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Characterisation of the *katA* gene encoding a catalase and evidence for at least a second catalase activity in *Staphylococcus xylosus*, bacteria used in food fermentation

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Abstract

The catalase gene *katA* of *Staphylococcus xylosus* was cloned. It encodes a protein of 494 amino acids with a molecular mass of 56.9 kDa, closely related to monofunctional catalases. A *katA* mutant still showed a relatively high catalase activity demonstrating that *S. xylosus* possesses more than one enzyme. By Southern blot analysis using a *katA* probe, a second genetic locus distinct from *katA* was detected that probably contained the additional catalase gene. To analyse *katA* expression, a transcriptional fusion of the *katA* promoter region to a promoterless β-galactosidase gene was integrated into the genome of *S. xylosus*. *katA* expression is induced upon entry into stationary phase, by oxygen and hydrogen peroxide. Iron and manganese depletion induced *katA* transcription. Comparing the resistance of *S. xylosus* wild-type and the *katA* mutant strain to hydrogen peroxide clearly showed that KatA is essential for *S. xylosus* to cope with hydrogen peroxide stress. Therefore, *S. xylosus* has at least two differentially expressed catalases.

Keywords: *Staphylococcus xylosus*; Catalase; *katA* gene; *katA* expression; *katA* mutant

1. Introduction

Cellular metabolism of molecular oxygen produces reactive and potentially toxic oxygen species such as superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals [1]. For defence against these reactive oxygen species, organisms contain antioxidants and enzymes that repair oxidative damage. Catalases catalyse the dismutation of hydrogen peroxide into water and oxygen and play an important role in reducing the formation of highly reactive hydroxyl radical which arises from H_2O_2 via the Fenton reaction [1]. Catalases are subdivided into three groups based on a survey of the properties and sequences of the enzymes [1]. The first grouping concerns the monofunctional catalases which are normally proteins with molecular masses of approximately 220–350 kDa and are formed by four identical subunits, each containing one (proto-)haem group [1]. Their active centre and NADPH-binding site have been described in detail [2]. The second group includes the bifunctional catalases with both catalase and peroxidase activity [1]. The third group is constituted by the non-haem, Mn-containing catalases [1].

In many bacteria, multiple catalase isoenzymes are present and each enzyme is encoded by a different gene and regulated differently. In *Escherichia coli*, *katE* and *katG* encode the monofunctional HPII and the bifunctional HPI catalases, respectively. HPII is the principal catalase in stationary phase cultures grown aerobically, whereas HPI is expressed under both aerobic and anaerobic conditions and its synthesis is induced when cells are exposed to sub-lethal levels of H_2O_2 [3]. Recently, the *katA* gene encoding the sole catalase of *Staphylococcus aureus* has been described and was shown to be regulated by the ferric uptake regulator homologue, PerR [4,5].

Staphylococcus xylosus is a facultatively anaerobic bacterium used as starter culture in association with lactic acid bacteria (LAB) for fermented meat products. The main contribution of LAB is acidification, which prevents

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growth of food poisoning bacteria and is the prerequisite for coagulation of soluble meat proteins to form a firm matrix [6]. *S. xylosus* ensures colour development by its nitrate reductase activity and contributes to the typical aroma, mainly by modulating the level and the nature of volatile products coming from lipid oxidation [7–9]. During fermentation, hydrogen peroxide may be formed and accumulated and may lead to rancidity and discoloration by attacking haem pigments [10]. *S. xylosus* inhibits the formation of hydrogen peroxide by its catalase activity and so could prevent colour and aroma imperfections in sausages. In this paper, we describe the characterisation of the *katA* gene from *S. xylosus*, encoding a monofunctional catalase. The effects of growth phase, oxygen, hydrogen peroxide and metals on *katA* expression were investigated. A *katA* mutant was obtained by allelic exchange and characterised.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains used are listed in Table 1. The temperature-sensitive shuttle vector pBT1 [11] and the *lacH* promoter probe plasmid pLP1 [12] were used. The *ermB* cassette from plasmid pEC4 was used to interrupt the *katA* gene in *S. xylosus* [11].

2.2. Media and culture conditions

S. xylosus was grown at 30°C in a complex medium (CM) (meat extract, 10 g l^{-1}; yeast extract, 5 g l^{-1}; NaCl, 5 g l^{-1}; Na_2HPO_4, 2 g l^{-1}) prepared in 67 mM phosphate buffer pH 6.0. *E. coli* was grown aerobically at 37°C in Luria–Bertani medium. When needed, media were supplemented with 2.5 μg ml^{-1} erythromycin or 100 μg ml^{-1} ampicillin. Highly aerated cultures were incubated on a rotary shaker at 170 rpm and the volume of cultures did not exceed 10% of total Erlenmeyer volume to ensure good aeration. For low aeration, bacteria were grown in tubes filled to 85% with low stirring (15 rpm) on a shaker-incubator. To study the effect of metals, heavy metals were removed from the medium with Chelex-100 (Bio-Rad Laboratories, Hercules, CA, USA) as recommended by the manufacturer and, when needed, ultrapure 0.1 mM $MnSO_4$ or 0.1 mM $FeSO_4$ (Sigma) was added. For this experiment, cells were grown with low aeration to prevent Fe^{2+} oxidation.

2.3. DNA preparation, transformation and molecular techniques

Chromosomal DNA from *S. xylosus* was isolated according to the classical procedure [13]. Plasmid DNA was introduced into *S. xylosus* by electroporation with glycine-treated electrocompetent cells [11]. DNA manipulations, plasmid DNA isolation and transformation of *E. coli* were performed according to standard procedures [13].

2.4. Southern blot analysis

DNA was digested with different restriction enzymes, fractionated on 0.8% agarose gel and transferred to a nylon membrane (N+, Amersham). A 762-bp internal *katA* fragment to be used as the probe was amplified by polymerase chain reaction (PCR) from plasmid pK71 with primers 5′-CAGTGGTTAACACCCAAACGG-3′ (primer SON1; positions 2136–2156) and 5′-CAGGCGAACGTGGTGCAGG-3′ (primer SON2; positions 2880–2898) and labelled with the Dig High Prime labeling system (Roche). Hybridisation was carried out overnight at 55°C, after which the membrane was washed twice with 2×saline sodium citrate (SSC) and 0.1% sodium dodecyl sulfate at room temperature and twice with 1×SSC at 37°C. The hybridised probe was detected by the DIG luminescent detection kit (Roche).

2.5. Construction of a katA–lacH transcriptional fusion

A *katA–lacH* transcriptional fusion was constructed and integrated into the *S. xylosus* chromosome. Briefly, a fragment containing the *crb–katA* intergenic region was obtained by PCR with pK71 DNA as template and primers 5′-CCTGTCGACTTTATTTTAATCATCAAATAAATG-3′ (primer PKAT6; positions 3193–3217; the *Sal*I re-

Table 1
Bacterial strains used

Strain	Genotype or characteristics	Reference or source
S. xylosus[a]		
C2a	Wild-type	[14]
TX350	*katA*::*ermB*	This study
TX300	*'lacR ΔlacP 'lacH*	[12]
TX302	*'lacR ΔlacP* P_{vegII}-*lacH*	[12]
TX356	*'lacR ΔlacP katA-lacH*	This study
E. coli		
DH5α	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	[27]
UM255	*pro leu rpsL hsdM hsdR endI lacY katG2katE*12::*Tn10 recA*	[28]

[a]All the *S. xylosus* strains are derived from *S. xylosus* DSM20267 cured of the endogenous plasmid pSX267 [14].

striction site is underlined) and 5′-CCTGGATCCATGG-TTATGGACTTACAACC-3′ (primer PKAT7; positions 3703–3682; the *Bam*HI restriction site is underlined). The *Bam*HI–*Sal*I fragment was cloned into the *lacH* promoter probe plasmid pLP1. The *Bam*HI–*Sal*I sequence was verified by sequencing on both strands. The plasmid containing the *katA–lacH* fusion was designated pLP24. The β-galactosidase-deficient TX300 derivative of the wild-type strain [12] was transformed with plasmid pLP24. The *katA–lacH* was integrated as described by Jankovic et al. [12], yielding strain TX356. PCR analyses of the *lac* region confirmed the correct integration of *katA–lacH* into the chromosome (data not shown).

2.6. Construction of a katA mutant by gene replacement

To construct a *katA* mutant, the *Bam*HI–*Hin*dIII *katA* fragment from pK71 was moved to the *Staphylococcus–E. coli* shuttle vector, pBT1. In the resulting plasmid, the unique *Kpn*I and *Sst*I restriction sites located at the beginning of the *katA* gene were used to introduce an *ermB* cassette (Fig. 1B). The *katA* inactivation plasmid, pBtKe, was introduced into *S. xylosus* wild-type C2a. By a double-crossover event, the inactivated copy of gene was introduced into the genome as described by Brückner [11].

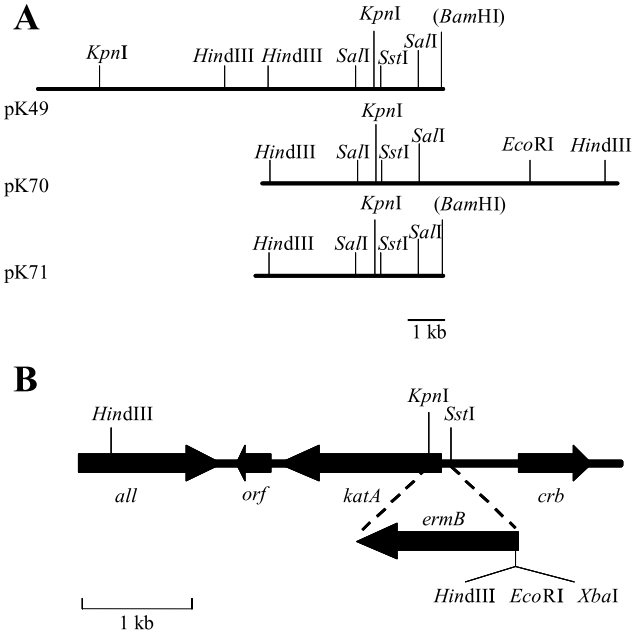

Fig. 1. Restriction maps of plasmids pK49, pK70 and pK71 (A) and genetic organisation of the insert of pK71 (B). A: *Bam*HI restriction sites are located at the cloning site and were created with the sequence of the insert and the flanking region of pBR322. B: Genes are shown by arrows. Relevant restriction sites are labelled. In the strain *S. xylosus* TX350, the *Kpn*I–*Sst*I fragment was replaced by the *ermB* cassette shown by dotted lines. Introduction of the *ermB* cassette generated a *Hin*dIII, an *Eco*RI and an *Xba*I restriction site.

By raising the temperature to 40°C, the cells were cured of the plasmid. The inactivation of the *katA* gene in the genome of *S. xylosus* was verified by PCR analyses (data not shown). The resulting strain was named TX350.

2.7. Preparation of crude extracts

Crude extracts were prepared by repeatedly vortexing with glass beads followed by removal of unbroken bacteria by centrifugation [14]. Protein concentration was estimated by using the Bradford dye-binding procedure (Bio-Rad protein assay, Bio-Rad), with bovine serum albumin as the standard.

2.8. Enzyme assays

β-Galactosidase activity was assayed according to Jankovic et al. [12]. Quantitative determination of catalase activity was performed by monitoring the decomposition of hydrogen peroxide by catalase spectrophotometrically at 240 nm as described previously [15]. One unit of catalase decomposed 1 μmol of H_2O_2 at 25°C in 1 min. Catalase plate assays were performed by placing a drop of 3% H_2O_2 onto the edge of colonies. A positive reaction was indicated by formation of O_2 bubbles.

2.9. Zone of inhibition assays

For zone of inhibition assays, cells were grown to an OD_{600} of 1.0 (exponential phase) or 7.0 (stationary phase), and 100 μl was added to 2 ml pre-warmed 0.7% top agar and layered onto CM plates containing 25 ml of medium. After the top agar had hardened, a 7-mm filter paper disk containing 10 μl of 0.88 M H_2O_2 was placed in the centre of the plate. Plates were incubated for 48 h at 30°C and the diameter of the zone of growth inhibition was measured. All assays were performed two times in duplicate, and the values were then averaged. Results were reproducible with a range of ±1 mm.

2.10. Survival studies

Cells were grown with high aeration in CM medium to an OD of 1.0 (exponential phase) and treated with H_2O_2 ranging from 0 to 400 mM for 60 min. The reaction was immediately stopped by the addition of 4000 U ml^{-1} of bovine catalase to the treated cultures. The numbers of colony-forming units were determined by plating serial dilutions onto CM agar plates and counted after 48 h of incubation.

2.11. Nucleotide sequence accession number

The DNA sequence reported here is available from the EMBL, GenBank and DDBJ databases under accession number AJ295151.

3. Results

3.1. Cloning the S. xylosus katA gene by complementation of E. coli UM255

The *katA* gene was isolated from an *S. xylosus* gene library constructed by cloning partially *Sau*3AI-restricted *S. xylosus* genomic DNA into the *E. coli* vector pBR322 and stored as 56 pools of plasmid DNAs prepared from 50 transformants [14]. The *E. coli* strain UM255, deficient for catalase activity, was transformed with each pool and the library was screened for catalase activity. Three positive clones from three independent pools were obtained. Restriction maps of the three plasmids, designated pK49, pK70, and pK71 (Fig. 1A), revealed that the plasmids shared the same *Hin*dIII–*Sal*I chromosomal region. Therefore, plasmids pK49, pK70 and pK71 contained the same catalase gene, designated *katA*. Only the insertion of plasmid pK71 was studied further.

3.2. Nucleotide sequence analysis of the katA gene

The complete insert of pK71 consisting of 4634 bp was sequenced. Four open reading frames (ORFs), three complete and one truncated, were found (Fig. 1B). The third ORF, encoding a polypeptide of 494 amino acids with a theoretical M_r of 56.9 kDa and a p*I* of 5.46, is clearly the *katA* gene as its deduced amino acid sequence revealed high similarity with prokaryotic and eukaryotic catalases. The highest similarities (80–82%) were obtained with staphylococcal catalases: *S. aureus* KatA [AJ000472], *S. aureus* subsp. *anaerobius* KatB [AJ000471] and *Staphylococcus warneri* catalase [AB045340]. More than 63% similarity was observed with catalases of *Streptomyces coelicolor* CatA [X96981], *Bacteroides fragilis* KatB [U18676], *Neisseria meningitidis* [AL162752], *Vibrio fischeri* KatA [AF011784] and *Haemophilus influenzae* HktE [U32774]. Comparison of the deduced amino acid sequence of *S. xylosus* KatA with the sequence of bovine liver catalase (BLC) revealed that the three residues involved in the active site of the BLC were conserved in *S. xylosus* KatA at the His-53, Ser-92 and Asn-126 positions [16]. Furthermore, the proximal haem site of BLC is conserved in *S. xylosus* KatA and comprises Pro-314, Arg-332 and Tyr-336 as ligands. Of the five distal haem site ligands in BLC, the Val-73 residue was replaced by Met-52 while the others were conserved as Arg-91, Thr-93, Phe-131 and Phe-139. In addition, the NADPH-binding site residues of BLC [2], Arg-202 and His-234, seemed to be conserved in KatA as Arg-181 and His-213, but Asp-212, Tyr-214, Lys-236 and His-286 were replaced in KatA by His-191, Phe-193, Arg-215 and Lys-283, respectively. The changes detected in KatA in comparison with BLC have also been described in the *Proteus mirabilis* catalase and, according to biochemical results and modelling, they do not appear to influence either the specific activity or interaction of the enzyme with NADPH [17].

The deduced amino acid sequence of the truncated ORF1, designated *all*, is similar to allantoinases that are implicated in the assimilation of allantoin (purine catabolism) [18]. ORF2 is located on the same strand as *katA* and shared no significant similarity with any sequence in the databases. The deduced amino acid sequence of ORF4 displayed low similarities with carboxypeptidases and was named *crb*.

3.3. Construction of a KatA-deficient S. xylosus mutant

A KatA-deficient strain of *S. xylosus*, designated TX350, was constructed by exchanging the 5′-end of *katA* for an erythromycin resistance gene, *ermB* (Fig. 1B). By this replacement, the first 60 codons of *katA* including the start codon and ribosome-binding site were removed resulting in a complete block of KatA production. Surprisingly, the KatA mutant still exhibited catalase activity. Comparing catalase activities in the wild-type and the TX350 *katA* mutant strain revealed that about 60% of the wild-type catalase activity remained in the mutant. It appeared, therefore, that *S. xylosus* has more than one catalase.

To determine whether the gene encoding the additional enzyme is similar to *katA*, Southern blot analysis was performed using a *katA*-specific probe and chromosomal DNAs from the wild-type and the *katA* mutant strain. A 20-kb *Eco*RI fragment is detected in the wild-type, which is substantially shortened in the TX350 DNA due to the introduction of an *Eco*RI restriction site within the *ermB* cassette (data not shown). In addition to the *katA*-containing band, a second weaker hybridisation signal of about 4.5 kb was detected in both strains, most likely due to a second catalase gene. Using other restriction enzymes, equivalent hybridisation patterns were obtained (data not shown). These results strongly suggest that *S. xylosus* has two paralogous catalase genes, but the presence of additional, less similar genes cannot be ruled out. In the following, we will refer to the second, not yet characterised catalase gene as *katB*.

3.4. Regulation of katA expression in S. xylosus

Due to the multiple catalases in *S. xylosus*, it was necessary to use a reporter gene to assess the *katA* expression pattern under different growth conditions. For that purpose, the *crb–katA* intergenic region harbouring the *katA* promoter was cloned in front of the promoterless *S. xylosus* β-galactosidase gene *lacH* [12]. The reporter construct was subsequently integrated into the *S. xylosus* chromosome thereby replacing the endogenous lactose operon [12]. In the resulting strain *S. xylosus* TX356, *katA* promoter-directed β-galactosidase activity was measured in cultures grown as specified in Table 2. As a control for

Table 2
Regulation of β-galactosidase expression directed by the *katA* promoter and regulation of KatB catalase activity in a KatA-deficient strain

Growth conditions	Treatment/addition	β-Galactosidase activity[a] in TX356 (P_{katA}-*lacH*)	Catalase activity[b] in TX350 (*katA–ermB*)
Low aeration, exp. phase[c]	–	2.2 ± 0.5	< 1[d]
Low aeration, stat. phase[e]	–	8 ± 1	158 ± 20
High aeration, exp. phase	–	5 ± 1	317 ± 21
High aeration, stat. phase	–	18 ± 3	1370 ± 51
High aeration, exp. phase	50 mM H_2O_2	50 ± 6	260 ± 19
Low aeration, stat. phase	Chelex	89 ± 3	913 ± 136

[a]β-Galactosidase activity is expressed as nmol nitrophenol released per minute and mg of protein.
[b]Catalase activity is expressed as μM H_2O_2 decomposed per minute and mg of protein.
[c]Enzyme activities were measured in the middle of the exponential (exp.) growth phase.
[d]Below detection level.
[e]Enzyme activities were measured 2 h after the onset of the stationary (stat.) growth phase.

the applicability of β-galactosidase as a reporter under these conditions, *S. xylosus* TX302 harbouring the constitutive promoter P_{vegII} from *Bacillus subtilis* in front of the *lacH* gene [12] was used. As expected, P_{vegII}-directed β-galactosidase activity was highest during growth, declined in stationary phase, and was not induced by the conditions outlined below (data not shown). From the β-galactosidase activities summarised in Table 2, we deduced that *katA* is expressed weakly during growth and about four-fold higher during stationary phase. Expression of the gene is induced by oxygen slightly more than two-fold. Hydrogen peroxide added to highly aerated cultures and metal depletion of the growth medium by Chelex treatment were found to be the most efficient stimuli (Table 2), increasing *katA* promoter-directed β-galactosidase activity about 10-fold. Addition of iron and manganese to the Chelex-treated cultures reduced *katA* expression back to the untreated values (data not shown). Therefore, lack of iron and manganese is responsible for *katA* induction.

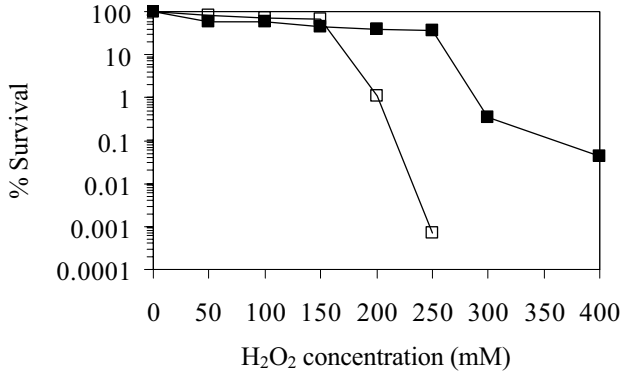

Fig. 2. Survival of exponential phase cells of *katA* mutant (open squares) and wild-type (closed squares) following addition of H_2O_2 for 60 min. Survival is expressed as (the number of colony-forming units in the sample challenged with H_2O_2 divided by the number of colony-forming units in the untreated sample)×100. Data points are the means of at least three independent experiments.

3.5. Catalase KatB activity in a KatA-deficient S. xylosus strain

The KatA deletion strain TX350 provided the opportunity to measure the remaining catalase activity, KatB, and to compare these data with *katA* expression. Catalase activity in the KatA-deficient strain was measured under the same conditions as for the β-galactosidase determination mentioned above. The results of these measurements, summarised in Table 2, revealed an expression pattern distinct from *katA*. Induction of KatB activity during stationary was more than 100-fold under conditions of low aeration. Oxygen enhanced KatB activity more than 300-fold during the exponential growth phase and almost nine-fold during stationary phase. On the other hand, metal depletion induced *katB* less efficiently than *katA* and addition of H_2O_2 under high aeration did not stimulate KatB activity at all (Table 2). Thus, *S. xylosus* has two catalase genes that are differently expressed.

3.6. Sensitivity of S. xylosus TX350 (katA) to hydrogen peroxide

The growths of *S. xylosus* TX350 (*katA*) and the parental strain in high aeration were similar (data not shown). *S. xylosus* TX350 was tested for its sensitivity to H_2O_2 using a zone of inhibition assay. When exponential phase cells or stationary phase cells were tested, the zone of inhibition of *S. xylosus* TX350 was larger than that of *S. xylosus* C2a (38 mm versus 23 mm for exponential phase cells and 19 mm versus 12 mm for stationary phase cells). In addition the survival of *S. xylosus* TX350 to various concentrations of H_2O_2 was compared with that of *S. xylosus* C2a (Fig. 2). Up to 150 mM H_2O_2, the survival of *S. xylosus* TX350 was almost similar to *S. xylosus* C2a. However, in the presence of 200 mM H_2O_2, *S. xylosus* TX350 was killed, whereas survival of *S. xylosus* C2a was only slightly reduced (Fig. 2; 1% TX350 survival versus 40% C2a survival). This implies

that in spite of the presence of (an)other catalase(s), KatA is essential for a optimal resistance to exogenous H_2O_2.

4. Discussion

Contrary to *S. aureus*, which synthesises only a single catalase KatA [5], *S. xylosus* possesses more than one enzyme. The catalase KatA of *S. xylosus* is highly homologous to other staphylococcal catalases and the active centre and NADPH-binding site of monofunctional catalases are conserved. The genetic organisation surrounding the *katA* gene, however, is different in *S. xylosus* and *S. aureus* [5]. The *S. aureus katA* gene is preceded by a gene encoding a γ-aminobutyrate permease and followed by the *rpmG* and *rpsN* genes encoding homologues of a 50S ribosomal protein, L33, and a 30S ribosomal protein, S14, [AP003362] [19]. Genes encoding allantoinase or a carboxypeptidase are in close vicinity to *S. xylosus katA*. In contrast to the *katA* region, the genetic organisation around *sod* genes encoding another antioxidant enzyme, superoxide dismutase, is conserved in the two species [20]. Therefore, the gene from *S. xylosus* isolated in this study might represent the catalase gene that is missing in *S. aureus*. Isolation and analysis of the second catalase gene of *S. xylosus* will eventually reveal if it is located in the same genomic neighbourhood as the *S. aureus* counterpart.

In *S. xylosus*, transcription of *katA* is induced upon entry into stationary phase, by oxygen and particularly by hydrogen peroxide. Transcription of *katA* is downregulated by iron and especially by manganese. In *B. subtilis*, the major vegetative catalase KatA is also induced following exposure to hydrogen peroxide or by entry into stationary phase when iron and manganese are low [21]. *B. subtilis* KatA was shown to be part of the peroxide stimulon which is regulated by a Fur homologue, the peroxide regulon repressor PerR [22]. In *B. subtilis*, PerR appears to require a divalent metal ion to activate its DNA-binding activity, and both iron and manganese can serve this role [23]. In *S. aureus*, both Fur and PerR regulate the transcription of *katA*, with Fur acting as an iron-responsive activator of transcription [5,24]. PerR acts as a manganese-dependent transcriptional repressor, and the PerR regulon is induced during growth in media containing elevated levels of iron. In *S. xylosus*, a putative Per box was identified in positions 3422–3438, which matched 15 of the 17 bases (bold face) of the *S. aureus* PerR box (**ATTATAATTATTATAAT**) consensus sequence [5], suggesting that a similar regulation could take place in *S. xylosus*. Comparing *katA* expression with the expression of the other catalase(s) present in *S. xylosus* clearly showed that these genes are differentially expressed. In many bacteria such as *E. coli*, catalase genes are subject to various control mechanisms; synthesis of HPI catalase has been shown to be under the positive control of OxyR [25] whereas synthesis of HPII catalase is controlled by RpoS [26]. Further work will be necessary to determine the molecular mechanisms of differential catalase expression in *S. xylosus*.

Despite the relatively high residual level of catalase activity measured in the *katA* mutant, KatA was shown to be essential for resistance to elevated levels of hydrogen peroxide. In many bacteria such as *Rhizobium meliloti*, *Pseudomonas aeruginosa* or *B. subtilis*, catalases are necessary to protect cells against oxidative stress [1].

In summary, in an attempt to analyse the antioxidant capacities of *S. xylosus*, catalase KatA was characterised. The presence of other catalase(s) was shown and further work is necessary to clone the corresponding gene(s). Catalase KatA may not be the major catalase in *S. xylosus*, but it appears essential for optimal resistance to hydrogen peroxide. Work is in progress to understand its role in the oxidation of unsaturated free fatty acids.

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