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ORIGINAL PAPER

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# Characterisation of oxidative stress-resistant mutants of *Streptococcus thermophilus* CNRZ368

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**Abstract** During industrial processes, the dairy organism *Streptococcus thermophilus* is exposed to stress conditions. Its ability to survive and grow in an aerobic environment indicates that it must possess defensive mechanisms against reactive oxygen species. To identify the genes involved in oxidative stress defence, a collection of mutants was generated by random insertional mutagenesis and screened for menadione sensitivity and resistance. Results obtained for resistant clones allowed the identification of eight loci. The insertions affected genes whose homologues in other bacteria were previously identified as being involved in stress response (*deoB*, *gst*) or transcription regulation (*rggC*) and five ORFs of unknown function. The tolerance of the eight mutants to air-exposure, methyl viologen and $H_2O_2$ was studied. Real-time quantitative PCR was used to analyse the transcript level of mutated genes and revealed that most were down-regulated during oxidative stress.

**Keywords** *Streptococcus thermophilus* · Oxidative stress · Real-time quantitative RT-PCR

## Introduction

*Streptococcus thermophilus* is widely used as a starter strain in the production of yogurt, Swiss-type cheeses and Mozarella cheese. During the production or conservation of these products, *S. thermophilus* is exposed to potentially stressful environmental conditions, including contact with oxygen. To thrive in such an environment, *S. thermophilus* must possess defensive mechanisms against the deleterious effects of oxygen caused by the production of reactive oxygen species.

The key enzymes of the oxidative stress response are generally well conserved throughout the bacterial world (Koonin et al. 2000) and, as in other bacteria, streptococci are equipped with superoxide dismutase (SOD), NADH oxidase, peroxidase and glutathione reductase. SOD activity represents the major mechanism of defence against superoxide stress by converting superoxide anions to molecular oxygen and $H_2O_2$ (Fridovich 1997). Four types of SOD can be distinguished in bacteria, depending on the metal cofactor (Cu–Zn, Fe, Mn or Ni). Most streptococci possess only a MnSOD encoded by the *sodA* gene (Poyart et al. 2001; Jakubovics et al. 2002), whereas in *S. pneumoniae* two co-factored SOD (MnSOD, FeSOD) have been found (Yesilkaya et al. 2000). To remove $O_2$, streptococci also utilise the action of NADH oxidase or pyruvate oxidase (Gibson et al. 2000; Pericone et al. 2003). In *S. mutans*, two distinct NADH oxidases have been identified, catalysing either the two-electron reduction of $O_2$ to $H_2O_2$ (Nox1) or the four-electron reduction of $O_2$ to $H_2O$ (Nox2; Higuchi et al. 1999).

In the genus *Streptococcus*, $H_2O_2$ generated by SOD or NADH oxidase is metabolised by various peroxidase enzymes. For example, protection against $H_2O_2$ and organic peroxides in *S. pyogenes* involves a glutathione peroxidase and an alkyl hydroperoxide reductase (King et al. 2000) and, in *S. parasanguis*, a thiol peroxidase (Spatafora et al. 2002). Moreover, streptococci contain Dpr that provides a means to tolerate $H_2O_2$, by binding free intracellular iron and thus inhibiting the Fenton chemistry-catalysed formation of toxic hydroxyl radicals (Yamamoto et al. 2000).

*Streptococcus*, however, lacks some proteins, such as catalase and the global regulator OxyR, that have been shown, through the analysis of multiple bacterial species, to protect against oxidative stress. Moreover, in streptococci the pool of enzymes involved in oxidative stress defence can vary from one species to another. For instance, the $H_2O_2$ scavenger NADH peroxidase is

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absent in *S. pneumoniae* (Hoskins et al. 2001; Tettelin et al. 2001) and *S. suis* (Niven and Ekins 2001). Thus, although the *Streptococcus* oxidative-stress defence mechanism shares common characteristics with other bacteria, it also provides a good model for novel oxidative stress response mechanisms in bacteria.

In order to better understand the ability of *S. thermophilus* to cope with reactive oxygen species, mutants from the strain CNRZ368 were generated, screened and characterised. In a previous study, menadione-sensitive mutants were analysed and revealed that genes involved in cell wall metabolism, exopolysaccharide translocation, tRNA modification and iron metabolism also play a role in cell survival during oxidative stress (Thibessard et al. 2004). In this work, a genetic and phenotypic analysis of menadione-resistant mutants is presented.

## Materials and methods

### Bacterial strains, plasmid and growth conditions

Bacterial strains used in this study were derived from *S. thermophilus* CNRZ368 purchased from the INRA-CNRZ368 strain collection (Jouy en Josas, France). Depending on the experiments, *S. thermophilus* CNRZ368 and its derivatives were cultivated at 30°C or 42°C, in milk medium (for pre-cultures), TPPY (Bracquart 1981) or M17 (Terzaghi and Sandine 1975) media. When required, 2 μg/ml erythromycin was added.

The plasmid pGh9:IS*S1* (Maguin et al. 1996) used for insertional mutagenesis served to generate recombinant plasmids. *E. coli* EC101 (Leenhouts 1995), a TG1-derived strain [*supE hsd-5 thi* Δ(*lac-proAB*) F′ (*traD6proAB*$^+$ *lacI*$^q$*lacZ*ΔM15)] containing the pWV01 *repA* gene, was transformed with pGh9:IS*S1* and derivatives. The resulting transformants were selected at 37°C on Luria–Bertani medium containing 150 μg/ml erythromycin.

### Insertional mutagenesis and isolation of menadione-resistant mutants

Mutagenesis with pGh9:IS*S1* and cloning of the chromosomal target of the plasmid were performed as described by Thibessard et al. (2002). The screening procedure used to select menadione-sensitive and resistant mutants is detailed by Thibessard et al. (2004) and is briefly described below. Two experimental procedures were performed, differing mainly in the length of menadione exposure. For long-term menadione exposure, mutant pre-cultures were inoculated onto M17 agar plates containing 2 μg/ml erythromycin and various concentrations of menadione (0–120 μg/ml). Plates were incubated for 20 h at 42°C. For short-term menadione exposure, mutant pre-cultures were inoculated in liquid TPPY medium and grown for 3.5 h at 42°C. Then, each culture was exposed to various menadione concentrations (0–15 mg/ml). After 3 h incubation at 42°C, cells were dropped on TPPY agar plates containing erythromycin and incubated for 20 h at 42°C. For each procedure, clones were considered resistant when they grew on a plate on which less than 10% of the clones were growing.

### General DNA techniques

DNA manipulations were performed according to Sambrook et al. (1989). Sequencing was carried out with the DNA sequencing kit (Applied Biosystems) on an ABI Prism 377 genetic analyser (Applied Biosystems). Sequence data were analysed with BLAST, ProDom, the TIGRfam model, TMHMM and HMMTOP software. DNA sequences obtained in this work were deposited in GenBank under accession numbers from AY598747 to AY598752.

### RNA manipulation

To extract total RNA, *S. thermophilus* CNRZ368 was cultivated in TPPY medium containing 200 mM MOPS and incubated at 42°C to an optical density at 600 nm ($OD_{600}$) = 0.6. The culture was subdivided into three aliquots. One was kept at 42°C without addition of $H_2O_2$ (control experiment); and in the other two, 1 mM $H_2O_2$ was added for 20 min or 45 min. Total RNA was extracted with a guanidinium thiocyanate and phenol–chloroform step (Chomczynski and Sacchi 1987) using the Tri-Reagent (Sigma-Aldrich). DNA-free RNA was obtained by the action of DNase I (0.5 units/μg RNA) for 15 min at 37°C. The absence of contaminating genomic DNA was checked by PCR, directly on RNA samples.

cDNA was generated from 2.5 μg of RNA by the action of 100 units of MMLV-reverse transcriptase at 37°C for 60 min in the presence of hexamer oligonucleotide random primers at a concentration of 100 ng/μl (Amersham Pharmacia Biotech).

Primers for real-time RT-PCR (Table 1) were designed with Primer3 software and purchased from MWG Biotech. Amplification of specific products was performed with the iCycler (BioRad) with the following program: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 35 cycles at 95°C for 15 s and at 55°C for 1 min. The amplification reactions were carried out with qPCR Mastermix for Sybr Green I (Eurogentec) in a final volume of 25 μl containing reaction buffer (1×), a 1:66,000 dilution of Sybr Green I, 400 nM (each) forward and reverse primer, 10 nM fluorescein and cDNA template (83 ng or 28 ng, depending on the experiment). A standard curve for efficiency of PCR determination was plotted for each primer set with a serially diluted cDNA template. Melting curve analysis was performed with 0.5°C increments every 10 s from 55°C to 90°C to check that the cDNA amplification did not lead to

**Table 1** Real-time RT-PCR primers

| PCR template gene | Primer used for real-time RT PCR | |
|---|---|---|
| | Name | Gene sequence |
| *ldh* | ldh.F | AAGCTATCCTTGACGATGAA |
| | ldh.R | AATAGCAGGTTGACCGATAA |
| *osrA* (*deoB*) | osrA.F | ACCCAACTTATGCTGGTACA |
| | osrA.R | AAATGTCCAACTGGAATCAA |
| *osrB* (*gst*) | osrB.F | GCGCAATTGTAGTTCTACTTG |
| | osrB.R | AAGGACATTCAAAGCTGTTCT |
| *osrC* (*rggC*) | osrC.F | ACTTGTTTTCTGCGGAATACT |
| | osrC.R | TCCGAACGATGACACATTT |
| *osrD* | osrD.F | ACCGTCGTGAATTTCTTAAA |
| | osrD.R | CTTGAACGATCCTGATTAGC |
| *osrE* | osrE.F | ACATGGTGTTTCTGGTAA |
| | osrE.R | TTCTCCAATTCAATGGTTGT |
| *osrF* | osrF.F | TTCGAATAGCTTCGGATTTA |
| | osrF.R | TAAGCCTCCAATAACAGCAT |
| *osrG* | osrG.F | TCCCAAATAGCCCAATAGTA |
| | osrG.R | ATAGGCGGGAACTCTCTAAA |
| *osrH* (*orfP*) | osrH.F | TGAGGATGACTCTGATGACA |
| | osrH.R | AAAGGCATCCTCTCCATAAT |

secondary products. Quantification of tested gene expression was done using the comparative ΔΔCt method (Livak and Schmittgen 2001). Cycle threshold (Ct) values were defined as the cycle number at which the fluorescence exceeded a fixed threshold value above the baseline.

### Determination of tolerance to other oxidative stress

*Exposure to air in liquid medium* *S. thermophilus* and its derivatives were grown in TPPY at 42°C to the stationary phase. Each culture was divided into five parts. Four of them were shaken (200 rpm) in a ratio of liquid to air space of approximately 3/1 and the fifth was not shaken (control experiment). Appropriate dilutions of each aliquot were spread on TPPY. The fraction of surviving colony-forming units (CFU) was calculated by dividing the CFU/ml count after growth under aerated conditions by the CFU/ml count for the control experiment.

*Exposure to methyl viologen* Overnight cultures in milk medium were diluted 100-fold into TPPY. After 8 h growth, cultures were diluted 150-fold in fresh TPPY medium, split and exposed to different concentrations of methyl viologen (Sigma-Aldrich). After 14 h incubation at 42°C, the $OD_{600}$ was measured and the growth ratio was determined by comparing the final OD obtained with and without treatment.

*Exposure to $H_2O_2$* Overnight cultures in milk medium were diluted 1,000-fold in TPPY containing different concentrations of $H_2O_2$. Following 14 h incubation at 42°C, the $OD_{600}$ was measured and the growth ratio was determined by comparing the final OD with and without treatment.

## Results

### Selection of menadione-resistant mutants

A collection of 2,112 mutants was constructed by insertional mutagenesis (Thibessard et al. 2002). Two different procedures (see Materials and methods) assessing resistance to menadione, a superoxide-generating compound, allowed the selection of eight clones exhibiting a menadione-resistance phenotype. These clones were named *osrA* to *osrH* (for oxidative stress resistance locus) and were further characterised.

### Identification of genes involved in menadione-resistant phenotype

Replicative transposition of IS*S1*, carried by the pGh9:IS*S1* plasmid, resulted in the integration of the entire plasmid in the chromosome, flanked by two direct copies of IS*S1*. Chromosomal targets of pGh9:IS*S1* insertion were recovered on replicative plasmids as described by Thibessard et al. (2002) and sequenced. In each case, the plasmid interrupted an ORF and the corresponding sequence was used to establish DNA and protein identity with database sequences.

Six out of the eight target gene products (OsrA to OsrF) showed sequence similarities with proteins with an *E*-value lower than $10^{-10}$ for BLASTX searches. The last two sequences (OsrG, OsrH) showed no significant identity to any database sequence. According to their putative function, target genes were separated into four classes (from I to IV) and renamed where relevant (Table 2).

#### *Class I: genes already known to be involved in lactic acid bacteria stress defence mechanism*

The first class comprised *osrA* and *osrB* mutants, both disrupted in ORFs whose products share homology with proteins already involved in stress response.

The sequence disrupted in the *osrA* mutant encodes a protein exhibiting 100% identity with DeoB from *S. thermophilus* NCBF2393, and is renamed *deoB*. The DeoB protein is a phosphopentomutase, that catalyses the phosphotransfer between the C1 and C5 carbon atoms of pentose. This enzyme is involved in the purine salvage pathway and assimilation of exogenous free bases or nucleosides from the environment (Nygaard 1993). In *Lactococcus lactis*, *deoB* mutations confer higher sensitivity to UV stress (Duwat et al. 1997) and increased resistance to acid and heat stress, demonstrating the importance of purine metabolism in stress defence (Rallu et al. 2000).

The disrupted ORF in the *osrB* mutant corresponds to *gst*, a gene localised in ICE *St1*, an integrative and conjugative element of *S. thermophilus* CNRZ368 (Burrus et al. 2002). The Gst (OsrB) protein shows 33%

**Table 2** Characterisation of *osr* ORFs. The definite gene name is in brackets when applicable. Organism, gene name and, in brackets, accession number and length are given for the protein sequence showing the best alignment score. Percentage of identity and the portion of the protein sharing this identity (numbers correspond to the amino acid positions) are indicated for the protein showing the best alignment score to each *S. thermophilus* Osr protein. Insertion site corresponds to the deduced amino acid position of the pGh9:IS *S1*-generated disruption in the full-length protein showing the best alignment score to the Osr proteins

| Gene name | Accession number | Gene encoding the most similar product | Amino acid identity | Insertion site | Putative ORF function | Class |
|---|---|---|---|---|---|---|
| *osrA* (*deoB*) | AY598747 | *S. thermophilus* NCFB2393 *deoB* (AAM93387, 403) | 98% (133–403) | 133 | Phophopentomutase | I |
| *osrB* (*gst*) | AJ278471 | *Yersinia pestis gltP* (AH0031, 438) | 33% (9–403) | 404 | Proton/sodium-dicarboxylate symporter | |
| *osrC* (*rggC*) | AY598748 | *S. mutans rgg* (AAN59160, 285) | 57% (3–284) | 242 | Transcriptional regulator | II |
| *osrD* | AY598749 | *S. pneumoniae* R6 *spr1179* (AAK99982, 334) | 78% (1–174) | 48 | Conserved hypothetical protein | III |
| *osrE* | AY598750 | *Lactococcus lactis ORF00050* (T43121, 614) | 23% (3–399) | 59 | Conserved hypothetical protein | |
| *osrF* | AY598751 | *P. horikoshii* PH1052 (H71098, 307) | 35% (3–303) | 169 | Conserved hypothetical protein | |
| *osrG* | AY598752 | – | 778 nt [a] | – | – | IV |
| *osrH* (*orfP*) | AJ278471 | – | 1317 nt [a] | – | – | |

[a]Sequence analysed length (in nucleotides, nt) for the mutants showing no homology.

identity with a proton-glutamate/aspartate symport protein from *Yersinia pestis* and with other proteins belonging to the proton/sodium-dicarboxylate symporter protein family (SDF). Hydropathy profiles of Gst, determined using TMHMM (Sonnhammer et al. 1998) and HMMTOP (Tusnady and Simon 1998), revealed the presence of ten trans-membrane regions, as observed in proteins belonging to the SDF family (Storck et al. 1992). The link between glutamate transport and stress response has already been suggested in *L. lactis* (Rallu et al. 2000). The short intergenic region and the lack of a rho-independent transcriptional terminator between *osrB* and the downstream ORF suggest that these two ORFs form an operon. This adjacent ORF corresponds to the already-named *orfX* gene of ICE *St1* (Burrus et al. 2002) and encodes a protein showing 49% identity with a hypothetical protein BAC15033 from *Oceanobacillus ihyensis*. These proteins belong to the RimK family, comprising a potential alpha-L-glutamate ligase, whose function is to add glutamate residues to maturing proteins. Thus, *gst* and *orfX*, like most operon-organised genes, seem to be involved in the same function: Gst could import glutamate residues needed for OrfX activity.

### *Class II: gene involved in transcriptional regulation*

In the *osrC* mutant, pGh9:IS*S1* disrupted an ORF (*rggC*$_2$, 699 bp) whose hypothetical product shares 54% identity with the C-terminal region of Rgg from *S. mutans*. This ORF is preceded by another ORF (*rggC*$_1$, 198 bp) in the same orientation, whose hypothetical product shares 77% identity with the N-terminal part of the same protein. This apparent frameshift was confirmed by sequencing this region directly on the *S. thermophilus* CNRZ368 chromosome. A putative Shine–Dalgarno sequence located 8 bp upstream of *rggC*$_1$ was observed, suggesting this ORF was probably translated. In constrast, no canonical Shine–Dalgarno sequence-preceding *rggC*$_2$ was detected. A full-length protein (RggC) might be translated, implying a translational or transcriptional frameshifting. This event could occur between positions 136 and 144, where a 9-mer (TTT TTT TTT), could serve as a substrate, as described by Larsen et al. (2000). The sequence of RggC$_1$ displays a characteristic helix-turn-helix DNA-binding motif (Schell 1993) expected for Rgg-like proteins. These proteins constitute a family of transcriptional regulators that coordinate the expression of various functions in Gram-positive bacteria.

### *Class III: genes of unknown function*

The *osrD* mutant was found to be disrupted in a gene encoding a putative protein that shares 78% identity with the conserved hypothetical protein spr1179 from *S. pneumoniae* R6. OsrD and spr1179 proteins show significant identity with predicted iron-dependent peroxidases belonging to the family of dye-decolorising (or DyP-type) peroxidases. DyP peroxidases are characterised by an atypical heme-binding region (Sugano et al. 2000). Sequence analysis of OsrD indicated the presence of a twin-arginine translocation (TAT) signal sequence followed by a membrane-spanning hydrophobic region. The TAT domain allows translocation of a folded protein with a bound cofactor across the membrane (Berks et al. 2000). Analysis of the surrounding sequence in the *S. thermophilus* LMG18311 genome suggests that the *osrD* gene and the downstream ORF are part of an operon. This downstream ORF encodes a putative lipoprotein involved in the transport of iron, necessary for peroxidase activity. Thus, once again, both *osrD* and the downstream ORF seem to contribute to the same function.

The OsrE protein shows 23% identity with a hypothetical protein encoded by the *L. lactis* pMRC01 plasmid. These two proteins present a putative catalytic domain typical of kinase protein.

The *osrF* product shows 33% identity with the hypothetical protein PH1052 of the archaean, *Pyrococcus horikoshii*.

### *Class IV: genes with no known homologue*

No sequence identities were detected for the *osrG* and *osrH* genes, which are thus considered as specific to *S. thermophilus*. Therefore, no hypothesis can be drawn about their function. The *osrH* gene is carried by ICE *St1* from *S. thermophilus* CNRZ368 and was named *orfP* by Burrus et al. (2002).

## Menadione-resistant mutant growth rates

Growth rate is an important parameter for the industrial use of *S. thermophilus*. In TPPY medium, seven out of the eight mutants exhibited a doubling time similar to that of the parental CNRZ368 strain ($32.9 \pm 2.1$ min). Only the *deoB* mutant grew faster ($24.8 \pm 2.8$ min). Therefore, these mutations do not negatively affect growth.

Entry into the stationary phase was also analysed for the eight mutants. Only the *deoB* and *orfP* mutants showed a delayed entry into the stationary phase ($OD_{600} = 1.94 \pm 0.07$, $1.95 \pm 0.06$, respectively), compared with the reference strain ($OD_{600} = 1.66 \pm 0.11$). Under the conditions used, entry into the stationary phase was caused by culture medium acidification. In order to check whether such a delay could be correlated with impairment of lactic acid production, the pH decrease in the TPPY medium was monitored during growth of both *deoB* and *orfP* mutants. Results of this analysis showed no difference, compared with the reference strain. This point suggested a better resistance of *deoB* and *orfP* mutants to acid stress and was supported by the pH values observed during entry into the stationary phase (pH 4.98 for the reference strain, pH 4.78 for the *deoB* mutant, pH 4.82 for the *orfP* mutant).

Viability during the stationary phase in TPPY medium was estimated for each mutant 2.5 h and 24 h after entry into the stationary phase. The reference strain exhibited $23.7 \pm 3.9\%$ and $0.31 \pm 0.14\%$ survival after 2.5 h and 24 h, respectively. No significant difference could be shown between mutant and reference strains. Thus, *deoB*, *gst*, *rggC*, *osrD*, *osrE*, *osrF*, *osrG* and *orfP* gene integrity was not essential for survival during the stationary phase.

## Survival of menadione-resistant mutants with other oxidising agents

The *osr* mutant survival with various oxidising agents was examined. Survival curves of stationary-phase cells exposed to air were compared (Fig. 1). The mutants showed an increased survival compared with the reference strain, all of them displaying at least a 2-fold higher resistance after a 320-min air exposure. Since survival rates in the stationary phase without air exposure were similar (see above), the observed difference may be due to air resistance.

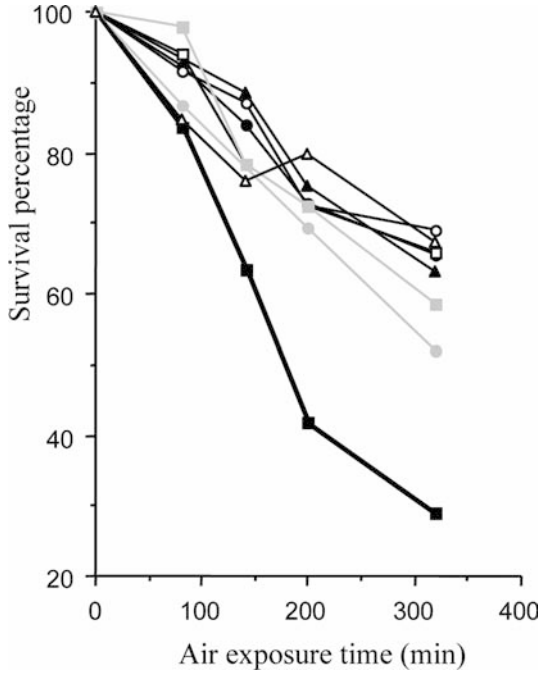


**Fig. 1** Survival of *osr* mutant and reference strains to air exposure. During air exposure, all strains are grown in TPPY liquid medium at 42°C under agitation (200 rpm). Each experiment was performed in triplicate and typical results are presented. The *osrG* mutant, whose response was not reproducible, is missing in the graph. *Filled squares* Reference strain, *open circles deoB* mutant, *open triangles gst* mutant, *grey circles rggC* mutant, *grey squares osrD* mutant, *filled triangles osrE* mutant, *filled circles osrF* mutant, *open squares orfP* mutant

The survival of the mutants with various concentrations of methyl viologen (Fig. 2a) and $H_2O_2$ (Fig. 2b) was also estimated. The resistance phenotype of the *osr* mutants with menadione was extended not only to another superoxide-generating compound (methyl viologen) but also to $H_2O_2$. Four of the mutants (*gst*, *rggC*, *osrG*, *orfP*) were markedly more resistant than the reference strain, especially at high doses of both oxidising compounds. Considering the spontaneous or catalysed dismutation of superoxide radicals into $H_2O_2$, the observed superoxide resistance of these mutants could be at least partially due to $H_2O_2$ resistance. Although they also exhibited an increased resistance to both oxidising agents, the *osrD* and *osrE* mutants survived less well, compared with the other strains. In contrast, *deoB* displayed a strong relative resistance to methyl viologen and only a weak resistance to $H_2O_2$, whereas *osrF* showed a high resistance to $H_2O_2$ and only a slightly increased survival with methyl viologen. These data suggest that DeoB and OsrF affect the sensitivity level by two distinct pathways.

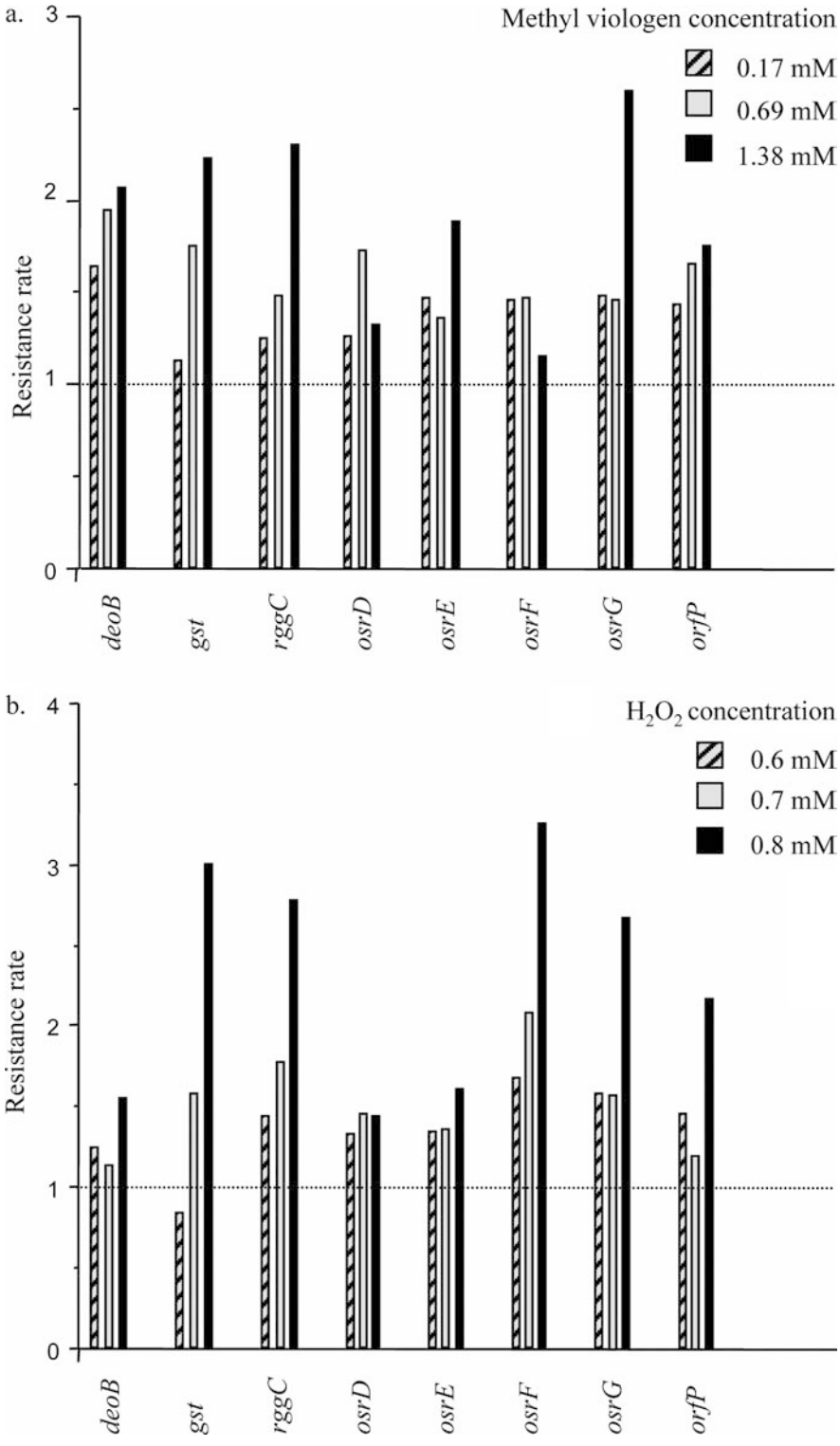


**Fig. 2** Effects of oxidising agents on the survival of *S. thermophilus* strains. All strains were grown in TPPY liquid medium supplemented with the indicated concentration of **a** methyl-viologen and **b** $H_2O_2$. The growth ratio was calculated as the ratio of the $OD_{600}$ obtained with and without oxidative stress exposure. To obtain the resistance rate, the growth ratio of each mutant was divided by the growth ratio of the reference strain. Each experiment was done in duplicate and typical results are presented. The reference strain $OD_{600}$ was 0.72 without oxidative stress and was, respectively, 0.42, 0.33, 0.27 with 0.17, 0.69, 1.38 mM methyl viologen for the experiment shown in **a**. The reference strain $OD_{600}$ was 1.10 without oxidative stress and was, respectively, 0.46, 0.24, 0.11 with 0.6, 0.7, 0.8 mM $H_2O_2$ for the experiment shown in **b**

## Expression of *osr* genes under stress conditions

Real-time quantitative RT-PCR was used to analyse changes in the levels of *deoB*, *gst*, *rggC*, *osrD*, *osrE*, *osrF*, *osrG* and *orfP* gene transcripts after exposure to $H_2O_2$ for 20 min and 45 min in *S. thermophilus* CNRZ368.

The *S. thermophilus ldh* gene encoding lactate dehydrogenase was used as a reference gene. To confirm that *ldh* was constitutively expressed in the presence of $H_2O_2$, the amount of *ldh* cDNA was measured in at least two separate RT-PCR experiments with three independent RNA samples from cells treated with $H_2O_2$ or not treated (control experiment). No significant difference in gene expression level was detected and the *ldh* gene was then considered as a relevant reference. PCR efficiencies were approximately the same for all genes studied,

ranging from 95% to 100% (data not shown). The quantity of cDNA for each gene was normalised to the quantity of *ldh* cDNA present in each RNA preparation and was ranked relative to the quantity of cDNA in the non-stressed conditions.

Transcripts were detected for each of the *osr* genes, demonstrating that all the genes were active. The transcriptional expression of these genes was analysed in the absence or presence of 1 mM $H_2O_2$ at two different exposure times (20 min, 45 min). During analysis, only 2-fold and better differences were considered as significant. Following 45 min exposure, the *deoB*, *gst*, *osrD*, *osrE* and *osrF* transcript levels were about 5-fold lower than in non-stress conditions and about 2- to 3-fold lower for the *osrP* gene (Table 3). Therefore, these genes were down-regulated during oxidative stress. For the *rggC* and *osrG* genes, the differences were not significant.

## Discussion

In this study, we identified eight novel loci involved in *S. thermophilus* behaviour under various oxidative stress conditions, named *osr* (for oxidative stress resistance). In each case, the plasmid disrupted an ORF. Analysis of the region surrounding the *deoB*, *gst*, *osrD*, *osrE*, *osrF* and *osrG* genes suggests that these genes belong in operons. Thus, the resistance phenotype of the corresponding disrupted mutants can be the result of inactivation of the listed genes or/and the result of a polar effect on neighbouring genes. Nevertheless, genes clustered in operons are generally involved in a common process and, from the sequence data available, the operons disrupted in this study do not seem to be an exception. Thus, our mutants should be considered as mutants impaired in a process rather than mutants of one particular ORF. The link between the disrupted loci and behaviour under oxidative stress conditions is discussed below.

*deoB* (encoding a phosphopentomutase) was previously shown in *L. lactis* to be involved in defence against various stress conditions, e.g. UV (Duwat et al. 1997), acid and thermal (Rallu et al. 2000). Moreover, disruption of *deoD*, potentially co-transcribed with *deoB*, confers increased heat tolerance in *S. thermophilus* (Varcamonti et al. 2003). A common feature of *deoB* and *deoD* mutations is the modulation of the guanine nucleotide pool involved in the biosynthesis of (p)ppGpp. This molecule, known to be involved in the stringent response, is proposed to play a role in other stress-signal transduction by Duwat et al. (1999) and Rallu et al. (2000). The *deoB* disruption could lead to an increased ppGpp level, as shown for a *S. thermophilus deoD* mutant (Varcamonti et al. 2003). Such a ppGpp increase would induce a constitutive pre-adapted state, which could explain stress resistance.

In *S. thermophilus* CNRZ368, disruption of *gst* led to an oxidative stress-resistant phenotype and transcription of this gene was down-regulated under oxidative stress. The involvement of glutamate in stress response has already been reported in *L. lactis* (Rallu et al. 2000). Moreover, in *S. mutans*, the transcription of genes involved in glutamate synthesis (aconitase hydratase, isocitrate dehydrogenase, citrate synthase) is repressed under acid and osmotic stresses (Chia et al. 2001). It is possible that glutamate acts as a stress sensor, a reduction of its concentration inducing stress resistance, maybe via the establishment of a stringent response. Furthermore, the gene downstream of *gst*, *orfX*, belongs to the RimK family whose *E. coli* member adds glutamate residues to maturing proteins. The *E. coli rimK* gene has already been linked to oxidative stress defence in different studies (Kang et al. 1989; Zheng et al. 2001), although its action in stress response is still not determined.

The *rggC* gene, encoding a Rgg-like transcriptional regulator, contains a frameshift mutation. The production of a full-length RggC protein could arise by a recoding event, requiring either a ribosome or RNA polymerase slippage to an alternative frame in a region composed of nine thymines, as observed in the *Thermus thermophilus dnaX* gene (Larsen et al. 2000). Thus, disruption of the $rggC_2$ ORF could abolish RggC full-protein production. The Rgg-like protein family consists of activators and/or repressors that are, so far, confined to Gram-positive bacteria (Chaussee et al. 2001; Rawlinson et al. 2002) and are involved in the regulation of various functions (Sulavik and Clewell 1996; Chaussee et al. 1999). However, this study involves for the first time a Rgg-like protein in oxidative stress response. RggC probably acts in regulating, and more probably in repressing, the expression of oxidative-stress defence genes.

The last five genes are of unknown function. An oxidative stress-resistant phenotype can now be linked to the disruption of these genes. Moreover, the recognition

**Table 3** Relative *osr* gene expression under $H_2O_2$ stress. The ΔΔCt method (Livak and Schmittgen 2001) was used to calculate gene expression changes relative to the non-stressed condition, normalised against *ldh*. For each assayed gene, a gene expression level of 100 was assigned for the non-stressed condition. Gene expression in stress conditions was given as a fraction of the expression level in the non-stressed condition. The amount of each cDNA was measured in two separate RT-PCR experiments with independent RNA samples

| Gene name | Relative gene expression | | |
|---|---|---|---|
| | 0 min | 20 min | 45 min |
| *deoB* | 100 | 13.6 ± 4.5 | 16.2 ± 3.3 |
| *gst* | 100 | 34.0 ± 17.3 | 19.8 ± 1.2 |
| *rggC* | 100 | 69.6 ± 22.4 | 59.1 ± 22.7 |
| *osrD* | 100 | 17.2 ± 8.9 | 13.8 ± 2.3 |
| *osrE* | 100 | 30.1 ± 17.6 | 6.4 ± 1.5 |
| *osrF* | 100 | 19.6 ± 12.8 | 13.6 ± 0.1 |
| *osrG* | 100 | 55.8 ± 44.0 | 58.8 ± 64.2 |
| *orfP* | 100 | 63.8 ± 10.8 | 35.8 ± 11.7 |

of specific domains allows the formulation of some hypotheses regarding their function and localisation. The OsrD protein sequence contains an iron-dependent peroxidase domain. The neighbouring gene encodes a predicted lipoprotein involved in iron transport necessary for the peroxidase activity. In the mutant disrupted in this locus, the inactivation of the putative peroxidase could result in an increased level of reactive oxygen species, which could lead to a constitutive pre-adapted state. Such a state would improve survival under oxidative stress conditions. The *osrD* phenotype could also be explained by a depletion of intracellular iron concentration, reducing the catalysis of the Fenton reaction (and consequently the reactive oxygen species production) and thus increasing cell survival.

Determination of the expression level of the studied genes under oxidative stress conditions indicated that most of them (*deoB*, *gst*, *osrD*, *osrE*, *osrF*, *orfP*) are down-regulated. The ratios in gene expression in the absence and presence of $H_2O_2$ range from 2 to 15. Small variation in gene expression seems to be a common feature in streptococci (Smoot et al. 2001; Voyich et al. 2003), suggesting a complex level of regulation in these organisms. Moreover, it would appear that *S. thermophilus* modulates gene expression in response to the cell oxidation state, probably thanks to proteins capable of sensing and responding to such signals. The *osr* genes could form part of an oxidative-stress response regulation, which regulator(s) might be a PerR homologue (King et al. 2000; Ricci et al. 2002), RggC or another unidentified regulator.

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