious explanation for the low degree of homology between catalases of the two *Rhizobium* species, and we cannot conclude that the SNU003 *Rhizobium* sp. is phylogenetically very distant from *R. meliloti.*

Only the KatA activity of *R. meliloti* is inducible by H_2O_2 as in *E. coli* (34), in which only HPI is inducible by H_2O_2. Thus, the *katA* gene is homologous to the *E. coli katE* gene encoding the HPII catalase but is upregulated by exposure to hydrogen peroxide, as is peroxide-inducible *E. coli katG* encoding the HPI catalase. The same genetic characteristics have been reported for the *H. influenzae hktE* gene (5). The induction of KatA synthesis following treatment with 1 mM H_2O_2 was accompanied by resistance to killing by 4 mM H_2O_2. The high sensitivity of the *katA* cells to H_2O_2, even after pretreatment, shows that KatA plays the major role in protection against H_2O_2. Further studies are necessary to determine whether other H_2O_2 induction conditions could induce alternative protective factors, as previously described for *E. coli* (14).

Wild-type *R. meliloti* seems to be much more resistant to H_2O_2 than *R. leguminosarum* bv. phaseoli, since a characteristic V-shaped survival curve was observed at a concentration of 2 mM for *R. leguminosarum*, whereas twice this concentration (4 mM) is required to achieve a similar effect for the *R. meliloti* strain (10). Moreover, at a concentration of 3 mM, a drastic effect is seen for wild-type *R. leguminosarum*, which is unable to recover, whereas a similar effect was observed at a concentration of 4 mM only when the *katA* gene was mutated.

Both catalases KatA and KatB were detected in periplasmic extracts of *R. meliloti*. Expression of a periplasmic monofunctional catalase has also been detected in pathogenic *B. abortus* (48). The export of a catalase to the periplasm without cleaved signal sequence in *B. abortus* was suggested to be reminiscent of the ATP-dependent import of catalase into the eucaryotic peroxisome (48). The eucaryotic minimal peroxisome-targetting signal (Ser/Ala)-(Lys/Arg/His)-(Leu/Ile), named the SKL sequence, is located in the C termini of a wide range of peroxisomal proteins and is present in *R. meliloti* KatA. The presence of this SKL sequence in *katA* which could be associated with the export to the periplasm is consistent with the presence of KatA in this compartment. A periplasmic location for multiple catalases has also been described in the case of phytopathogenic *Pseudomonas syringae* (29), even if at least one of the enzymes is periplasmic as well as cytoplasmic (30). It is reasonable to assume that periplasmic catalase activity would benefit the organism against exogenous H_2O_2, especially that which would be produced during plant-pathogen interactions (7). Indeed, one of the rapid processes characteristic of hypersensitive responses of plants to pathogens is the striking release of reactive oxygen species, termed the oxidative burst (37). In several aspects, the *Rhizobium*-legume symbiosis can be considered a controlled incompatible reaction (43), and many experiments suggest that exopolysaccharides could act as suppressors of the host plant response (for a review, see reference 2). However, the possibility of an oxidative burst, involving H_2O_2, taking place in the early stages of the infection thread formation could also be suggested, since the plant exerts control over the number of nodules and most infections can be aborted by a hypersensitive-like response (52). Our results demonstrate that a mutation of the H_2O_2-inducible KatA of *R. meliloti* has no effect on the number of plant nodules, showing that the mutant still copes with a potential H_2O_2 burst. However, other H_2O_2-scavenging systems like the periplasmic KatB enzyme can supply the deficiency of the KatA enzyme in *R. meliloti*. The fact that this *katA* mutant has a Fix^+ phenotype does not support the hypothesis of an important protective role of the KatA in the nitrogen fixation process. Thus, the cloning of the gene encoding KatB and experiments with a double *katA katB* mutant are needed to give a clear-cut answer to the role of catalase in the maintenance of functional nodules.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1993. Current protocols in molecular biology. Greene Publishing Associates, New York.
2. **Baron, C., and P. C. Zambryski.** 1995. The plant response in pathogenesis, symbiosis, and wounding: variations on a common theme? Annu. Rev. Genet. **29:**107–129.
3. **Becana, M., and R. V. Klucas.** 1992. Transition metals in legume root nodules: iron-dependent free radical production increases during nodule senescence. Proc. Natl. Acad. Sci. USA **89:**8958–8962.
4. **Beringer, J. E., J. L. Beynon, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug resistance transposon Tn5 to *Rhizobium*. Nature (London) **176:**633–634.
5. **Bishai, W. R., H. O. Smith, and G. J. Barcak.** 1994. A peroxide/ascorbate-inducible catalase from *Haemophilus influenzae* is homologous to the *Escherichia coli katE* gene product. J. Bacteriol. **176:**2914–2921.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Brown, S. M., M. L. Howell, M. L. Vasil, A. J. Anderson, and D. J. Hassett.** 1995. Cloning and characterization of the *katB* gene of *Pseudomonas aeruginosa* encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. J. Bacteriol. **177:**6536–6544.
8. **Christman, M. F., G. Storz, and B. N. Ames.** 1989. *OxyR*, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA **86:**3484–3488.
9. **Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich.** 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem. **140:**532–537.
10. **Crockford, A. J., G. A. Davis, and H. D. Williams.** 1995. Evidence for cell-density-dependent regulation of catalase activity in *Rhizobium leguminosarum* bv. *phaseoli*. Microbiology **141:**843–851.
11. **Dalton, D. A., L. Langeberg, and N. C. Treneman.** 1993. Correlations between the ascorbate-glutathione pathway and effectiveness in legume root nodules. Physiol. Plant. **87:**365–370.
12. **Dalton, D. A., C. J. Post, and L. Langeberg.** 1991. Effects of ambient oxygen and of fixed nitrogen on concentrations of glutathione, ascorbate and associated enzymes in soybean root nodules. Plant Physiol. **96:**812–818.
13. **Davies, M. J., and A. Puppo.** 1992. Direct evidence of a globin-derived radical in leghaemoglobin treated with peroxides. Biochem. J. **281:**197–201.
14. **Demple, B., and J. Halbrook.** 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature (London) **304:**466–468.
15. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
16. **Finan, T. M., E. K. Hartwieg, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159:**120–124.
17. **Finan, T. M., B. Kunkel, G. F. DeVos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167:**66–72.
18. **Fita, I., and M. G. Rossmann.** 1985. The active center of catalase. J. Mol. Biol. **185:**21–37.
19. **Fita, I., and M. G. Rossmann.** 1985. The NADPH binding site on beef liver catalase. Proc. Natl. Acad. Sci. USA **82:**1604–1608.

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20. **Francis, A. J., and M. Alexander.** 1972. Catalase activity and nitrogen fixation in legume root nodules. Can. J. Microbiol. **18:**861–864.
21. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
22. **Glazebrook, J.** 1991. Genetic techniques in *Rhizobium meliloti*. Methods Enzymol. **204:**398–418.
23. **Halliwell, B., and J. M. C. Gutteridge.** 1986. Oxygen free-radicals and iron in relation to biology and medicine. Some problems and concepts. Arch. Biochem. Biophys. **246:**501–508.
24. **Heimberger, A., and A. Eisenstark.** 1988. Compartmentalization of catalases in *Escherichia coli*. Biochem. Biophys. Res. Commun. **154:**392–397.
25. **Honeycutt, R. J., M. McClelland, and B. W. S. Sobral.** 1993. Physical map of the genome of *Rhizobium meliloti* 1021. J. Bacteriol. **175:**6945–6952.
26. **Ivanova, A., C. Miller, G. Glinsky, and A. Eisenstark.** 1994. Role of *rpo*S (*kat*F) in *oxy*R-independent regulation of hydroperoxidase I in *Escherichia coli*. Mol. Microbiol. **12:**571–578.
27. **Jenkins, D. E., S. A. Chaissons, and A. Matin.** 1990. Starvation-induced cross-protection against osmotic challenge in *Escherichia coli*. J. Bacteriol. **172:**2779–2781.
28. **Jones, D. P.** 1982. Intracellular catalase function: analysis of the catalytic activity by product formation in isolated liver cells. Arch. Biochem. Biophys. **214:**806–814.
29. **Klotz, M. G., and S. W. Hutcheson.** 1992. Multiple periplasmic catalases in phytopathogenic strains of *Pseudomonas syringae*. Appl. Environ. Microbiol. **58:**2468–2473.
30. **Klotz, M. G., Y. C. Kim, J. Katsuwon, and A. J. Anderson.** 1995. Cloning, characterization and phenotypic expression in *Escherichia coli* of *catF*, which encodes the catalytic subunit of catalase isozyme CatF of *Pseudomonas syringae*. Appl. Microbiol. Biotechnol. **43:**656–666.
31. **Kuykendall, L. D., and G. H. Elkan.** 1976. *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. Appl. Environ. Microbiol. **32:**511–519.
32. **Le Rudulier, D., K. Gloux, and N. Riou.** 1991. Identification of an osmotically induced periplasmic glycine betaine-binding protein from *Rhizobium meliloti*. Biochim. Biophys. Acta **1061:**197–205.
33. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
34. **Loewen, P. C., J. Switala, and B. L. Triggs-Raine.** 1985. Catalases HPI and HPII in *Escherichia coli* are induced independently. Arch. Biochem. Biophys. **243:**144–149.
35. **Long, S. R.** 1989. *Rhizobium*-legume nodulation: life together in the underground. Cell **56:**203–214.
36. **Mayfield, J. E., and M. R. Duvall.** 1996. Anomalous phylogenies based on bacterial catalase gene sequences. J. Mol. Evol. **42:**469–471.
37. **Mehdy, M. C.** 1994. Active oxygen species in plant defense against pathogen. Plant Physiol. **105:**467–472.
38. **Murshudov, G. N., W. R. Melik-Adamyan, A. I. Grebenko, V. V. Barynin, A. A. Vagin, B. K. Vainshtein, Z. Dauter, and R. S. Wilson.** 1992. Three-dimensional structure of the catalase from *Micrococcus lysodeikticus* at 1.5 Å resolution. FEBS Lett. **312:**127–131.
39. **Murthy, M. R. N., T. J. Reid III, A. Sicignano, N. Tanaka, and M. G. Rossmann.** 1981. Structure of beef liver catalase. J. Mol. Biol. **152:**465–499.
40. **Puppo, A., and J. Rigaud.** 1986. Superoxide dismutase: an essential role in the protection of the nitrogen fixation process? FEBS Lett. **201:**187–189.
41. **Puppo, A., J. Rigaud, and D. Job.** 1981. Role of the superoxide anion in the leghemoglobin autoxidation. Plant Sci. Lett. **22:**353–360.
42. **Rigaud, J.** 1976. Effet des nitrates sur la fixation d'azote par des nodules de Haricot (*Phaseolus vulgaris* L.) Physiol. Vég. **14:**297–308.
43. **Rolfe, B. G., and P. M. Gresshoff.** 1988. Genetic analysis of legume nodule initiation. Annu. Rev. Physiol. Plant Mol. Biol. **39:**297–319.
44. **Rosenberg, C., and T. Huguet.** 1984. The pAtC58 plasmid is not essential for tumor induction. Mol. Gen. Genet. **196:**533–536.
45. **Ruvkun, G. B., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) **189:**85–88.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
48. **Sha, Z., T. J. Stabel, and J. E. Mayfield.** 1994. *Brucella abortus* catalase is a periplasmic protein lacking a standard signal sequence. J. Bacteriol. **176:** 7375–7377.
49. **Triggs-Raine, B. L., B. W. Doble, M. R. Mulvey, P. A. Sorby, and P. C. Loewen.** 1988. Nucleotide sequence of *katG*, encoding catalase HPI of *Escherichia coli*. J. Bacteriol. **170:**4415–4419.
50. **Vainshtein, B. K., W. R. Melik-Adamyan, V. V. Barynin, A. A. Vagin, A. I. Grebenko, V. V. Borisov, K. S. Bartels, I. Fita, and M. G. Rossmann.** 1986. Three-dimensional structure of catalase from *Penicillium vitale* at 2.0 Å resolution. J. Mol. Biol. **188:**49–61.
51. **van Slooten, J. C., E. Cervantes, W. J. Broughton, C. H. Wong, and J. Stanley.** 1990. Sequence and analysis of the *rpoN* sigma factor gene of *Rhizobium* sp. strain NGR234, a primary coregulator of symbiosis. J. Bacteriol. **172:**5563–5574.
52. **Vasse, J., F. de Billy, and G. Truchet.** 1993. Abortion of infection during a *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. Plant J. **4:**555–566.
53. **Von Ossowski, I., G. Hausner, and P. C. Loewen.** 1993. Molecular evolutionary analysis based on the amino acid sequence of catalase. J. Mol. Evol. **37:**71–76.
54. **Von Ossowski, I., M. R. Mulvey, P. A. Leco, A. Borys, and P. C. Loewen.** 1991. Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. J. Bacteriol. **173:**514–520.
55. **Yanagi, M., and K. Yamasato.** 1993. Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107:**115–120.
56. **Yarosh, O. K., T. C. Charles, and T. M. Finan.** 1989. Analysis of C_4-dicarboxylic acid transport genes in *Rhizobium meliloti*. Mol. Microbiol. **3:**813–823.

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