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**Identification of *Streptococcus thermophilus* CNRZ368 genes involved in  
defense against superoxide stress**

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## ABSTRACT

To better understand the defense mechanism of *Streptococcus thermophilus* against superoxide stress, molecular analysis of 10 menadione-sensitive mutants, obtained by insertional mutagenesis, was undertaken. This analysis allowed the identification of 10 genes that, with respect to their putative function, were classified in 5 categories (i) those involved in cell wall metabolism, (ii) those involved in exopolysaccharide translocation, (iii) those involved in RNA modification (iv) those involved in iron homeostasis and (v) those whose function is still unknown.

The behavior of the 10 menadione sensitive mutants exposed to heat shock was investigated. Data from these experiments allowed us to distinguish genes whose action might be specific to oxidative stress defense (*tgt*, *ossF*, *ossG*) from those whose action may be generalized to other stressful conditions (*mreD*, *rodA*, *pbp2b*, *cpsX*, *iscU*).

Among the mutants, two harboured an independently inserted copy of pGh9:ISS1 in two loci close to each other. More precisely, these two loci are homologous to *sufD* and *iscU* genes, involved in the biosynthesis of iron-sulfur clusters. This region, called the *suf* region, was further characterized in *S. thermophilus* CNRZ368 by sequencing and by construction of  $\Delta$ *sufD* and *iscU*<sub>97</sub> non polar mutants. Streptonigrin sensitivity level of both mutants suggested that these two genes were involved in iron metabolism.

## INTRODUCTION

Oxygen is an essential element for the viability of most living organisms through its involvement in oxidative phosphorylation that takes place during the aerobic respiration process. However, oxygen can also be harmful, due to the production, especially during respiration, of toxic derivatives namely ROS (reactive oxygen species) such as hydrogen peroxide, hydroxyl and superoxide radicals (for a review, (25)). Accumulation of these molecules, triggers lipid, protein and nucleic acid oxidation, causing lethal cellular damage (for reviews (7, 25, 53)). In response to such attack, aerobic micro-organisms have evolved anti-oxidant mechanisms (eg catalase, peroxidase and superoxide dismutase activities) to protect and adapt cells against ROS excess (6, 18, 24, 65, 68).

Although they do not achieve aerobic respiration, anaerobic micro-organisms can be subject to ROS. The strict anaerobes *Bacteroides fragilis* and *Clostridium perfringens* are able to survive in the presence of a low amount of oxygen, due to the induction of an adaptative oxidative stress response that is activated under aerobic conditions (13, 46-49). The peroxide response of *B. fragilis* shares some characteristics with that of *E. coli* such as the involvement of the redox-sensitive transcriptional activator OxyR (46) and some antioxidant activities under its control (47, 49).

The lactic acid bacterium (LAB) *Streptococcus thermophilus* is a Gram-positive bacterium widely used in dairy factories, mainly as a starter of fermentation in yogurt and cheese production. During fermentation processes, *S. thermophilus* is exposed to oxygen and to its deleterious effects that can lead to a reduction of cell viability and consequently may have repercussions on texture, flavor and safety of the final product.

*S. thermophilus* is an anaerobic aerotolerant organism that can grow in presence of oxygen and survive to low concentrations of superoxide and hydroxyl radicals (59). This suggests that it displays a defense system that provides protection against the effects of ROS. In the literature, data about its oxidative stress response are scarce. The existence of at least one

ROS defense system and its inducibility by hydrogen peroxide are established in *S. thermophilus* CNRZ368 (58). This organism possesses a single manganese-containing superoxide dismutase (19) and a glutathione reductase (43). However, no catalase activity was found. In other streptococci, antioxidant enzymes were reported to play a role in the defense against oxidative stress (27, 33, 44, 66) and in pathogenesis (45, 67, 69). The aim of this work was to identify genes involved in the oxidative stress defense of *S. thermophilus* CNRZ368 without presuming their function. For this purpose a collection of mutants was created by random insertional mutagenesis and the mutants were screened for their sensitivity to menadione (a superoxide generating agent). The locus disrupted in 10 *S. thermophilus* menadione sensitive clones was identified and the potential physiological role of the mutated genes in oxidative stress defense is discussed. The implication of these 10 loci during heat shock was also evaluated to assess their specificity to oxidative stress defense. Moreover, the characterization of two of these mutants, disrupted in *sufD* and *iscU* genes which may participate in Fe-S clusters assembly or repair, is more detailed. The results presented in this paper indicate that the inactivation of *sufD* and *iscU* genes caused a higher sensitivity to superoxide stress and suggest that Fe-S cluster repair or assembly could play an important role in the oxidative stress defense of anaerobic bacteria.

81

## 82 MATERIALS AND METHODS

83 **Bacterial strains, growth conditions.** *S. thermophilus* CNRZ368 was purchased from the  
84 INRA-CNRZ strain collection (Jouy en Josas, France). Depending on the experiments,  
85 *S. thermophilus* CNRZ368 and its derivatives were cultivated at 30°C or 42°C in milk  
86 medium (for pre-cultures), TPPY (12) or M17 (55) media, without shaking. Erythromycin was  
87 added at 2 µg.ml<sup>-1</sup>, when required. Streptonigrin sensitivity was monitored using a TPPYC  
88 medium which contained a reduced concentration of metallic dications (including Fe<sup>2+</sup>).  
89 TPPYC was performed according to the protocol described by Visca *et al.* (62) and modified as

90 follow: Chelex beads (20 g/l, sigma) were added to TPPY medium and stirred at 4°C for 48 h  
91 and the medium was then supplemented with 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>.  
92 Recombinant plasmids were transformed into *E. coli* EC101, a TG1 strain [*supE hsd-5 thi Δ(lac-*  
93 *proAB)* F' (*traD6 proAB<sup>+</sup> lacI<sup>f</sup> lacZΔM15*)] containing a chromosomal copy of the pWV01 *repA*  
94 gene (34) and selected at 37°C on Luria-Bertani (LB) (50) agar plates containing 150 µg/ml of  
95 erythromycin.

96

97 **Insertional mutagenesis.** The collection of mutants was made by insertional mutagenesis using the  
98 plasmid pGh9:ISSI that carries Em<sup>R</sup> gene (35). The mutagenesis procedure and the cloning of the  
99 fragments flanking the pGh9:ISSI insertion locus were carried out on *S. thermophilus* CNRZ368  
100 genome, as previously described (56).

101

102 **Selection of mutants impaired in their ability to tolerate menadione.** For selection of  
103 menadione sensitive clones, two different procedures, differing mainly by the length of the  
104 menadione-exposure, were assayed. Both assays were preceded by a pre-culture in milk medium  
105 containing 2 µg/ml erythromycin: pre-cultures of the 2112 mutants were performed in 96-well  
106 microtitre plates.

107 For “long” time menadione exposure, each pre-culture was transferred, using a hedgehog, onto  
108 M17 agar plates containing 2 µg/ml erythromycin and increasing concentrations of menadione  
109 (from 0 µg/ml to 120 µg/ml). After 20h incubation at 42°C, the presence (or the absence) of  
110 colonies was observed.

111 For “short” time menadione exposure, each pre-culture was diluted (with a 1/100 ratio) in 5  
112 different 96-well microtitre plates containing liquid TPPY medium supplemented with 2 µg/ml  
113 erythromycin (in final volume of 100µl, resulting in an approximately 1/1 liquid to air space  
114 ratio). After a 3h30 growth period at 42°C, 5 different concentrations of menadione (from  
115 0 mg/ml to 15 mg/ml) were added to each of the 5 microtitre plates. Microtitre plates were

incubated at 42°C for 3h. The cultures were then transferred on TPPY agar plates containing erythromycin (2 µg/ml) using a hedgehog. The ability of the mutants to survive to the menadione stress was assessed, after 20 h incubation at 42°C, by observation of the presence or the absence of colonies.

For each experiment, clones were considered as sensitive when they did not grow on a plate where more than 90% of the clones were growing. Both procedures were applied twice and the results had been compiled to reveal the mutants raising the more severe phenotype.

**DNA manipulations.** Preparation of chromosomal, plasmid and recombinant DNA and Southern analysis were performed according to standard protocols (50). All cloning junctions and cloned PCR products were checked by sequencing using Dye-Terminator chemistry on an ABI Prism 377 Genetic Analyser (PE Biosystems). Sequence data were analysed using Genbank, BLAST (2, 3), TMHMM (51) and HMMTOP (61) software. For all analysed sequences, The DNA sequences reported in this paper have been deposited in GenBank under accession numbers AY386239, AY386240, AY386241, AY386242, AY386243, AY386244, AY386245.

**Construction of the *ΔsufD* and *iscU<sub>97</sub>* mutants.** Oligonucleotides used to construct the *ΔsufD* and *iscU<sub>97</sub>* mutants were purchased from MWG Biotech AG (Ebersberg, Germany). To construct non-polar truncated *ΔsufD* mutant, the 5' and 3' ends of the *sufD* gene were independently amplified by PCR. The 5' end was amplified by using the primers GAGCGTGTT**GAATTCC**ACCG containing an *EcoRI* site, symbolized in bold characters and AACTTA**ACTGCAGC**ACCGTCT containing a *PstI* site. The 3' end was amplified by using the primers GGTGTTCCAG**GAATTCAA**ATAACC containing an *EcoRI* site and TGGTCTGTCATCAGGTCG. This last primer does not contain any *PstI* site, but the PCR product generated does contain a *PstI* restriction site. These two PCR products were digested

by *Pst*I and ligated with each other. The resulting fragment was amplified by PCR, then digested by *Eco*RI and cloned into pGh9. The final construct (pGh9 $\Delta$ *su*f*D*) was used to transform *S. thermophilus* CNRZ368. For IscU protein inactivation, the strategy consisted in replacing the TGT codon encoding the conserved Cys<sub>97</sub> by a TAT codon encoding a Tyr. This was achieved by replacing, in oligonucleotides, the 3 underlined nucleotides of the sequence (AGG TGT ACA) generating an *Eco*RV restriction site (AGA **TAT** CCA). Thus, the three amino-acids Gly<sub>96</sub> Cys<sub>97</sub> Tyr<sub>98</sub> were replaced by Arg<sub>96</sub> Tyr<sub>97</sub> Thr<sub>98</sub> in the mutated protein. Two internal and complementary primers containing the mutation (GCTGGTGATGATATCACCATTTCACA and GAAATGGTGATATCATCACCAGCAAAGG) were used with two additional external primers (AAGCGGAATTCAAGCATCCACTCAGGC containing an *Eco*RI site and TGTGACAAGCTTGTAGAGGCAATTCTT containing an *Hind*III site), respectively, to amplify 5' and 3' end of *iscU*. The two PCR products of 515 bp and 262 bp were digested by *Eco*RI and *Eco*RV and by *Hind*III and *Eco*RV respectively before being ligated to each other. The resulting fragment was cloned into pGh9 digested by *Eco*RI and *Hind*III. The final construct, (pGh9*iscU*<sub>97</sub>) was used to transform *S. thermophilus* CNRZ368. Preparation of *S. thermophilus* competent cells was performed as previously described by O'sullivan and Fitzgerald (41). The following gene replacements were done as previously described (8) but in TPPY medium and at 30°C/42°C as permissive and restrictive temperature, respectively.

## **Physiological characterization of *S. thermophilus* mutant strains.**

**Exposure to methyl viologen.** For “short” time exposure, cultures of CNRZ368 and its derivative strains were grown in TPPY medium. At an OD<sub>600</sub> of 0.6, cultures were divided into two subcultures (in a ratio of liquid to air space of approximately 1/1). Methyl viologen (paraquat; Sigma-Aldrich) was added to one of them with a final concentration of 50 mM. After 3 h incubation at 42°C, cultures were diluted to appropriate concentrations and spread onto



TPPY agar plates. For “long” time methyl viologen exposure, strains were grown in TPPY medium until OD<sub>600</sub> of 0.6. Cultures were then diluted to a final theoretical OD<sub>600</sub> of 0.001 in TPPY medium (in a ratio of liquid to air space of approximately 5/6) supplemented with 1.7 mM methyl viologen, or not for control experiment. After 18h incubation at 42°C, cultures were diluted in TPPY to appropriate concentrations and spread onto TPPY agar plates. In both procedures, methyl viologen sensitivity was estimated by comparing CFU/ml obtained with treated cultures, with respect to non treated cultures.

**Heat shock.** The ability of *S. thermophilus* and its derivatives to respond to a heat stress was assessed as follow. Each strain is grown at 42°C in TPPY medium until early log phase (OD<sub>600</sub> = 0.3). Then the culture was divided into two. One part was kept at 42°C (control experiment); the other one was immediately transferred to 60°C for 15 min. Appropriate dilutions were spread on TPPY agar plates. The fraction of surviving cells was calculated by dividing the surviving CFU/ml by the count for the control experiment.

**Streptonigrin sensitivity.** Streptonigrin sensitivity was monitored on cells grown overnight in TPPYC medium at 42°C. Cells were harvested by centrifugation, washed twice in TPPYC medium, resuspended in TPPYC, diluted to OD<sub>600</sub> = 0.01 and cultivated in TPPYC for 9 h at 42°C. Then, the cultures were diluted again to OD<sub>600</sub> = 0.005 and divided into 4 parts which were all incubated for 21 h at 42°C. One part was supplemented with 0.4% (v/v) dimethyl formamide (control experiment), the 3 other parts were supplemented with different concentrations of streptonigrin (1 µg/ml, 2 µg/ml and 4 µg/ml dissolved in dimethyl formamide). As a control experiment, cultures were also carried out in TPPYC supplemented with 1 mM of 2'2-dipyridyl (an Fe chelator). In each experiment, OD<sub>600</sub> value was used to estimate the cellular density after 21h incubation. Relative optic density was determined by

comparing cellular density obtained with and without streptonigrin. All experiments were performed in triplicate.

## RESULTS

### **1. Selection of *S. thermophilus* CNRZ368 mutants with altered oxidative stress response.**

A collection of 2112 mutants of *S. thermophilus* CNRZ368 was generated by insertional mutagenesis using the plasmid pGh9:ISSI (35, 56). This plasmid carries an erythromycin resistance gene for its selection and the insertional element ISSI that allows random integration of the plasmid into *S. thermophilus* genome. The 2112 mutants obtained were screened to select clones sensitive to menadione (a superoxide-generating compound). Two distinct selection procedures were used to identify mutants susceptible to menadione (see materials and methods). The characterization of 10 mutants, disrupted in loci originally named *ossA* to *ossJ* (for oxidative stress sensitive locus) is described in this paper.

**2. Sensitivity of *S. thermophilus* CNRZ368 mutants to methyl viologen.** Previously, we demonstrated that *ossB* and *ossC* mutants (previously named 6G4 and 18C3, respectively) are compromised in their ability to survive superoxide stress (57). To confirm that the other 8 mutants selected were sensitive to superoxide stress, their sensitivity to methyl viologen was examined (Fig. 1). In this experiment, we used the non-polar mutants *ΔsufD* and *iscU<sub>97</sub>* constructed in this work (see further) rather than the potential polar clones *ossI* and *ossJ*. Short-term methyl viologen exposure revealed the extreme sensitivity of *ossG*, as well as the 4 to 5-fold increased sensitivity of *ossD*, *ossH* and *iscU<sub>97</sub>* mutants (Fig. 1A). Although less severe the *ΔsufD* mutant exhibited about 2-fold less survival following such stress. Under these conditions, the other 3 mutants (*ossA*, *ossE* and *ossF*) did not exhibit any significant sensitivity, however long term methyl viologen exposure confirmed their sensitivity. Indeed, long term methyl viologen exposure (Fig. 1B) showed that the *ossA* mutant strain displayed a

relative survival more than 10-fold lower than that of the wild type strain. The *ossE* and *ossF* mutants also showed a 2-fold lower growth compared to the wild type strain.

**3. Survival of the mutants exposed to heat stress.** To assess the specificity of these 10 loci to oxidative stress defense, the mutants ability to survive to heat shock was estimated. Five out of the 10 mutants (*ossA*, *ossB*, *ossC*, *ossD*, and *ossJ*) isolated as oxidative stress sensitive clones also showed a reduced survival to heat shock compared with WT (Fig. 2). These data suggest that the genes impaired in these mutants are not specific to oxidative stress defense process but are probably involved in a more global response to stresses.

In contrast, the *ossE*, *ossF* and *ossG* mutants did not differ significantly from the wild type, indicating that the genes disrupted in these strains did not influence cells ability to respond to heat shock. Interestingly, the *ossG* mutant, that was highly sensitive to methyl viologen, showed no significant decrease of survival to heat shock compared with WT, suggesting that *ossG* gene, essential for oxidative stress defense, could be specific to such a stress.

**4. Identification of genes implicated in oxidative stress tolerance.** The disrupted locus was identified for each of the 10 mutants by cloning and sequencing the junctions as previously described (56). All of the insertions were mapped within coding sequences. Sequence similarity searches and analysis revealed similar sequences in other bacteria (see Table 1) with an E-value lower than  $10^{-10}$  for BLASTX searches. In 9/10 cases, data about the function of these genes were available. To facilitate their description, these genes were grouped into 5 classes (from I to V) according to the putative function and renamed when relevant (Table 1).

**Class I: Genes potentially involved in cell wall structure and cell shape determination.**

The *ossA*, *ossB* and *ossC* putative gene products shared sequence identity with MreD, RodA and Pbp2b, respectively, which putatively direct the elongation of the lateral cell wall in

*E. coli*, conferring the cell its characteristic rod shape (5, 28, 29). The *ossA* mutant was defective in a gene encoding a protein, with 30% identity with MreD of *Streptococcus mutans*. Sequence analysis of the surrounding region indicated that *S. thermophilus mreD* gene (*ossA*) was clustered with a gene encoding a putative protein similar to MreC. Moreover, hydrophopathy profiles of MreD (OssA) determined by using TMHMM and HMMTOP showed 5 regions of sufficient length and hydrophobicity to be membrane-spanning segments (data not shown), suggesting that this protein is the counterpart of MreD. The relatively low identity (30%) observed between *S. thermophilus* and *S. mutans* MreD was in agreement with the general idea that the structure of this protein is more highly conserved than its primary sequence (17). To study the effect of *mreD* disruption in *S. thermophilus*, TPPY cultures of the wild type and *mreD* mutant strains were observed under light microscopy. In contrast to wild type cells, *mreD* mutant cells formed very long chains, up to 180 cells per chain (Fig. 3). Moreover, a reduction of about 25% of the cell size was observed in the mutant. Growth of the *mreD* mutant and of the wild type in TPPY medium were monitored by measuring the OD<sub>600</sub>. Both strains displayed a similar doubling time of 30 min, but the maximal OD<sub>600</sub> of the *mreD* mutant (OD<sub>max</sub> = 1.7 ± 0.1) was lower than that of the wild type (OD<sub>max</sub> = 2.4 ± 0.2) showing that the *mreD* mutant entered stationary phase earlier than wild type cells. The characterization of *rodA* and *pbp2b* mutants of *S. thermophilus* CNRZ368 is described in a previous paper (57).

**Class II: Gene potentially involved in exopolysaccharide translocation.** The sequence disrupted in the mutant *ossD* was partially determined. Its analysis indicated that the C-terminal portion of the predicted encoded protein showed 98% identity to CpsX, a putative membrane protein from *S. thermophilus* MR-1C. The role of CpsX is still unknown but the conserved location of *cpsX* near *eps* genes of *S. thermophilus* (14) and the presence of a putative gene related to an ABC transport system in its vicinity argue in favor of CpsX being

involved in exopolysaccharide translocation (15). Moreover, the TMHMM and HMMTOP software predictions indicated that CpsX (OssD) from *S. thermophilus* CNRZ368 would contain highly hydrophobic domains compatible with a location in the cytoplasmic membrane. Although the *cpsX* function is not known yet, our work indicate that its disruption is associated with sensitivity not only to superoxide stress but also to heat shock.

**Class III: Genes involved in RNA maturation.** In the *ossE* and *ossF* mutants, the plasmid inserted into genes whose predicted products share sequence identity with enzymes involved in RNA maturation. The *ossE* gene encodes a putative tRNA guanine transglycosylase (TGT) which shares 92% identity with the putative *Streptococcus pneumoniae* TGT. TGT enzyme takes part in the process which removes the guanine residue at position 34 (wobble position of the anticodon loop) of tRNA<sup>His</sup>, tRNA<sup>Asp</sup>, tRNA<sup>Asn</sup> and tRNA<sup>Tyr</sup> and replaces it by the modified residue queuosine (39, 40).

*ossF* potentially encodes a protein that shows 77 % identity with a putative RNA methyltransferase of *Streptococcus agalactiae* (Gbs0448) belonging to the TrmA family. Enzymes of the TrmA family catalyze the formation of the 5-methyluridine in position 54 (localized in the TΨC loop) of tRNA (9). The presence of a TRAM-like domain (RNA binding domain) in the N-terminal part of OssF supports the idea of an interaction between this protein and RNA.

**Class IV: Gene of unknown function.** The *ossG* product shows 67% identity with the product of a gene of unknown function (*smu.1428c*) of *S. mutans* (Table 1). Although *ossG* function is not known yet, the mutation of this gene is now associated with a sensitivity phenotype to superoxide stress. The TMHMM and HMMTOP software predictions suggest the existence of two transmembrane domains in the N-terminal region of OssG, making of OssG a potential membrane protein.

297

298 **Class V: Genes involved in iron metabolism.**

299 **(i) Ferric iron transport.** The putative product of *ossH* shows 55% identity with FatD from  
300 *S. pneumoniae*, a potential membrane spanning permease of an Fe<sup>3+</sup> ABC-transporter. Analysis  
301 of the adjacent sequence reveals the presence of a gene encoding a putative FatC similar protein  
302 downstream of *fatD* (*ossH*), in the same orientation. The *fatC* gene is also clustered with *fatD* in  
303 *S. pneumoniae* genome. Hydropathy profile of FatD (OssH) from *S. thermophilus*, assessed with  
304 TMHMM and HMMTOP, reveals that this protein displays ten putative membrane spanning  
305 segments compatible with its expected cytoplasmic membrane location.

306

307 **(ii) [Fe-S] cluster biosynthesis.** The sequence affected in the *ossI* mutant encodes a product  
308 that shows 75% identity with Sag0142, a putative protein deduced from the complete genome  
309 sequence of *S. agalactiae* and similar to SufD-like proteins. Analysis of totally sequenced  
310 microbial genomes reveals that *sufD*-like genes are conserved in bacteria and also in some  
311 archaea such as *Halobacterium* sp. NRC-1, *Thermoplasma acidophilum* and *Aeropyrum*  
312 *pernix* (23). In *E. coli* and *Erwinia chrysanthemi*, the *sufD* gene belongs to the *sufABCDSE*  
313 cluster identified as being involved in iron metabolism and more precisely in [Fe-S] cluster  
314 biogenesis and/or repair (37, 42). The *ossJ* gene product shows sequence identity to the  
315 *gbs0140* product from *S. agalactiae* (Table 1), probably encoding a IscU protein, a NifU  
316 family protein. NifU proteins have been proposed to serve as scaffold proteins in [Fe-S]  
317 cluster assembly in *Synechocystis* PCC6803 and would function as a mediator for iron-sulfur  
318 cluster delivery (38).

319 In some genomes including that of *S. pyogenes*, *nifU*-like genes are clustered with the *suf*  
320 genes (23). To determine if *sufD* (*ossI*) and *iscU* (*ossJ*) from *S. thermophilus* were genetically  
321 linked together, the chromosomal DNA regions flanking the pGhost9:ISSI insertion site of  
322 both mutants were sequenced until overlapping. Examination of the genes in the

neighborhood of *sufD* and *iscU* revealed that they both belong to a cluster of 5 genes all orientated in the same direction. This cluster, named *sufCDS iscU sufB* cluster, is shown in Fig. 4. The highest sequence identities of these gene products are found with putative proteins from *S. agalactiae* (Table 2). They also share sequence similarity with proteins encoded by *E. coli* and *Er. chrysanthemi* *suf* gene clusters. The putative SufC, SufD, SufS and SufB proteins encoded by the *S. thermophilus* cluster present respectively 72%, 48%, 65%, and 57% similarity to their *E. coli* counterparts. Moreover, IscU presents 47% similarity with IscU from *E. coli*.

Sequences corresponding to promoter regions were searched by inspection of the total sequence of the *sufCDS iscU sufB* cluster. A unique extended -10 sequence (5'-TTTGGTATAAT-3') is found upstream *sufC*, the first ORF of the cluster. A potential rho-independent transcriptional terminator is identified 63 bp downstream *sufB*, the last ORF of the cluster ( $\Delta G = -10$  kcal/mol). These features, together with the short or missing intergenic regions and the same orientation of the genes, suggest that these 5 ORFs form an operon, as it is the case for the *Er. chrysanthemi* *suf* locus.

## **5. Molecular characterization of the *sufCDS iscU sufB* cluster.**

**5.1. Construction of  $\Delta$ *sufD* and *iscU*<sub>97</sub> mutants.** Since *S. thermophilus* *suf* genes are potentially clustered in an operon, non polar  $\Delta$ *sufD* and *iscU*<sub>97</sub> mutants were constructed and analysed. The *sufD* mutation consists of an internally in frame deletion leading to the loss of 43% of the whole protein (see materials and methods and Fig. 4). Sequence comparison of NifU family proteins from various organisms indicates that *S. thermophilus* IscU protein contains the three highly conserved cystein residues that have been shown to be the assembly site of a "transitory" [2Fe-2S] center within NifU (70). Thus, the construction of a null *iscU* mutant was achieved by replacing one of these conserved residues, the Cys<sub>97</sub>, by a Tyr (see

materials and methods and Fig. 4). Sequencing of these two constructs confirmed that they did not contain any secondary mutation.

## **5.2. Role of *sufD* and *iscU* in iron metabolism.**

To investigate the possibility that *ΔsufD* and *iscU*<sub>97</sub> mutations impair growth due to the lack of functional [Fe-S] cluster biosynthesis, the mutants were grown in TPPY medium and compared to wild type. Growth of the *ΔsufD* and *iscU*<sub>97</sub> mutants showed no significant difference compared to the WT strain. Assuming that the high iron concentration of the TPPY medium could allow self assembling of the [Fe-S] clusters (71), and consequently could be responsible for the absence of difference in growth between strains, the same experiment was conducted in TPPYC, a divalent cation depleted medium. Under these conditions, the mutants displayed a growth rate similar of that of the WT strain but their maximal OD<sub>600</sub> was lower than that of the wild type, showing their premature entry in stationary phase (Table 3). Moreover, this difference disappeared when the cultures were grown on TPPYC supplemented by 100 μM of FeSO<sub>4</sub>. Taken together, these results suggest that (i) *iscU* and *sufD* genes of *S. thermophilus* are involved in iron metabolism and (ii) their presence would be of particular importance under iron-limiting conditions.

To confirm the involvement of *sufD* and *iscU* in iron metabolism, the iron intracellular concentration of *ΔsufD* and *iscU*<sub>97</sub> was checked by measuring the sensitivity of these strains to streptonigrin (an iron-activated antibiotic). As shown in Fig. 5 A, the *iscU* mutant was more sensitive to streptonigrin than the wild type strain whereas the *ΔsufD* mutant was more resistant. Moreover, addition of 2,2'-dipyridyl (a ferrous iron chelator) abolished the difference of growth between the strains (Fig. 5 B) indicating that iron was responsible for the streptonigrin sensitivity observed without 2,2'-dipyridyl. Taken altogether these results suggest that both *iscU* and *sufD* of *S. thermophilus* play a role in iron metabolism.



## Discussion

To investigate the oxidative stress defense system of *S. thermophilus*, a collection of random insertion mutants was screened to select clones with high sensitivity to menadione. Using this approach, we aimed to identify genes involved in superoxide stress defense without presuming their function. It can be noted that no gene encoding a protein considered, in other organisms, as major antioxidant enzyme were found using this approach. This can be due to the fact that (i) the collection of mutants was non-exhaustive (ii) the corresponding mutant could have a weak phenotype (iii) such mutations could be lethal (iv) except from SOD, most anti-oxidant enzymes are protective against H<sub>2</sub>O<sub>2</sub> and not O<sub>2</sub><sup>-</sup>. The genes identified in the present work were classified into 5 groups according to their function: genes potentially involved in cell wall metabolism, exopolysaccharide translocation, tRNA modification, and iron metabolism and gene of unknown function.

Perhaps the most striking observation of this study is that 5 out of the 10 sensitive mutants selected are disrupted in a gene encoding an membrane protein (OssH, MreD, RodA, PBP2b and CpsX). This could reflect that the cell envelope is the main target of the stress applied. Among these mutants, three (*mreD*, *rodA* and *pbp2b*) are impaired in genes potentially encoding proteins described in *E. coli* as playing a role in peptidoglycan biosynthesis during cell elongation (28, 52, 63). Phenotypic analysis of these three *S. thermophilus* mutants showed that they all present a reduced cell size associated with a modification of the cell shape, as observed in *rodA*, *pbp2* and *mre* mutants of *E. coli* or *Salmonella typhimurium* (5, 20, 28, 29, 63, 64). Moreover, the 3 *S. thermophilus* mutants also presented an increased sensitivity to heat shock compared to the wild type strain suggesting that the protective role of MreD, RodA and PBP2b is not specific to oxidative stress but could be enlarged to a more global stress response. In support of this hypothesis is the existence in *Lactococcus lactis* of mutations in *pbp* genes that negatively affect acid tolerance of a *guaA* mutant (16).

The major function assigned to cell wall is the maintenance of cell integrity by protecting cells not only against osmotic stress but also against other stress conditions. For instance, in LAB, it has already been shown that compromised biosynthesis of peptidoglycan results in an increased sensitivity to either UV (22) or acid (11) stress. Additionally, disruption of *mreD*, *rodA* or *pbp2b* may cause a modification of the peptidoglycan architecture in *S. thermophilus*, reducing the cell protection against aggressions such as oxidative and heat stress.

Transfer RNA modification is a universal feature of all living organisms, although some described modifications have no assigned function yet. Modifying enzymes affect the tRNA precursor either by chemical modification of an existing residue (in the base or in the sugar) or by nucleotide substitution. These modifications, in particular those present in the anticodon region can affect translation efficiency and fidelity (for a review (10)). In this study, two clones disrupted in the loci *tgt* and *ossF*, encoding proteins showing high identity with tRNA modifying enzymes were selected for their high sensitivity to oxidative stress. This sensitivity could suggest that the expression of genes involved in oxidative stress defense may require tRNA maturation or, that such modifications could help tRNA stabilization under stress conditions. In agreement with the former hypothesis *Shigella flexneri* pathogenicity is regulated by *virF* mRNA translation efficiency under TGT activity control (21). Additionally, several environmental conditions are suggested to influence tRNA modification: in the case of queuosine (whose synthesis and substitution is carried out by several enzymes among which is TGT), synthesis is sensitive to the presence of iron (31) and TGT requires oxygen for its full activity (54). Therefore, further investigations on this topic should provide an interesting new regulatory model and could contribute towards the emerging observation that translational regulation plays an important role in oxidative stress response, a role mainly ascribed to transcriptional regulation thus far.

Iron is essential for cellular metabolism, due to its requirement as a cofactor for a large number of enzymes. However, an excess of iron is toxic because of its involvement in the Fenton reaction that enhances the formation of ROS. In this work, a gene homologous to *fatD*, encoding a putative Fe<sup>3+</sup> ABC-transporter was found to be involved in *S. thermophilus* oxidative stress response. To test whether or not *S. thermophilus* FatD is indeed an iron transporter, the sensitivity of the *fatD* mutant to streptonigrin (an iron activated-antibiotic) was assessed. No significant difference between the mutant and the wild type strains was detected (data not shown) suggesting that disruption of *fatD* would have no effect on intracellular iron concentration. Thus, either FatD is not a major iron transporter under the experimental procedure we used or *S. thermophilus fatD* encodes an ABC transporter for another metallic cation. A possible cation could be Mn<sup>2+</sup> known to have a protective function against oxidative damage either via direct dismutase activity, such as reported in other organisms (4, 26, 60), or via its role as a cofactor of the only SOD reported into *S. thermophilus* (19). Thus, inactivation of a Mn<sup>2+</sup> ABC transporter could trigger sensitivity to oxidative stress.

[Fe-S] clusters are oxygen sensitive prosthetic groups, incorporated into proteins via covalent bonds to cysteines. Iron-sulfur proteins, which are found in more than 120 classes of enzymes and proteins (30), are known to play important physiological roles in electron transfer, metabolic reactions and in transcriptional regulation (30, 32). Indeed, because of their intrinsic redox properties, they are likely to sense oxygen or its derivatives as it is the case in SoxR and FNR transcriptional regulators, responding to oxidative stress and oxygen availability, (for a review (32)). IscR is also a iron-sulfur transcriptional regulator that is suggested to respond to Fe-S cluster limiting conditions and potentially to hydrogen peroxide in *E. coli* (32). Although it has been shown that [Fe-S] cluster assembly can occur spontaneously *in vitro* under anaerobic conditions (36), this process can also be catalyzed enzymatically *in vivo*. Genes of the *suf* operon and of the *isc* cluster (iron sulfur cluster) are

conserved and encode [Fe-S] cluster biosynthesis and/or repair pathways (23). More precisely, it has been recently proposed that Isc proteins are necessary for biosynthesis, while Suf proteins are essentially involved in repair of oxidatively damaged [Fe-S] clusters (37). Study of *ΔsufD* and *iscU<sub>97</sub>* non-polar mutants indicates that SufD and IscU proteins are required for growth in an iron-deficient medium. SufD and IscU inactivation conferred an increased resistance and sensitivity respectively to streptonigrin. These results indicate that *S. thermophilus* SufD and IscU are involved in intracellular iron balance. Additionally, SufD and IscU of *S. thermophilus*, similar to SufD and NifU family proteins from other microorganisms, are encoded by genes clustered with *sufB*, *sufC* and *sufS* homologues, also potentially involved in [Fe-S] cluster repair. Hence the participation of *S. thermophilus* SufD and IscU proteins in iron intracellular balance is probably the result of their involvement in [Fe-S] cluster repair, as in *Er. chrysanthemi* (37). The NifU family protein function is to provide a scaffold for assembly of clusters mediated by NifS activity, and such clusters can then be transferred into apo forms of [Fe-S] cluster containing proteins (1, 70). The role of the SufD in [Fe-S] cluster biosynthesis is not well established. However, in *S. thermophilus*, the increased resistance to streptonigrin of the *ΔsufD* mutant suggests that its intracellular iron concentration is lower than in the wild type. At least two alternative hypotheses can be drawn: (i) SufD could be directly involved in the uptake or assimilation of extracellular iron, (ii) SufD could inhibit the synthesis and/or repair of the [Fe-S] clusters. In this latter case, SufD inactivation would result in an increase synthesis of [Fe-S] clusters, and consequently in a reduced intracellular free iron concentration. Additionally, *S. thermophilus* *sufD* and *iscU* mutants displayed an increased sensitivity to superoxide radicals compared to wild type. Thus, SufD and IscU are likely to play a major role in oxidative stress defense, probably resulting from their predicted role in [Fe-S] cluster assembly and/or repair. The involvement of the *suf* operon in oxidative stress resistance during the early steps of infection by *Er. chrysanthemi* has also been reported (37), as well as

the induction of the *suf* gene cluster of *E. coli* by the hydrogen peroxide response regulator OxyR (72). In spite of a possible spontaneous formation, our data suggest that under oxidative stress, [Fe-S] cluster assembly and/or repair becomes a limiting step in the formation of iron-sulfur proteins, and that the cell requires an additional, dedicated system, encoded by the *suf* gene cluster, to optimize [Fe-S] cluster formation or repair.

Conclusion: The strategy used in this work allowed the identification of genes involved in *S. thermophilus* oxidative stress defense system. Data concerning the function of some loci is already available in the literature, whereas the role of others is not known. In both cases, the exact roles of these genes in the defense of *S. thermophilus* against oxidative stress remains to be elucidated.

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## LEGENDS FIGURES

**FIG. 1. Relative methyl viologen sensitivity.** (A). Results of short term methyl viologen exposure. (B) Results of long term methyl viologen exposure (see materials and methods). The relative methyl viologen sensitivity was determined by comparing viability with and without treatment. Levels are shown as values relative to the survival level of the wild type strain. All experiments were performed at least in duplicate. Error bars show two standard deviations of the data.

**FIG. 2. Survival to heat shock.** The heat shock consisted of 15 min treatment at 60°C. The percentage of surviving cells corresponds to the number of colony forming units (CFUs) counted on plates after the heat-shock exposure divided by the number of CFU before treatment. Error bars show two standard deviations of the data. The dotted line represents the surviving percentage observed for the wild type strain and the grey band comprises 2 standard deviations of the data.

**FIG. 3. Influence of *mreD* disruption on chain length.** Cell number per chains was determined for 70 chains of the wild type strain (■) and of the *mreD* mutant (■) grown in liquid TPPY medium.

**FIG. 4. Schematic map of the *S. thermophilus* *sufCDS iscU sufB* cluster.** Genes are indicated by grey arrows, and the positions of putative start and stop codons are given (in bp) below the map. The site of insertion of the pGh9:ISSI plasmid in *sufD* (*ossI*) and *iscU* (*ossJ*) disrupted mutants is indicated by the solid black triangles. The broken arrow indicates the position of the *suf* cluster putative promoter. Putative rho-independent terminator is represented as hairpin loop. Fragments used for generation of the non-polar mutants are indicated as open boxes. The star indicates the position of the *EcoRV* restriction site causing the Cys<sub>97</sub> replacement by a Tyr in the *iscU*<sub>97</sub> mutant.

**FIG. 5. Streptonigrin tolerance.** *S. thermophilus* strain tolerance to streptonigrin was assessed by measuring OD<sub>600</sub> after growth in TPPYC medium containing various concentrations of streptonigrin without (A) or supplemented with 1 mM of 2,2'-dipyridyl (B) (see material and methods for details). Relative OD<sub>600nm</sub> was determined by comparing the OD<sub>600</sub> obtained with or without streptonigrin. Wild type strain (○ and ●); *iscU*<sub>97</sub> (□ and ■); *ΔsufD* (Δ and ▲).

TABLE 1. Products of *oss* genes: database similarities and putative fonctions.

Gene name <sup>a</sup>	Accession number	Gene encoding the most similar product <sup>c</sup>	amino acid identity <sup>d</sup>	Insertion site <sup>e</sup>	Putative function	Class
<i>ossA</i> ( <i>mreD</i> )	AY386242	<i>S. mutans mreD</i> (168)	30% (1-168)	127	Cell shape maintenance	I
<i>ossB</i> ( <i>rodA</i> )	AF399832	<i>S. mutans rodA</i> (408)	65% (1-391)	304	Peptidoglycan elongation	
<i>ossC</i> ( <i>pbp2b</i> ) <sup>b</sup>	AF399833	<i>S. thermophilus</i> Sfi6 <i>pbp2b</i> (704)	98% (641-704)	640	Peptidoglycan elongation	
<i>ossD</i> ( <i>cpsX</i> ) <sup>b</sup>	AY386241	<i>S. thermophilus cpsX</i> (378)	98% (212-378)	264	Membrane translocation of exopolysaccharide	II
<i>ossE</i> ( <i>tgt</i> ) <sup>b</sup>	AY386243	<i>S. pneumoniae tgt</i> (380)	92% (1-333)	99	tRNA-guanine transglycosylase	III
<i>ossF</i> <sup>b</sup>	AY386244	<i>S. agalactiae gbs0448</i> (451)	77% (1-204)	51	RNA methyltransferase	
<i>ossG</i>	AY386239	<i>S. mutans smu.1428c</i> (284)	67% (1-280)	170	Unknown conserved function	IV
<i>ossH</i> ( <i>fatD</i> )	AY386240	<i>S. pneumoniae fatD</i> (324)	55% (22-323)	119	Iron ABC transporter	V
<i>ossI</i> ( <i>sufD</i> )	AY386245	<i>S. agalactiae sag0142</i> (420)	76% (1-420)	144	Assembly of Fe/S clusters	
<i>ossJ</i> ( <i>iscU</i> )	AY386245	<i>S. agalactiae gbs0140</i> (147)	78% (1-141)	57	Assembly of Fe/S clusters	

a. Initial gene name with, in brackets and when applicable, the definitive gene name.

b. Disrupted ORF of *S. thermophilus* CNRZ368 that have been partially sequenced.

c. Organism, gene name and, in brackets, length of the protein sequence giving the best alignment score.

d. Percentage of sequence identity with the protein showing the best alignment score to each *S. thermophilus* Oss protein. In brackets is indicated the portion of the protein sharing this identity (numbers correspond to the amino acid positions).

e. The numbers correspond to the deduced amino acid position of the pGh9:ISS1-generated disruption in the full length protein showing the best alignment score to the Oss proteins.



TABLE 2. *suf* cluster gene products of *S. thermophilus* CNRZ368

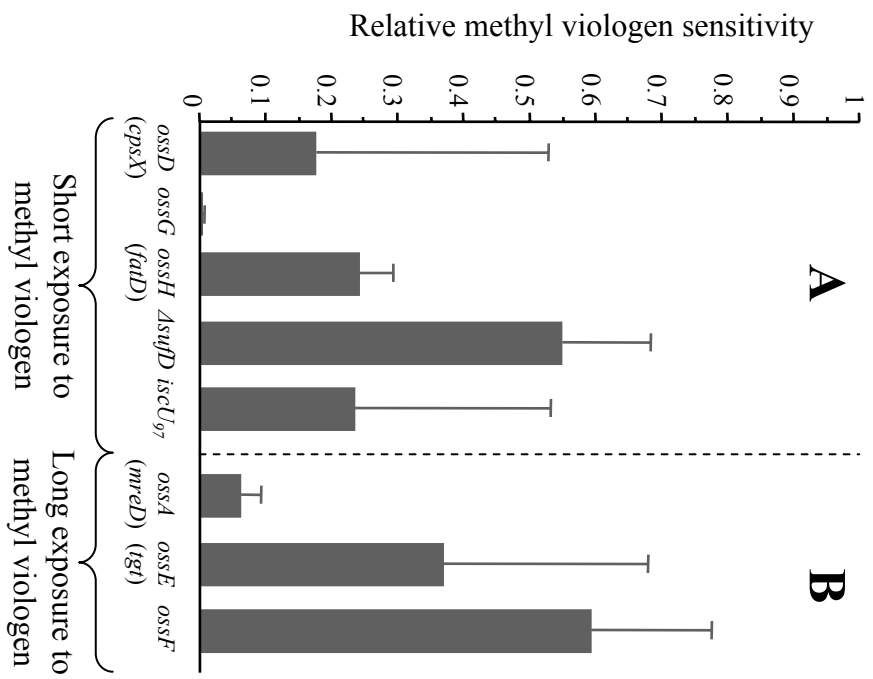
Gene product	Closest similar protein (organism, accession number) <sup>a</sup>	Identity/similarity(%)	Function
SufC	CAD45782 ( <i>S. agalactiae</i> NEM316)	88/94	Putative ABC transporter (ATP-binding protein)
SufD	AAM99050 ( <i>S. agalactiae</i> 2603V/R)	76/87	Unknown, conserved protein
SufS	CAD45784 ( <i>S. agalactiae</i> NEM316)	79/89	Similar to SufS
IscU	CAD45785 ( <i>S. agalactiae</i> NEM316)	78/90	Similar to NifU family proteins
SufB	CAD45786 ( <i>S. agalactiae</i> NEM316)	89/94	Unknown, conserved protein

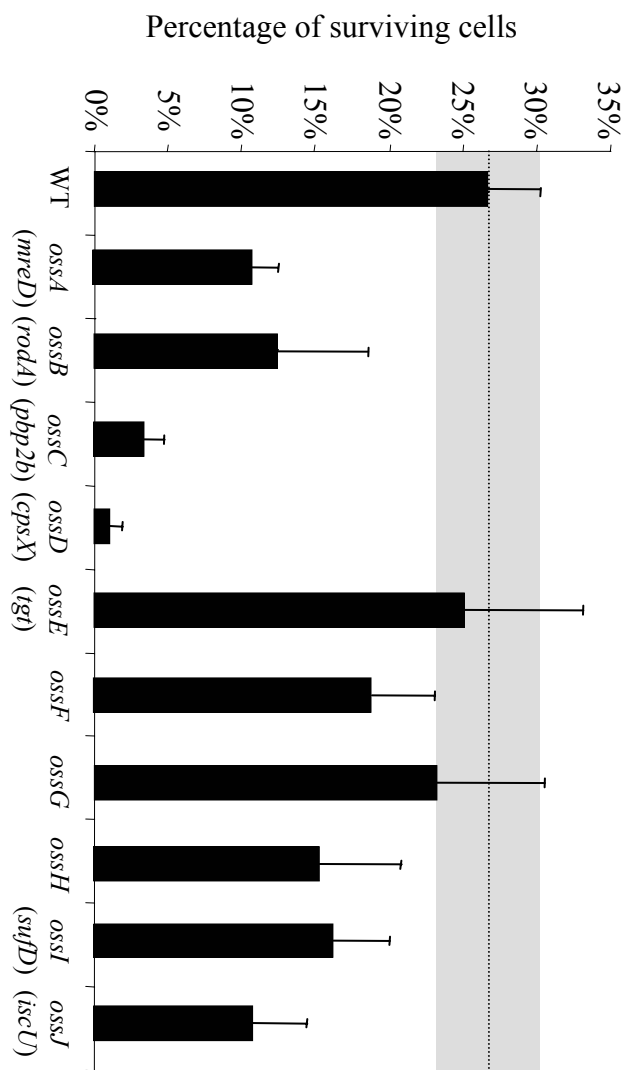
a. Accession number of the protein giving the best alignment score. In brackets, the name of the organism is indicated.

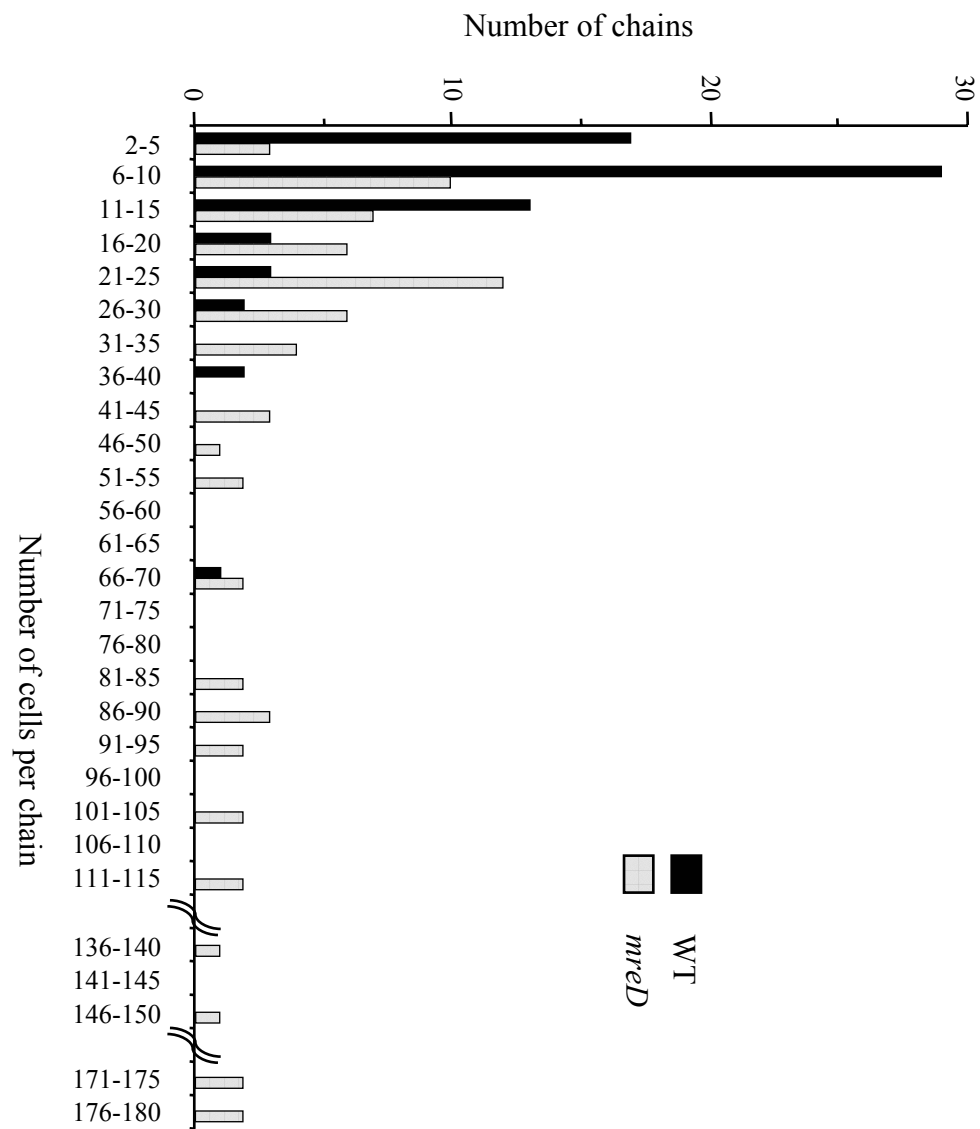
TABLE 3. Growth of wild type and mutant strains in TPPYC and in TPPYC supplemented with iron.

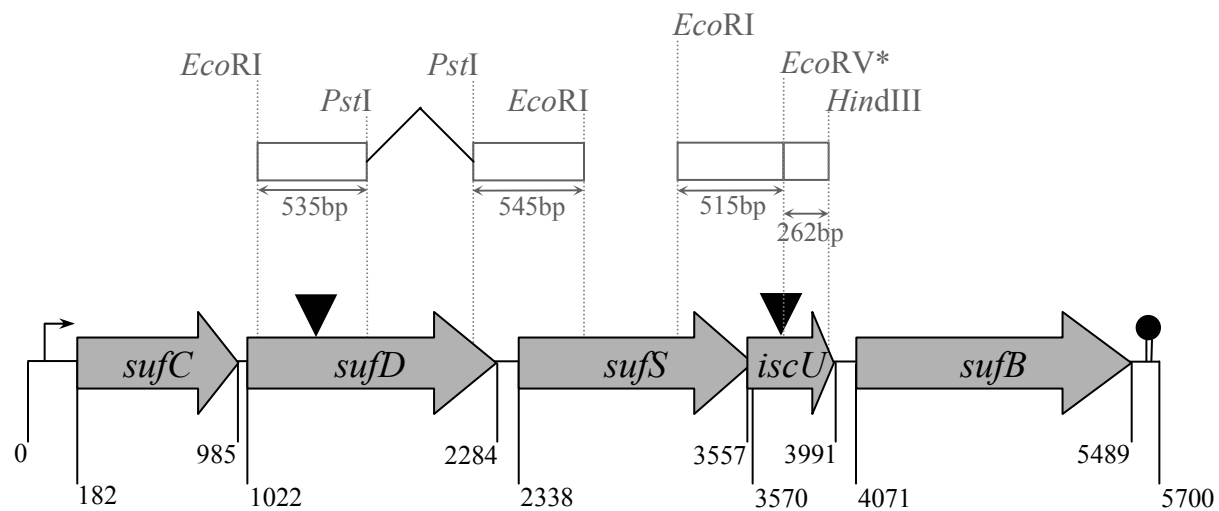
Strain	Final OD <sub>600</sub> resulting from growth <sup>a</sup> in:	
	TPPYC	TPPYC + FeSO <sub>4</sub>
WT	0.96	1.32
<i>ΔsufD</i>	0.58	1.48
<i>iscU<sub>97</sub></i>	0.58	1.15

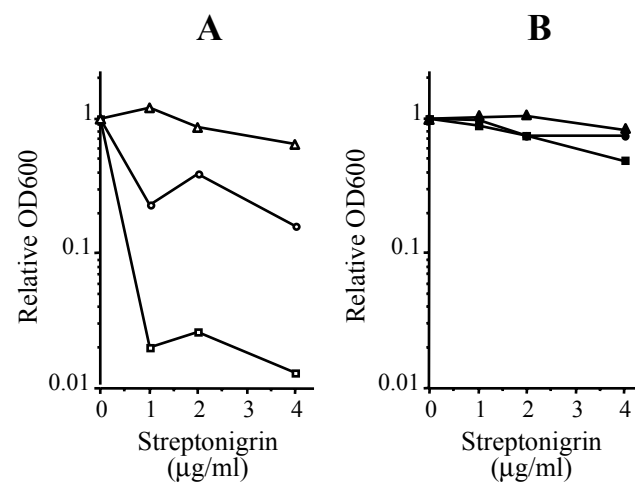
<sup>a</sup> Cells were grown in milk medium for 15°h at 42°C and diluted in FeSO<sub>4</sub>-deficient medium (TPPYC medium supplemented with MgCl<sub>2</sub> (1 mM) and CaCl<sub>2</sub> (1 mM)). After one night growth, cells were washed twice and resuspend either in FeSO<sub>4</sub>-deficient medium or supplemented with 100 μM FeSO<sub>4</sub>. The growth of the cells was then followed by measuring the OD<sub>600</sub>. The OD<sub>600</sub> were measured after 24h incubation at 42°C. This experiment was done in triplicate.











**Identification of *Streptococcus thermophilus* CNRZ368 genes involved in  
defense against superoxide stress**

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Key words: *Streptococcus thermophilus*, superoxide stress, insertional mutagenesis, iron-  
sulfur cluster, RNA maturation, cell wall

Running title: *S. thermophilus* oxidative stress defense genes

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## ABSTRACT

To better understand the defense mechanism of *Streptococcus thermophilus* against superoxide stress, molecular analysis of 10 menadione-sensitive mutants, obtained by insertional mutagenesis, was undertaken. This analysis allowed the identification of 10 genes that, with respect to their putative function, were classified in 5 categories (i) those involved in cell wall metabolism, (ii) those involved in exopolysaccharide translocation, (iii) those involved in RNA modification (iv) those involved in iron homeostasis and (v) those whose function is still unknown.

The behavior of the 10 menadione sensitive mutants exposed to heat shock was investigated. Data from these experiments allowed us to distinguish genes whose action might be specific to oxidative stress defense (*tgt*, *ossF*, *ossG*) from those whose action may be generalized to other stressful conditions (*mreD*, *rodA*, *pbp2b*, *cpsX*, *iscU*).

Among the mutants, two harboured an independently inserted copy of pGh9:ISS1 in two loci close to each other. More precisely, these two loci are homologous to *sufD* and *iscU* genes, involved in the biosynthesis of iron-sulfur clusters. This region, called the *suf* region, was further characterized in *S. thermophilus* CNRZ368 by sequencing and by construction of  $\Delta$ *sufD* and *iscU*<sub>97</sub> non polar mutants. Streptonigrin sensitivity level of both mutants suggested that these two genes were involved in iron metabolism.

## INTRODUCTION

Oxygen is an essential element for the viability of most living organisms through its involvement in oxidative phosphorylation that takes place during the aerobic respiration process. However, oxygen can also be harmful, due to the production, especially during respiration, of toxic derivatives namely ROS (reactive oxygen species) such as hydrogen peroxide, hydroxyl and superoxide radicals (for a review, (25)). Accumulation of these molecules, triggers lipid, protein and nucleic acid oxidation, causing lethal cellular damage (for reviews (7, 25, 53)). In response to such attack, aerobic micro-organisms have evolved anti-oxidant mechanisms (eg catalase, peroxidase and superoxide dismutase activities) to protect and adapt cells against ROS excess (6, 18, 24, 65, 68).

Although they do not achieve aerobic respiration, anaerobic micro-organisms can be subject to ROS. The strict anaerobes *Bacteroides fragilis* and *Clostridium perfringens* are able to survive in the presence of a low amount of oxygen, due to the induction of an adaptative oxidative stress response that is activated under aerobic conditions (13, 46-49). The peroxide response of *B. fragilis* shares some characteristics with that of *E. coli* such as the involvement of the redox-sensitive transcriptional activator OxyR (46) and some antioxidant activities under its control (47, 49).

The lactic acid bacterium (LAB) *Streptococcus thermophilus* is a Gram-positive bacterium widely used in dairy factories, mainly as a starter of fermentation in yogurt and cheese production. During fermentation processes, *S. thermophilus* is exposed to oxygen and to its deleterious effects that can lead to a reduction of cell viability and consequently may have repercussions on texture, flavor and safety of the final product.

*S. thermophilus* is an anaerobic aerotolerant organism that can grow in presence of oxygen and survive to low concentrations of superoxide and hydroxyl radicals (59). This suggests that it displays a defense system that provides protection against the effects of ROS. In the literature, data about its oxidative stress response are scarce. The existence of at least one

ROS defense system and its inducibility by hydrogen peroxide are established in *S. thermophilus* CNRZ368 (58). This organism possesses a single manganese-containing superoxide dismutase (19) and a glutathione reductase (43). However, no catalase activity was found. In other streptococci, antioxidant enzymes were reported to play a role in the defense against oxidative stress (27, 33, 44, 66) and in pathogenesis (45, 67, 69). The aim of this work was to identify genes involved in the oxidative stress defense of *S. thermophilus* CNRZ368 without presuming their function. For this purpose a collection of mutants was created by random insertional mutagenesis and the mutants were screened for their sensitivity to menadione (a superoxide generating agent). The locus disrupted in 10 *S. thermophilus* menadione sensitive clones was identified and the potential physiological role of the mutated genes in oxidative stress defense is discussed. The implication of these 10 loci during heat shock was also evaluated to assess their specificity to oxidative stress defense. Moreover, the characterization of two of these mutants, disrupted in *sufD* and *iscU* genes which may participate in Fe-S clusters assembly or repair, is more detailed. The results presented in this paper indicate that the inactivation of *sufD* and *iscU* genes caused a higher sensitivity to superoxide stress and suggest that Fe-S cluster repair or assembly could play an important role in the oxidative stress defense of anaerobic bacteria.

81

## 82 MATERIALS AND METHODS

83 **Bacterial strains, growth conditions.** *S. thermophilus* CNRZ368 was purchased from the  
84 INRA-CNRZ strain collection (Jouy en Josas, France). Depending on the experiments,  
85 *S. thermophilus* CNRZ368 and its derivatives were cultivated at 30°C or 42°C in milk  
86 medium (for pre-cultures), TPPY (12) or M17 (55) media, without shaking. Erythromycin was  
87 added at 2 µg.ml<sup>-1</sup>, when required. Streptonigrin sensitivity was monitored using a TPPYC  
88 medium which contained a reduced concentration of metallic dications (including Fe<sup>2+</sup>).  
89 TPPYC was performed according to the protocol described by Visca *et al.* (62) and modified as

90 follow: Chelex beads (20 g/l, sigma) were added to TPPY medium and stirred at 4°C for 48 h  
91 and the medium was then supplemented with 1 mM MgCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>.  
92 Recombinant plasmids were transformed into *E. coli* EC101, a TG1 strain [*supE hsd-5 thi Δ(lac-*  
93 *proAB)* F' (*traD6 proAB<sup>+</sup> lacI<sup>f</sup> lacZΔM15*)] containing a chromosomal copy of the pWV01 *repA*  
94 gene (34) and selected at 37°C on Luria-Bertani (LB) (50) agar plates containing 150 µg/ml of  
95 erythromycin.

96

97 **Insertional mutagenesis.** The collection of mutants was made by insertional mutagenesis using the  
98 plasmid pGh9:ISSI that carries Em<sup>R</sup> gene (35). The mutagenesis procedure and the cloning of the  
99 fragments flanking the pGh9:ISSI insertion locus were carried out on *S. thermophilus* CNRZ368  
100 genome, as previously described (56).

101

102 **Selection of mutants impaired in their ability to tolerate menadione.** For selection of  
103 menadione sensitive clones, two different procedures, differing mainly by the length of the  
104 menadione-exposure, were assayed. Both assays were preceded by a pre-culture in milk medium  
105 containing 2 µg/ml erythromycin: pre-cultures of the 2112 mutants were performed in 96-well  
106 microtitre plates.

107 For “long” time menadione exposure, each pre-culture was transferred, using a hedgehog, onto  
108 M17 agar plates containing 2 µg/ml erythromycin and increasing concentrations of menadione  
109 (from 0 µg/ml to 120 µg/ml). After 20h incubation at 42°C, the presence (or the absence) of  
110 colonies was observed.

111 For “short” time menadione exposure, each pre-culture was diluted (with a 1/100 ratio) in 5  
112 different 96-well microtitre plates containing liquid TPPY medium supplemented with 2 µg/ml  
113 erythromycin (in final volume of 100µl, resulting in an approximately 1/1 liquid to air space  
114 ratio). After a 3h30 growth period at 42°C, 5 different concentrations of menadione (from  
115 0 mg/ml to 15 mg/ml) were added to each of the 5 microtitre plates. Microtitre plates were

incubated at 42°C for 3h. The cultures were then transferred on TPPY agar plates containing erythromycin (2 µg/ml) using a hedgehog. The ability of the mutants to survive to the menadione stress was assessed, after 20 h incubation at 42°C, by observation of the presence or the absence of colonies.

For each experiment, clones were considered as sensitive when they did not grow on a plate where more than 90% of the clones were growing. Both procedures were applied twice and the results had been compiled to reveal the mutants raising the more severe phenotype.

**DNA manipulations.** Preparation of chromosomal, plasmid and recombinant DNA and Southern analysis were performed according to standard protocols (50). All cloning junctions and cloned PCR products were checked by sequencing using Dye-Terminator chemistry on an ABI Prism 377 Genetic Analyser (PE Biosystems). Sequence data were analysed using Genbank, BLAST (2, 3), TMHMM (51) and HMMTOP (61) software. For all analysed sequences, The DNA sequences reported in this paper have been deposited in GenBank under accession numbers AY386239, AY386240, AY386241, AY386242, AY386243, AY386244, AY386245.

**Construction of the *ΔsufD* and *iscU<sub>97</sub>* mutants.** Oligonucleotides used to construct the *ΔsufD* and *iscU<sub>97</sub>* mutants were purchased from MWG Biotech AG (Ebersberg, Germany). To construct non-polar truncated *ΔsufD* mutant, the 5' and 3' ends of the *sufD* gene were independently amplified by PCR. The 5' end was amplified by using the primers GAGCGTGTTGAATTCCACCG containing an *EcoRI* site, symbolized in bold characters and AACTTAACTGCAGCACCGTCT containing a *PstI* site. The 3' end was amplified by using the primers GGTGTTCCAGAATTCAAATAACC containing an *EcoRI* site and TGGTCTGTCATCAGGTCG. This last primer does not contain any *PstI* site, but the PCR product generated does contain a *PstI* restriction site. These two PCR products were digested

by *Pst*I and ligated with each other. The resulting fragment was amplified by PCR, then digested by *Eco*RI and cloned into pGh9. The final construct (pGh9 $\Delta$ *su**fD*) was used to transform *S. thermophilus* CNRZ368. For IscU protein inactivation, the strategy consisted in replacing the TGT codon encoding the conserved Cys<sub>97</sub> by a TAT codon encoding a Tyr. This was achieved by replacing, in oligonucleotides, the 3 **underlined** nucleotides of the sequence (**AGG TGT ACA**) generating an *Eco*RV restriction site (**AGA TAT CCA**). Thus, the three amino-acids Gly<sub>96</sub> Cys<sub>97</sub> Tyr<sub>98</sub> were replaced by Arg<sub>96</sub> Tyr<sub>97</sub> Thr<sub>98</sub> in the mutated protein. Two internal and complementary primers containing the mutation (**GCTGGTGATGATATCACCATTTCACAA** and **GAAATGGTGATATCATCACCAGCAAAGG**) were used with two additional external primers (**AAGCGGAATTCAAGCATCCACTCAGGC** containing an *Eco*RI site and **TGTGACAAGCTTGTAGAGGCAATTCTT** containing an *Hind*III site), respectively, to amplify 5' and 3' end of *iscU*. The two PCR products of 515 bp and 262 bp were digested by *Eco*RI and *Eco*RV and by *Hind*III and *Eco*RV respectively before being ligated to each other. The resulting fragment was cloned into pGh9 digested by *Eco*RI and *Hind*III. The final construct, (pGh9*iscU*<sub>97</sub>) was used to transform *S. thermophilus* CNRZ368. Preparation of *S. thermophilus* competent cells was performed as previously described by O'sullivan and Fitzgerald (41). The following gene replacements were done as previously described (8) but in TPPY medium and at 30°C/42°C as permissive and restrictive temperature, respectively.

#### **Physiological characterization of *S. thermophilus* mutant strains.**

**Exposure to methyl viologen.** For “short” time exposure, cultures of CNRZ368 and its derivative strains were grown in TPPY medium. At an OD<sub>600</sub> of 0.6, cultures were divided into two subcultures (in a ratio of liquid to air space of approximately 1/1). Methyl viologen (paraquat; Sigma-Aldrich) was added to one of them with a final concentration of 50 mM. After 3 h incubation at 42°C, cultures were diluted to appropriate concentrations and spread onto

TPPY agar plates. For “long” time methyl viologen exposure, strains were grown in TPPY medium until OD<sub>600</sub> of 0.6. Cultures were then diluted to a final theoretical OD<sub>600</sub> of 0.001 in TPPY medium (in a ratio of liquid to air space of approximately 5/6) supplemented with 1.7 mM methyl viologen, or not for control experiment. After 18h incubation at 42°C, cultures were diluted in TPPY to appropriate concentrations and spread onto TPPY agar plates. In both procedures, methyl viologen sensitivity was estimated by comparing CFU/ml obtained with treated cultures, with respect to non treated cultures.

**Heat shock.** The ability of *S. thermophilus* and its derivatives to respond to a heat stress was assessed as follow. Each strain is grown at 42°C in TPPY medium until early log phase (OD<sub>600</sub> = 0.3). Then the culture was divided into two. One part was kept at 42°C (control experiment); the other one was immediately transferred to 60°C for 15 min. Appropriate dilutions were spread on TPPY agar plates. The fraction of surviving cells was calculated by dividing the surviving CFU/ml by the count for the control experiment.

**Streptonigrin sensitivity.** Streptonigrin sensitivity was monitored on cells grown overnight in TPPYC medium at 42°C. Cells were harvested by centrifugation, washed twice in TPPYC medium, resuspended in TPPYC, diluted to OD<sub>600</sub> = 0.01 and cultivated in TPPYC for 9 h at 42°C. Then, the cultures were diluted again to OD<sub>600</sub> = 0.005 and divided into 4 parts which were all incubated for 21 h at 42°C. One part was supplemented with 0.4% (v/v) dimethyl formamide (control experiment), the 3 other parts were supplemented with different concentrations of streptonigrin (1 µg/ml, 2 µg/ml and 4 µg/ml dissolved in dimethyl formamide). As a control experiment, cultures were also carried out in TPPYC supplemented with 1 mM of 2’2-dipyridyl (an Fe chelator). In each experiment, OD<sub>600</sub> value was used to estimate the cellular density after 21h incubation. Relative optic density was determined by

comparing cellular density obtained with and without streptonigrin. All experiments were performed in triplicate.

## RESULTS

### **1. Selection of *S. thermophilus* CNRZ368 mutants with altered oxidative stress response.**

A collection of 2112 mutants of *S. thermophilus* CNRZ368 was generated by insertional mutagenesis using the plasmid pGh9:ISSI (35, 56). This plasmid carries an erythromycin resistance gene for its selection and the insertional element ISSI that allows random integration of the plasmid into *S. thermophilus* genome. The 2112 mutants obtained were screened to select clones sensitive to menadione (a superoxide-generating compound). Two distinct selection procedures were used to identify mutants susceptible to menadione (see materials and methods). The characterization of 10 mutants, disrupted in loci originally named *ossA* to *ossJ* (for oxidative stress sensitive locus) is described in this paper.

**2. Sensitivity of *S. thermophilus* CNRZ368 mutants to methyl viologen.** Previously, we demonstrated that *ossB* and *ossC* mutants (previously named 6G4 and 18C3, respectively) are compromised in their ability to survive superoxide stress (57). To confirm that the other 8 mutants selected were sensitive to superoxide stress, their sensitivity to methyl viologen was examined (Fig. 1). In this experiment, we used the non-polar mutants *ΔsufD* and *iscU<sub>97</sub>* constructed in this work (see further) rather than the potential polar clones *ossI* and *ossJ*. Short-term methyl viologen exposure revealed the extreme sensitivity of *ossG*, as well as the 4 to 5-fold increased sensitivity of *ossD*, *ossH* and *iscU<sub>97</sub>* mutants (Fig. 1A). Although less severe the *ΔsufD* mutant exhibited about 2-fold less survival following such stress. Under these conditions, the other 3 mutants (*ossA*, *ossE* and *ossF*) did not exhibit any significant sensitivity, however long term methyl viologen exposure confirmed their sensitivity. Indeed, long term methyl viologen exposure (Fig. 1B) showed that the *ossA* mutant strain displayed a



relative survival more than 10-fold lower than that of the wild type strain. The *ossE* and *ossF* mutants also showed a 2-fold lower growth compared to the wild type strain.

**3. Survival of the mutants exposed to heat stress.** To assess the specificity of these 10 loci to oxidative stress defense, the mutants ability to survive to heat shock was estimated. Five out of the 10 mutants (*ossA*, *ossB*, *ossC*, *ossD*, and *ossJ*) isolated as oxidative stress sensitive clones also showed a reduced survival to heat shock compared with WT (Fig. 2). These data suggest that the genes impaired in these mutants are not specific to oxidative stress defense process but are probably involved in a more global response to stresses.

In contrast, the *ossE*, *ossF* and *ossG* mutants did not differ significantly from the wild type, indicating that the genes disrupted in these strains did not influence cells ability to respond to heat shock. Interestingly, the *ossG* mutant, that was highly sensitive to methyl viologen, showed no significant decrease of survival to heat shock compared with WT, suggesting that *ossG* gene, essential for oxidative stress defense, could be specific to such a stress.

**4. Identification of genes implicated in oxidative stress tolerance.** The disrupted locus was identified for each of the 10 mutants by cloning and sequencing the junctions as previously described (56). All of the insertions were mapped within coding sequences. Sequence similarity searches and analysis revealed similar sequences in other bacteria (see Table 1) with an E-value lower than  $10^{-10}$  for BLASTX searches. In 9/10 cases, data about the function of these genes were available. To facilitate their description, these genes were grouped into 5 classes (from I to V) according to the putative function and renamed when relevant (Table 1).

**Class I: Genes potentially involved in cell wall structure and cell shape determination.**

The *ossA*, *ossB* and *ossC* putative gene products shared sequence identity with MreD, RodA and Pbp2b, respectively, which putatively direct the elongation of the lateral cell wall in

*E. coli*, conferring the cell its characteristic rod shape (5, 28, 29). The *ossA* mutant was defective in a gene encoding a protein, with 30% identity with MreD of *Streptococcus mutans*. Sequence analysis of the surrounding region indicated that *S. thermophilus mreD* gene (*ossA*) was clustered with a gene encoding a putative protein similar to MreC. Moreover, hydrophopathy profiles of MreD (OssA) determined by using TMHMM and HMMTOP showed 5 regions of sufficient length and hydrophobicity to be membrane-spanning segments (data not shown), suggesting that this protein is the counterpart of MreD. The relatively low identity (30%) observed between *S. thermophilus* and *S. mutans* MreD was in agreement with the general idea that the structure of this protein is more highly conserved than its primary sequence (17). To study the effect of *mreD* disruption in *S. thermophilus*, TPPY cultures of the wild type and *mreD* mutant strains were observed under light microscopy. In contrast to wild type cells, *mreD* mutant cells formed very long chains, up to 180 cells per chain (Fig. 3). Moreover, a reduction of about 25% of the cell size was observed in the mutant. Growth of the *mreD* mutant and of the wild type in TPPY medium were monitored by measuring the OD<sub>600</sub>. Both strains displayed a similar doubling time of 30 min, but the maximal OD<sub>600</sub> of the *mreD* mutant (OD<sub>max</sub> = 1.7 ± 0.1) was lower than that of the wild type (OD<sub>max</sub> = 2.4 ± 0.2) showing that the *mreD* mutant entered stationary phase earlier than wild type cells. The characterization of *rodA* and *pbp2b* mutants of *S. thermophilus* CNRZ368 is described in a previous paper (57).

**Class II: Gene potentially involved in exopolysaccharide translocation.** The sequence disrupted in the mutant *ossD* was partially determined. Its analysis indicated that the C-terminal portion of the predicted encoded protein showed 98% identity to CpsX, a putative membrane protein from *S. thermophilus* MR-1C. The role of CpsX is still unknown but the conserved location of *cpsX* near *eps* genes of *S. thermophilus* (14) and the presence of a putative gene related to an ABC transport system in its vicinity argue in favor of CpsX being

involved in exopolysaccharide translocation (15). Moreover, the TMHMM and HMMTOP software predictions indicated that CpsX (OssD) from *S. thermophilus* CNRZ368 would contain highly hydrophobic domains compatible with a location in the cytoplasmic membrane. Although the *cpsX* function is not known yet, our work indicate that its disruption is associated with sensitivity not only to superoxide stress but also to heat shock.

**Class III: Genes involved in RNA maturation.** In the *ossE* and *ossF* mutants, the plasmid inserted into genes whose predicted products share sequence identity with enzymes involved in RNA maturation. The *ossE* gene encodes a putative tRNA guanine transglycosylase (TGT) which shares 92% identity with the putative *Streptococcus pneumoniae* TGT. TGT enzyme takes part in the process which removes the guanine residue at position 34 (wobble position of the anticodon loop) of tRNA<sup>His</sup>, tRNA<sup>Asp</sup>, tRNA<sup>Asn</sup> and tRNA<sup>Tyr</sup> and replaces it by the modified residue queuosine (39, 40).

*ossF* potentially encodes a protein that shows 77 % identity with a putative RNA methyltransferase of *Streptococcus agalactiae* (Gbs0448) belonging to the TrmA family. Enzymes of the TrmA family catalyze the formation of the 5-methyluridine in position 54 (localized in the TΨC loop) of tRNA (9). The presence of a TRAM-like domain (RNA binding domain) in the N-terminal part of OssF supports the idea of an interaction between this protein and RNA.

**Class IV: Gene of unknown function.** The *ossG* product shows 67% identity with the product of a gene of unknown function (*smu.1428c*) of *S. mutans* (Table 1). Although *ossG* function is not known yet, the mutation of this gene is now associated with a sensitivity phenotype to superoxide stress. The TMHMM and HMMTOP software predictions suggest the existence of two transmembrane domains in the N-terminal region of OssG, making of OssG a potential membrane protein.

297

298 **Class V: Genes involved in iron metabolism.**

299 **(i) Ferric iron transport.** The putative product of *ossH* shows 55% identity with FatD from  
300 *S. pneumoniae*, a potential membrane spanning permease of an Fe<sup>3+</sup> ABC-transporter. Analysis  
301 of the adjacent sequence reveals the presence of a **gene encoding a putative FatC similar protein**  
302 downstream of *fatD* (*ossH*), in the same orientation. The *fatC* gene is also clustered with *fatD* in  
303 *S. pneumoniae* genome. Hydropathy profile of FatD (OssH) from *S. thermophilus*, assessed with  
304 TMHMM and HMMTOP, reveals that this protein displays ten putative membrane spanning  
305 segments compatible with its expected cytoplasmic membrane location.

306

307 **(ii) [Fe-S] cluster biosynthesis.** The sequence affected in the *ossI* mutant encodes a product  
308 that shows 75% identity with Sag0142, a putative protein deduced from the complete genome  
309 sequence of *S. agalactiae* and **similar** to SufD-like proteins. Analysis of totally sequenced  
310 microbial genomes reveals that *sufD*-like genes are conserved in bacteria and also in some  
311 archaea such as *Halobacterium* sp. NRC-1, *Thermoplasma acidophilum* and *Aeropyrum*  
312 *pernix* (23). In *E. coli* and *Erwinia chrysanthemi*, the *sufD* gene belongs to the *sufABCDSE*  
313 cluster identified as being involved in iron metabolism and more precisely in [Fe-S] cluster  
314 biogenesis and/or repair (37, 42). The *ossJ* **gene product shows sequence identity to the**  
315 ***gbs0140* product** from *S. agalactiae* (Table 1), probably encoding a IscU protein, a NifU  
316 family protein. NifU proteins have been proposed to serve as scaffold proteins in [Fe-S]  
317 cluster assembly in *Synechocystis* PCC6803 and would function as a mediator for iron-sulfur  
318 cluster delivery (38).

319 In some genomes including that of *S. pyogenes*, *nifU*-like genes are clustered with the *suf*  
320 genes (23). To determine if *sufD* (*ossI*) and *iscU* (*ossJ*) from *S. thermophilus* were genetically  
321 linked together, the chromosomal DNA regions flanking the pGhost9:ISSI insertion site of  
322 both mutants were sequenced until overlapping. Examination of the genes in the

neighborhood of *sufD* and *iscU* revealed that they both belong to a cluster of 5 genes all orientated in the same direction. This cluster, named *sufCDS iscU sufB* cluster, is shown in Fig. 4. The highest **sequence identities** of these gene products are found with putative proteins from *S. agalactiae* (Table 2). They also share sequence similarity with proteins encoded by *E. coli* and *Er. chrysanthemi* *suf* gene clusters. The putative SufC, SufD, SufS and SufB proteins encoded by the *S. thermophilus* cluster present respectively 72%, 48%, 65%, and 57% similarity to their *E. coli* **counterparts**. Moreover, IscU presents 47% similarity with IscU from *E. coli*.

Sequences corresponding to promoter regions were searched by inspection of the total sequence of the *sufCDS iscU sufB* cluster. A unique extended -10 sequence (5'-TTTGGTATAAT-3') is found upstream *sufC*, the first ORF of the cluster. A potential rho-independent transcriptional terminator is identified 63 bp downstream *sufB*, the last ORF of the cluster ( $\Delta G = -10$  kcal/mol). These features, together with the short or missing intergenic regions and the same orientation of the genes, suggest that these 5 ORFs form an operon, as it is the case for the *Er. chrysanthemi* *suf* locus.

## **5. Molecular characterization of the *sufCDS iscU sufB* cluster.**

**5.1. Construction of  $\Delta$ *sufD* and *iscU*<sub>97</sub> mutants.** Since *S. thermophilus* *suf* genes are potentially clustered in an operon, non polar  $\Delta$ *sufD* and *iscU*<sub>97</sub> mutants were constructed and analysed. The *sufD* mutation consists of an internally in frame deletion leading to the loss of 43% of the whole protein (see materials and methods and Fig. 4). Sequence comparison of NifU family proteins from various organisms indicates that *S. thermophilus* IscU protein contains the three highly conserved cystein residues that have been shown to be the assembly site of a "transitory" [2Fe-2S] center within NifU (70). Thus, the construction of a null *iscU* mutant was achieved by replacing one of these conserved residues, the Cys<sub>97</sub>, by a Tyr (see

materials and methods and Fig. 4). Sequencing of these two constructs confirmed that they did not contain any secondary mutation.

## **5.2. Role of *sufD* and *iscU* in iron metabolism.**

To investigate the possibility that *ΔsufD* and *iscU*<sub>97</sub> mutations impair growth due to the lack of functional [Fe-S] cluster biosynthesis, the mutants were grown in TPPY medium and compared to wild type. Growth of the *ΔsufD* and *iscU*<sub>97</sub> mutants showed no significant difference compared to the WT strain. Assuming that the high iron concentration of the TPPY medium could allow self assembling of the [Fe-S] clusters (71), and consequently could be responsible for the absence of difference in growth between strains, the same experiment was conducted in TPPYC, a divalent cation depleted medium. Under these conditions, the mutants displayed a growth rate similar of that of the WT strain but their maximal OD<sub>600</sub> was lower than that of the wild type, showing their premature entry in stationary phase (Table 3). Moreover, this difference disappeared when the cultures were grown on TPPYC supplemented by 100 μM of FeSO<sub>4</sub>. Taken together, these results suggest that (i) *iscU* and *sufD* genes of *S. thermophilus* are involved in iron metabolism and (ii) their presence would be of particular importance under iron-limiting conditions.

To confirm the involvement of *sufD* and *iscU* in iron metabolism, the iron intracellular concentration of *ΔsufD* and *iscU*<sub>97</sub> was checked by measuring the sensitivity of these strains to streptonigrin (an iron-activated antibiotic). As shown in Fig. 5 A, the *iscU* mutant was more sensitive to streptonigrin than the wild type strain whereas the *ΔsufD* mutant was more resistant. Moreover, addition of 2,2'-dipyridyl (a ferrous iron chelator) abolished the difference of growth between the strains (Fig. 5 B) indicating that iron was responsible for the streptonigrin sensitivity observed without 2,2'-dipyridyl. Taken altogether these results suggest that both *iscU* and *sufD* of *S. thermophilus* play a role in iron metabolism.

## Discussion

To investigate the oxidative stress defense system of *S. thermophilus*, a collection of random insertion mutants was screened to select clones with high sensitivity to menadione. Using this approach, we aimed to identify genes involved in superoxide stress defense without presuming their function. It can be noted that no gene encoding a protein considered, in other organisms, as major antioxidant enzyme were found using this approach. This can be due to the fact that (i) the collection of mutants was non-exhaustive (ii) the corresponding mutant could have a weak phenotype (iii) such mutations could be lethal (iv) except from SOD, most anti-oxidant enzymes are protective against H<sub>2</sub>O<sub>2</sub> and not O<sub>2</sub><sup>-</sup>. The genes identified in the present work were classified into 5 groups according to their function: genes potentially involved in cell wall metabolism, exopolysaccharide translocation, tRNA modification, and iron metabolism and gene of unknown function.

Perhaps the most striking observation of this study is that 5 out of the 10 sensitive mutants selected are disrupted in a gene encoding an membrane protein (OssH, MreD, RodA, PBP2b and CpsX). This could reflect that the cell envelope is the main target of the stress applied. Among these mutants, three (*mreD*, *rodA* and *pbp2b*) are impaired in genes potentially encoding proteins described in *E. coli* as playing a role in peptidoglycan biosynthesis during cell elongation (28, 52, 63). Phenotypic analysis of these three *S. thermophilus* mutants showed that they all present a reduced cell size associated with a modification of the cell shape, as observed in *rodA*, *pbp2* and *mre* mutants of *E. coli* or *Salmonella typhimurium* (5, 20, 28, 29, 63, 64). Moreover, the 3 *S. thermophilus* mutants also presented an increased sensitivity to heat shock compared to the wild type strain suggesting that the protective role of MreD, RodA and PBP2b is not specific to oxidative stress but could be enlarged to a more global stress response. In support of this hypothesis is the existence in *Lactococcus lactis* of mutations in *pbp* genes that negatively affect acid tolerance of a *guaA* mutant (16).

The major function assigned to cell wall is the maintenance of cell integrity by protecting cells not only against osmotic stress but also against other stress conditions. For instance, in LAB, it has already been shown that compromised biosynthesis of peptidoglycan results in an increased sensitivity to either UV (22) or acid (11) stress. Additionally, disruption of *mreD*, *rodA* or *pbp2b* may cause a modification of the peptidoglycan architecture in *S. thermophilus*, reducing the cell protection against aggressions such as oxidative and heat stress.

Transfer RNA modification is a universal feature of all living organisms, although some described modifications have no assigned function yet. Modifying enzymes affect the tRNA precursor either by chemical modification of an existing residue (in the base or in the sugar) or by nucleotide substitution. These modifications, in particular those present in the anticodon region can affect translation efficiency and fidelity (for a review (10)). In this study, two clones disrupted in the loci *tgt* and *ossF*, encoding proteins showing high identity with tRNA modifying enzymes were selected for their high sensitivity to oxidative stress. This sensitivity could suggest that the expression of genes involved in oxidative stress defense may require tRNA maturation or, that such modifications could help tRNA stabilization under stress conditions. In agreement with the former hypothesis *Shigella flexneri* pathogenicity is regulated by *virF* mRNA translation efficiency under TGT activity control (21). Additionally, several environmental conditions are suggested to influence tRNA modification: in the case of queuosine (whose synthesis and substitution is carried out by several enzymes among which is TGT), synthesis is sensitive to the presence of iron (31) and TGT requires oxygen for its full activity (54). Therefore, further investigations on this topic should provide an interesting new regulatory model and could contribute towards the emerging observation that translational regulation plays an important role in oxidative stress response, a role mainly ascribed to transcriptional regulation thus far.



Iron is essential for cellular metabolism, due to its requirement as a cofactor for a large number of enzymes. However, an excess of iron is toxic because of its involvement in the Fenton reaction that enhances the formation of ROS. In this work, a gene homologous to *fatD*, encoding a putative Fe<sup>3+</sup> ABC-transporter was found to be involved in *S. thermophilus* oxidative stress response. To test whether or not *S. thermophilus* FatD is indeed an iron transporter, the sensitivity of the *fatD* mutant to streptonigrin (an iron activated-antibiotic) was assessed. No significant difference between the mutant and the wild type strains was detected (data not shown) suggesting that disruption of *fatD* would have no effect on intracellular iron concentration. Thus, either FatD is not a major iron transporter under the experimental procedure we used or *S. thermophilus fatD* encodes an ABC transporter for another metallic cation. A possible cation could be Mn<sup>2+</sup> known to have a protective function against oxidative damage either via direct dismutase activity, such as reported in other organisms (4, 26, 60), or via its role as a cofactor of the only SOD reported into *S. thermophilus* (19). Thus, inactivation of a Mn<sup>2+</sup> ABC transporter could trigger sensitivity to oxidative stress.

[Fe-S] clusters are oxygen sensitive prosthetic groups, incorporated into proteins via covalent bonds to cysteines. Iron-sulfur proteins, which are found in more than 120 classes of enzymes and proteins (30), are known to play important physiological roles in electron transfer, metabolic reactions and in transcriptional regulation (30, 32). Indeed, because of their intrinsic redox properties, they are likely to sense oxygen or its derivatives as it is the case in SoxR and FNR transcriptional regulators, responding to oxidative stress and oxygen availability, (for a review (32)). IscR is also a iron-sulfur transcriptional regulator that is suggested to respond to Fe-S cluster limiting conditions and potentially to hydrogen peroxide in *E. coli* (32). Although it has been shown that [Fe-S] cluster assembly can occur spontaneously *in vitro* under anaerobic conditions (36), this process can also be catalyzed enzymatically *in vivo*. Genes of the *suf* operon and of the *isc* cluster (iron sulfur cluster) are

conserved and encode [Fe-S] cluster biosynthesis and/or repair pathways (23). More precisely, it has been recently proposed that Isc proteins are necessary for biosynthesis, while Suf proteins are essentially involved in repair of oxidatively damaged [Fe-S] clusters (37). Study of  $\Delta$ *sufD* and *iscU*<sub>97</sub> non-polar mutants indicates that SufD and IscU proteins are required for growth in an iron-deficient medium. SufD and IscU inactivation conferred an increased resistance and sensitivity respectively to streptonigrin. These results indicate that *S. thermophilus* SufD and IscU are involved in intracellular iron balance. Additionally, SufD and IscU of *S. thermophilus*, similar to SufD and NifU family proteins from other microorganisms, are encoded by genes clustered with *sufB*, *sufC* and *sufS* homologues, also potentially involved in [Fe-S] cluster repair. Hence the participation of *S. thermophilus* SufD and IscU proteins in iron intracellular balance is probably the result of their involvement in [Fe-S] cluster repair, as in *Er. chrysanthemi* (37). The NifU family protein function is to provide a scaffold for assembly of clusters mediated by NifS activity, and such clusters can then be transferred into apo forms of [Fe-S] cluster containing proteins (1, 70). The role of the SufD in [Fe-S] cluster biosynthesis is not well established. However, in *S. thermophilus*, the increased resistance to streptonigrin of the  $\Delta$ *sufD* mutant suggests that its intracellular iron concentration is lower than in the wild type. At least two alternative hypotheses can be drawn: (i) SufD could be directly involved in the uptake or assimilation of extracellular iron, (ii) SufD could inhibit the synthesis and/or repair of the [Fe-S] clusters. In this latter case, SufD inactivation would result in an increase synthesis of [Fe-S] clusters, and consequently in a reduced intracellular free iron concentration. Additionally, *S. thermophilus* *sufD* and *iscU* mutants displayed an increased sensitivity to superoxide radicals compared to wild type. Thus, SufD and IscU are likely to play a major role in oxidative stress defense, probably resulting from their predicted role in [Fe-S] cluster assembly and/or repair. The involvement of the *suf* operon in oxidative stress resistance during the early steps of infection by *Er. chrysanthemi* has also been reported (37), as well as

the induction of the *suf* gene cluster of *E. coli* by the hydrogen peroxide response regulator OxyR (72). In spite of a possible spontaneous formation, our data suggest that under oxidative stress, [Fe-S] cluster assembly and/or repair becomes a limiting step in the formation of iron-sulfur proteins, and that the cell requires an additional, dedicated system, encoded by the *suf* gene cluster, to optimize [Fe-S] cluster formation or repair.

Conclusion: The strategy used in this work allowed the identification of genes involved in *S. thermophilus* oxidative stress defense system. Data concerning the function of some loci is already available in the literature, whereas the role of others is not known. In both cases, the exact roles of these genes in the defense of *S. thermophilus* against oxidative stress remains to be elucidated.

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## LEGENDS FIGURES

**FIG. 1. Relative methyl viologen sensitivity.** (A). Results of short term methyl viologen exposure. (B) Results of long term methyl viologen exposure (see materials and methods). The relative methyl viologen sensitivity was determined by comparing viability with and without treatment. Levels are shown as values relative to the survival level of the wild type strain. All experiments were performed at least in duplicate. Error bars show two standard deviations of the data.

**FIG. 2. Survival to heat shock.** The heat shock consisted of 15 min treatment at 60°C. The percentage of surviving cells corresponds to the number of colony forming units (CFUs) counted on plates after the heat-shock exposure divided by the number of CFU before treatment. Error bars show two standard deviations of the data. The dotted line represents the surviving percentage observed for the wild type strain and the grey band comprises 2 standard deviations of the data.

**FIG. 3. Influence of *mreD* disruption on chain length.** Cell number per chains was determined for 70 chains of the wild type strain (■) and of the *mreD* mutant (■) grown in liquid TPPY medium.

**FIG. 4. Schematic map of the *S. thermophilus* *sufCDS iscU sufB* cluster.** Genes are indicated by grey arrows, and the positions of putative start and stop codons are given (in bp) below the map. The site of insertion of the pGh9:ISSI plasmid in *sufD* (*ossI*) and *iscU* (*ossJ*) disrupted mutants is indicated by the solid black triangles. The broken arrow indicates the position of the *suf* cluster putative promoter. Putative rho-independent terminator is represented as hairpin loop. Fragments used for generation of the non-polar mutants are indicated as open boxes. The star indicates the position of the *EcoRV* restriction site causing the Cys<sub>97</sub> replacement by a Tyr in the *iscU*<sub>97</sub> mutant.

**FIG. 5. Streptonigrin tolerance.** *S. thermophilus* strain tolerance to streptonigrin was assessed by measuring OD<sub>600</sub> after growth in TPPYC medium containing various concentrations of streptonigrin without (A) or supplemented with 1 mM of 2,2'-dipyridyl (B) (see material and methods for details). Relative OD<sub>600nm</sub> was determined by comparing the OD<sub>600</sub> obtained with or without streptonigrin. Wild type strain (○ and ●); *iscU*<sub>97</sub> (□ and ■); *ΔsufD* (Δ and ▲).

TABLE 1. Products of *oss* genes: database similarities and putative functions.

Gene name <sup>a</sup>	Accession number	Gene encoding the most similar product <sup>c</sup>	amino acid identity <sup>d</sup>	Insertion site <sup>e</sup>	Putative function	Class
<i>ossA</i> ( <i>mreD</i> )	AY386242	<i>S. mutans mreD</i> (168)	30% (1-168)	127	Cell shape maintenance	I
<i>ossB</i> ( <i>rodA</i> )	AF399832	<i>S. mutans rodA</i> (408)	65% (1-391)	304	Peptidoglycan elongation	
<i>ossC</i> ( <i>pbp2b</i> ) <sup>b</sup>	AF399833	<i>S. thermophilus</i> Sfi6 <i>pbp2b</i> (704)	98% (641-704)	640	Peptidoglycan elongation	
<i>ossD</i> ( <i>cpsX</i> ) <sup>b</sup>	AY386241	<i>S. thermophilus cpsX</i> (378)	98% (212-378)	264	Membrane translocation of exopolysaccharide	II
<i>ossE</i> ( <i>tgt</i> ) <sup>b</sup>	AY386243	<i>S. pneumoniae tgt</i> (380)	92% (1-333)	99	tRNA-guanine transglycosylase	III
<i>ossF</i> <sup>b</sup>	AY386244	<i>S. agalactiae gbs0448</i> (451)	77% (1-204)	51	RNA methyltransferase	
<i>ossG</i>	AY386239	<i>S. mutans smu.1428c</i> (284)	67% (1-280)	170	Unknown conserved function	IV
<i>ossH</i> ( <i>fatD</i> )	AY386240	<i>S. pneumoniae fatD</i> (324)	55% (22-323)	119	Iron ABC transporter	V
<i>ossI</i> ( <i>sufD</i> )	AY386245	<i>S. agalactiae sag0142</i> (420)	76% (1-420)	144	Assembly of Fe/S clusters	
<i>ossJ</i> ( <i>iscU</i> )	AY386245	<i>S. agalactiae gbs0140</i> (147)	78% (1-141)	57	Assembly of Fe/S clusters	

a. Initial gene name with, in brackets and when applicable, the definitive gene name.

b. Disrupted ORF of *S. thermophilus* CNRZ368 that have been partially sequenced.

c. Organism, gene name and, in brackets, length of the protein sequence giving the best alignment score.

d. Percentage of sequence identity with the protein showing the best alignment score to each *S. thermophilus* Oss protein. In brackets is indicated the portion of the protein sharing this identity (numbers correspond to the amino acid positions).

e. The numbers correspond to the deduced amino acid position of the pGh9:ISS1-generated disruption in the full length protein showing the best alignment score to the Oss proteins.

TABLE 2. *suf* cluster gene products of *S. thermophilus* CNRZ368

Gene product	Closest similar protein (organism, accession number) <sup>a</sup>	Identity/ similarity(%)	Function
SufC	CAD45782 ( <i>S. agalactiae</i> NEM316)	88/94	Putative ABC transporter (ATP-binding protein)
SufD	AAM99050 ( <i>S. agalactiae</i> 2603V/R)	76/87	Unknown, conserved protein
SufS	CAD45784 ( <i>S. agalactiae</i> NEM316)	79/89	Similar to SufS
IscU	CAD45785 ( <i>S. agalactiae</i> NEM316)	78/90	Similar to NifU family proteins
SufB	CAD45786 ( <i>S. agalactiae</i> NEM316)	89/94	Unknown, conserved protein

a. Accession number of the protein giving the best alignment score. In brackets, the name of the organism is indicated.

TABLE 3. Growth of wild type and mutant strains in TPPYC and in TPPYC supplemented with iron.

Strain	Final OD <sub>600</sub> resulting from growth <sup>a</sup> in:	
	TPPYC	TPPYC + FeSO <sub>4</sub>
WT	0.96	1.32
<i>ΔsufD</i>	0.58	1.48
<i>iscU<sub>97</sub></i>	0.58	1.15

<sup>a</sup> Cells were grown in milk medium for 15°h at 42°C and diluted in FeSO<sub>4</sub>-deficient medium (TPPYC medium supplemented with MgCl<sub>2</sub> (1 mM) and CaCl<sub>2</sub> (1 mM)). After one night growth, cells were washed twice and resuspend either in FeSO<sub>4</sub>-deficient medium or supplemented with 100 μM FeSO<sub>4</sub>. The growth of the cells was then followed by measuring the OD<sub>600</sub>. The OD<sub>600</sub> were measured after 24h incubation at 42°C. This experiment was done in triplicate.