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Role of *oxyR* from *Sinorhizobium meliloti* in Regulating the Expression of Catalases

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Abstract The process of symbiotic nitrogen fixation results in the generation of reactive oxygen species such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The response of rhizobia to these toxic oxygen species is an important factor in nodulation and nitrogen fixation. In *Sinorhizobium meliloti*, one *oxyR* homologue and three catalase genes, *katA*, *katB*, and *katC* were detected by sequence analysis. This *oxyR* gene is located next to and divergently from *katA* on the chromosome. To investigate the possible roles of *oxyR* in regulating the expression of catalases at the transcriptional level in *S. meliloti*, an insertion mutant of this gene was constructed. The mutant was more sensitive and less adaptive to H_2O_2 than the wild type strain, and total catalase/peroxidase activity was reduced approximately fourfold with the OxyR mutation relative to controls. The activities of KatA and KatB and the expression of *katA*::*lacZ* and *katB*::*lacZ* promoter fusions were increased in the mutant strain compared with the parental strain grown in the absence of H_2O_2, indicating that *katA* and *katB* are repressed by OxyR. However, when exposed to H_2O_2, *katA* expression was also increased in both *S. meliloti* and *Escherichia coli*. When exposed to H_2O_2, OxyR is converted from a reduced to an oxidized form in *E. coli*. We concluded that the reduced form of OxyR functions as a repressor of *katA* and *katB* expression. Thus, in the presence of H_2O_2, reduced OxyR is converted to the oxidized form of OxyR that then results in increased *katA* expression. We further showed that *oxyR* expression is autoregulated via negative feedback.

Key words *Sinorhizobium meliloti*; OxyR; catalase; *katA*; H_2O_2

Sinorhizobium meliloti, a gram-negative bacterium found in soil, infects the *Medicago* species to form symbiotic nitrogen-fixing nodules through complex interactions [1]. In the early stage of interactions between rhizobia and legumes, reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (HO·), are generated. This ROS generation is similar to that seen when pathogens infect host plants [2,3]. It has been confirmed that H_2O_2 plays a supportive role in nodule initiation by mediating Nod factor responses between *Sesbania rostrata* and *Azorhizobium caulinodans* [4]. Through the analysis of mutations in catalase genes, H_2O_2 has also been found to inhibit infection thread formation and nodule development [5–7]. In the genome of *S. meliloti* Rm1021, there are three distinct catalases: two monofunctional catalases (KatA and KatC) homologous to *Escherichia coli* HPII (KatE), and one bifunctional catalase-peroxidase (KatB), which is a homologue of *E. coli* HPI (KatG). Investigation of catalase expression in free-living cells has indicated that *katA* is induced at the transcription level under oxidative stress conditions, while *katB* gene is constitutively expressed [5–7].

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In *E. coli*, the LysR family regulator, OxyR, acts as a sensor and transducer of oxygen stress, via critical redox-sensitive Cys199 and C208 residues. These residues can be oxidized by H_2O_2 to form a disulfide bond and are reduced for recycling by GrxA and GSH [8–10]. Only the oxidized form of the protein is able to activate the expression of downstream genes and negatively autoregulate its expression by binding to specific regions in promoters [11]. It is known that *E. coli* possesses two catalases, HPI (encoded by *katG*) and HPII (encoded by *katE*) [12]. HPI is transcriptionally induced during the logarithmic phase in response to low concentrations of H_2O_2, and requires the transcriptional regulator, OxyR [13]. Conversely, HPII is induced during the transition from the exponential phase to the stationary phase by RpoS, an alternative sigma factor, which is neither regulated by OxyR nor induced by H_2O_2 [14–17]. It is not known whether OxyR similarly regulates the expression of catalase genes in *S. meliloti*.

When the genome of *S. meliloti* Rm1021 was sequenced [18], 84 putative LysR family genes were predicted, including one OxyR homologous gene, which has been oriented to *katA*.

In this study, we constructed an insertion mutant of *oxyR* of *S. meliloti*, as well as *katA*, *katB*, and *oxyR* promoter::*lacZ* fusions, and subsequently detected the activity of these promoter fusions in free-living cells. Our results demonstrate that OxyR is capable of regulating the expression of *katA* as well as regulating its own expression.

Materials and Methods

Bacterial strains, phage, plasmids and growth media

The bacterial strains, phage and plasmids used in this work are listed in **Table 1**. Luria-Bertani (LB) medium was used for the growth of *E. coli*. The LB used for *S. meliloti* was supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC). Agar (1.5%) was used for solid media. Jenson's medium was used according to previously

Table 1 Bacterial strains, phage and plasmids

Strains, plasmids or phage	Relative characteristics	Source or reference
E. coli strains		
DH5α	*supE44 ΔlacU169(ϕ80lacZ DM15)hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
MT616	*pro-82 thi-1 endA1 supE44 hsdR17$^-$ hsdM$^+$ recA56*, *chl*^{R}, carrying pRK600	[19]
MC4100	*19 F$^-$ araD139 Δ(argF-lacU169)rpsL150(str^R)relA1 flbB5301 deoC1 ptsF25 rbsR*	[20]
GSO47	MC4100, *oxyR::kan*^{R}	[20]
S. meliloti strains		
Rm1021	*str*^{R}, derivative of *SU47*	[21]
OxyRsm	Rm1021, *oxyR::pK19mob2ΩHMB*, *neo*^{R}	This work
Phage		
ϕM12		[22]
Plasmids		
pK19mob2ΩHMB	*kan*^{R}	Dr. Haiping CHENG's Laboratory (NY)
pGD926	*tet*^{R}, *IncP lacZ* fusion vector	[23]
pHC359	*kan*^{R}, *oxyR::pK19mob2ΩHMB*	This work
pGL5	*tet*^{R}, *oxyR::lacZ* promoter fusion in pGD926	This work
pGL6	*tet*^{R}, *katA::lacZ* promoter fusion in pGD926	This work
pGL9	*tet*^{R}, *katB::lacZ* promoter fusion in pGD926	This work
pMD18-T	T-vector, *amp*^{R}	TaKaRa
pMB393	*spe*^{R}	[24]
pMUL1	*amp*^{R}, *spe*^{R}, *pMB393-pMD18T-oxyR*	This work

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published methods [25]. The following antibiotics were used at the indicated concentrations: 25 μg/ml kanamycin, 10 μg/ml chloramphenicol, 100 μg/ml ampicillin, 200 μg/ml neomycin, 500 μg/ml streptomycin, 10 μg/ml tetracycline, and 100 μg/ml spectinomycin.

Construction of *oxyR* mutants, plasmids and promoter::*lacZ* fusions

The suicide plasmid pK19mob2ΩHMB and polymerase chain reaction (PCR) products (P_1, P_2) were used to construct one insertion mutant Sm359 with the help of MT616. This mutant was lysed by ϕM12, and the resulting lysate was transduced into Rm1021 to obtain a single insertion mutant, named OxyRsm, which was identified by PCR with primer pairs P_3/P_4 that could incise a 1 kb fragment [**Fig. 1(B)**]. One primer was complementary to pK19mob2ΩHMB (P_3) and the other primer was complementary to *oxyR* in the *S. meliloti* genome (P_4). The reactions were carried out with the DNA polymerase *Taq* (TaKaRa, Dalian, China). Genomic DNA was extracted with a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). A pair of primers (P_5/P_6) was designed to amplify a fragment carrying the complete *S. meliloti oxyR* sequence and the generated product was cloned into the pMD18-T vector. The obtained plasmid was recombined into pMB393, which produced the plasmid pMUL1. Three pairs of primers (P_7/P_8, P_9/P_{10}, P_{11}/P_{12}) carrying *Bam*HI and *Hin*dIII sites were designed and used to amplify the sequences of *oxyR*, *katA* and *katB* promoter regions by PCR. These sequences were subsequently ligated into pGD926 digested by *Bam*HI and *Hin*dIII, generating pGL5 (*oxyR* promoter::*lacZ* fusion), pGL6 (*katA* promoter::*lacZ* fusion) and pGL9 (*katB* promoter::*lacZ* fusion), respectively. All restriction enzymes and systems were purchased from Promega. All molecular techniques were carried out according to the published techniques of Sambrook and Russell [26]. The primers used in this work are listed in **Table 2**.

Peroxide hypersensitive test

Rm1021 and OxyRsm were inoculated into 3 ml of LB/MC liquid medium with antibiotics and incubated at 28 °C for 2 days. A range of dilutions (indicated below) of 3 ml LB/MC top agar with 0.3 ml bacterial culture were mixed

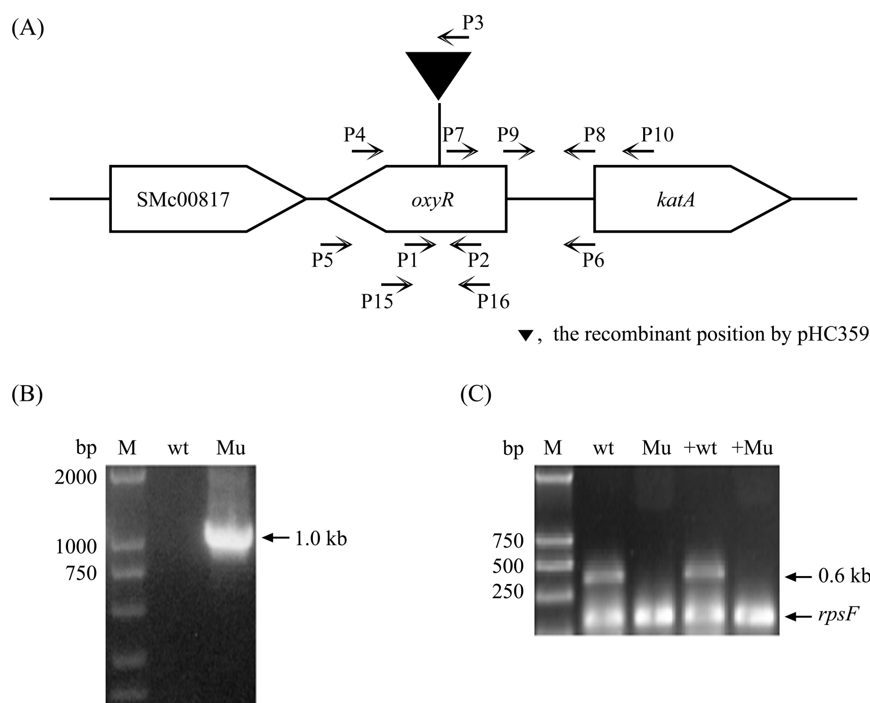

Fig. 1 Identification of *oxyR* mutants

(A) The physical map of the *S. meliloti oxyR* region. Small arrows indicate the positions of the primers and open arrows indicate the direction of transcription. (B) Identification of OxyRsm by PCR with P_3 and P_4 primers. (C) Identification of OxyRsm by RT-PCR with P_{15} and P_{16}. M, DL2000 marker; wt, wild type; Mu, OxyRsm; +wt, nodule induced by Rm1021; nodule induced by OxyRsm.

Table 2 Primers used for PCR in this study

Primer	Sequence	Position
P_1	5′-GGTTCCACGT***AAGCTT***CCACCGTCAAGGGCGAGGAAG-3′	840272–840293
P_2	5′-GCGATTACCC***TGTACA***CCTCCGCCACGGCCATATAGA-3′	840596–840617
P_3	5′-CCTGGCCTTTTGCTGGCCT-3′	pK19mob2ΩHMB 2436–2455
P_4	5′-CCCATGTCAGCCAGCCGG-3′	840176–840194
P_5	5′-GGGGTACCGTTCTGGTGCGCCAGCTT-3′	840429–840447
P_6	5′-CGGGATCCGGACCGTCGAATTCCTGAAG-3′	839148–839168
P_7	5′-CG***GGATCC***CGCCACGGCCATATAGAAG-3′	840594–840613
P_8	5′-CCC***AAGCTT***CACATCCCTCCTGTTTCGATG-3′	840289–840310
P_9	5′-CG***GGATCC***CGGTTCTGGTGCGCCAGCTT-3′	840116–840136
P_{10}	5′-CCC***AAGCTT***CCTTCGAGAGTGCCCGTATT-3′	840355–840375
P_{11}	5′-CCC***AAGCTT***CTCCGTCGAGGTGCCCTC-3′	1344997–1345015
P_{12}	5′-CGG***GGATCC***GTGCCAGCGCTGTGCCAG-3′	1344165–1344183
P_{15}	5′-CGGACAAATACGGGCACTCT-3′	840349–840369
P_{16}	5′-GTTTGCAAGTGCGTCCATGT-3′	840944–840964

"AAGCTT" is the *Hin*dIII site; "TGTACA" is the *Bsr*GI site; "GGATCC" is the *Bam*HI site.

and poured onto LB/MC agar plates. Three pieces of sterilized filter paper (5 mm in diameter) were placed on each plate, and 10 µl of H_2O_2 (8.8 M) was pipetted onto each piece of filter paper. After plates were incubated at 28 °C for two days, the diameters of the inhibition zones were measured [27]. The A_{600} of fresh bacterial culture was determined for survival fractions. In addition, 10 µl of H_2O_2 (8.8 M) was added into 1 ml of rhizobium culture and incubated at 28 °C for 30 min or 1 h. The mixture was then diluted to $1:10^2$, $1:10^4$, $1:10^6$, $1:10^8$, or $1:10^{10}$ with liquid LB/MC. After 5 µl of each dilution was spotted onto LB/MC plates with antibiotics, the plates were incubated at 28 °C for 3 days, at which time the number of colonies was counted.

RNA extraction and RT-PCR

To extract total RNA, the cell suspensions (1 ml) of Rm1021 or OxyRsm (A_{600}=1.0) in LB/MC medium were transferred into 1.5 ml tubes and centrifuged at 10,000 *g* for 1 min according to the instructions for SV total RNA isolation system (trial size; Promega). The fresh nodules were frozen in liquid nitrogen and pulverized to extract total RNA as described for rhizobia. After total RNA was treated with RNase-free DNase I and RNase inhibitor, PCR was performed to confirm that there was no genomic DNA contamination. The primers of *S. meliloti oxyR* were designated as P_{15}/P_{16} to identify the mutant that could incise a 0.6 kb fragment for the wild-type strain [**Fig. 1(C)**].

The reverse-transcript PCR (RT-PCR) system was obtained from TaKaRa (Dalian, Liaoning, China). The reactions were performed as follows: RT reaction at 42 °C for 30 min, inactivation of RTase at 94 °C for 2 min, PCR reaction (30 cycles) at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min.

Catalase and β-galactosidase activity assay

Rhizobia were grown at A_{600}=2.0 for crude protein extraction in order to detect total catalase activity with a CAT (catalase) kit (Jiancheng, Nanjing, China). The crude proteins (50 mg) extracted from rhizobia grown at A_{600}= 0.8 were separated by native PAGE gel for the detection of catalase activity [5]. β-galactosidase activity in crude extracts of bacteria was determined by measuring the hydrolysis of *o*-nitrophenyl-β-*D*-galactoside as described by Miller [28].

Sequence and structure analysis

BlastP (National Center of Biological Information, NCBI) and ClustalW (European Bioinformatics Institute, EBI) programs were used to search for homologues and align sequences. The putative promoter sequences were analyzed with the Berkeley *Drosophila* Genome Project (BDGF) Neural Network Promoter Prediction. The senior

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structure was predicted on the Swiss-Model server, and structure alignments were completed with the EMBL DALI (3D structure alignment) server.

Results and Discussion

Identification and mutagenesis of the *oxyR* gene in *S. meliloti*

In *S. meliloti* Rm1021, ORF SMc00818 encoding a peptide of 311 amino acids, shows homology to known OxyR proteins from *Brucella abortus* (53%), *Agrobacterium tumefaciens* (41%) and *E. coli* (39%). The multiple sequence alignments performed with the ClustalW program from EBI revealed two important features. The analysis indicated that the N-terminal domain of *S. meliloti oxyR* contains a typical helix-turn-helix DNA binding motif, HFXXAAXXXXSQPXLSXQI (X represents non-conserved amino acid residue) [29], and that there are two conserved cysteine residues in the central domain, C199 and C208, which are thought to be involved in activation of the protein by oxidation [12].

When the *E. coli* OxyR structures were input as templates, the structures of both the oxidized and reduced forms of OxyR from *S. meliloti* were predicted. No differences were noted between the oxidized form of OxyR proteins from *S. meliloti* and *E. coli*. An open loop appeared in the reduced form of OxyR from *E. coli*, whereas a closed loop was found for *S. meliloti* OxyR (data not shown). The structure alignments of both proteins were performed and the Z-scores were 37.0 at oxidized status and 35.1 at reduced status. Thus, the predicted structures of *S. meliloti* OxyR are also highly homologous to those from *E. coli*.

OxyRsm is more sensitive and less adaptive to H_2O_2 in free-living cells

In many bacteria, OxyR inactivation alters the responses to oxidative stress. We therefore determined the level of resistance to H_2O_2 in OxyRsm (mutant strain), Rm1021 (parental strain), and the mutant strain complemented with the plasmid pMUL1 (carrying a functional OxyR) by performing the inhibition zone test with 1 μl, 5 μl and 10 μl of 8.8 M H_2O_2. With the parental strain, Rm1021, the average diameters of inhibition zones were 19, 24 and 26 mm, respectively. However, markedly larger inhibition zones with 38, 51 and 57 mm in diameter, respectively, were seen with the mutant strain. This increase was attenuated with the complemented strain OxyRsm/pMUL1, in which the diameters of the inhibition zones were 23, 28 and 31 mm, respectively [**Fig. 2(A)**]. These results indicate that the mutant strain, OxyRsm, is more sensitive to H_2O_2 than the parental strain Rm1021, and that the phenotype of OxyRsm can be restored by introduction of the pMUL1 plasmid, which carried constitutively-expressed OxyR.

To test whether the mutant strain is able to adapt to H_2O_2, the wild-type strain Rm1021, mutant strain OxyRsm and complemented strain in liquid cultures were treated

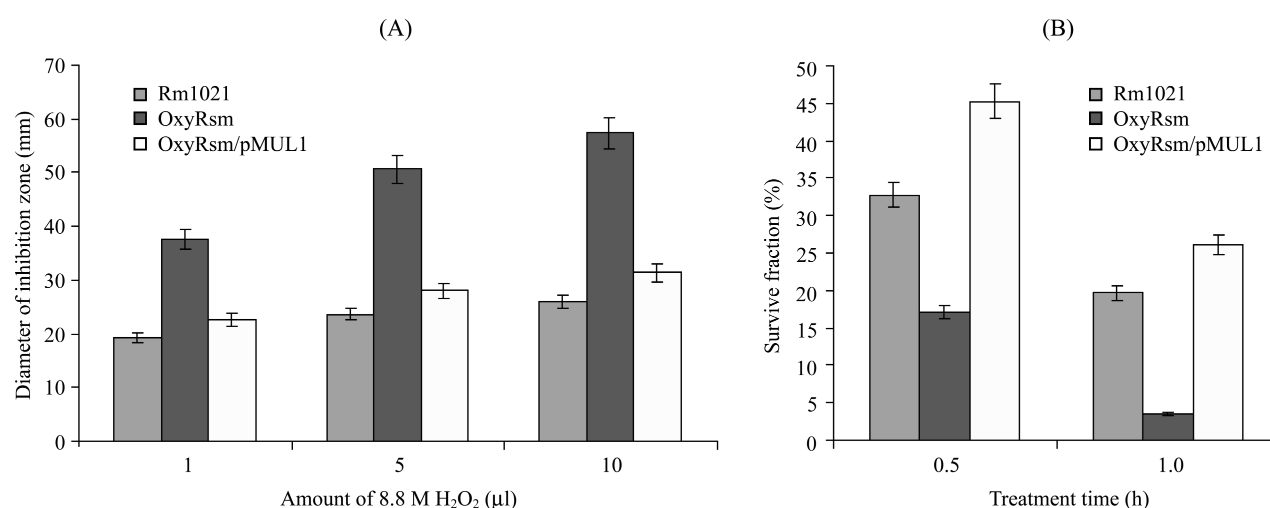

Fig. 2 Response and adaptation of Rm1021, OxyRsm and OxyRsm/pMUL1 to H_2O_2

(A) The diameter of inhibition zones of Rm1021, OxyRsm and OxyRsm/pMUL1. (B) Survival fractions of Rm1021, OxyRsm and OxyRsm/pMUL1. All experiments were repeated three times.

with the final concentration of 88 mM H_2O_2. The cultures were incubated at 28 °C, diluted and plated. The findings shown in **Fig. 2(B)** indicate that functional OxyR is required for adaptation. The survival fractions were 32.76% and 19.70% for the wild-type strain, 45.24% and 26.09% for the complemented strain and 17.07% and 3.57% for the mutant strain after incubation for 30 min or 1 h, respectively. These results indicate that the mutant strain was more sensitive and less adaptive to H_2O_2 than the wild-type strain.

OxyR acts as a repressor of *katA* and *katB* expression in *S. meliloti*

That the mutant strain OxyRsm is more sensitive and less adaptive to H_2O_2 than its parental strain suggests that OxyR might be involved in regulating catalase expression. We therefore determined the total catalase/peroxidase levels in the wild type and the mutant strains. The total catalase/peroxidase activity in Rm1021 and OxyRsm was 880 U/g and 230 U/g, respectively [**Fig. 3(A)**]. These results indicate that the total catalase/peroxidase activity was reduced approximately fourfold with OxyR mutation, and are consistent with the inhibition zone and survival fraction test findings (**Fig. 2**).

Native PAGE gel assays showed increases in KatA and KatB activities with the *oxyR* mutant [**Fig. 3(B)**]. To confirm the native gel analysis, we determined the *katA* and *katB* expression levels using *katA* and *katB* promoter::*lacZ* fusions in Rm1021 and OxyRsm. The results shown in **Table 3** indicate that in the absence of H_2O_2, β-galactosidase activity increased 11.8-fold for *katA* and 2.85-fold for *katB* in the mutant strain, compared with the wild-type strain (**Table 3**, No. 2, 5, 3, 6). These results imply that there was a compensatory decrease in catalase/peroxidase activity in response to the increase in KatA and KatB levels, most likely due to the decreased expression of other unknown catalase genes.

In comparing *katA* expression in the presence and absence of H_2O_2, we found that β-galactosidase activity in *katA*::*lacZ* fusion in the presence of H_2O_2 was 6.2-fold higher than in the absence of H_2O_2 in the wild type strain (**Table 3**, No. 2). OxyR can act either as a transcriptional activator or repressor depending on its oxidation state. Furthermore, when exposed to H_2O_2, OxyR is converted from a reduced form to an oxidized form. Our findings suggest that the reduced OxyR may function as a repressor for the expression of *katA*. Accordingly, conversion of reduced OxyR to oxidized OxyR in the presence of H_2O_2 may induce *katA* expression.

Reduced OxyR represses *S. meliloti katA* in *E. coli*

Tseng *et al.* reported that in *Neisseria gonorrhoeae*, *oxyR* acts as a repressor for the expression of *kat* genes. The difference between the present findings with *S. meliloti* and those with *N. gonorrhoeae* may be due to species-specific differences in the promoters of *kat* genes in these organisms [30]. Thus we examined the expression of *katA* promoter::*lacZ* fusions in the *E. coli* strain

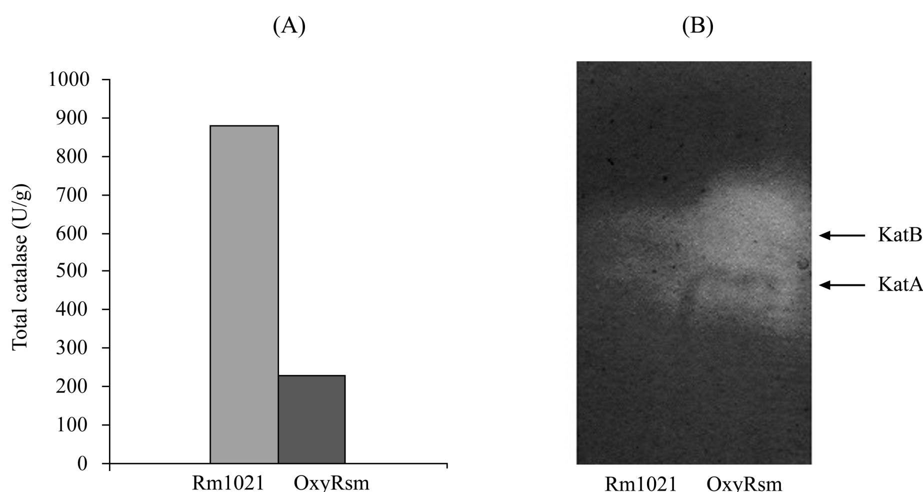

Fig. 3 Catalase activity assay of Rm1021 and OxyRsm

(A) Total catalase/peroxidase activity was determined (rhizobia A_{600}=2.0). (B) KatA and KatB activities were assayed by native PAGE (rhizobia A_{600}= 0.8).

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Table 3 β-galactosidase activity of promoter::*lacZ* fusions in Rm1021 and OxyRsm

Experiment No.	Strain	Relevant genotype chromosome/plasmid	H_2O_2– *	H_2O_2+ **
1	*S. meliloti* Rm1021/pGD926	wt/promoter-less lacZ	0	0
2	*S. meliloti* Rm1021/pGL6	wt/P_{katA}-lacZ	48±9	295±29
3	*S. meliloti* Rm1021/pGL9	wt/P_{katB}-lacZ	237±20	187±9
4	*S. meliloti* OxyRsm/pGD926	oxyR/promoter-less lacZ	0	0
5	*S. meliloti* OxyRsm /pGL6	oxyR/P_{katA}-lacZ	566±26	590±16
6	*S. meliloti* OxyRsm /pGL9	oxyR/P_{katB}-lacZ	676±35	452±30
7	*E. coli* MC4100/pGD926	wt/promoter-less lacZ	0	0
8	*E. coli* MC4100/pGL6	wt/P_{katA}-lacZ	11±1	39±3
9	*E. coli* GSO47/pGD926	oxyR/promoter-less lacZ	0	0
10	*E. coli* GSO47/pGL6	oxyR/P_{katA}-lacZ	64±2	69±1

* the β-galactosidase of pGL6 and pGL9 was detected at the exponential phase (A_{600}=0.8); ** 50 mM H_2O_2 was added into rhizobial cultures and incubated at 28 °C for 30 min.

MC4100 (wild-type) and GSO47 (*oxyR::kan*) with MC4100/pGD926 and GSO47/pGD926 as the controls. Our findings indicate that β-galactosidase activity of the *katA::lacZ* fusion was 5.8-fold higher in the GSO47 (*oxyR::kan*) strain than in the MC4100 (wild type) strain (**Table 3**, No. 8, 10). By the comparison of the *katA* expression with or without 50 mM H_2O_2 treatment, we found that the β-galactosidase activity of the *katA*::*lacZ* fusion was 6.2-fold higher following treatment with H_2O_2 than without treatment in the wild-type strain (**Table 3**, No. 8) and had no effect on the *oxyR* mutant strain (**Table 3**, No. 10). Therefore our findings indicate that *S. meliloti katA* can be repressed by *E. coli*-reduced OxyR.

Our data suggest that reduced OxyR represses the expression of *S. meliloti katA* and *katB* in free-living cells. In the presence of H_2O_2, however, reduced OxyR is converted to oxidized OxyR, resulting in the induction of *katA* and *katB* expression.

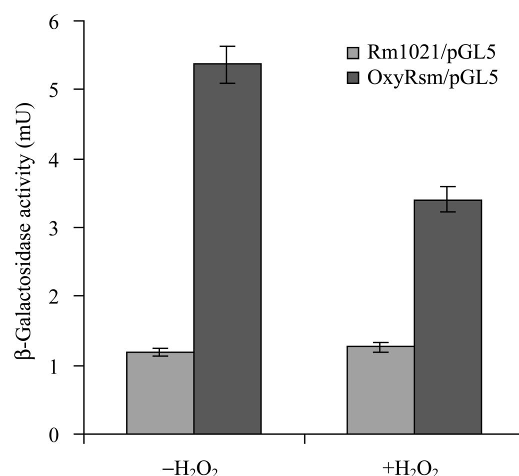

Fig. 4 Expression of *oxyR* promoter::*lacZ* fusion in *S. meliloti*
Rhizobia were grown at stationary phase, A_{600}=2.0.

OxyR negatively autoregulates its expression

OxyR belongs to the LysR family of transcriptional regulators. One of the common features of the genes in this family is their ability to control expression via autoregulation. The regulation of OxyR expression was investigated using an OxyR-lacZ fusion. β-galactosidase activity was determined in the wild-type strain Rm1021 and in the mutant strain, OxyRsm, harboring the fusion plasmid pGL5. The results clearly indicate that the lack of functional OxyR resulted in a 4-fold increase in β-galactosidase activity (**Fig. 4**). This suggests that, as in *E. coli*, the OxyR protein in *S. meliloti* negatively regulates its own expression [11]. We also investigated the response of the OxyR promoter to H_2O_2 stress and found that the β-galactosidase activity was not affected when cells were treated with 50 mM H_2O_2 (**Fig. 4**). This result revealed that exposing *S. meliloti* to H_2O_2 has no effect on OxyR concentration.

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