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Characterization of the Single Superoxide Dismutase of *Staphylococcus xylosus*

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Staphylococcus xylosus **is a facultative anaerobic bacterium used as a starter culture for fermented meat products. In an attempt to analyze the antioxidant capacities of this organism, the superoxide dismutase (SOD) was characterized. *S. xylosus* contains a single cytoplasmic SOD, which was not inhibited by H_2O_2. The SOD activity in crude extracts was completely lost upon metal depletion, but it could be recovered by manganese and very weakly by iron. It is therefore suggested that the *S. xylosus* SOD is a manganese-preferring enzyme. The corresponding gene, *sod*, was isolated from a genomic library of *S. xylosus* DNA and complemented the growth defect of an *Escherichia coli* SOD-deficient mutant. As deduced from the nucleotide sequence, *sod* encodes a protein of 199 amino acids with a molecular mass of 22.5 kDa. Two transcriptional start sites 25 and 120 bp upstream of the *sod* start codon were identified. A terminator-like structure downstream of the gene suggested a monocistronic *sod* mRNA. Regulation of *sod* expression was studied using fusions of the *sod* promoters to a genomic promoterless β-galactosidase gene. The *sod* expression was not affected by manganese and increased slightly with paraquat. It was induced during stationary phase in a complex medium but not in a chemically defined medium. To investigate the physiological role of SOD, a mutant devoid of SOD activity was constructed. Growth experiments showed that *sod* is not essential for aerobic growth in complex medium. However, in chemically defined medium without leucine, isoleucine, and valine, the *sod* mutant hardly grew, in contrast to the wild-type strain. In addition, the mutant was sensitive to hyperbaric oxygen and to paraquat. Therefore, *sod* plays an important role in the protection of *S. xylosus* from oxidative stress.**

The presence of oxygen in the environment is potentially toxic to all forms of life. This toxicity is mediated by reactive oxygen species (ROS), generated as by-products during the univalent reduction of oxygen to water, which can damage DNA, proteins, and lipids (28). These ROS include superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). As a defense against oxidative stress, most bacteria contain superoxide dismutases (SODs) (EC 1.15.1.1), which detoxify $O_2^{\cdot -}$ to H_2O_2, which in turn is broken down to water by catalases. The SODs are metalloenzymes that are classified according to their metal cofactor. There are three main classes of SODs in bacteria, the manganese SOD (MnSOD), the iron SOD (FeSOD), and the copper zinc SOD (CuZnSOD). Recently, a new class of SOD has been described, the nickel SOD (NiSOD) (33, 34, 57). Usually, FeSODs and MnSODs exhibit strict metal cofactor specificity (6) and can be distinguished by their sensitivity to H_2O_2. However, a small group of Mn/FeSODs, named cambialistic, is active with either manganese or iron incorporated into the same active site and exhibits variable sensitivity to H_2O_2 (47, 55).

Some bacteria, such as *Escherichia coli* or *Staphylococcus aureus*, possess more than one SOD (4, 15). *E. coli* has three SODs, which differ in their location and temporal expression: two SODs, the FeSOD and the MnSOD, are present in the cytoplasm (32, 56), while the CuZnSOD is located in the periplasm (29). Expression of the FeSOD is thought to be constitutive, but the levels of the MnSOD fluctuate, increasing in response to $O_2^{\cdot -}$ and upon changes in growth phase (17). In *S. aureus*, where two SODs are detected, the major enzyme, characterized as a MnSOD, is inducible by a variety of conditions (15). In contrast, the second and less-characterized SOD enzyme appears to be expressed constitutively (15).

Staphylococcus xylosus is an anaerobic facultative bacterium used as a starter culture for fermented meat products. It ensures color development by its nitrate reductase activity and protects the cured color by its catalase activity (38, 51). It also contributes to aroma, mainly by modulating the level and the nature of volatile compounds coming from lipid oxidation (5, 40, 41, 52). Antioxidant activities of *S. xylosus* (e.g., catalase and SOD) are thought to be involved in the development of the sensorial qualities (45). Therefore, it is crucial to characterize these enzymes and to construct mutants with the corresponding genes to understand their role. In this study, we describe the physiological and molecular characterization of the single SOD from *S. xylosus*. The corresponding gene was cloned and sequenced, and its regulation and its role were investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. The temperature-sensitive shuttle vector pBT2 (10) and the *lacH* promoter probe plasmid pLP1 (31) were used. The *ermB* cassettes from plasmid pEC5 and plasmid pEC7 were used, respectively, to interrupt the *sod* and the *zurR* genes in *S. xylosus* (10).

Media and culture conditions. *S. xylosus* was grown at 30°C in a complex medium (CM) (meat extract [10 g/liter], yeast extract [5 g/liter], NaCl [5 g/liter],

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TABLE 1. Bacterial strains used

Strain	Genotype or characteristics	Reference or source
S. xylosus[a]		
C2a	Wild type	22
TX300	*'lacR ΔlacP 'lacH*	31
TX302	*'lacR ΔlacP* P_{vegII}*-lacH*	31
TX351	*sod*::*ermB*	This study
TX352	*zurR*::*ermB*	This study
TX353	*'lacR ΔlacP* $P1_{sod}$ $P2_{sod}$*-lacH*	This study
TX354	*'lacR ΔlacP* $P1_{sod}$*-lacH*	This study
TX355	*'lacR ΔlacP* $P2_{sod}$*-lacH*	This study
E. coli		
DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	23
GC4468	F^- Δ*lacU169 rpsL*	R. D'Ari
QC779	As GC4468, (*sodA*::MudPR13)25 Φ(*sodB*-kan)1-Δ_2	D. Touati

[a] All *S. xylosus* strains are derived from *S. xylosus* DSM20267 (48) cured of the endogenous plasmid pSX267 (22).

Na_2HPO_4 [2 g/liter]) prepared in 0.067 M phosphate buffer, pH 6.0, or in chemically defined medium (CDM) (27). When needed, media were supplemented with erythromycin (2.5 μg/ml). To study the effect of metals, heavy metals were removed from the medium with Chelex-100 (Bio-Rad Laboratories, Hercules, Calif.) as recommended by the manufacturer, and, when needed, ultrapure $MnSO_4$ (Sigma) was added. Aerobic cultures were incubated on a rotary shaker at 170 rpm, and the volume of cultures did not exceed 1/10 of the total Erlenmeyer volume to ensure good aeration. Low aeration was performed by growing the cells in tubes filled up to 85% (total volume, 7 ml) with slow stirring (15 rpm) on a shaker-incubator. *E. coli* was grown aerobically at 37°C in Luria-Bertani medium supplemented, when needed, with ampicillin (100 μg/ml) and 0.05 mM paraquat (Sigma).

Determination of SOD and β-galactosidase activities in crude extracts. For preparation of crude extracts, cells were washed once in SOD buffer containing 10 mM Tris, pH 7.0, or in β-galactosidase buffer (31). The washed cells were resuspended in 500 μl of the same buffer, 1 g of glass beads (150 to 212 μm) was added, and the samples were vortexed twice for 1 min with ice cooling in between. After centrifugation (10 min, 8,000 × *g*, 4°C), the supernatants were collected and kept on ice. Subsequently, partially broken cells including glass beads were resuspended three times in 500 μl of buffer, vortexed, and centrifuged as described before. The combined supernatants (2 ml corresponding approximately to 2 mg of protein) were kept on ice, and aliquots were tested for SOD or β-galactosidase activity. The SOD activity was measured using a RANSOD kit (Randox, Co., Antrim, United Kingdom) with 5 to 30 μg of cellular protein. The β-galactosidase activity was assayed with 15 to 400 μg of cellular protein according to the method of Jankovic et al. (31). The concentration of protein was determined by the method of Bradford with bovine serum albumin as a standard (7).

Visualization of SOD activity on nondenaturing polyacrylamide gels. The SOD activity on 12.5% nondenaturing polyacrylamide gels was visualized by nitroblue tetrazolium negative staining (2). Inhibition experiments with SOD isoenzymes were done with H_2O_2 or KCN as described previously (2).

Metal depletion and reconstitution of crude extracts. Metal depletion was performed by dialyzing crude extracts against metal depletion buffer (20 mM 8-hydroxyquinoline, 2.5 M guanidium chloride, 5 mM Tris-HCl [pH 3.8], 0.1 mM EDTA) (35). Reconstitution of metal-depleted crude extracts was performed with either 0.1 mM $MnCl_2$ or 1 mM $Fe(NH_4)_2(SO_4)_2$ (35).

DNA preparation, transformation, and molecular techniques. Chromosomal DNA from *S. xylosus* was isolated according to the method of Marmur (39). Plasmid DNA was introduced into *S. xylosus* by electroporation with glycine-treated electrocompetent cells (10). DNA manipulations, Southern blotting, plasmid DNA isolation, and transformation of *E. coli* were performed according to standard procedures (46).

PCR conditions used for cloning the *sod* gene. Chromosomal DNA from *S. xylosus* was amplified by PCR with two degenerate primers, SOD1 and SOD2 (Table 2) (43). The PCR mix contained 100 pmol of each primer, about 300 ng of chromosomal DNA, a 0.2 mM concentration of each dNTP, 1× *Taq* polymerase buffer, and 1 U of *Taq* polymerase (Appligene). The reaction was cycled 30 times through the following temperature profile: denaturation at 95°C for 2 min, annealing of primers at 37°C for 1 min, and extension at 72°C for 1 min, followed by a final extension step of 10 min at 72°C. A 468-bp amplified fragment was obtained and sequenced. From this sequence, two specific primers, SOD4 and SOD9 (Table 2), were designed and used to screen a *S. xylosus* gene library (11).

RNA isolation and primer extension analysis. Preparation of RNA and primer extension reactions were done as described previously (1). The primer P3 (Table 2) complementary to the *sod* coding sequence was labeled at the 5′ end with infrared dye IRD700. The DNA primer was elongated, and the products were analyzed on an 8% polyacrylamide–urea gel with a Li-Cor DNA sequencer to determine the transcriptional start site.

Construction of mutants by gene replacement. To inactivate the *sod* gene in *S. xylosus*, the plasmid pBSe was constructed in three steps. First, a 1-kb fragment was obtained by PCR from the *sod* nucleotide sequence region with the primers S1 and S2 (Table 2). The amplified fragment contained about two-thirds of the *zurM* gene and the complete *zurR* gene. It was digested with *Eco*RI and *Sst*I and inserted in the temperature-sensitive shuttle vector pBT2 digested by the same enzymes. Second, the *ermB* cassette coming from the plasmid pEC5 was inserted between the *Sst*I and *Kpn*I restriction sites of the previous construct. Finally, a 1-kb fragment was obtained by PCR from the *sod* nucleotide sequence region with the primers S3 and S4 (Table 2). The amplified fragment contained the *sod* gene without its Shine Dalgarno sequence, its start codon, and its first 22 nucleotides, and a part of the *stpB* gene. It was digested by *Kpn*I and *Sal*I and inserted between the *Kpn*I and *Sal*I sites of the previous construct, yielding the plasmid pBSe.

To inactivate the *zurR* gene, the plasmid pBZe was constructed in three steps. A 1.5-kb fragment was obtained by PCR from the *sod* nucleotide sequence region with the primers Z1 and Z2 (Table 2). It contained the *zurA* gene and the majority of the *zurM* gene. It was digested with *Eco*RI and *Kpn*I and introduced into plasmid pBT2, digested by the same enzymes. Then, another 1.5-kb fragment containing a deleted *zurR* gene was obtained by PCR using the primers Z3 and S4 (Table 2) and inserted between the *Kpn*I and *Sal*I sites of the previous construct. Finally, the *ermB* cassette was introduced into the *Kpn*I site in the same orientation as the *zurA*, *zurM*, and *zurR* genes, yielding the plasmid pBZe.

Staphylococcus xylosus C2a was then transformed with the plasmid pBSe or pBZe. By a double-crossover event, the inactivated copy of the gene was introduced into the genome as described by Brückner (10). The cells were cured from the plasmid by raising the temperature to 40°C. The inactivation of the *sod* and *zurR* genes in the genome of *S. xylosus* was verified by Southern blot and PCR analyses (data not shown).

Integration of promoters in front of the chromosomal β-galactosidase gene *lacH*. The *S. xylosus* promoters that were analyzed with the promoter probe system are shown below (see Fig. 4). A fragment containing the complete *sod* promoter $P1/2_{sod}$ was obtained by PCR with pS41 DNA as a template and the primers PSOD1 and PSOD2 (Table 2). The fragments containing only the promoter $P1_{sod}$ or $P2_{sod}$ were obtained using the primers PSOD1 and PSOD3 or PSOD2 and PSOD4 (Table 2), respectively. The *Bam*HI-*Sal*I fragments were

TABLE 2. Sequences of the primers used

Primer	Sequence[a]	Position[b]
SOD1	CCNTAYNCNTAYGAYGCNYTNGARCC	
SOD2	ARRTARTANGCRTGYTCCCANACRTC	
SOD4	TGCATTGGAACCACACATTGATC	
SOD9	GTGTTCCCAAACATCTAATCCTAAG	
P3	GTGTTTGCCATGGTGAATTTCC	2620–2641
S1	CCTGAATTCCTTGGCAGATGGCTTCAACC	1371–1393
S2	CTTGAGCTCTACTAGTATATCTCAAATTGAGC	2403–2428
S3	CCTGGTACCTTATGGTTTTGATGCATTGGAACC	2570–2598
S4	CCTGTCGACTTCTGTTTGCGAAGTTTTTCTACC	3550–3575
Z1	CCTGAATTCATTGTATAGTATAGAACGAGGTG	227–251
Z2	CCTGGTACCAGGTGATATATTCATATAAAACG	1760–1785
Z3	CCTGGTACCGTGAAATGCTTGATATATTCGTCTC	1940–1965
PSOD1	CCTGGATCCATAGGTCTTTCGTATGAATTAAATTG	2340–2366
PSOD2	CCTGTCGACTTTAAATAAAGCATAAC	2508–2524
PSOD3	CCTGTCGACTTTAGTTACTCTACTAGTATATC	2414–2437
PSOD4	CCTGGATCCTAAAGATGACAAATATTATTCAATAAC	2432–2459

[a] Restriction sites introduced at the 5′ end of primers for further digestions are underlined.

[b] Positions refer to the sequence found under EMBL accession no. AJ276960.

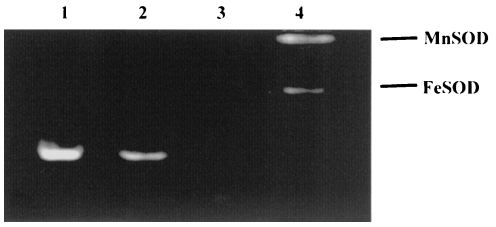


FIG. 1. Detection of SOD activity in *E. coli* and *S. xylosus* on a nondenaturing polyacrylamide gel stained for SOD activity. Lanes: 1, *S. xylosus* C2a; 2, *E. coli* QC779 (*sodAsodB*)/pS41 with *S. xylosus sod* gene; 3, *E. coli* QC779; 4, *E. coli* GC4468. Lanes contain crude extracts of aerobic cultures (15 μg of proteins were loaded for *S. xylosus* C2a and 30 μg were loaded for *E. coli* strains).

cloned into the *lacH* promoter probe plasmid pLP1 (see Fig. 4). Successful integration of promoter fragments into pLP1 was detected on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-containing agar plates at 30°C. Promoter-containing plasmids were designated pLP21 ($P1/2_{sod}$), pLP22 ($P1_{sod}$) and pLP23 ($P2_{sod}$). Promoter sequences were verified by sequencing on both strands.

Then, the β-galactosidase-deficient derivative of the wild-type strain, the TX300 strain, was transformed with the plasmid pLP21, pLP22, or pLP23. By a double-crossover event, integration of promoters in front of chromosomal *lacH* gene was done as described by Jankovic et al. (31). The PCR analysis of the *lac* region confirmed the integration of promoter-containing *lacH* genes into the chromosome (data not shown).

Nucleotide sequence accession number. The *S. xylosus* sequence determined in this study is available from the EMBL database under accession no. AJ276960.

RESULTS

Cloning the *sod* gene. To isolate the SOD-encoding gene, a PCR-based approach with degenerate primers was used. These degenerate primers were previously used to detect *sod* genes in different bacterial species (43). An amplified fragment was obtained and sequenced. Its deduced amino acid sequence showed a high level of similarity to SODs. Therefore, two specific primers were designed to screen an *S. xylosus* gene library stored as pools of plasmids (11). One pool of plasmid DNAs gave a fragment of the expected size. To identify the plasmid harboring the *sod* gene, *E. coli* QC779 (*sodA sodB*), which is deficient in both cytoplasmic SODs, was transformed with the plasmid mixture and plated on a rich medium with paraquat, a generator of superoxide. Without a functional cytoplasmic SOD, *E. coli* is unable to grow under these conditions (13). Several colonies grew, and their plasmid DNA was analyzed. All transformants harbored identical plasmids. One plasmid, designated pS41, containing an insertion of about 3.6 kb, was further studied.

The SOD activity encoded by the pS41 plasmid in *E. coli* QC779 was visualized on a nondenaturing polyacrylamide gel (Fig. 1). As expected, no SOD activity was detected in *E. coli* QC779 cell extracts without cloned *S. xylosus* DNA. The SOD activity encoded by the pS41 plasmid was found at the same position as the SOD of *S. xylosus*. Therefore, the gene, named *sod*, encoding the SOD of *S. xylosus* was probably cloned on the plasmid pS41.

Nucleotide sequence of the *sod* region. The complete insert of pS41 consisting of 3,595 bp was sequenced. Four complete open reading frames (ORFs) and a truncated ORF were found on one strand (Fig. 2). The fourth ORF, encoding a polypeptide of 199 amino acids with a theoretical M_r of 22.5 and a pI of 4.67, is clearly the *sod* gene, since the deduced amino acid sequence of its product revealed a high level of similarity to the SOD family of proteins. The highest levels of identity were observed with the following SODs: the MnSOD (SodA) of *S. aureus* (accession no. AF121672) (91% identity), the SOD of *Staphylococccus carnosus* (accession no. AJ295150) (87% identity), the MnSODs of *Bacillus subtilis* (accession no. D86856), *Bacillus licheniformis* (accession no. AJ002279), *Bacillus stearothermophilus* (accession no. P00449), and *Bacillus caldotenax* (accession no. P28760) (68 to 72% of identical residues). The critical residues in SODs commonly used to predict the metal specificity of the enzymes (42) suggest that the *S. xylosus* SOD requires Mn rather than Fe as a metal cofactor. The SOD enzyme from *S. xylosus* (PsiPred Prediction; Protein Bioinformatics Group, Department of Biological Sciences, University of Warwick, Coventry, United Kingdom) showed a secondary structure typical of Mn/FeSODs, with two domains, the N-terminal one with two α-helices and the C-terminal domain with two α-helices, followed by three β-strands and two α-helices (data not shown).

Upstream of *sod*, three genes are detected that resemble *S. aureus* and *Listeria monocytogenes* operons involved in zinc homeostasis (16, 26; J. A. Lindsay and S. J. Foster, unpublished data). The products of the first two ORFs, designated ZurA and ZurM, revealed sequence similarity to a typical ABC protein and the transmembrane protein, forming an ABC transporter of *S. aureus* (accession no. AF121672) and *L. monocytogenes* (accession no. AF104349). The gene product of the third gene, *zurR*, belongs to the family of ferric uptake regulation proteins which includes Fur, Zur, and PerR, regulating, respectively, iron uptake, zinc uptake, and the peroxide stress response (12, 19). The *S. xylosus* ZurR proteins show 80% identity with the zinc uptake regulation protein homolog Zur of *S. aureus* (accession no. AF121672) and 51% identity with the ZurR of *Listeria monocytogenes* (accession no. AF104349). In *S. aureus*, the operon involved in zinc homeostasis is followed by the *sodA* gene. Therefore, the three genes upstream of the *S. xylosus sod* may also encode components involved in zinc uptake and regulation.

The deduced amino acid sequence corresponding to the truncated ORF5 downstream of *sod* displays similarities to penicillin-binding proteins, the targets of β-lactam antibiotics, involved in the final stages of peptidoglycan biosynthesis. The highest level of identity (83%) was obtained with the penicillin-binding protein Pbp2b of *S. aureus* (accession no. AF098901). Therefore, ORF5 was designated *stpB*.

Determination of *sod* transcriptional start sites. To define the transcriptional start site of the *sod* gene, the 5′ end of the *sod* transcript was mapped. A primer specific to the coding

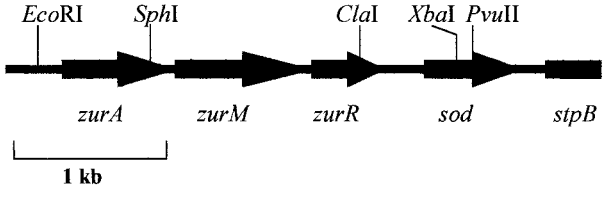


FIG. 2. Genetic organization surrounding the *sod* gene from *S. xylosus*. Genes are shown by arrows. Relevant restriction sites are labeled.

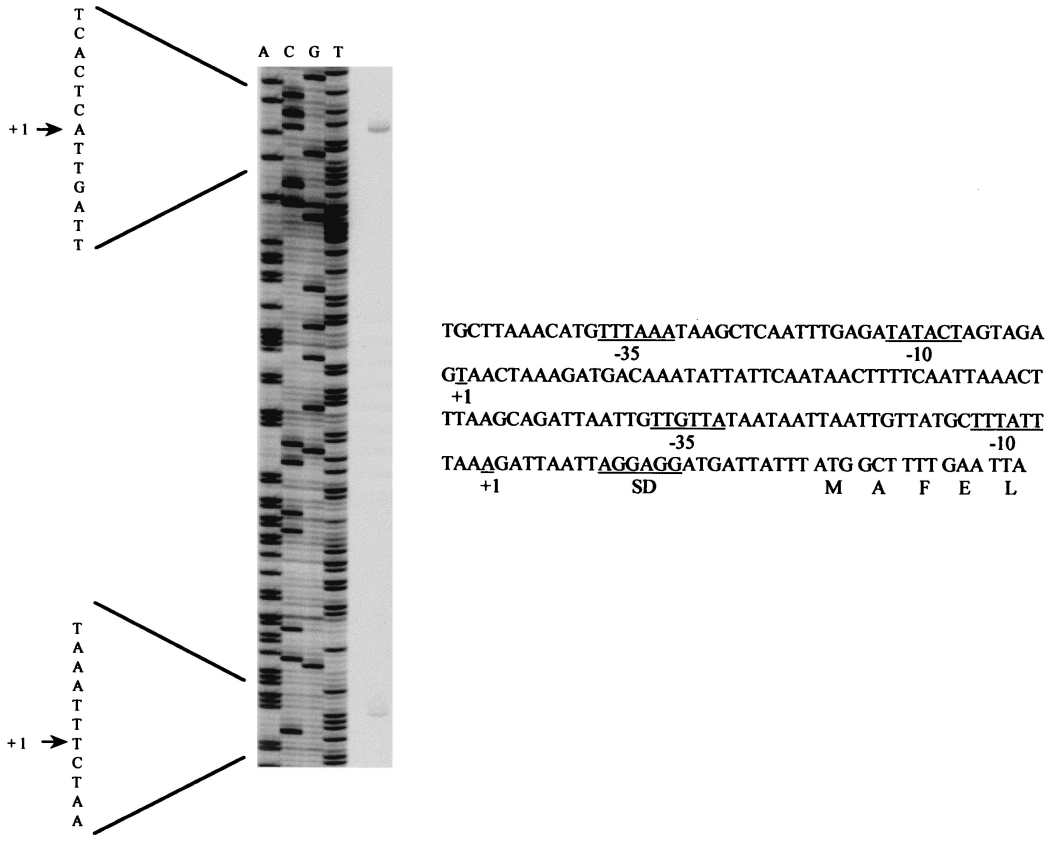
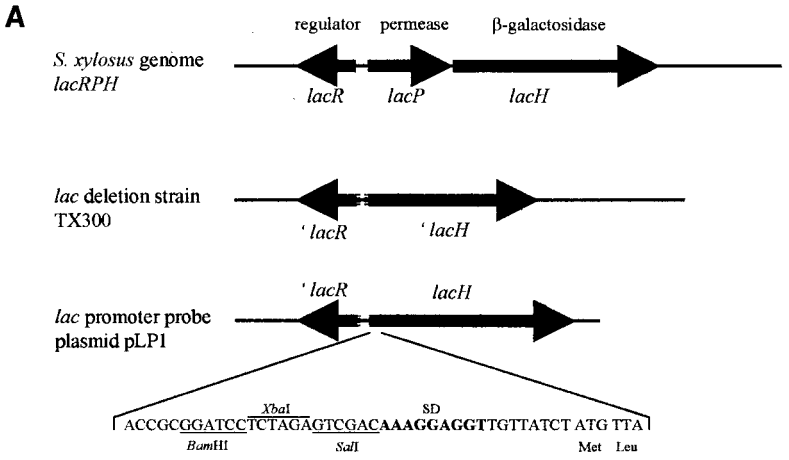


FIG. 3. Primer extension of *sod* transcription. Total RNA was prepared from *S. xylosus* C2a cells grown in nonbuffered CM medium. Thirteen micrograms of total RNA was used to extend a *sod*-specific primer, P3, by reverse transcriptase. One-thirtieth of the primer extension reaction was analyzed on an 8% polyacrylamide–urea gel alongside a sequencing reaction performed with the same primer. Possible sites (+1) are shown by arrows.

region was annealed with total RNA and extended in a primer extension assay. RNA was prepared from cells grown in CM medium and harvested at different times during growth. Two primer extension products were obtained under all conditions (data not shown). As an example, the reaction with RNA from cells in exponential growth phase is shown in Fig. 3. The sizes of the reverse transcripts placed the transcriptional start points, respectively, 25 bp and 120 bp upstream of the *sod* start codon. Upstream of the first start point, a putative *E. coli* sigma 70-like sequence was found matching four of the six bases (boldface) with the −35 (**TTGACA**) consensus sequence and five of the six bases with the −10 (**TATAAT**) consensus sequence (Fig. 3). The respective boxes of the second promoter fitted less perfectly to the consensus sequences (Fig. 3). To verify that *sod* possesses two functional promoters, the genomic reporter gene system described for *S. xylosus* (31) was used. Each presumed promoter was integrated in front of the promoterless β-galactosidase gene *lacH* by homologous recombination (Fig. 4). The resulting strains, *S. xylosus* TX354 containing the first promoter $P1_{sod}$ and *S. xylosus* TX355 containing the second promoter $P2_{sod}$, gave rise to blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside agar plates, substantiating the presence of two promoters in front of *sod*. A terminator-like structure immediately downstream of the *sod* reading frame suggests that the *sod* mRNA of *S. xylosus* is monocistronic.

Metal cofactor requirements of *S. xylosus* SOD. In *S. xylosus*, a single SOD was detected on a nondenaturing polyacrylamide gel (Fig. 5). This SOD was not inhibited by KCN (data not shown) or H_2O_2 (Fig. 5), suggesting that the enzyme is a MnSOD. To determine the exact nature of the cofactor of the *S. xylosus* SOD, metal depletion and reconstitution experiments were performed. The enzyme in crude extracts was depleted of metal (Fig. 5). The resulting inactive apoenzyme was dialyzed against Mn or Fe. A high activity (5.1 ± 0.2 U/mg of protein) was recovered with Mn. The Mn-reconstituted enzyme was not inhibited by H_2O_2 (Fig. 5). A very weak activity (0.4 ± 0.2 U/mg of protein) was recovered with Fe, and a faint band with the same mobility as the Mn-reconstituted enzyme was revealed after electrophoresis (data not shown).

SOD activity and *sod* expression in *S. xylosus*. For many bacteria, the level of SOD activity fluctuates depending on the growth phase, presence of superoxide generators, and metal availability. For *S. xylosus*, only a slight increase of SOD activity was observed during stationary phase when cultures were grown in CM or CDM medium (Table 3). The strains *S. xylosus* TX353, TX354, and TX355 containing, respectively, $P1/2_{sod}$, $P1_{sod}$, and $P2_{sod}$ in front of the *lacH* gene enabled monitoring of *sod* expression throughout different growth conditions by measurement of the level of β-galactosidase activity. The strain *S. xylosus* TX302 harboring the constitutive promoter from *B. subtilis*, P_{vegII} in front of the *lacH* gene (31) was used to



B

Promoter

P1/2$_{sod}$ (plasmid pLP21; strain TX353)
GGATCCATAGGTCTTTCGTATGAATTAAATTGAATTTTTAAAATTGTTGCTTAAACATGTTTAAATAAGCTCAATTTGAGATATACT
*Bam*HI -35 -10
AGTAGAGTAACTAAAGATGACAAATATTATTCAATAACTTTTCAATTAAACTTTAAGCAGATTAATTGTTGTTATAATAATTAATTG
+1 -35
TTATGCTTTATTTAAAGTCGAC
-10 +1 *Sal*I

P1$_{sod}$ (plasmid pLP22; strain TX354)
GGATCCATAGGTCTTTCGTATGAATTAAATTGAATTTTTAAAATTGTTGCTTAAACATGTTTAAATAAGCTCAATTTGAGATATACT
*Bam*HI -35 -10
AGTAGAGTAACTAAAGTCGAC
+1 *Sal*I

P2$_{sod}$ (plasmid pLP23; strain TX355)
GGATCCTAAAGATGACAAATATTATTCAATAACTTTTCAATTAAACTTTAAGCAGATTAATTGTTGTTATAATAATTAATTGTTATG
*Bam*HI -35
CTTTATTTAAAGTCGAC
-10 +1 *Sal*I

FIG. 4. Genetic organization of the genomic reporter system for *S. xylosus* (A) and nucleotide sequences of the promoters integrated in front of the β-galactosidase gene *lacH* (B). (A) The genetic organization of the wild-type *S. xylosus* lactose utilization genes *lacR, lacP*, and *lacH* is shown in the first line. The *lac* deletion strain TX300 harbors versions of *'lacR* and *'lacH* truncated at their 5′-ends and lacks *lacP*. The promoterless β-galactosidase genes *lacH* and *'lacR* are contained on the promoter probe plasmid pLP1. The nucleotide sequence of the region preceding the reporter gene *lacH* is shown below pLP1. The three restriction sites *Bam*HI, *Xba*I, and *Sal*I are available for insertion of promoter fragments. (B) The promoter regions relevant for transcription initiation and regulation are shown. Putative RNA polymerase binding sites are underlined. Transcriptional start sites are shown in boldface. Designations of the plasmids harboring the shown promoters and corresponding strains are shown in parenthesis.

verify that there was no variation of β-galactosidase activity under all growth conditions (data not shown). In agreement with SOD activity measures, expression of *sod* in the three strains was slightly increased in the stationary phase when cells were grown in CDM (Table 3). However, when cells were grown in CM, a 4-fold to 10-fold increase of *sod* expression was noticed during the stationary phase (Table 3). P2$_{sod}$ and especially P1$_{sod}$ were induced by stationary phase (Table 3). The low level of SOD activity measured from the stationary-phase cells grown in CM could be explained in part by the weak concentration of manganese in the medium, since in CM supplemented by 0.1 mM $MnSO_4$, SOD activity was higher in the stationary phase (6.5 ± 0.1 U/mg of protein) than in the non-supplemented medium (3.2 ± 0.4 U/mg of protein).

Superoxide anions may be formed intracellularly by the presence of paraquat, an aromatic compound that undergoes redox cycling within cells (24). When paraquat was added to *S. xylosus* C2a grown in CM or CDM, a less than twofold increase in SOD activity over that of the untreated control was observed (Table 4). This correlated with the same increase in *sod* expression compared to the untreated culture (Table 4).

When cultures were grown in CM or CDM treated by Chelex in order to remove heavy metals, SOD activity, especially in CM, was lower than in the untreated media (Table 5).

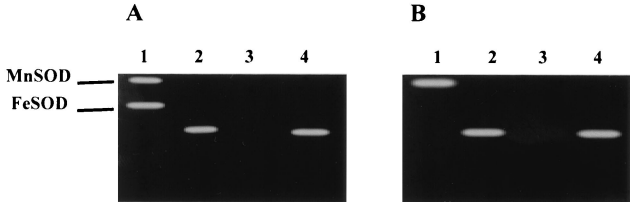


FIG. 5. Activity of reconstituted SOD from *S. xylosus* with manganese. (A) No inhibitors. (B) with H_2O_2. Lanes: 1, crude extract of *E. coli* DH5α; 2, crude extract of *S. xylosus*; 3, apoenzyme of *S. xylosus*, 4, Mn-reconstituted SOD of *S. xylosus*. Thirty micrograms of proteins were loaded in each lane.

TABLE 3. Effect of growth phase on SOD activity and on β-galactosidase expression directed by P1/2$_{sod}$ (TX353), P1$_{sod}$ (TX354), and P2$_{sod}$ (TX355) in *S. xylosus*

Growth conditions[a]	SOD activity[b] (U/mg of protein) of *S. xylosus* wild-type strain C2a		β-Galactosidase activity[b] (nmol of nitrophenol produced/min/mg of protein) of strain:					
			TX353		TX354		TX355	
	CM	CDM	CM	CDM	CM	CDM	CM	CDM
Exponential growth phase	2.4 ± 0.1	3.5 ± 0.1	135 ± 12	257 ± 44	51 ± 7	120 ± 22	105 ± 17	143 ± 8
Stationary growth phase	3.2 ± 0.4	4.3 ± 0.1	973 ± 62	337 ± 113	525 ± 33	256 ± 42	474 ± 28	215 ± 8

[a] Cells were grown aerobically in CM medium or CDM medium during 4 h to an optical density at 600 nm of 0.8 (exponential growth phase) or during 14 h to an optical density at 600 nm of 7.0 (stationary growth phase).
[b] Values are the means ± standard deviations for at least three independent experiments.

Addition of 0.1 mM $MnSO_4$ to the treated media restored SOD activities (Table 5). No relevant variations of *sod* expression were noticed under all conditions (Table 5).

SOD activity in a *zurR* mutant. *zurR* encodes a putative metalloregulatory protein and belongs to the family of ferric uptake regulation proteins. In *E. coli*, the *sodA* gene encoding the MnSOD is regulated transcriptionally by Fur (53) and post-translationally in a metal-dependent fashion (6, 44). Therefore, we measured SOD activity in a *zurR* mutant constructed by allelic exchange. No change of SOD activity with the *zurR* mutant from that of the wild type strain grown in CM or CDM media was observed (data not shown).

Physiological characterization of a *sod* mutant. To investigate the physiological role of the *sod* gene, a *sod* mutant was constructed by allelic exchange. The *sod* mutant was completely devoid of SOD activity (data not shown), proving the presence of a single expressed *sod* gene. The aerobic growth of the *sod* mutant in CM was similar to that of the wild-type strain (Fig. 6B). However, the addition of hyperbaric O_2 in the exponential growth phase resulted in a significant decrease of growth of the *sod* mutant compared to the wild-type strain (Fig. 6A). The same effect was noticed when 50 μM paraquat was added in the exponential growth phase (Fig. 6B), while the wild-type strain was unaffected at this concentration (Fig. 6B). In addition, growth of the *sod* mutant in CDM with all amino acids was at a slightly lower level than that of the wild-type strain, and when Leu, Ile and Val were lacking, this difference was dramatically increased (Fig. 6C). The *sod* mutant was practically unable to grow, whereas the growth of the wild-type strain was almost unaffected.

DISCUSSION

We determined in this study that *S. xylosus* contains a single SOD. In contrast, *S. aureus* has at least two SODs: SodA and SodX. SodX was suggested to be cell wall associated and implicated in virulence (14). This difference between the two species could be explained by the fact that *S. aureus* as a potential pathogen has to deal with highly microbiocidal reactive oxygen metabolites produced during the oxidative burst by phagocytes, whereas *S. xylosus* is rarely associated with human or animal infections.

The SOD of *S. xylosus* is a manganese-preferring enzyme. In crude extracts, SOD activity was completely recovered by manganese and very weakly by iron. This result questioned the cambialistic nature of *S. xylosus* SOD, as it has already been shown that some cambialistic SODs are less active with iron than with manganese (47, 55). However, *S. xylosus* SOD is not inhibited by hydrogen peroxide, and its amino acid sequence exhibits strong similarity to SodA of *S. aureus* which was shown to be a manganese-requiring enzyme (15). Furthermore, manganese was necessary to sustain SOD activity in the stationary phase, as was also observed by Inaoka et al. with *B. subtilis* (30).

In *S. xylosus*, upstream of the *sod* gene a putative zinc uptake and regulation operon (*zurA, -M*, and *-R*) was detected. This genetic organization seems to be conserved in other staphylococcal species, such as *S. aureus* and *Staphylococcus epidermidis* (25, 26; Lindsay and Foster, unpublished data). In *S. xylosus*, ZurR did not appear to regulate *sod* expression.

In *E. coli*, the *sodA* sequence contains two putative promoters, but only one has been found to be functional under normal aerobic growth conditions (50). In *B. subtilis*, nucleotide sequence analysis indicated that *sodA* possesses six putative promoters (30). According to our results, the *S. xylosus sod* gene possesses two functional promoters. In either a chemically defined media or a complex medium, a weak induction effect of paraquat was observed, confirming the results obtained by measuring the levels of SOD activity. For *S. aureus*, addition of paraquat leads to an approximately fourfold induction of *sod*

TABLE 4. Effect of paraquat on SOD activity and on β-galactosidase expression directed by P1/2$_{sod}$ (TX353), P1$_{sod}$ (TX354), and P2$_{sod}$ (TX355) in *S. xylosus*

Growth conditions[a]	SOD activity[b] (U/mg of protein) of *S. xylosus* wild-type strain C2a		β-Galactosidase activity[b] (nmol of nitrophenol produced/min/mg of protein) of strain:					
			TX353		TX354		TX355	
	CM	CDM	CM	CDM	CM	CDM	CM	CDM
No paraquat	2.3 ± 0.4	2.5 ± 0.2	168 ± 10	225 ± 24	75 ± 10	95 ± 9	99 ± 18	129 ± 9
With paraquat	4.2 ± 0.3	3.6 ± 0.2	239 ± 21	408 ± 54	106 ± 4	166 ± 12	111 ± 15	175 ± 12

[a] Cells were grown aerobically in CM medium or CDM medium during 4 h to an optical density at 600 nm of 0.8, and 200 μM paraquat was added as indicated. After 2 h of further growth (optical density at 600 nm = 2.0), cells were harvested and disrupted with glass beads in order to determine SOD and β-galactosidase activities.
[b] Values are the means ± standard deviations for at least three independent experiments.

TABLE 5. Effect of metals on SOD activity and on β-galactosidase expression directed by P1/2$_{sod}$ (TX353), P1$_{sod}$ (TX354), and P2$_{sod}$ (TX355) in *S. xylosus*

Treatment[a]	SOD activity[b] (U/mg of protein) in *S. xylosus* wild-type strain C2a		β-galactosidase activity[b] (nmol of nitrophenol produced/min/mg of protein) of strain:					
			TX353		TX354		TX355	
	CM	CDM	CM	CDM	CM	CDM	CM	CDM
None	3.7 ± 0.4	3.2 ± 0.3	349 ± 35	349 ± 35	63 ± 8	153 ± 14	99 ± 26	138 ± 10
Chelex	0.6 ± 0.1	2.4 ± 0.1	288 ± 47	310 ± 10	85 ± 6	110 ± 13	141 ± 6	120 ± 12
Chelex + $MnSO_4$	2.6 ± 0.5	4.3 ± 0.1	239 ± 10	298 ± 17	75 ± 7	137 ± 9	133 ± 25	157 ± 6

[a] Cells were grown during 6 h (optical density at 600 nm = 0.6) with low aeration in CM medium or CDM medium treated or not by Chelex 100; 0.1 mM $MnSO_4$ was added as indicated.
[b] Values are the means ± standard deviations for at least three independent experiments.

expression (15), whereas for *B. subtilis* or *L. monocytogenes*, the addition of paraquat does not induce *sod* expression (30, 54). In a complex medium and not in a chemically defined medium, *sod* expression for *S. xylosus* was induced in stationary-growth phase, as was also observed for *S. aureus* when cells were grown in BHI medium (15), for *E. coli* (3), and for *L. monocytogenes* (54). This could reflect the need for greater protection from accumulated toxic oxidants as the cells age. However, it remains unclear why no induction was observed in a chemically defined medium. In *S. xylosus*, manganese did not play a role at a transcriptional level, as was shown, for instance, for *E. coli sodA* (49). Manganese appears to be necessary only at the posttranslational step of metal insertion at the active site.

The *sod* gene is not essential for aerobic growth of *S. xylosus*, suggesting the presence of other protective functions in *S. xylosus*. The *sod S. xylosus* mutant shares phenotypes similar to those of *sodA sodB E. coli* mutants, which are also sensitive to hyperbaric O_2 and to paraquat and which exhibit multiple

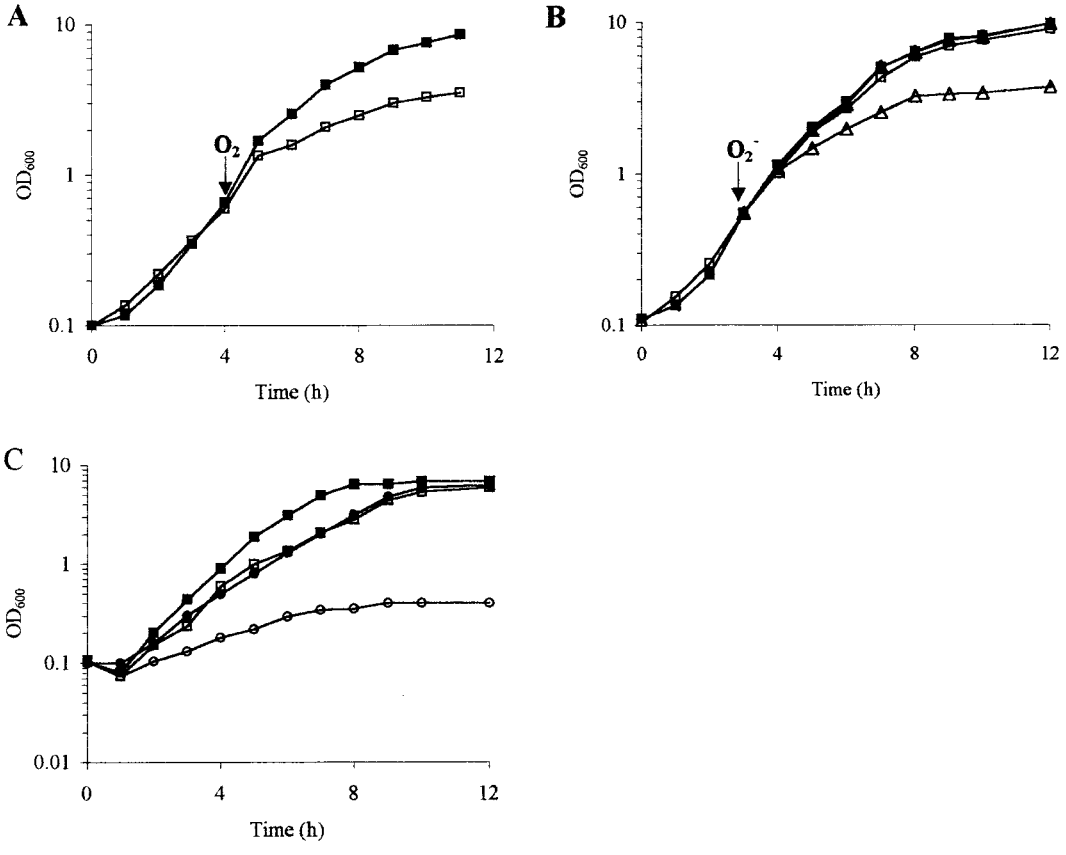


FIG. 6. Growth of the *S. xylosus* wild-type strain (C2a) and an *S. xylosus sod* mutant (TX351) in the presence of hyperbaric oxygen or paraquat or in a chemically defined medium (CDM). Closed symbols represent the growth curves of *S. xylosus* C2a, and open symbols represent the growth curves of *S. xylosus* TX351. (A) Growth in the presence of hyperbaric oxygen (squares). Cells were grown aerobically in MC medium. In exponential growth phase, a continuous bubbling of pure oxygen in the medium was applied at a pressure of 0.2 bar (shown by O_2 with an arrow). (B) Growth in the presence of paraquat. Cells were grown aerobically in MC medium. In exponential growth phase, paraquat was added (shown by an arrow) at a final concentration of 0 μM (squares) or 50 μM (triangles). (C) Growth in CDM. Cells were grown aerobically in CDM (squares) or in CDM without Leu, Ile, and Val (circles). Results are representative of two independent experiments. OD_{600}, optical density at 600 nm.

amino acid auxotrophy (13). For *E. coli*, this multiple amino acid auxotrophy results from different superoxide targets. One clearly identified target is the dihydroxyacid-dehydratase, which contains a 4Fe-4S cluster and which catalyzes the penultimate step in the biosynthesis of branched-chain amino acids (8, 9, 36). Other enzymes implicated in general metabolic pathways containing 4Fe-4S clusters were found to be inactivated by superoxides and protected by SOD (18, 20, 21, 37). In *S. xylosus*, the lack of SOD could lead to comparable damage.

In conclusion, *S. xylosus* possesses one single SOD, closely related to MnSODs. The *sod* gene is not essential for aerobic growth but appears to be important in the protection of cell constituents against oxidative stress. The role of SOD in the inhibition of the oxidation of unsaturated free fatty acids is being studied. This will lead to an understanding of the contribution of SOD to the antioxidant properties of *S. xylosus*.

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