Implication of (Mn)superoxide dismutase of *Enterococcus faecalis* in oxidative stress responses and survival inside macrophages

Nicolas Verneuil,¹ Alain Mazé,¹ Maurizio Sanguinetti,²
Jean-Marie Laplace,¹ Abdellah Benachour,¹ Yanick Auffray,¹
Jean-Christophe Giard¹ and Axel Hartke¹

Correspondence

Axel Hartke

axel.hartke@unicaen.fr

¹Laboratoire de Microbiologie de l'Université de Caen, EA956 USC INRA 2017, 14032 CAEN Cedex, France

²Institute of Microbiology, Catholic University of Sacred Heart, L. go F. Vito 1, 00168, Rome, Italy

The gene encoding the manganese-containing superoxide dismutase (MnSOD) of *Enterococcus faecalis* was characterized. It is transcribed monocistronically from an upstream promoter identified by rapid amplification of cDNA ends (RACE)-PCR. A *sodA* mutant was constructed and characterized. Growth of the mutant strain was not significantly different from that of its wild-type counterpart in standing and aerated cultures. However, the mutant was more sensitive towards menadione and hydroperoxide stresses. The response to H₂O₂ stress was analysed in more detail, and the mode of killing of this oxidant was different under anaerobic and aerobic conditions. Cultures grown and challenged under anaerobic conditions were highly sensitive to treatment with 35 mM H₂O₂. They were largely protected by the iron chelator deferoxamine, which suggested that killing was mainly due to an enhanced Fenton reaction. In contrast, neither strain was protected by the iron chelators deferoxamine and diethylenetriaminepentaacetic acid when grown and challenged under aerobic conditions, which suggested that inactivation of the cells by H₂O₂ was due to another killing mode. The *sodA* mutant was more sensitive under these conditions, showing that MnSOD is also important for protecting the cells from damage under aerobic conditions. Finally, the MnSOD of *Ent. faecalis* may be considered to be a virulence factor, since survival of the corresponding mutant strain was highly affected inside mouse peritoneal macrophages.

Received 14 February 2006

Revised 13 June 2006

Accepted 15 June 2006

INTRODUCTION

Reactive oxygen species (ROS), including superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH[•]) have many deleterious effects on living organisms, ranging from DNA-strand damage to peroxidation of membrane lipids (Imlay, 2003). Sources of reactive oxygen intermediates are abundant, and include incomplete reduction of oxygen during respiration, exposure to radiation or to redox-active compounds, and the respiratory burst of phagocytes. Aerobic organisms, however, have developed several enzymic and non-enzymic mechanisms to detoxify these very active compounds. Enzymically, ROS are removed mainly by the action of superoxide dismutase, catalase, glutathione peroxidase, NADH peroxidase, and glutathione reductase.

Abbreviations: Cm, chloramphenicol; DAPE, diaminopimelate epimerase; Em, erythromycin; MnSOD, manganese-containing superoxide dismutase; RACE-PCR, rapid amplification of cDNA ends PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; tBOOH, *tert*-butylhydroperoxide.

Superoxide dismutases (SODs) are metalloenzymes that catalyse the conversion of O₂^{•-} to H₂O₂ and molecular oxygen, and therefore form one of the major defence mechanisms of the cell against oxidative stress (Hassan, 1989). There are three main classes of SOD in bacteria, the manganese SOD (MnSOD, SODA), the iron SOD (FeSOD, SODB), and the copper-zinc SOD (CuZnSOD, SODC). The presence of SODs in virtually all facultative and aerobic organisms and several anaerobes suggests that O₂^{•-} presents a problem for organisms growing in the presence of O₂. Although *in vitro* experiments have established that O₂^{•-} does not react detectably with biomolecules (Imlay, 2002, 2003), characterization of mutants lacking cytosolic SOD has provided the first *in vivo* evidence that excess O₂^{•-} causes DNA damage (Farr *et al.*, 1986; Keyer *et al.*, 1995). Several studies conducted with *Escherichia coli* have provided strong support for the theory that O₂^{•-} triggers the Fenton reaction (H₂O₂ + Fe²⁺ → O₂^{•-} + Fe³⁺ + OH⁻) by the oxidative release of iron from iron-containing molecules, such as those containing [4Fe-4S] clusters, thereby increasing the pool of free iron available to catalyse hydroxyl

radical production (Gardner & Fridovich, 1992; Keyer *et al.*, 1995; Keyer & Imlay, 1996). O₂^{•-} is a powerful oxidant that reacts with biomolecules, including DNA, at virtually diffusion-limited rates. Thus, the higher free iron levels in SOD mutants also explain why they are more sensitive to killing by H₂O₂ than their wild-type counterparts (Carlioz & Touati, 1986). This effect of H₂O₂ can be blocked by cell-permeable iron chelators, confirming that the killing is mediated by Fenton chemistry (Imlay & Linn, 1988).

Enterococcus faecalis, a Gram-positive facultative anaerobic bacterium, belongs to the normal part of the intestinal flora. It is emerging as a major component of hospital-acquired infections, such as urinary tract, surgical wound, abdominal, pelvic and neonatal infections. Furthermore, it is an important cause of endocarditis and of mortality due to enterococcal bacteraemia (Gilmore *et al.*, 2002). Many strains of *Ent. faecalis* are resistant to most antibiotics, and many have acquired resistance to vancomycin, rendering conventional therapies insufficient for serious infections (Kak & Chow, 2002).

The oxidative stress response of *Ent. faecalis* has been analysed in our laboratory. Physiological experiments have revealed that this bacterium is able to strongly resist H₂O₂ treatment, demonstrating that it is well equipped with anti-oxidant defences (Flahaut *et al.*, 1998). *Ent. faecalis* contains a single MnSOD that is induced by oxygen (Britton *et al.*,

1978). The purified enzyme has a mass of 45 kDa and is a homodimer. SOD has been shown to be a virulence factor for several pathogenic micro-organisms (Yesilkaya *et al.*, 2000; Poyart *et al.*, 2001; Narasipura *et al.*, 2003, 2005; Giles *et al.*, 2005). In this study, we constructed and characterized a *sodA* deletion mutant of *Ent. faecalis*. We show that MnSOD plays a central role in the oxidative stress response in this bacterium, and we present data indicating the presence of sophisticated mechanisms allowing *Ent. faecalis* to rapidly adjust its intracellular free-iron levels depending on the oxygen status of the environment. Finally, we show that the survival of the SODA mutant inside mouse peritoneal macrophages is severely affected.

METHODS

Reagents. Deferoxamine mesylate, diethylenetriaminepentaacetic acid, menadione sodium bisulfite, paraquat (methyl viologen), *tert*-butylhydroperoxide (tBOOH) and PMSF were purchased from Sigma. IPTG, X-Gal and oligonucleotides were purchased from Eurobio. Restriction enzymes, *Taq* DNA polymerase, dNTPs and [α -³²P]dATP were purchased from Amersham Biosciences. PCR reactions were performed with PCR Master Mix (Eppendorf). T4 DNA ligase was purchased from Roche Diagnostics.

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *Ent. faecalis* strain JH2-2 (Jacob & Hobbs, 1974; Yagi & Clewell, 1980) and its derivatives were grown at 37 °C in M17 medium

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant characteristics and oligonucleotide sequence	Reference or source
<i>Ent. faecalis</i>		
JH2-2	Fus ^R Rif ^R , plasmid-free wild-type strain	Jacob & Hobbs (1974); Yagi & Clewell (1980)
Δ <i>sodA</i>	JH2-2 isogenic derivative <i>sodA</i> mutant	This work
<i>E. coli</i>		
EC101	Kan ^R <i>supE thi (lacproAB) (F' traD36 proAB lacI^f ZAM15) repA</i>	Law <i>et al.</i> (1995)
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Lac [F' proAB lacI^f ZAM15 Tn10 (Tet^R)]</i>	Stratagene
Plasmids		
pORI ₁₉₋₁	pWV01 derivative, Em ^R Ori ⁺ RepA ⁻ lacZ'	Law <i>et al.</i> (1995)
pG ⁺ host ₃	pWV01 derivative, Cm ^R repA ^{ts} , (previously named pVE6007)	Maguin <i>et al.</i> (1992)
pMAD	ori pE194 ^{ts} , Em ^R , Amp ^R , <i>bgaB</i>	Arnaud <i>et al.</i> (2004)
Oligonucleotides		
<i>RACE1</i>	GGTGAATCTTGGTTTGGTGTTG	
<i>RACE2</i>	GCCACCATTGTTACGAACAGCTGTGC	
<i>RACE3</i>	GATTTTTCGCCTAATTCTGG	
<i>sod1</i>	GACTAACTTAACTGCAGCGAT	
<i>sod2</i>	CCAGTTAACAAGGTACCAGAATG	
<i>sod3</i>	CTGAGGATCCTCATCAAAGCTATCAAATGTTTC	
<i>sod4</i>	CTGAGGATCCCTGCTTTGAAAACAGCTGCAAC	
<i>sodMad1</i>	GCTATGGATCCTTTTTTTAAGAACCAGGG	
<i>sodMad2</i>	GCTATCCATGGATAATGTTTCTGTTTCATTGC	
<i>mad1F</i>	TCTAGCTAATGTTACGTTACAC	
<i>mad2R</i>	TCATAATGGGGAAGGCCATC	

(Terzaghi & Sandine, 1975) supplemented with 0.5% glucose (GM17). Standing and aerated cultures were grown in 30 ml tubes and 100 ml Erlenmeyer flasks, respectively, filled with 10 ml GM17 medium. Aerated cultures were incubated at 37 °C with vigorous shaking (150 r.p.m.). In the case of anaerobic cultures, the GM17 medium was bubbled with nitrogen for 3 h prior to use, and the absence of oxygen was controlled with a Clark electrode (Jeulin). When required, erythromycin (100 µg ml⁻¹) was added. *E. coli* strains were cultured with vigorous shaking at 37 °C in LB medium (Sambrook *et al.*, 1989) with ampicillin (100 µg ml⁻¹) or erythromycin (300 µg ml⁻¹), when required.

Challenge conditions. Exponentially growing culture (10 ml) (OD₆₀₀ 0.5) was harvested by centrifugation and resuspended in 10 ml of either fresh GM17 medium (H₂O₂ challenges) or 0.9% (w/v) NaCl (menadione and tBOOH challenges) containing the stressing agent. These cultures were placed into a 37 °C water bath, and at the desired time point, samples were taken and rapidly diluted in 0.9% NaCl. Viability was determined by spreading 0.5 ml of appropriate serial dilutions on GM17 agar (1.5%, w/v) and c.f.u. were determined after 48 h incubation at 37 °C. Killing by H₂O₂ was assessed as the percentage of surviving colonies in comparison with unchallenged cells.

General molecular methods. Molecular cloning and other standard techniques were performed essentially as described by Sambrook *et al.* (1989). *E. coli* and *Ent. faecalis* were transformed by electroporation using Gene Pulser Apparatus (Bio-Rad Laboratories). Plasmids and PCR products were purified using Qiagen kits.

Mapping of the transcriptional start site. The 5' end of *sodA* mRNA was mapped from a 5' rapid amplification of cDNA ends (RACE)-PCR product obtained with the 3'/5' RACE kit (Roche Diagnostics). For this purpose, we used total RNA extracted from *Ent. faecalis* JH2-2 cells cultured with aeration and harvested 1 h after the onset of stationary phase. Briefly, the first strand cDNA was

synthesized using the primer RACE1, AMV reverse transcriptase and the deoxynucleotide mixture of the 3'/5' RACE kit as recommended by the manufacturer. After purification and dA-tailing of the cDNA, a nested PCR using the oligo dT-anchor primer and the specific *sodA* RACE2 primer was carried out. The PCR product was purified and sequenced by the dideoxy chain-termination method with the ABI Prism sequencing system (PE Biosystems) using primer RACE3 (Table 1).

Analysis of mRNA by Northern blot experiments. Total RNA of *Ent. faecalis* JH2-2 strain was isolated using the RNeasy Midi kit (Qiagen). For Northern blots, 10 µg per lane of total RNA was electrophoretically resolved and transferred onto Hybond-N⁺ membranes (Amersham Biosciences) using standard procedures (Sambrook *et al.*, 1989). Sizes of transcripts were estimated by comparison with an RNA ladder (0.56–9.4 kb) (Amersham Biosciences). Membrane-bound nucleic acids were hybridized at a temperature of 55 °C in 1 M sodium phosphate buffer (pH 7.0) containing 5% (w/v) SDS with a single-strand labelled probe complementary to *sodA* mRNA. After hybridization, membranes were washed twice in 2 × saline sodium citrate (SSC), 0.1% SDS (10 min), twice in 0.5 × SSC, 0.1% SDS (10 min) at 55 °C, and exposed to a storage phosphor screen (Packard Instrument Company).

Preparation of the single-strand labelled probe was as follows. First, a DNA fragment was amplified by PCR from chromosomal DNA of *Ent. faecalis* JH2-2 with the primers *sod1* and *sod2* (see Table 1 for the sequences of the primers). The probe was then synthesized by elongating the specific oligonucleotide *sod2* (Fig. 1) with *Taq* DNA polymerase, 2 mM each of dCTP, dGTP and dTTP, 2 µCi (74 kBq) [α -³²P]dATP and 10 ng of the previous PCR DNA fragment as template. Thirty cycles of 20 s at 94 °C, 30 s at 52 °C and 45 s at 72 °C were performed.

Construction of a Δ *sodA* mutant. A *sodA* mutant was constructed by a gene replacement event using a method based on the

Fig. 1. Schematic representation of the genetic organization of the *sodA* chromosomal region of *Ent. faecalis*. Large arrows represent the ORFs, and their orientation shows the transcriptional direction. The gene upstream of *sodA* encodes a hypothetical protein (Hp) and the downstream gene a DAPE. The nucleotide sequences of the *sodA* promoter region and the putative Rho-independent terminator are shown. The transcriptional initiation nucleotides (+1) and the putative -35 and -10 motifs are indicated. The Northern blot (NB) demonstrates the monocistronic organization of the *sodA* gene. The transcriptional start site was determined by 5' RACE-PCR. The sequence in the electropherogram was obtained using primer RACE3 and cDNA from 5' A-tailed cDNA.

conditional replication of the pORI₁₉₋₁/pG⁺host₃ system (Law *et al.*, 1995). A 425 bp *sodA* internal DNA fragment was amplified by PCR using total DNA of *Ent. faecalis* JH2-2, and primers *sod1* and *sod2* containing *Pst*I and *Kpn*I sites, respectively (Fig. 1, Table 1). This PCR fragment was digested by the two enzymes and then cloned into pORI₁₉₋₁ previously digested by *Pst*I and *Kpn*I, and finally transformed into electrocompetent cells of *E. coli* EC101. The resulting plasmid was used as DNA template to perform a second PCR using primers *sod3* and *sod4*, each harbouring a *Bam*HI site at the 5' end (Fig. 1, Table 1). Amplification with these oligonucleotides introduced two stop codons and a central deletion of 8 bp in the coding sequence of *sodA*. The resulting PCR product was digested with *Bam*HI, purified, and ligated to obtain a circular plasmid which was then used to transform *E. coli* EC101. Recombinant pORI₁₉₋₁ plasmids carrying the JH2-2 chromosomal mutated DNA fragment were used to transform *Ent. faecalis* JH2-2 in which plasmid pG⁺host₃ (pVE6007) (Maguin *et al.*, 1992) encoding a thermo-sensitive RepA protein had previously been introduced. After electroporation, the two plasmids were maintained together at the permissive temperature of 30 °C by plating cells on GM17 agar medium containing erythromycin (Em) and chloramphenicol (Cm). Several clones were grown for 1 h at 30 °C in GM17 broth without antibiotic and then incubated for 3 h at 42 °C before being plated on GM17 agar medium containing only Em. After 48 h at 42 °C, some Em^R clones were transformed with the helper plasmid pG⁺host₃. After 100 generations at 30 °C on GM17 broth containing Cm, the resulting transformants were grown for 1 h at 30 °C on GM17 without antibiotic and transferred to 42 °C for 3 h, before being plated on solid GM17 medium and incubated at 42 °C. Following this step, Em-sensitive clones were screened for the presence of the mutated *sodA* allele by PCR and restriction analysis.

Complementation of the *sodA* mutant. For the complementation assays, a DNA fragment containing the entire *sodA* gene as well as upstream (1012 bp) and downstream (1100 bp) sequences, obtained by PCR (Triple Master Polymerase Mix, Eppendorf) using primers *sodMad1* and *sodMad2*, was cloned into plasmid pMAD (Table 1). Recombinant plasmid (1 µg) was finally used to transform competent cells of the *Ent. faecalis* *sodA* mutant. After electroporation, 300 µl of cell suspension was plated onto GM17 agar containing 150 µg Em ml⁻¹ and 80 µg X-Gal ml⁻¹. The plates were incubated for 48 h at 30 or 37 °C. In both cases, a few dark-blue colonies were obtained and analysed for the presence of the plasmid by PCR using primers *mad1F* and *mad2R*. Several of these blue colonies were then cultured twice in GM17Ery liquid medium at 45 °C overnight. In a next step, the cultures were used to inoculate (0.05 %, v/v) GM17 liquid medium without antibiotic. The tubes were incubated for 4 h at 30 °C, followed by incubation at 42 °C overnight. This step was repeated four to five times. Serial dilutions of the culture were plated on GM17 agar containing 80 µg X-Gal ml⁻¹ and incubated for 48–72 h at 45 °C. Among a vast majority of dark- and light-blue colonies, 0.1–0.3 % white colonies were present, and represented candidate clones resulting from a double-crossover event. These white colonies were isolated on GM17 agar with or without Em. Antibiotic-sensitive clones were analysed by PCR for the presence of an intact *sodA* gene.

Preparation of cell extracts, PAGE, and SOD activity detection. Cultures of *Ent. faecalis* JH2-2 and its *ΔsodA* derivative were harvested by centrifugation, the pellets were washed once in 50 mM Tris, 1 mM EDTA (pH 7.0), and finally resuspended in 50 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.14 mM PMSF (pH 7.0). Glass beads (0.1–0.25 mm diameter) were added, and the cells were disrupted by vortexing for 5 min. The samples were chilled on ice, and the extraction procedure repeated. After centrifugation for 10 min at 6700 g to remove unbroken cells, the supernatants were transferred

to new tubes. Protein concentration was determined by the method of Lowry *et al.* (1951). Non-denaturing PAGE was carried out according to the protocol of Laemmli (1970), except that SDS and mercaptoethanol were omitted. Equal amounts of protein (30 µg) were loaded in each lane. In each assay, one gel was stained with Coomassie brilliant blue for total protein detection, and a second one was used to determine SOD activity according to the protocol of Beauchamp & Fridovich (1971).

Survival assays in mouse peritoneal macrophages. Survival of *Ent. faecalis* in mouse peritoneal macrophages was tested using an *in vivo-in vitro* infection model, as described previously (Verneuil *et al.*, 2004). Briefly, the *Ent. faecalis* *ΔsodA* mutant and JH2-2 wild-type strain were grown aerobically at 37 °C in brain heart infusion (BHI) for 16 h. Then, the bacteria were harvested by centrifugation and resuspended in a volume of PBS sufficient for injection. Male BALB/c mice (10 weeks old; Harlan Italy) were infected with 10⁷–10⁸ cells of each strain by intraperitoneal injection. After a 6 h infection period, the peritoneal macrophages were collected by peritoneal lavage, centrifuged and suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10 mM HEPES, 2 mM glutamine, 10 % bovine fetal serum, and 1 × non-essential amino acids, supplemented with vancomycin (10 µg ml⁻¹) and gentamicin (150 µg ml⁻¹). The cell suspension was dispensed into 24-well tissue-culture plates, incubated at 37 °C under 5 % CO₂ for 2 h, and bacterial survival was monitored at 24, 48 and 72 h after infection.

RESULTS

Genomic context and mapping of the transcriptional start site of the *sodA* gene

Analysis of the *Ent. faecalis* V583 sequence (The Institute of Genomic Research, <http://www.tigr.org>) revealed the presence of only one gene (*EF0463*) that encoded a protein highly similar to SODs from evolutionarily closely and distantly related organisms. The corresponding polypeptide of 202 amino acids had a predicted molecular mass of 22.7 kDa and a calculated pI of 4.8. The four Mn²⁺ metal ion binding residues characteristic of MnSOD were conserved (Parker and Blake, 1988). Eight nucleotides upstream of the initiation codon ATG and 9 nt downstream of the TAA stop codon, a potential ribosomal binding site (RBS) sequence (AGGGGGA) and a 27 bp inverted repeat ($\Delta G_{25\text{ °C}} = -46.6 \text{ kcal mol}^{-1}$ ($-194.8 \text{ kJ mol}^{-1}$), which could function as a Rho-independent terminator, were found, respectively (Fig. 1). The enterococcal *sodA* gene is flanked 193 nt upstream by a divergently transcribed gene encoding a hypothetical protein and 148 nt downstream by a gene encoding a putative diaminopimelate epimerase (DAPE) transcribed in the same direction as the *sodA* gene. The presence of the Rho-independent strong terminator between the two genes strongly suggested a monocistronic operon structure for the *sodA* gene, which was confirmed by Northern blot analysis. Indeed, a unique transcript of approximately 0.6 kb was detected (Fig. 1).

The transcriptional start site was determined by 5' RACE-PCR. It was identified as G, T or T located between 47 and 49 nt upstream of the start codon (Fig. 1). Upstream of this start point, a putative extended -10 box was found that

matched nine of the ten bases (TGNTATAAT, matches underlined) with the consensus sequence (Burr *et al.*, 2000). A potential -35 box was also present 17 bp upstream of the second T of the extended -10 box. However, only the first three bases matched with the consensus sequence (TTGACA).

Phenotypic characterization of a *sodA* deletion mutant

To examine possible phenotypic effects of SODA deficiency, the wild-type gene was replaced with a mutated copy of *sodA* containing stop codons and a central deletion as described in

Methods. Fig. 3 (A) shows that no residual SODA activity could be detected in the $\Delta sodA$ mutant strain. The growth behaviours of the wild-type and mutant strains in standing culture were essentially identical (Fig. 2A), and under aerated conditions the $\Delta sodA$ mutant showed only a slight retardation in growth compared with the wild-type strain. This experiment was repeated, but in the presence of 2 mM H_2O_2 . As can be seen from Fig. 2(A), both strains were similarly blocked in growth during the first 3 h under these conditions, but the mutant was more affected than the wild-type strain beyond this time point. Indeed, the wild-type strain regained a comparable growth rate ($\mu = 1.2 \text{ h}^{-1}$) and entered stationary phase at a final OD_{600} comparable to

Fig. 2. (A) Comparison of growth between the *Ent. faecalis* wild-type strain JH2-2 (triangles) and the isogenic $\Delta sodA$ strain (circles) under aerobic (solid symbols) and anaerobic (open symbols) conditions, and aerobic growth in the presence of 2 mM H_2O_2 (dotted lines). All data represent mean values derived from at least three independent experiments. (B and C) Survival of the wild-type (○) and the $\Delta sodA$ strain (△) in the presence of 50 mM menadione (B) and 155 mM tBOOH (C). (D) Survival of the wild-type (○) and the $\Delta sodA$ (△) mutant strain of a 30 min exposure to different concentrations of H_2O_2 . Stress exposure was carried out with agitation. All data represent mean values derived from at least three independent experiments.

Fig. 3. Complementation experiments of the Δ sodA mutant. (A) Zymogram of SOD activity. Lane 1, wild-type strain; lane 2, Δ sodA mutant; lanes 3–5, three SODA mutant strains complemented with a wild-type copy of the *sodA* gene. (B) Survival of wild-type (○), Δ sodA mutant (△) and complemented strains (▲) of exposure to 25 mM H_2O_2 . The three complemented strains shown in (A) gave essentially the same results.

that of unstressed cells, whereas the Δ sodA strain continued over the entire experiment to grow at a significantly reduced growth rate ($\mu = 0.6 \text{ h}^{-1}$, instead of 0.9 h^{-1} for unstressed cells) in the presence of H_2O_2 .

Next we analysed survival after treatment with different oxidants. Wild-type cells were completely resistant to exposure to 50 mM of the redox-cycling agent menadione, whereas this treatment killed nearly 99.99 % of the Δ sodA mutant cells after 30 min of exposure (Fig. 2B). It is noteworthy that the most commonly used redox-cycling agent paraquat did not significantly affect the viability or growth rate of the wild-type or the Δ sodA mutant. Indeed, after 30 min of treatment, 90 % viable cells could be recovered in both cases (results not shown). The Δ sodA mutant was several orders of magnitude more sensitive than the isogenic parental strain to the organic hydroperoxide tBOOH (Fig. 2C). In the case of H_2O_2 , different concentrations were analysed, since in *E. coli*, it has been shown that killing by H_2O_2 is bimodal (Imlay & Linn, 1986), i.e. low (1–2 mM for mode-one killing) and high (>20 mM for mode-two killing) concentrations of H_2O_2 are more lethal than intermediate concentrations. This was not the case for *Ent. faecalis*, since the lethality of H_2O_2 increased with increasing concentrations of this oxidant for the two strains (Fig. 2D).

Complementation assays

In order to verify that the observed physiological effects were the consequence of the deleted *sodA* gene, a complemented strain was constructed using the pMAD vector. This shuttle vector carries a *lacZ* gene of *Bacillus stearothermophilus*, which allows quick colorimetric blue/white discrimination on X-Gal plates of bacteria which have lost the plasmid (Arnaud *et al.*, 2004). It has recently been shown that this vector is a valuable tool for the construction of deletion mutants in several Gram-positive bacteria, although not in streptococci, enterococci and lactococci, in which the thermosensitive (ts) pE194 origin of replication is obviously

non-functional (Arnaud *et al.*, 2004). Despite this restriction, we tried to use the pMAD vector as a suicide plasmid for *Ent. faecalis*. After transformation with the vector harbouring the wild-type *sodA* gene as well as surrounding sequences, only a few dark-blue colonies were obtained, despite the use of a high amount of recombinant plasmid (see Methods for more details). This result was expected for a non-replicative suicide plasmid. However, several subsequent observations seemed to indicate that the pE194 origin is at least partially functional and even thermo-sensitive in *Ent. faecalis*. First, the plasmid was very rapidly lost from the cells if the culture had been grown at 45°C , the restrictive temperature for the pE194ts origin. Indeed, after overnight growth at 37°C without selection, the vast majority of colonies were white, and the analysed clones harboured exclusively the mutated allele of the *sodA* gene. This result may be explained by assuming that the plasmid was replicative under these conditions. Second, when grown for several generations at 45°C (origin pE194 non-functional) in the presence of Em, it was very difficult to lose the plasmid. This result may be explained by assuming that the plasmid was integrated into the chromosome by homologous recombination. Only by successive cycles of growth at 30°C followed by 45°C , as described in Methods (but not with growth at 30 or 37°C alone), could white colonies be obtained after four to five cycles. Several showed the presence of the wild-type allele, as judged by PCR analysis using primers *sod1* and *sod2*. Finally, using 1 μ l of supernatant of crude extracts of a culture obtained from a blue colony in a transformation experiment resulted in a high number of transformants in *E. coli*. The combined results demonstrated that the pMAD vector is also an interesting tool for *Ent. faecalis*, and a growing number of constructions have now been successfully done with this tool in our laboratory.

Three of the complemented *sodA* mutants were analysed for SOD activity, and all three were positive (Fig. 3A). Furthermore, complementation restored resistance to H_2O_2

exposure (Fig. 3B). This demonstrates that the important physiological alterations described in this study were due to the inactivation of the *sodA* gene.

Sensitivity to H₂O₂ is dependent on the oxygen status of the environment

Sensitivity towards H₂O₂ was, particularly for the wild-type strain, highly dependent on the level of oxygen in the

environment. In the absence of O₂ during growth and challenge, the survival after 20 min of treatment of the wild-type strain was comparable with that of the Δ *sodA* mutant (Fig. 4A, B). When these experiments were conducted in the presence of O₂, the *Ent. faecalis* wild-type strain displayed a tremendous increase in survival of nearly three orders of magnitude after 20 min of treatment compared with the results obtained under anaerobiosis (Fig. 4A). The Δ *sodA* mutant showed this oxygen effect only in the first 10 min

Fig. 4. Survival in the presence of 25 mM H₂O₂ of *Ent. faecalis* wild-type (A) and Δ *sodA* mutant (B) strains grown and challenged under aerated (open symbols) and strict anaerobic (closed symbols) conditions in the presence (squares) or absence (circles) of deferoxamine. The results obtained using aerobic cultures in the presence of deferoxamine were repeated using the cell-penetrant iron chelator diethylenetriaminepentaacetic acid, and comparable results were obtained. All data represent mean values derived from at least three independent experiments, and SD (bars) is indicated. (C) Decrease in survival of *Ent. faecalis* wild-type strain JH2-2 with increasing time of exposure to anaerobiosis before challenge. Cultures were challenged either immediately (1) or exposed for 90 s (2), 150 s (3), 5 min (4) or 10 min (5) to nitrogen, and then challenged without agitation with 35 mM H₂O₂ for 30 min. Survival of the Δ *sodA* mutant strain after exposure for 10 min to anaerobiosis prior to challenge is also shown (6). In all cases, cells were cultured with agitation. After centrifugation they were resuspended in fresh GM17 medium. (D) Results obtained with cultures of the wild-type (1, 2) and Δ *sodA* mutant (3, 4) grown under strictly anaerobic conditions and immediately challenged (20 min) with agitation with 35 mM H₂O₂. These experiments were conducted in the absence (1, 3) and presence (2, 4) of deferoxamine. Survival of the wild-type strain grown and challenged under aerobic conditions is also shown (5).

of treatment, but thereafter aerobically grown cells were nearly as sensitive as anaerobically grown cells after 20 min of treatment (Fig. 4B).

The transition from a resistant to a sensitive cellular state seemed to be a very rapid process in *Ent. faecalis*. This was shown by a kinetic study using wild-type cells grown with agitation and harvested in the exponential growth phase. In these experiments, the time of exposure to anaerobiosis before the H₂O₂ challenge was varied between 0 and 10 min (Fig. 4C). The sensitivity of the wild-type strain increased in a time-dependent manner in these experiments. After 10 min of exposure to anaerobiosis, the cells were as sensitive as cultures grown and challenged under anaerobiosis. Interestingly, the contrary, i.e. the transition from a sensitive to a resistant cellular state, seemed to be even more rapid. This was obvious from the experiments conducted with cultures grown under nitrogen atmosphere and immediately challenged under aerobic conditions (Fig. 4D). Unexpectedly, the wild-type and mutant cells were as resistant or slightly more resistant, respectively, as cultures grown and challenged under aerobic conditions (compare Fig. 4A, D).

The iron chelator deferoxamine protects cells under anaerobic but not under aerobic conditions

Deferoxamine is an iron chelator that binds free Fe³⁺ and, in the presence of oxygen, both binds and promotes the oxidation of Fe²⁺. It has been shown that this compound enters into *E. coli* cells and protects them from damage by H₂O₂ by binding the intracellular free iron that would otherwise catalyse the Fenton reaction (Keyer & Imlay, 1996). The effect of deferoxamine in *Ent. faecalis* was tested, and the results are shown in Fig. 4(A, B). When cells were grown and challenged under strict anaerobic conditions, the wild-type and, to a lesser degree, the Δ sodA mutant strains were highly protected by 0.1 mM of the chelator. After 20 min of treatment, survival increased by three and two orders of magnitude, respectively, for the two strains in comparison with cultures not treated with the chelator. In contrast, virtually no protection by deferoxamine using 0.1 mM or even 20 mM, or by 1 mM diethylenetriaminepentaacetic acid, another cell-penetrant iron chelator (Touati, 2002), was observed with cultures challenged under aerobic conditions. However, in contrast to the cultures grown and challenged under aerobic conditions which were not protected by iron chelators, cultures of the wild-type and the Δ sodA mutant grown under nitrogen atmosphere and immediately challenged under aerobic conditions were slightly protected by deferoxamine (Fig. 4D).

SODA is necessary for survival within murine macrophages

Ent. faecalis has been identified in recent years as a major component of hospital-acquired infections. In general,

non-professional phagocytes are the first defence against invading pathogens, and the ability to avoid or survive phagocytic attack is thus very important for a pathogen. Since, during phagocytosis, phagocytic cells generate superoxide and other ROS, which are involved in antibacterial activity (Hassett & Cohen, 1989), we were interested to compare the intracellular survival of the Δ sodA mutant and the wild-type strain inside infected mouse peritoneal macrophages. The uptake was similar for both strains, since 6×10^5 and 4×10^5 c.f.u. were recovered immediately after infection with the wild-type and the Δ sodA mutant, respectively. As can be seen from Fig. 5, the Δ sodA mutant was killed more rapidly over the 72 h time-course of the experiment than its wild-type counterpart. At the end of the experiment, the ratio of the survival of the wild-type and the mutant strain was nearly 400. This demonstrated that the MnSOD of *Ent. faecalis* is also important for survival in the toxic macrophage environment.

DISCUSSION

Ent. faecalis contains a single MnSOD that has been well characterized at the biochemical level (Gregory & Fridovich, 1973; Britton *et al.*, 1978). This enzyme has a molecular mass of 45 000 Da and is composed of two subunits. It contains 1.3 atoms of manganese per molecule (Britton *et al.*, 1978), and it is rapidly induced when cells are shifted from anaerobiosis to hyperbaric oxygen conditions (Gregory & Fridovich, 1973). However, no genetic studies investigating the involvement of this enzyme in the oxidative stress response of *Ent. faecalis* have been performed.

A mutant was constructed by replacing the wild-type gene with a mutated copy of *sodA*. The resulting mutant had no

Fig. 5. Time-course of intracellular survival of the *Ent. faecalis* wild-type (Δ) and Δ sodA mutant strain ($\square$) within murine peritoneal macrophages. The results represent the mean $\pm$ SD of viable intracellular bacteria per 10^5 macrophages of three independent experiments with three wells.

significant growth defect in comparison with its wild-type counterpart when grown in standing or aerated cultures. It has been shown for other bacterial species that the sensitivity of SOD mutants to oxygen varies greatly, ranging from no effect to a strong effect on aerobic growth (Touati, 2002).

Ent. faecalis is known as a potent producer of extracellular $O_2^{\cdot-}$ when exposed to aerobic conditions (Huycke *et al.*, 1996). Biosynthesis of demethylmenaquinone by this bacterial species is supposed to be responsible for this phenomenon (Huycke *et al.*, 2001). $O_2^{\cdot-}$ is relatively non-reactive and impermeant to cell membranes, but under mildly acidic conditions it spontaneously dismutates to H_2O_2 . Due to its fermentative metabolism and the formation of mainly lactic acid, *Ent. faecalis* acidifies its environment and consequently favours the formation of H_2O_2 from $O_2^{\cdot-}$. H_2O_2 can diffuse rapidly through cell membranes. A higher sensitivity of SOD mutants to exogenous H_2O_2 has been reported (Carlioz & Touati, 1986; Poyart *et al.*, 2001). Since only insignificant differences in growth characteristics were observed between the wild-type and the $\Delta sodA$ mutant, it may be concluded that the quantity of ROS produced by *Ent. faecalis* is too low to cause significant damage. We showed that a H_2O_2 concentration of 2 mM was necessary to induce significant inhibition of growth, and that the $\Delta sodA$ mutant was more sensitive to this treatment.

As expected, the $\Delta sodA$ mutant was also more sensitive to the redox-cycling agent menadione. In contrast, the wild-type as well as the mutant strain were highly resistant to paraquat in growth and survival experiments. It has been shown for *Streptococcus thermophilus* (Chang & Hassan, 1997), *Bacillus subtilis* (Inaoka *et al.*, 1998) and *Listeria monocytogenes* (Pedras Vasconcelos & Deneer, 1994) that paraquat fails to act as an inducer for SODs; however, effects on growth or survival were not studied. As demonstrated in *E. coli*, paraquat penetrates cells by means of active transport (Touati, 2002). The absence of such a transport system in *Ent. faecalis* may explain its extraordinary resistance to this agent.

The survival of the *Ent. faecalis* $\Delta sodA$ mutant was also more affected by exposure to peroxides (tBOOH and H_2O_2), at least when oxygen was present. A higher sensitivity of SOD mutants to H_2O_2 has also been reported for *E. coli* (Carlioz & Touati, 1986) and *Streptococcus agalactiae* (Poyart *et al.*, 2001). However, as we showed for *Ent. faecalis*, sensitivity towards H_2O_2 of the wild-type strain varied greatly with oxygen conditions, and in the absence of oxygen the wild-type strain was nearly as sensitive as the $\Delta sodA$ mutant.

Under anaerobic conditions, deferoxamine protected the wild-type and the mutant strain efficiently against the lethal effects of H_2O_2 exposure. Due to the known action of deferoxamine as a potent iron chelator, it can be supposed that cells exposed to strict anaerobiosis have high chelator-accessible iron levels. This iron may be the basis, via the Fenton reaction, for the generation of the highly reactive and deleterious $OH^{\cdot}$ radical, and this may explain the high

sensitivity to H_2O_2 under anoxic conditions. On the other hand, no protection by either deferoxamine or diethyltriaminepentaacetic acid was observed with cells grown in the presence of oxygen, suggesting that wild-type and $\Delta sodA$ mutant cells have very low chelator-accessible intracellular iron or, alternatively, low Fe^{2+} content due to a rapid auto-oxidation under these conditions of Fe^{2+} to Fe^{3+} (Welch *et al.*, 2002). This suggestion is supported by the experiments conducted with deferoxamine on cultures grown under anaerobiosis and immediately challenged with vigorous agitation. These cultures were slightly protected by deferoxamine, demonstrating that the cells still contained traces of Fenton-reactive iron. In conclusion, there may be less Fenton reaction under aerobic conditions, which is in good agreement with the much better survival of the wild-type strain under aerobic in comparison with anaerobic conditions. However, the SODA-deficient strain did not show such an impressive increase in survival when challenged with H_2O_2 in the presence of oxygen.

The shift experiments showed that the transition between a H_2O_2 -resistant and a peroxide-sensitive cellular state was very rapid in *Ent. faecalis*. From these observations it may be speculated that *Ent. faecalis* has sophisticated mechanisms for the regulation of intracellular iron. The ability to reduce or rapidly eliminate Fenton-reactive iron from the cells or the region around the chromosomal DNA when *Ent. faecalis* senses oxygen may correspond to an efficient survival strategy against oxidative stress. One explanation may be found in the sequestration of iron by the *E. coli* protein Dps (Almiron *et al.*, 1992). This protein has structural similarity to the iron-storage protein ferritin (Grant *et al.*, 1998; Zhao *et al.*, 2002), and the latter authors have found that Dps prevents DNA damage in *E. coli* through its capacity to bind $Fe(II)$, and prevents the formation of $OH^{\cdot}$. Two Dps family proteins are present in the genome of the *Ent. faecalis* V583 strain.

Our results demonstrated that SODA of *Ent. faecalis* plays a central role in the oxidative stress response, not only towards superoxide but also peroxides. Several studies have shown that *Ent. faecalis* can survive relatively well in mouse peritoneal macrophages, whereas *E. coli* is rapidly killed under these conditions (Gentry-Weeks *et al.*, 1999; Verneuil *et al.*, 2004). This ability may play a pivotal role in infection and disease. Indeed, it has been hypothesized that survival and sequestration within macrophages contribute to the pathogenesis of *Ent. faecalis* infections (Jett *et al.*, 1994). NADPH oxidase, which is present in the phagolysosomal membrane, catalyses the conversion of oxygen to $O_2^{\cdot-}$, which is subsequently converted to ROS. Exposure to these ROS is the main stress situation that a pathogen has to resist inside an infected host. Relatively little is known of the activities that permit *Ent. faecalis* wild-type cells to withstand the oxidative burst. Recently, *hypR* has been identified as the main transcriptional regulator of the oxidative stress response of *Ent. faecalis*, and survival of a *hypR* mutant is severely affected within macrophages (Verneuil *et al.*, 2004).

We showed in this study that the MnSOD of *Ent. faecalis* is important to resist these conditions. An increased susceptibility to killing by mouse bone-marrow-derived macrophages of a $\Delta sodA$ mutant has also been demonstrated for *S. agalactiae* (Poyart *et al.*, 2001). Interestingly, it has been shown by real-time PCR experiments that HypR seems to slightly regulate expression of *sodA* in *Ent. faecalis* (Verneuil *et al.*, 2005), suggesting that the reduced survival capacity of the *hypR* mutant may be due, at least partly, to the decrease in *sodA* expression.

Considering the results of the survival of both strains after H_2O_2 treatment in the presence or absence of O_2 , the inactivation of the bacteria inside the phagocytic cells resembles survival data obtained under aerated conditions. Phagocytic cells contain oxygen, the basis for the formation of ROS by these cells (Hassett & Cohen, 1989). We showed that *Ent. faecalis* is characterized by a low content of chelator-accessible iron under these conditions, and this could be at the basis of its high resistance to the oxidative burst inside macrophages. Identification of the mechanisms allowing this bacterium to regulate its intracellular iron content is an exciting challenge from a fundamental point of view, but may prove important for clinical purposes also.

ACKNOWLEDGEMENTS

We would like to extend special thanks for the outstanding technical assistance of Annick Blandin. The expert technical assistance of Béatrice Gillot, Marie-Jeanne Pigny and Valerio Cardinali is also greatly appreciated. We thank also Michel Débabouillé (Institut Pasteur, Paris) for the generous gift of plasmid pMAD, and Anuradha Alahari for English correction.

REFERENCES

- Almiron, M. A. J., Link, D., Furlong, D. & Kolter, R. (1992). A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes Dev* **6**, 2646–2654.
- Arnaud, M., Chastanet, A. & Débarbouillé, M. (2004). A new vector for efficient allelic replacement in naturally non transformable low GC% Gram-positive bacteria. *Appl Environ Microbiol* **70**, 6887–6891.
- Beauchamp, C. & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**, 276–287.
- Britton, L., Malinowski, D. P. & Fridovich, I. (1978). Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparisons with other organisms. *J Bacteriol* **134**, 229–236.
- Burr, T., Mitchell, J., Kolb, A., Minchin, S. & Busby, S. (2000). DNA sequence elements located immediately upstream of the –10 hexamer in *Escherichia coli* promoters: a systematic study. *Nucleic Acids Res* **28**, 1864–1870.
- Carlioz, A. & Touati, D. (1986). Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? *EMBO J* **5**, 623–630.
- Chang, S. K. & Hassan, H. M. (1997). Characterisation of superoxide dismutase in *Streptococcus thermophilus*. *Appl Environ Microbiol* **63**, 3732–3735.
- Farr, S. B., D'Ari, R. & Touati, D. (1986). Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. *Proc Natl Acad Sci U S A* **83**, 8268–8272.
- Flahaut, S., Laplace, J. M., Frère, J. & Auffray, Y. (1998). The oxidative stress response in *Enterococcus faecalis*: relationship between H_2O_2 tolerance and H_2O_2 stress proteins. *Lett Appl Microbiol* **26**, 259–264.
- Gardner, P. R. & Fridovich, I. (1992). Inactivation-reactivation of aconitase in *Escherichia coli*. A sensitive measure of superoxide radical. *J Biol Chem* **267**, 8757–8763.
- Gentry-Weeks, C. R., Karkhoff-Schweizer, R., Pikis, A., Estay, M. & Keith, J. M. (1999). Survival of *Enterococcus faecalis* in mouse peritoneal macrophages. *Infect Immun* **67**, 2160–2165.
- Giles, S. S., Batinic-Haberle, I., Perfect, J. R. & Cox, G. M. (2005). *Cryptococcus neoformans* mitochondrial superoxide dismutase: an essential link between antioxidant function and high-temperature growth. *Eukaryot Cell* **4**, 46–54.
- Gilmore, M. S., Coburn, P. S., Nallapareddy, S. R. & Murray, B. E. (2002). Enterococcal virulence. In *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance*, pp. 301–354. Edited by M. S. Gilmore. Washington, DC: American Society for Microbiology.
- Grant, R. A., Filman, D. J., Finkel, S. E., Kolter, R. & Hogle, J. M. (1998). The crystal structure of Dps, a ferritin homolog that binds and protects DNA. *Nat Struct Biol* **5**, 294–303.
- Gregory, E. M. & Fridovich, I. (1973). Induction of superoxide dismutase by molecular oxygen. *J Bacteriol* **114**, 543–548.
- Hassan, H. M. (1989). Microbial superoxide dismutases. *Adv Genet* **26**, 65–97.
- Hassett, D. J. & Cohen, M. S. (1989). Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *FASEB J* **3**, 2574–2582.
- Huycke, M. M., Joyce, W. & Wack, M. F. (1996). Augmented production of extracellular superoxide by blood isolates of *Enterococcus faecalis*. *J Infect Dis* **173**, 743–746.
- Huycke, M. M., Moore, D., Joyce, W., Wise, P., Shepard, L., Kotake, Y. & Gilmore, M. S. (2001). Extracellular superoxide production by *Enterococcus faecalis* requires demethylmenaquinone and is attenuated by functional terminal quinol oxidases. *Mol Microbiol* **42**, 729–740.
- Imlay, J. A. (2002). How oxygen damages microbes: oxygen tolerance and obligate anaerobiosis. *Adv Microb Physiol* **46**, 111–153.
- Imlay, J. A. (2003). Pathways of oxidative damage. *Annu Rev Microbiol* **57**, 395–418.
- Imlay, J. A. & Linn, S. (1986). Bimodal pattern of killing of DNA-repair-defective or anaerobically grown *Escherichia coli* by hydrogen peroxide. *J Bacteriol* **166**, 519–527.
- Imlay, J. A. & Linn, S. (1988). Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science* **240**, 640–642.
- Inaoka, T., Matsumura, Y. & Tsuchido, T. (1998). Molecular cloning and nucleotide sequence of the superoxide dismutase gene and characterization of its product from *Bacillus subtilis*. *J Bacteriol* **180**, 3697–3703.
- Jacob, A. E. & Hobbs, S. J. (1974). Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. *J Bacteriol* **117**, 360–372.
- Jett, B. D., Huycke, M. M. & Gilmore, M. S. (1994). Virulence of enterococci. *Clin Microbiol Rev* **7**, 462–478.
- Kak, V. & Chow, J. W. (2002). Acquired antibiotic resistances in Enterococci. In *The Enterococci: Pathogenesis, Molecular Biology, and*

- Antibiotic Resistance*, pp. 355–383. Edited by M. S. Gilmore. Washington, DC: American Society for Microbiology.
- Keyer, K. & Imlay, J. A. (1996).** Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A* **93**, 13635–13640.
- Keyer, K., Gort, A. S. & Imlay, J. A. (1995).** Superoxide and the production of oxidative DNA damage. *J Bacteriol* **177**, 6782–6790.
- Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G. & Leenhouts, K. (1995).** A system to generate chromosomal mutations in *Lactococcus lactis* allows fast analysis of targeted genes. *J Bacteriol* **177**, 7011–7018.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951).** Protein measurement with Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Maguin, E., Duwat, P., Hege, T. & Ehrlich, D. (1992).** New thermosensitive plasmid for Gram-positive bacteria. *J Bacteriol* **174**, 5633–5638.
- Narasipura, S. D., Ault, J. G., Behr, M. J., Chaturvedi, V. & Chaturvedi, S. (2003).** Characterization of Cu, Zn superoxide dismutase (SOD1) gene knock-out mutant of *Cryptococcus neoformans* var. *gattii*: role in biology and virulence. *Mol Microbiol* **47**, 1681–1694.
- Narasipura, S. D., Chaturvedi, V. & Chaturvedi, S. (2005).** Characterization of *Cryptococcus neoformans* variety *gattii* SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. *Mol Microbiol* **55**, 1782–1800.
- Parker, M. W. & Blake, C. C. (1988).** Iron and manganese containing superoxide-dismutases can be distinguished by analysis of their primary structures. *FEBS Lett* **229**, 377–382.
- Pedras Vasconcelos, J. A. & Deneer, H. G. (1994).** Expression of superoxide dismutase in *Listeria monocytogenes*. *Appl Environ Microbiol* **60**, 2360–2366.
- Poyart, C., Pellegrini, E., Guillot, O., Boumaila, C., Baptista, M. & Trieu-Cuot, P. (2001).** Contribution of Mn-cofactored superoxide dismutase (SODA) to the virulence of *Streptococcus agalactiae*. *Infect Immun* **69**, 5098–5106.
- Sambrook, J., Fritsch, E. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Terzaghi, B. E. & Sandine, W. (1975).** Improved medium for lactic streptococci and their bacteriophages. *Appl Environ Microbiol* **29**, 807–813.
- Touati, D. (2002).** Investigating phenotypes resulting from a lack of superoxide dismutase in bacterial null mutants. *Methods Enzymol* **349**, 145–154.
- Verneuil, N., Sanguinetti, M., Le Breton, Y., Posteraro, B., Fadda, G., Auffray, Y., Hartke, A. & Giard, J.-C. (2004).** Effects of *Enterococcus faecalis* *hypR* gene encoding a new transcriptional regulator on oxidative stress response and intracellular survival within macrophages. *Infect Immun* **72**, 4424–4431.
- Verneuil, N., Rincé, A., Sanguinetti, M., Auffray, Y., Hartke, A. & Giard, J.-C. (2005).** Implication of *hypR* in virulence and oxidative stress response of *Enterococcus faecalis*. *FEMS Microbiol Lett* **252**, 137–141.
- Welch, K. D., Davis, T. Z. & Aust, S. D. (2002).** Iron autooxidation and free radical generation: effects of buffers, ligands, and chelators. *Arch Biochem Biophys* **397**, 360–369.
- Yagi, Y. & Clewell, D. B. (1980).** Recombination-deficient mutant of *Streptococcus faecalis*. *J Bacteriol* **143**, 966–970.
- Yesilkaya, H., Kadioglu, A., Gingles, N., Alexander, J. E., Mitchell, T. & Andrew, P. W. (2000).** Role of manganese-containing superoxide dismutase in oxidative stress and virulence of *Streptococcus pneumoniae*. *Infect Immun* **68**, 2819–2856.
- Zhao, G., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T. M., Chiancone, E. & Chasteen, N. D. (2002).** Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells. A ferritin-like DNA-binding protein of *Escherichia coli*. *J Biol Chem* **277**, 27689–27696.