RESEARCH LETTER

Sinorhizobium meliloti rpoE2 is necessary for H_2O_2 stress resistance during the stationary growth phase

Maud Flechard, Catherine Fontenelle, Annie Trautwetter, Gwennola Ermel & Carlos Blanco

CNRS UMR6026 DUALS, Universite Rennes I, Rennes, France

Correspondence: Carlos Blanco, CNRS UMR6026 DUALS, Universite de Rennes I, Campus de Beaulieu, Bat13, Avenue du Général Leclerc, 35042 Rennes, Cedex, France. Tel.: +33 02 23 23 61 40; e-mail: carlos.blanco@univ-rennes1.fr

Received 23 July 2008; accepted 1 October 2008.
First published online 19 November 2008.

DOI:10.1111/j.1574-6968.2008.01401.x

Editor: Yaacov Okon

Keywords
transcription regulation; external superoxides; hydrogen peroxide; SodC; KatC; σE.

Abstract

RpoE2 is an extracytoplasmic σ factor produced by *Sinorhizobium meliloti* during stationary growth phase. Its inactivation affected the synthesis of the superoxide dismutase, SodC, and catalase, KatC. The absence of SodC within the cell did not result in an increased sensitivity to extracellular superoxides. In contrast, the absence of KatC affected the resistance of *S. meliloti* to H_2O_2 during the stationary growth phase. A *katC* strain behaved as an *rpoE2* strain during an H_2O_2 challenge, suggesting that the H_2O_2 sensitivity of the *rpoE2* strain resulted only from the lack of KatC in this strain.

Introduction

Like all living organisms, bacteria are periodically subjected to fluctuations of the physicochemical parameters of their surrounding medium. In contrast to the external milieu, cytoplasmic parameters are maintained at a nearly constant level by homeostatic mechanisms; otherwise, cell viability and proliferation are compromised. To maintain homeostasis, the cell needs to sense the fluctuations of the environmental parameters and has to produce responses to counteract them or to correct their deleterious effects. Various modes of signal transduction appeared during evolution to sense physicochemical parameters, involving two-component systems and also intracellular regulatory proteins. Most of the resulting regulations affect gene expression at the transcriptional level (Marles-Wright & Lewis, 2007). Alternative σ factors are used by most bacteria to control gene expression in response to the alteration of their environment. σS in enterobacteria and σB in *Bacillus subtilis* constitute the most-studied σ factors controlling stress responses (Wosten, 1998). Nevertheless, they are not universally represented in bacteria. An alternative involves another family of σ factors collectively known as σE. They are not involved in housekeeping functions but are involved in stress responses (Kazmierczak *et al.*, 2005). Such σ factors seem to be present in most bacteria. *Escherichia coli* has a unique σE factor, but other bacteria could possess a high number of σE representatives in their genome (Helmann, 2002). In such a case, the role of each σE factor is difficult to determine because they could have redundant functions (Mascher *et al.*, 2007).

In *Sinorhizobium meliloti*, 11 putative σE factors are annotated in the genome. One of them, σE2, was the subject of a transcriptomic analysis (Sauviac *et al.*, 2007). A regulon of 44 genes was predicted to be controlled by σE2 in that study, among which only two are involved in a stress response: *katC*, encoding a periplasmic catalase, and *rpoH2*, encoding a σ factor involved in a heat stress response. Nevertheless, no phenotypic difference was detected between the wild-type and a σE2 strain, suggesting that σE2 is not necessary for stress adaptation in *S. meliloti*.

In this study, we show that *S. meliloti rpoE2* is necessary for the production of two stationary growth-phase enzymes involved in detoxification of reactive oxygen species and

increasing the cell's H_2O_2 resistance level upon entry into the stationary growth phase.

Materials and methods

Bacterial strains and media

Sinorhizobium meliloti and *E. coli* strains (Table 1) were grown in Luria–Bertani (LB) medium (Miller, 1972). *Sinorhizobium meliloti* was also grown in GAS medium, which consisted of S medium (Pichereau *et al.*, 1998) supplemented with 10 mM galactose (G) and 10 mM aspartate (A) as carbon and nitrogen sources, respectively. In this case, cells were first grown in LB medium, harvested and washed in S medium before concentration to an $OD_{570\,nm}$ of 10. Cells were then inoculated in GAS media at a 100-fold dilution. *Sinorhizobium meliloti* and *E. coli* strains were grown aerobically at 30 and 37 °C, respectively; growth was followed by determining the $OD_{570\,nm}$. When appropriate, antibiotics were added as follows: tetracycline (5 μg mL^{-1} for *S. meliloti* and 10 μg mL^{-1} for *E. coli*), gentamycin (25 μg mL^{-1} for *S. meliloti* and 5 μg mL^{-1} for *E. coli*), streptomycin (100 μg mL^{-1}) and neomycin (50 μg mL^{-1}).

DNA manipulations

Chromosomal and plasmid DNA isolation and manipulation were carried out according to standard procedures (Sambrook *et al.*, 1989). All the cloning steps were performed in *E. coli* DH5α.

Gene inactivation

rpoE2, *sodC* and *katC* fragments carrying the corresponding ORF were amplified by PCR from *S. meliloti* 1021 total DNA using the oligonucleotides E2tetG and E2tetD, sodC5 and sodC3, and katC5 and katC3, respectively. The amplicons were then cloned into pGEMTeasy (Promega). For *rpoE2* and *sodC*, a Gm^R cassette was introduced into a unique restriction site present in the gene (KpnI and ApaI, respectively), and the mutated fragments were then transferred into pK18 *mobsacB* vector (Schafer *et al.*, 1994). For *katC*, an internal fragment of the ORF (SmaI–ApaI) was cloned into the pK18*mob* vector (Schafer *et al.*, 1994). None of these constructions are replicative in *S. meliloti,* into which they were delivered by triparental mating using strain MT616 for their mobilization. *sodC* and *rpoE2* mutants were selected on

Table 1. Bacterial strains and oligonucleotides

Strains	Genotype	Origin
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1*	
	Δ(lacZYA–argG)U169 deoR	Hanahan (1983)
MT616	MM294 pRK600 Cm^R	Finan
Sinorhizobium meliloti		
1021	SU47 SmR	F. Ausubel
R326	1021 *sodB*::Tet^R	L. Barra
R641	1021 *rpoE2*::Gm^R	This study
R635	1021 *rpoE2-uidA*-Neo^R, *rpoE2*+	This study
R815	1021 *sodC*::Gm^R	This study
R874	1021 *katC*::Neo^R	This study
R743	1021 *sodC–uidA*–Neo^R, *sodC*+	This study
R744	1021 *sodC–uidA*–Neo^R, *sodC*+, *rpoE2*::Gm^R	This study
R741	1021 *sodB–lacZ*–Tet^R, *sodB*+	L. Barra
R742	1021 *sodB–lacZ*–Tet^R, *sodB*+, *rpoE2*::Gm^R	This study
R872	1021 *katC–uidA*–Neo^R, *katC*+	This study
R878	1021 *katC–uid*–Neo^R, *katC*+, *rpoE2*::Gm^R	This study
R876	1021 *katA–uidA*–Neo^R, *katA*+	This study
R877	1021*katA–uidA*–Neo^R, *katA*+, *rpoE2*::Gm^R	This study
	Sequence (5′–3′)	Purpose
Oligonucleotides		
E2tetG	GGATCCCGGTCACGCTGTGGC	*rpoE2* cloning
E2tetD	GGATCCTATCGCCTGACCGTCCAG	*rpoE2* cloning
sodC5	AATGACCGCCTTCGCCC	*sodC* cloning
sodC3	CGTAAGTATCCCCCACCAC	*sodC* cloning
katC5	TGGTCGCCCTTGACGGCC	*katC* cloning
katC3	TCGGCTTTCCATGTGCGG	*katC* cloning
katA5	ATCACCACCACCGCCGGG	*katA–uidA* fusion
katA3	GCGCTTGTCGGGAATGGGCC	*katA–uidA* fusion

LB medium containing 0.3 M sucrose, streptomycin and gentamycin. Clones were screened for neomycin sensitivity and recombination was confirmed by PCR analysis. *katC* mutants were selected as streptomycin–neomycin-resistant clones, and checked by PCR analysis. The three mutations were transduced into the wild-type or *rpoE2* strains using ΦM12 (Finan *et al.*, 1984). Because of a high reversion frequency, the *rpoE2* mutation was transduced into the wild-type strain before each set of experiments.

Construction of *rpoE2–*, *sodC–*, *katC–* and *katA–uidA* fusions in a wild-type background

DNA restriction fragments from recombinant pGEMT vectors (BamHI–ApaI, ApaI and ApaI–NsiI for *rpoE2*, *sodC* and *katC*, respectively) carrying the 3′-deleted ORFs were cloned into pTH1946 (Cowie *et al.*, 2006). For the *katA* fusion, the region was amplified by PCR using katA5 and katA3 primers, cloned into pGEMTeasy and transferred to pTH1946 as an NsiI–ApaI fragment. The orientation that allowed gene fusion with *uidA* was selected. These constructions were transferred into *S. meliloti* by triparental mating, and did not replicate further. Recombinants were selected on LB streptomycin–neomycin plates. Single crossover insertions were verified by PCR and transduced into the wild-type or the *rpoE2* strain by ϕM12-mediated transduction (Finan *et al.*, 1984).

Enzyme assay

The proteins were assayed using the method of Bradford, β-glucuronidase and β-galactosidase as described previously (Miller, 1972; Bardonnet & Blanco, 1992).

Resistance to exogenous O_2^-

Strains were grown in GAS medium to the stationary growth phase, harvested by centrifugation, washed twice in 1 mM EDTA, 50 mM, pH 7.8, potassium phosphate buffer (PBS) and adjusted to a density of 10^8 cells mL^{-1}. Xanthine (final concentration of 1000 μM) was added to cell suspensions, along with 1000 U mL^{-1} of bovine liver catalase, to detoxify any H_2O_2 generated by spontaneous dismutation of the O_2^- produced during the xanthine oxidase reaction. Superoxide generation was initiated by the addition of 0.5 U mL^{-1} of xanthine oxidase. Periodically, aliquots were collected and 20 μM cytochrome *c* was added. The rate of cytochrome *c* reduction was monitored at 550 nm. To stop the reaction, cells were collected by centrifugation (15 000 ***g***, 3 min) and resuspended in the same volume of PBS. Cell survival was analysed over time by 10-fold serial dilution and plating on LB plates.

Challenges with pyrogallol were performed by adding 2 mM pyrogallol and 1000 U mL^{-1} of bovine liver catalase to the cell suspension in PBS. Cells were incubated with shaking at 30 °C, aliquots were collected at different time intervals, centrifuged to remove pyrogallol and resuspended in the same volume of PBS. Survival was determined as described above. The generation of superoxides was estimated by the auto-oxidation rate of pyrogallol at 420 nm and the reduction of cytochrome *c* at 550 nm.

H_2O_2 resistance assay

Cells were grown in GAS medium and collected in the mid-exponential or the stationary growth phase and adjusted to 10^9 cells mL^{-1} in S medium. H_2O_2 was added to a final concentration of 100 mM. To ensure the removal of H_2O_2, aliquots were collected over time, centrifuged (12 000 ***g***, 1 min) and cells were resuspended in the same volume of S medium. Survival analysis was as described before.

Results and discussion

Inactivation and induction of *rpoE2*

In order to investigate the implication of RpoE2 in resistance to oxidative stress, its gene was disrupted by inserting a gentamycin-resistance gene into its ORF. *rpoE2* probably forms an operon with a gene encoding a conserved hypothetical protein (SMc01505) but located upstream, and so any phenotype observed in the mutant strain could not have resulted from a polar effect.

The expression of the *rpoE2–uidA* fusion was analysed in GAS medium. As observed previously (Sauviac *et al.*, 2007), *rpoE2* expression increased when cells entered the stationary growth phase (data not shown). In contrast, rpoE2 expression did not increase when H_2O_2 (1 and 10 mM) or menadione (5 and 50 μM) was added to growth medium during the exponential or the stationary growth phase (data not shown).

RpoE2 is necessary for *sodC* expression during the stationary growth phase

The presence of superoxide dismutase (SOD) was assayed by nondenaturing polyacrylamide gel electrophoresis and detection of SOD activity. Cells were grown in GAS medium and collected in the mid-exponential or the stationary growth phase. The SOD content was identical in wild-type and *rpoE2* cells collected during exponential growth (data not shown). Only one SOD was observed in both strains, corresponding to SodB (Santos *et al.*, 2000). The extracts of the wild-type strain collected in the stationary growth phase contained two SODs: SodB and SodC (Fig. 1). In contrast, SodC activity was nearly abolished in the *rpoE2* strain (Fig. 1). This result suggests that *rpoE2* is necessary for *sodC* but not for *sodB* expression.

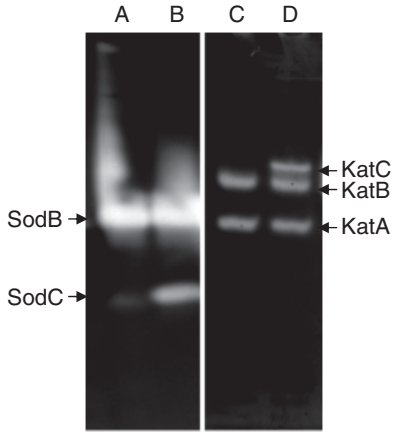

Fig. 1. SOD and catalase activity patterns of *Sinorhizobium meliloti* wild-type and *rpoE2* strains. Wild-type (B, D) and *rpoE2* strain (A, C) were grown in GAS medium to the stationary growth phase. Thirty micrograms of protein extract was subjected to electrophoresis through a native 10% (A, B) or 7.5% (C, D) polyacrylamide gel and stained for SOD activity (A, B) according to Beauchamp & Fridovich (1971) or catalase activity (C, D) (Clare *et al.*, 1984). The positions of KatA, KatB and KatC are according to Sigaud *et al.* (1999).

To confirm this result, *sodC–uidA* and *sodB–lacZ* transcriptional fusions were introduced into the wild-type and *rpoE2* strains by single crossing-over events, allowing the presence of both the fusion and a copy of the wild-type *sod* gene. In both strains, the expression of *sodB* appeared to be independent of *rpoE2* (Fig. 2). *sodC–uidA* expression was induced during the stationary phase of growth only in the wild-type strain (Fig. 2), suggesting that the lack of SodC activity in zymograms resulted from the absence of *sodC* transcription in the *rpoE2* strain. A consensus sequence ($GGAACNaN_{13-14}gcgTTt$) has been proposed for RpoE2-dependent promoters (Sauviac *et al.*, 2007). A motif corresponding to this consensus (**GGAACga**ccccggcagccgc**GCGTT**) is located 37 nucleotides upstream of *sodC* initiator codon, suggesting that RpoE2 exerts direct control on *sodC* transcription.

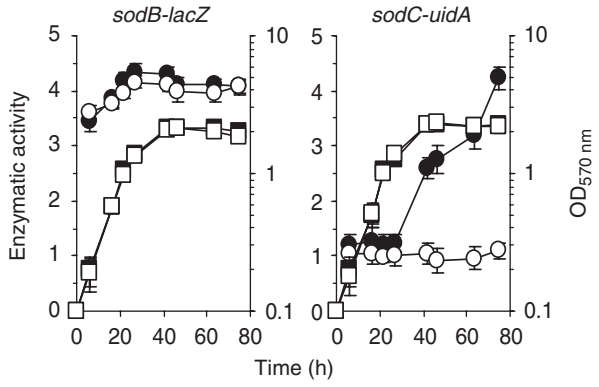

Fig. 2. Growth phase and RpoE2 dependence of *sodB* and *sodC* expression. The expression of *sodB–lacZ* and *sodC–uidA* fusions was analysed in a wild-type (closed symbols) and an *rpoE2* (open symbols) genetic background. Cells were grown in GAS medium, and the $OD_{570\,nm}$ (squares) and β-glucuronidase activity (circles) were determined periodically. β-glucuronidase and β-galactosidase activities are expressed in μmoles of substrate hydrolysed per minute per milligram of protein. Each value represents the average of at least three independent experiments; error bars show the SDs.

Resistance of *rpoE2* and *sodC* strains to superoxides

Wild-type, *rpoE2* and *sodC* strains were challenged with various superoxide-generating agents. The redox cycling agents menadione and paraquat induce intracellular superoxide production. When assayed by disc growth inhibition tests, they had the same inhibitory effect on the three strains (data not shown). Because SodC has been linked to resistance to extracytoplasmic superoxides in various strains, wild-type, *sodC* and *rpoE2* strains were also challenged with extracellular superoxides. Two methods were used to produce superoxides in the medium: the xanthine–xanthine oxidase reaction and the auto-oxidation of pyrogallol. No effect of extracellular superoxides produced by the xanthine –xanthine oxidase reaction was observed either in the wild-type strain or in the *sodC* and *rpoE2* strains (Fig. 3), with the reduction of cytochrome *c* indicating the effective production of superoxides. In contrast, pyrogallol exerted a lethal action on wild-type, *sodC* and *rpoE2* strains. The auto-oxidation of pyrogallol, which was monitored

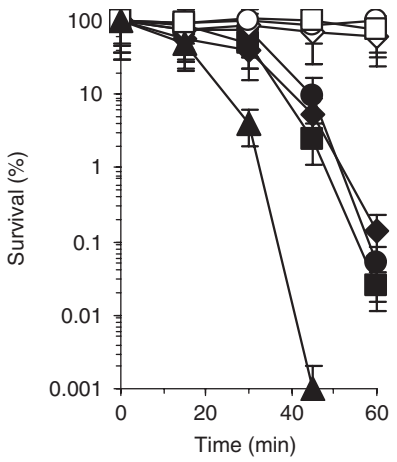

Fig. 3. Sensitivity of *Sinorhizobium meliloti* and its derivatives to extracellular superoxides. Wild-type (diamonds), *sodC* (squares), *sodB* (triangles) and *rpoE2* (circles) strains were grown to the stationary growth phase in GAS medium, and incubated in the presence of xanthine and xanthine oxidase (open symbols) or in the presence of pyrogallol (close symbols). The results are expressed as percent survival calculated for each strain by dividing the number of viable colonies obtained after the challenge by the number of colonies obtained before addition of a stress generator. The results are the average of three independent experiments; error bars represent the SDs.

spectroscopically throughout the experiments, together with the cytochrome *c* reduction, confirmed the production of superoxides. The assay was performed in the presence of catalase in the medium to ensure that lethality is not due to the action of H_2O_2 as described for *E. coli* (Korshunov & Imlay, 2002). Addition of 1000 U mL^{-1} of bovine SOD to the challenge test medium decreased the reduction of cytochrome *c* by 80%, whereas the sensitivity of parental *rpoE2* and *sodC* strains was not significantly modified. This result suggests that pyrogallol toxicity was not a result of its ability to generate superoxide extracellularly. *Sinorhizobium meliloti* possesses only one cytoplasmic SOD: SodB (Santos *et al.*, 2000; Davies & Walker, 2007). SodB inactivation resulted in a greater sensitivity to pyrogallol than that observed with parental or *sodC* strains (Fig. 3). In contrast, the *sodB* strain remained unaffected by superoxides produced by the xanthine–xanthine oxidase reaction, in accordance with the nonpermeability of biological membranes to superoxides at a neutral or a basic pH (Gort *et al.*, 1999). These results suggest that, in *S. meliloti*, pyrogallol promotes the production of intracellular superoxides by an unknown mechanism.

Superoxides are produced within the periplasm of gram-negative bacteria (Korshunov & Imlay, 2006), and also as antimicrobial agents during bacterial infection of eukaryotic cells. Thus, the infection process is impaired in *sodC* strains of *Salmonella typhimurium* (De Groote *et al.*, 1997; Pacello *et al.*, 2008), *Mycobacterium tuberculosis* (Piddington *et al.*, 2001) or *Brucella abortus* (Gee *et al.*, 2005). In some cases, *sodC* inactivation affects survival in challenge tests using a xanthine–xanthine oxidase reaction or pyrogallol (Schnell & Steinman, 1995; Gee *et al.*, 2005). In contrast, in other bacteria such as *E. coli* and *S. typhimurium,* mutants that lacked extracytoplasmic SOD were not sensitive to exogenous superoxides (Gort *et al.*, 1999). The absence of SodC in the *rpoE2* strain did not affect its symbiotic ability (Sauviac *et al.*, 2007). We observed that it did not affect its resistance to external superoxides. Hence, as in other bacteria, the role of *sodC* in *S. meliloti* and the phenotype associated with its inactivation await elucidation.

rpoE2 affects *katC* expression

Protein extracts of wild-type and *rpoE2* strains grown in GAS medium and collected in the stationary growth phase were analysed on a catalase activity gel. Three catalase activity bands were observed on extracts from *S. meliloti*, corresponding to KatA, KatB and KatC according to Sigaud *et al.* (1999) (Fig. 1). Extracts from the *rpoE2* strain showed KatA and KatB bands, but KatC was missing (Fig. 1).

To confirm the specific involvement of *rpoE2* in *katC* expression, *katC–uidA* or *katA–uidA* transcriptional fusions were introduced into wild-type or *rpoE2* strains, the resulting strains retaining a functional copy of both catalases. Cells were grown in GAS medium, and β-glucuronidase activity was determined throughout growth. In wild-type and *rpoE2* strains, *katA* expression was identical; it was low and constant during exponential growth and decreased slightly during the stationary growth phase as observed previously (Jamet *et al.*, 2003) (Fig. 4). This confirms that *rpoE2* is not involved in *katA* expression in accordance with the activity gel (Fig. 1) and also that the cells did not encounter a H_2O_2 stress during cultivation; otherwise, *katA* would be induced (Jamet *et al.*, 2005). In the wild-type strain, the expression of *katC* was low and constant during exponential growth, and then it increased as soon as the cells entered the stationary growth phase to reach an induced level fourfold greater than that observed during exponential growth, in agreement with the regulation of *katC* in *S. meliloti* RM5000 (Sigaud *et al.*, 1999). This increase was not observed in the *rpoE2* strain, suggesting that *rpoE2* is necessary for *katC* induction in the stationary growth phase (Fig. 4). This is in accordance with the presence of a putative RpoE2 promoter consensus sequence upstream of the *katC* gene (Sauviac *et al.*, 2007).

rpoE2 affects survival in response to H_2O_2 during the stationary growth phase

Sensitivity to 100 mM H_2O_2 was analysed in wild-type, *katC* and *rpoE2* strains grown in GAS medium. No difference was observed regardless of the strain when cells were collected in the exponential growth phase, all of them showing the same pattern of killing with a 3-log reduction of viable cells after 30 min of exposure (Fig. 5). Wild-type cells collected in the

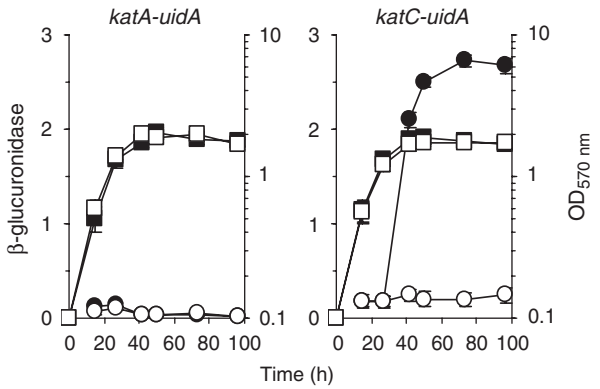

Fig. 4. RpoE2 dependence of *katA* and *katC* expression. The expression of *katA–uidA* or *katC–uidA* fusions was analysed in a wild-type (closed symbols) and an *rpoE2* genetic background (open symbols). The $OD_{570\,nm}$ (squares) and β-glucuronidase activity (circles) were determined periodically during growth in GAS medium. β-glucuronidase activity is expressed in μmoles of substrate hydrolysed per min per milligram of protein. Each value represents the average of at least three independent experiments; error bars show the SDs.

stationary phase of growth-acquired resistance to H_2O_2 as only 10% of the population was sacrificed after 30 min. In contrast to this, both *rpoE2* and *katC* cells remained as sensitive to H_2O_2 in the stationary phase of growth as they had been in the exponential phase (Fig. 5). This phenotype is in accordance with the expression patterns of *katC* and *rpoE2*. This suggests that the H_2O_2 sensitivity of the *rpoE2* strain during the stationary growth phase was due to the absence of KatC. This phenotype is similar to that reported for the σF mutant in *Caulobacter crescentus*, which is impaired in resistance to H_2O_2 exclusively during the stationary phase (Alvarez-Martinez *et al.*, 2006). The role of catalases in symbiosis has been widely studied. Strains deprived of one of them have no symbiotic defect (Sigaud *et al.*, 1999; Jamet *et al.*, 2003, 2005) as it has been observed for the *rpoE2* strain (Sauviac *et al.*, 2007). No role was previously attributed to these catalases in free-living bacteria. In this study, we show that KatC is necessary for resistance to H_2O_2 stress during the stationary growth phase.

Stability of *rpoE2* mutation

RpoE is essential in *E. coli*, and *rpoE* strain survival is maintained by the accumulation of compensatory mutations. In *S. meliloti*, *rpoE2* was described as a nonessential factor whose mutation confers no phenotype (Sauviac *et al.*, 2007). In this study, we report significant phenotypes concerning stress resistance during the stationary growth phase. These phenotypes were observed only when the *rpoE2* mutation was freshly transduced in a wild-type background. Conservation of the *rpoE2* strain on agar plates induced the development of phenotypic revertants that progressively lost the original phenotype. The *rpoE2* mutation did not alter survival in the stationary growth phase, but prolonged culturing resulted in progressive reversion of the phenotype. The reversion is not genetically linked to *rpoE2* because the *rpoE2* mutation transduced from revertants to wild-type cells yielded 100% transductants expressing the original *rpoE2* phenotype. Phenotypic reversion did not result from an increase in the mutation rate because rifampicin-resistant colonies appeared spontaneously at the same rate in *rpoE2* and wild-type cells during prolonged culture in the stationary growth phase. The distinct phenotypic traits of *rpoE2* strains observed in Sauviac's study (Sauviac *et al.*, 2007) and ours could also be explained by the use of different growth media that contained different concentrations of metals. Metals could play an important role in oxidative stress, either by being deleterious when they promote the Fenton reaction or by exerting a protective effect against oxygen radicals (Davies & Walker, 2007).

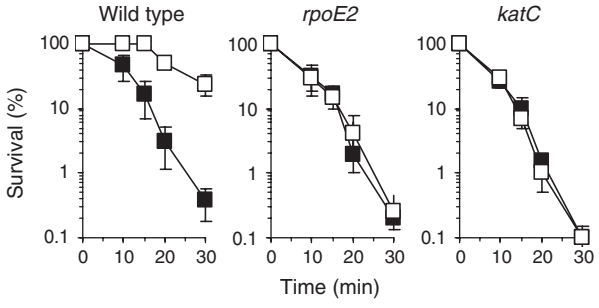

Fig. 5. Sensitivity of *Sinorhizobium meliloti* wild-type, *rpoE2* and *katC* strains to H_2O_2. All the strains were grown in GAS medium. Cells were collected in the exponential (closed symbols) or the stationary (open symbols) phase of growth and challenged with 100 mM H_2O_2. The data are represented as percent survival relative to unstressed cells ($t = 0$ min). The results are the mean of five independent experiments.

Acknowledgements

We thank S. Georgeault, C. Monnier, M. Uguet and M.C. Savary for technical assistance and D. Schuessler and R. Goude for improving the English. This work was supported by the CNRS and the Ministère de la Recherche.

References

Alvarez-Martinez CE, Baldini RL & Gomes SL (2006) A *Caulobacter crescentus* extracytoplasmic function sigma factor mediating the response to oxidative stress in stationary phase. *J Bacteriol* **188**: 1835–1846.

Bardonnet N & Blanco C (1992) *uidA* antibiotic resistance cassettes for insertion mutagenesis, gene fusion and genetic constructions. *FEMS Microbiol Lett* **93**: 243–248.

Beauchamp C & Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**: 276–287.

Clare DA, Duong MN, Darr D, Archibald F & Fridovich I (1984) Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal Biochem* **140**: 532–537.

Cowie A, Cheng J, Sibley CD, Fong Y, Zaheer R, Patten CL, Morton RM, Golding GB & Finan TM (2006) An integrated approach to functional genomics: construction of a novel reporter gene fusion library for *Sinorhizobium meliloti*. *Appl Environ Microbiol* **72**: 7156–7167.

Davies BW & Walker GC (2007) Disruption of sitA compromises *Sinorhizobium meliloti* for manganese uptake required for protection against oxidative stress. *J Bacteriol* **189**: 2101–2109.

De Groote MA, Ochsner UA, Shiloh MU, Nathan C, McCord JM, Dinauer MC, Libby SJ, Vazquez-Torres A, Xu Y & Fang FC (1997) Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *P Natl Acad Sci USA* **94**: 13997–14001.

Finan TM, Hartweig E, LeMieux K, Bergman K, Walker GC & Signer ER (1984) General transduction in *Rhizobium meliloti*. *J Bacteriol* **159**: 120–124.

Gee JM, Valderas MW, Kovach ME, Grippe VK, Robertson GT, Ng WL, Richardson JM, Winkler ME & Roop RM II (2005) The *Brucella abortus* Cu, Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. *Infect Immun* **73**: 2873–2880.

Gort AS, Ferber DM & Imlay JA (1999) The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. *Mol Microbiol* **32**: 179–191.

Helmann JD (2002) The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**: 47–110.

Jamet A, Sigaud S, Van de Sype G, Puppo A & Herouart D (2003) Expression of the bacterial catalase genes during *Sinorhizobium meliloti–Medicago sativa* symbiosis and their crucial role during the infection process. *Mol Plant Microbe In* **16**: 217–225.

Jamet A, Kiss E, Batut J, Puppo A & Herouart D (2005) The katA catalase gene is regulated by OxyR in both free-living and symbiotic *Sinorhizobium meliloti*. *J Bacteriol* **187**: 376–381.

Kazmierczak MJ, Wiedmann M & Boor KJ (2005) Alternative sigma factors and their roles in bacterial virulence. *Microbiol Mol Biol R* **69**: 527–543.

Korshunov S & Imlay JA (2002) A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria. *Mol Microbiol* **43**: 95–106.

Korshunov S & Imlay JA (2006) Detection and quantification of superoxide formed within the periplasm of *Escherichia coli*. *J Bacteriol* **188**: 6326–6334.

Marles-Wright J & Lewis RJ (2007) Stress responses of bacteria. *Curr Opin Struc Biol* **17**: 755–760.

Mascher T, Hachmann AB & Helmann JD (2007) Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. *J Bacteriol* **189**: 6919–6927.

Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, New York.

Pacello F, Ceci P, Ammendola S, Pasquali P, Chiancone E & Battistoni A (2008) Periplasmic Cu,Zn superoxide dismutase and cytoplasmic Dps concur in protecting *Salmonella enterica* serovar *Typhimurium* from extracellular reactive oxygen species. *Biochim Biophys Acta* **1780**: 226–232.

Pichereau V, Pocard J-A, Hamelin J, Blanco C & Bernard T (1998) Differential effects of dimethylsulfoniopropionate, dimethylsulfonioacetate, and other *S*-methylated compounds on the growth of *Sinorhizobium meliloti* at low and high osmolarities. *Appl Environ Microbiol* **64**: 1420–1429.

Piddington DL, Fang FC, Laessig T, Cooper AM, Orme IM & Buchmeier NA (2001) Cu, Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. *Infect Immun* **69**: 4980–4987.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Santos R, Herouart D, Puppo A & Touati D (2000) Critical protective role of bacterial superoxide dismutase in rhizobium–legume symbiosis. *Mol Microbiol* **38**: 750–759.

Sauviac L, Philippe H, Phok K & Bruand C (2007) An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti*. *J Bacteriol* **189**: 4204–4216.

Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G & Puhler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**: 69–73.

Schnell S & Steinman HM (1995) Function and stationary-phase induction of periplasmic copper–zinc superoxide dismutase and catalase/peroxidase in *Caulobacter crescentus*. *J Bacteriol* **177**: 5924–9592.

Sigaud S, Becquet V, Frendo P, Puppo A & Herouart D (1999) Differential regulation of two divergent *Sinorhizobium meliloti* genes for HPII-like catalases during free-living growth and protective role of both catalases during symbiosis. *J Bacteriol* **181**: 2634–2639.

Wosten MM (1998) Eubacterial sigma-factors. *FEMS Microbiol Rev* **22**: 127–150.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Effect of H_2O_2 on the expression of rpoE2–uidA.
Table S2. Influence of menadione on the expression of rpoE2–uidA.
Table S3. Influence of extracellular superoxides generated by xanthine–xanthine oxidase reaction on the expression of rpoE2–uidA.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.