

Identification of *Streptococcus thermophilus* CNRZ368 genes involved in

Annabelle Thibessard, Frédéric Borges, Annabelle Fernandez, Brigitte Gintz,
Bernard Decaris, Nathalie Leblond-Bourget

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ABSTRACT

19

20

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

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

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

INTRODUCTION

38

39 Oxygen is an essential element for the viability of most living organisms through its involvement
40 in oxidative phosphorylation that takes place during the aerobic respiration process. However,
41 oxygen can also be harmful, due to the production, especially during respiration, of toxic
42 derivatives namely ROS (reactive oxygen species) such as hydrogen peroxide, hydroxyl and
43 superoxide radicals (for a review, (25)). Accumulation of these molecules, triggers lipid, protein
44 and nucleic acid oxidation, causing lethal cellular damage (for reviews (7, 25, 53)). In response
45 to such attack, aerobic micro-organisms have evolved anti-oxidant mechanisms (eg catalase,
46 peroxidase and superoxide dismutase activities) to protect and adapt cells against ROS excess (6,
47 18, 24, 65, 68).

48 Although they do not achieve aerobic respiration, anaerobic micro-organisms can be subject to
49 ROS. The strict anaerobes *Bacteroides fragilis* and *Clostridium perfringens* are able to survive in
50 the presence of a low amount of oxygen, due to the induction of an adaptative oxidative stress
51 response that is activated under aerobic conditions (13, 46-49). The peroxide response of
52 *B. fragilis* shares some characteristics with that of *E. coli* such as the involvement of the redox-
53 sensitive transcriptional activator OxyR (46) and some antioxidant activities under its control
54 (47, 49).

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

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

64 ROS defense system and its inducibility by hydrogen peroxide are established in
65 *S. thermophilus* CNRZ368 (58). This organism possesses a single manganese-containing
66 superoxide dismutase (19) and a glutathione reductase (43). However, no catalase activity was
67 found. In other streptococci, antioxidant enzymes were reported to play a role in the defense
68 against oxidative stress (27, 33, 44, 66) and in pathogenesis (45, 67, 69).

69 The aim of this work was to identify genes involved in the oxidative stress defense of
70 *S. thermophilus* CNRZ368 without presuming their function. For this purpose a collection of
71 mutants was created by random insertional mutagenesis and the mutants were screened for
72 their sensitivity to menadione (a superoxide generating agent). The locus disrupted in 10
73 *S. thermophilus* menadione sensitive clones was identified and the potential physiological role
74 of the mutated genes in oxidative stress defense is discussed. The implication of these 10 loci
75 during heat shock was also evaluated to assess their specificity to oxidative stress defense.
76 Moreover, the characterization of two of these mutants, disrupted in *sufD* and *iscU* genes
77 which may participate in Fe-S clusters assembly or repair, is more detailed. The results
78 presented in this paper indicate that the inactivation of *sufD* and *iscU* genes caused a higher
79 sensitivity to superoxide stress and suggest that Fe-S cluster repair or assembly could play an
80 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⁻¹, when required. Streptonigrin sensitivity was monitored using a TPPYC
88 medium which contained a reduced concentration of metallic dications (including Fe²⁺).
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 MgCl₂ and 1 mM CaCl₂.
92 Recombinant plasmids were transformed into *E. coli* EC101, a TG1 strain [*supE hsd-5 thi Δ(lac-*
93 *proAB)* F' (*traD6 proAB⁺ lacI^f 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^R 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

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

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

123

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

132

133 **Construction of the *ΔsufD* and *iscU₉₇* mutants.** Oligonucleotides used to construct the
134 *ΔsufD* and *iscU₉₇* mutants were purchased from MWG Biotech AG (Ebersberg, Germany). To
135 construct non-polar truncated *ΔsufD* mutant, the 5' and 3' ends of the *sufD* gene were
136 independently amplified by PCR. The 5' end was amplified by using the primers
137 GAGCGTGTT**GAATTC**ACCG containing an *EcoRI* site, symbolized in bold characters
138 and AACTTAACT**GCAGC**ACCGTCT containing a *PstI* site. The 3' end was amplified by
139 using the primers GGTGTTCCAGA**ATTCAA**ATAACC containing an *EcoRI* site and
140 TGGTCTGTCATCAGGTCG. This last primer does not contain any *PstI* site, but the PCR
141 product generated does contain a *PstI* restriction site. These two PCR products were digested

142 by *Pst*I and ligated with each other. The resulting fragment was amplified by PCR, then
143 digested by *Eco*RI and cloned into pGh9. The final construct (pGh9 Δ *su*fD) was used to
144 transform *S. thermophilus* CNRZ368. For IscU protein inactivation, the strategy consisted in
145 replacing the TGT codon encoding the conserved Cys₉₇ by a TAT codon encoding a Tyr. This
146 was achieved by replacing, in oligonucleotides, the 3 underlined nucleotides of the sequence
147 (AGG TGT ACA) generating an *Eco*RV restriction site (AGA TAT CCA). Thus, the three
148 amino-acids Gly₉₆ Cys₉₇ Tyr₉₈ were replaced by Arg₉₆ Tyr₉₇ Thr₉₈ in the mutated protein. Two
149 internal and complementary primers containing the mutation
150 (GCTGGTGATGATATCACCATTTCACA and
151 GAAATGGTGATATCATCACCAGCAAAGG) were used with two additional external
152 primers (AAGCGGAATTCAAGCATCCACTCAGGC containing an *Eco*RI site and
153 TGTGACAAGCTTGTAGAGGCAATTCTT containing an *Hind*III site), respectively, to
154 amplify 5' and 3' end of *iscU*. The two PCR products of 515 bp and 262 bp were digested by
155 *Eco*RI and *Eco*RV and by *Hind*III and *Eco*RV respectively before being ligated to each other.
156 The resulting fragment was cloned into pGh9 digested by *Eco*RI and *Hind*III. The final
157 construct, (pGh9*iscU*₉₇) was used to transform *S. thermophilus* CNRZ368. Preparation of
158 *S. thermophilus* competent cells was performed as previously described by O'sullivan and
159 Fitzgerald (41). The following gene replacements were done as previously described (8) but
160 in TPPY medium and at 30°C/42°C as permissive and restrictive temperature, respectively.

161

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

163 **Exposure to methyl viologen.** For “short” time exposure, cultures of CNRZ368 and its
164 derivative strains were grown in TPPY medium. At an OD₆₀₀ of 0.6, cultures were divided into
165 two subcultures (in a ratio of liquid to air space of approximately 1/1). Methyl viologen
166 (paraquat; Sigma-Aldrich) was added to one of them with a final concentration of 50 mM. After
167 3 h incubation at 42°C, cultures were diluted to appropriate concentrations and spread onto

168 TPPY agar plates. For “long” time methyl viologen exposure, strains were grown in TPPY
169 medium until OD₆₀₀ of 0.6. Cultures were then diluted to a final theoretical OD₆₀₀ of 0.001 in
170 TPPY medium (in a ratio of liquid to air space of approximately 5/6) supplemented with 1.7 mM
171 methyl viologen, or not for control experiment. After 18h incubation at 42°C, cultures were
172 diluted in TPPY to appropriate concentrations and spread onto TPPY agar plates. In both
173 procedures, methyl viologen sensitivity was estimated by comparing CFU/ml obtained with
174 treated cultures, with respect to non treated cultures.

175

176 **Heat shock.** The ability of *S. thermophilus* and its derivatives to respond to a heat stress was
177 assessed as follow. Each strain is grown at 42°C in TPPY medium until early log phase
178 (OD₆₀₀ = 0.3). Then the culture was divided into two. One part was kept at 42°C (control
179 experiment); the other one was immediately transferred to 60°C for 15 min. Appropriate
180 dilutions were spread on TPPY agar plates. The fraction of surviving cells was calculated by
181 dividing the surviving CFU/ml by the count for the control experiment.

182

183 **Streptonigrin sensitivity.** Streptonigrin sensitivity was monitored on cells grown overnight
184 in TPPYC medium at 42°C. Cells were harvested by centrifugation, washed twice in TPPYC
185 medium, resuspended in TPPYC, diluted to OD₆₀₀ = 0.01 and cultivated in TPPYC for 9 h at
186 42°C. Then, the cultures were diluted again to OD₆₀₀ = 0.005 and divided into 4 parts which
187 were all incubated for 21 h at 42°C. One part was supplemented with 0.4% (v/v) dimethyl
188 formamide (control experiment), the 3 other parts were supplemented with different
189 concentrations of streptonigrin (1 µg/ml, 2 µg/ml and 4 µg/ml dissolved in dimethyl
190 formamide). As a control experiment, cultures were also carried out in TPPYC supplemented
191 with 1 mM of 2'2-dipyridyl (an Fe chelator). In each experiment, OD₆₀₀ value was used to
192 estimate the cellular density after 21h incubation. Relative optic density was determined by

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

195

196

RESULTS

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

198 A collection of 2112 mutants of *S. thermophilus* CNRZ368 was generated by insertional
199 mutagenesis using the plasmid pGh9:ISS1 (35, 56). This plasmid carries an erythromycin
200 resistance gene for its selection and the insertional element ISS1 that allows random
201 integration of the plasmid into *S. thermophilus* genome. The 2112 mutants obtained were
202 screened to select clones sensitive to menadione (a superoxide-generating compound). Two
203 distinct selection procedures were used to identify mutants susceptible to menadione (see
204 materials and methods). The characterization of 10 mutants, disrupted in loci originally named
205 *ossA* to *ossJ* (for oxidative stress sensitive locus) is described in this paper.

206

207 **2. Sensitivity of *S. thermophilus* CNRZ368 mutants to methyl viologen.** Previously, we

208 demonstrated that *ossB* and *ossC* mutants (previously named 6G4 and 18C3, respectively) are
209 compromised in their ability to survive superoxide stress (57). To confirm that the other 8
210 mutants selected were sensitive to superoxide stress, their sensitivity to methyl viologen was
211 examined (Fig. 1). In this experiment, we used the non-polar mutants *ΔsufD* and *iscU₉₇*
212 constructed in this work (see further) rather than the potential polar clones *ossI* and *ossJ*.
213 Short-term methyl viologen exposure revealed the extreme sensitivity of *ossG*, as well as the
214 4 to 5-fold increased sensitivity of *ossD*, *ossH* and *iscU₉₇* mutants (Fig. 1A). Although less
215 severe the *ΔsufD* mutant exhibited about 2-fold less survival following such stress. Under
216 these conditions, the other 3 mutants (*ossA*, *ossE* and *ossF*) did not exhibit any significant
217 sensitivity, however long term methyl viologen exposure confirmed their sensitivity. Indeed,
218 long term methyl viologen exposure (Fig. 1B) showed that the *ossA* mutant strain displayed a

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

221

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

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

233

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

241

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

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

245 *E. coli*, conferring the cell its characteristic rod shape (5, 28, 29). The *ossA* mutant was
246 defective in a gene encoding a protein, with 30% identity with MreD of *Streptococcus*
247 *mutans*. Sequence analysis of the surrounding region indicated that *S. thermophilus mreD*
248 gene (*ossA*) was clustered with a gene encoding a putative protein similar to MreC. Moreover,
249 hydrophopathy profiles of MreD (OssA) determined by using TMHMM and HMMTOP
250 showed 5 regions of sufficient length and hydrophobicity to be membrane-spanning segments
251 (data not shown), suggesting that this protein is the counterpart of MreD. The relatively low
252 identity (30%) observed between *S. thermophilus* and *S. mutans* MreD was in agreement with
253 the general idea that the structure of this protein is more highly conserved than its primary
254 sequence (17). To study the effect of *mreD* disruption in *S. thermophilus*, TPPY cultures of
255 the wild type and *mreD* mutant strains were observed under light microscopy. In contrast to
256 wild type cells, *mreD* mutant cells formed very long chains, up to 180 cells per chain (Fig. 3).
257 Moreover, a reduction of about 25% of the cell size was observed in the mutant. Growth of
258 the *mreD* mutant and of the wild type in TPPY medium were monitored by measuring the
259 OD₆₀₀. Both strains displayed a similar doubling time of 30 min, but the maximal OD₆₀₀ of the
260 *mreD* mutant (OD_{max} = 1.7 ± 0.1) was lower than that of the wild type (OD_{max} = 2.4 ± 0.2)
261 showing that the *mreD* mutant entered stationary phase earlier than wild type cells. The
262 characterization of *rodA* and *pbp2b* mutants of *S. thermophilus* CNRZ368 is described in a
263 previous paper (57).

264

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

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

276

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

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

290

291 **Class IV: Gene of unknown function.** The *ossG* product shows 67% identity with the
292 product of a gene of unknown function (*smu.1428c*) of *S. mutans* (Table 1). Although *ossG*
293 function is not known yet, the mutation of this gene is now associated with a sensitivity
294 phenotype to superoxide stress. The TMHMM and HMMTOP software predictions suggest
295 the existence of two transmembrane domains in the N-terminal region of OssG, making of
296 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 Fe³⁺ 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

323 neighborhood of *sufD* and *iscU* revealed that they both belong to a cluster of 5 genes all
324 orientated in the same direction. This cluster, named *sufCDS iscU sufB* cluster, is shown in
325 Fig. 4. The highest sequence identities of these gene products are found with putative proteins
326 from *S. agalactiae* (Table 2). They also share sequence similarity with proteins encoded by
327 *E. coli* and *Er. chrysanthemi suf* gene clusters. The putative SufC, SufD, SufS and SufB
328 proteins encoded by the *S. thermophilus* cluster present respectively 72%, 48%, 65%, and
329 57% similarity to their *E. coli* counterparts. Moreover, IscU presents 47% similarity with IscU
330 from *E. coli*.

331 Sequences corresponding to promoter regions were searched by inspection of the total
332 sequence of the *sufCDS iscU sufB* cluster. A unique extended -10 sequence
333 (5'-TTTGTTATAAT-3') is found upstream *sufC*, the first ORF of the cluster. A potential
334 rho-independent transcriptional terminator is identified 63 bp downstream *sufB*, the last ORF
335 of the cluster ($\Delta G = -10$ kcal/mol). These features, together with the short or missing
336 intergenic regions and the same orientation of the genes, suggest that these 5 ORFs form an
337 operon, as it is the case for the *Er. chrysanthemi suf* locus.

338

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

340 **5.1. Construction of Δ *sufD* and *iscU*₉₇ mutants.** Since *S. thermophilus suf* genes are
341 potentially clustered in an operon, non polar Δ *sufD* and *iscU*₉₇ mutants were constructed and
342 analysed. The *sufD* mutation consists of an internally in frame deletion leading to the loss of
343 43% of the whole protein (see materials and methods and Fig. 4). Sequence comparison of
344 NifU family proteins from various organisms indicates that *S. thermophilus* IscU protein
345 contains the three highly conserved cystein residues that have been shown to be the assembly
346 site of a "transitory" [2Fe-2S] center within NifU (70). Thus, the construction of a null *iscU*
347 mutant was achieved by replacing one of these conserved residues, the Cys₉₇, by a Tyr (see

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

350

351 **5.2. Role of *sufD* and *iscU* in iron metabolism.**

352 To investigate the possibility that Δ *sufD* and *iscU*₉₇ mutations impair growth due to the lack
353 of functional [Fe-S] cluster biosynthesis, the mutants were grown in TPPY medium and
354 compared to wild type. Growth of the Δ *sufD* and *iscU*₉₇ mutants showed no significant
355 difference compared to the WT strain. Assuming that the high iron concentration of the TPPY
356 medium could allow self assembling of the [Fe-S] clusters (71), and consequently could be
357 responsible for the absence of difference in growth between strains, the same experiment was
358 conducted in TPPYC, a divalent cation depleted medium. Under these conditions, the mutants
359 displayed a growth rate similar of that of the WT strain but their maximal OD₆₀₀ was lower
360 than that of the wild type, showing their premature entry in stationary phase (Table 3).
361 Moreover, this difference disappeared when the cultures were grown on TPPYC
362 supplemented by 100 μ M of FeSO₄. Taken together, these results suggest that (i) *iscU* and
363 *sufD* genes of *S. thermophilus* are involved in iron metabolism and (ii) their presence would
364 be of particular importance under iron-limiting conditions.

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

373

Discussion

374

375 To investigate the oxidative stress defense system of *S. thermophilus*, a collection of random
376 insertion mutants was screened to select clones with high sensitivity to menadione. Using this
377 approach, we aimed to identify genes involved in superoxide stress defense without
378 presuming their function. It can be noted that no gene encoding a protein considered, in other
379 organisms, as major antioxidant enzyme were found using this approach. This can be due to
380 the fact that (i) the collection of mutants was non-exhaustive (ii) the corresponding mutant
381 could have a weak phenotype (iii) such mutations could be lethal (iv) except from SOD, most
382 anti-oxidant enzymes are protective against H₂O₂ and not O₂⁻. The genes identified in the
383 present work were classified into 5 groups according to their function: genes potentially
384 involved in cell wall metabolism, exopolysaccharide translocation, tRNA modification, and
385 iron metabolism and gene of unknown function.

386 Perhaps the most striking observation of this study is that 5 out of the 10 sensitive mutants
387 selected are disrupted in a gene encoding an membrane protein (OssH, MreD, RodA, PBP2b
388 and CpsX). This could reflect that the cell envelope is the main target of the stress applied.
389 Among these mutants, three (*mreD*, *rodA* and *pbp2b*) are impaired in genes potentially
390 encoding proteins described in *E. coli* as playing a role in peptidoglycan biosynthesis during
391 cell elongation (28, 52, 63). Phenotypic analysis of these three *S. thermophilus* mutants
392 showed that they all present a reduced cell size associated with a modification of the cell
393 shape, as observed in *rodA*, *pbp2* and *mre* mutants of *E. coli* or *Salmonella typhimurium* (5,
394 20, 28, 29, 63, 64). Moreover, the 3 *S. thermophilus* mutants also presented an increased
395 sensitivity to heat shock compared to the wild type strain suggesting that the protective role of
396 MreD, RodA and PBP2b is not specific to oxidative stress but could be enlarged to a more
397 global stress response. In support of this hypothesis is the existence in *Lactococcus lactis* of
398 mutations in *pbp* genes that negatively affect acid tolerance of a *guaA* mutant (16).

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

405

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

424

425 Iron is essential for cellular metabolism, due to its requirement as a cofactor for a large number
426 of enzymes. However, an excess of iron is toxic because of its involvement in the Fenton
427 reaction that enhances the formation of ROS. In this work, a gene homologous to *fatD*, encoding
428 a putative Fe³⁺ ABC-transporter was found to be involved in *S. thermophilus* oxidative stress
429 response. To test whether or not *S. thermophilus* FatD is indeed an iron transporter, the
430 sensitivity of the *fatD* mutant to streptonigrin (an iron activated-antibiotic) was assessed. No
431 significant difference between the mutant and the wild type strains was detected (data not
432 shown) suggesting that disruption of *fatD* would have no effect on intracellular iron
433 concentration. Thus, either FatD is not a major iron transporter under the experimental procedure
434 we used or *S. thermophilus fatD* encodes an ABC transporter for another metallic cation. A
435 possible cation could be Mn²⁺ known to have a protective function against oxidative damage
436 either via direct dismutase activity, such as reported in other organisms (4, 26, 60), or via its role
437 as a cofactor of the only SOD reported into *S. thermophilus* (19). Thus, inactivation of a Mn²⁺
438 ABC transporter could trigger sensitivity to oxidative stress.

439

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

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

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

482

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

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488

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698

699

LEGENDS FIGURES

700

701 **FIG. 1. Relative methyl viologen sensitivity.** (A). Results of short term methyl viologen
702 exposure. (B) Results of long term methyl viologen exposure (see materials and methods).

703 The relative methyl viologen sensitivity was determined by comparing viability with and
704 without treatment. Levels are shown as values relative to the survival level of the wild type
705 strain. All experiments were performed at least in duplicate. Error bars show two standard
706 deviations of the data.

707

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

714

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

718

719 **FIG. 4. Schematic map of the *S. thermophilus* *sufCDS iscU sufB* cluster.** Genes are
720 indicated by grey arrows, and the positions of putative start and stop codons are given (in bp)
721 below the map. The site of insertion of the pGh9:ISS1 plasmid in *sufD* (*ossI*) and *iscU* (*ossJ*)
722 disrupted mutants is indicated by the solid black triangles. The broken arrow indicates the
723 position of the *suf* cluster putative promoter. Putative rho-independent terminator is
724 represented as hairpin loop. Fragments used for generation of the non-polar mutants are
725 indicated as open boxes. The star indicates the position of the *EcoRV* restriction site causing
726 the Cys₉₇ replacement by a Tyr in the *iscU*₉₇ mutant.

727

728 **FIG. 5. Streptonigrin tolerance.** *S. thermophilus* strain tolerance to streptonigrin was
729 assessed by measuring OD₆₀₀ after growth in TPPYC medium containing various
730 concentrations of streptonigrin without (A) or supplemented with 1 mM of 2,2'-dipyridyl (B)
731 (see material and methods for details). Relative OD_{600nm} was determined by comparing the
732 OD₆₀₀ obtained with or without streptonigrin. Wild type strain (○ and ●); *iscU*₉₇ (□ and ■);
733 Δ *sufD* (Δ and ▲).

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

735

Gene name ^a	Accession number	Gene encoding the most similar product ^c	amino acid identity ^d	Insertion site ^e	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>) ^b	AF399833	<i>S. thermophilus Sfi6 pbp2b</i> (704)	98% (641-704)	640	Peptidoglycan elongation	
<i>ossD</i> (<i>cpsX</i>) ^b	AY386241	<i>S. thermophilus cpsX</i> (378)	98% (212-378)	264	Membrane translocation of exopolysaccharide	II
<i>ossE</i> (<i>tgt</i>) ^b	AY386243	<i>S. pneumoniae tgt</i> (380)	92% (1-333)	99	tRNA-guanine transglycosylase	III
<i>ossF</i> ^b	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	

736

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

738 b. Disrupted ORF of *S. thermophilus* CNRZ368 that have been partially sequenced.739 c. Organism, gene name and, in brackets, length of the protein sequence giving the best alignment
740 score.741 d. Percentage of sequence identity with the protein showing the best alignment score to each
742 *S. thermophilus* Oss protein. In brackets is indicated the portion of the protein sharing this identity
743 (numbers correspond to the amino acid positions).744 e. The numbers correspond to the deduced amino acid position of the pGh9:ISS1-generated
745 disruption in the full length protein showing the best alignment score to the Oss proteins.

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

747

Gene product	Closest similar protein (organism, accession number) ^a	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

748

749 a. Accession number of the protein giving the best alignment score. In brackets, the name of the

750 organism is indicated.

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

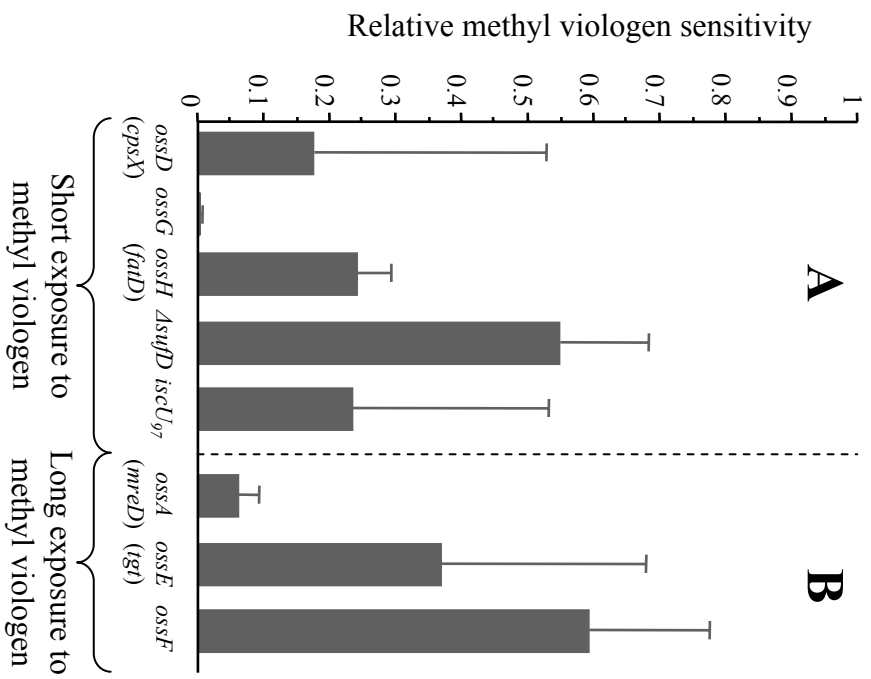
753

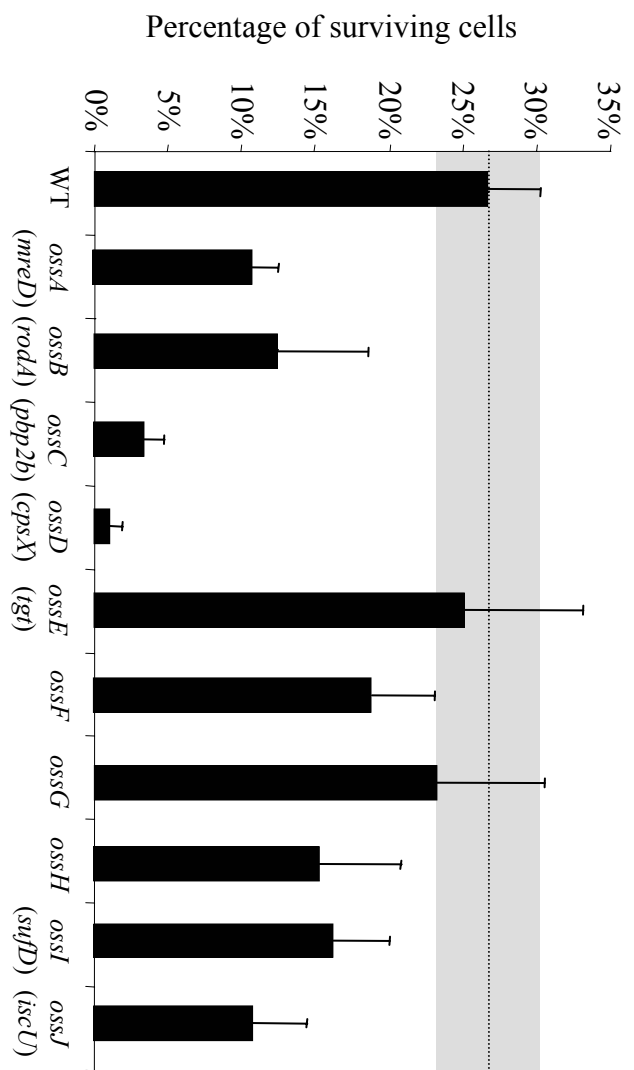
Strain	Final OD ₆₀₀ resulting from growth ^a in:	
	TPPYC	TPPYC + FeSO ₄
WT	0.96	1.32
<i>ΔsufD</i>	0.58	1.48
<i>iscU₉₇</i>	0.58	1.15

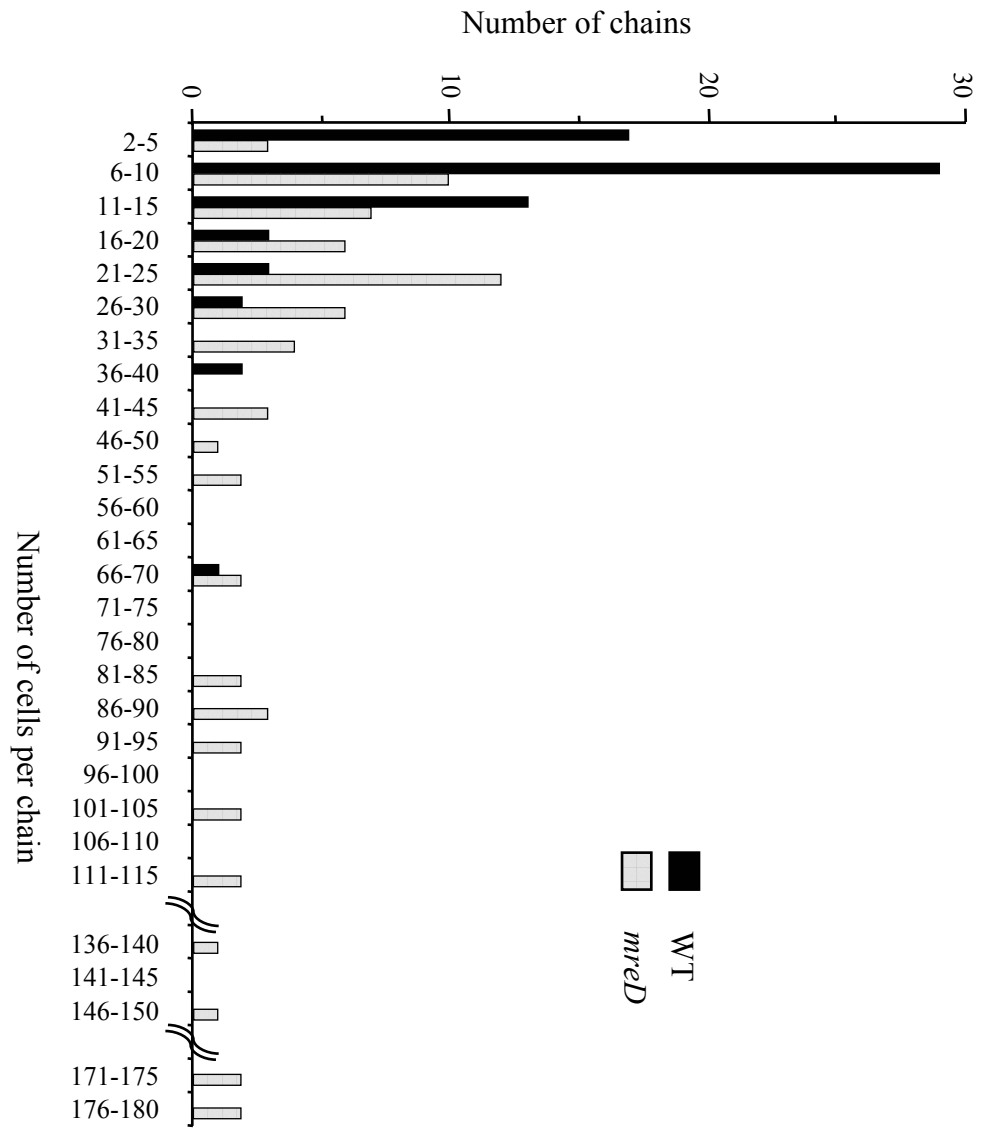
754

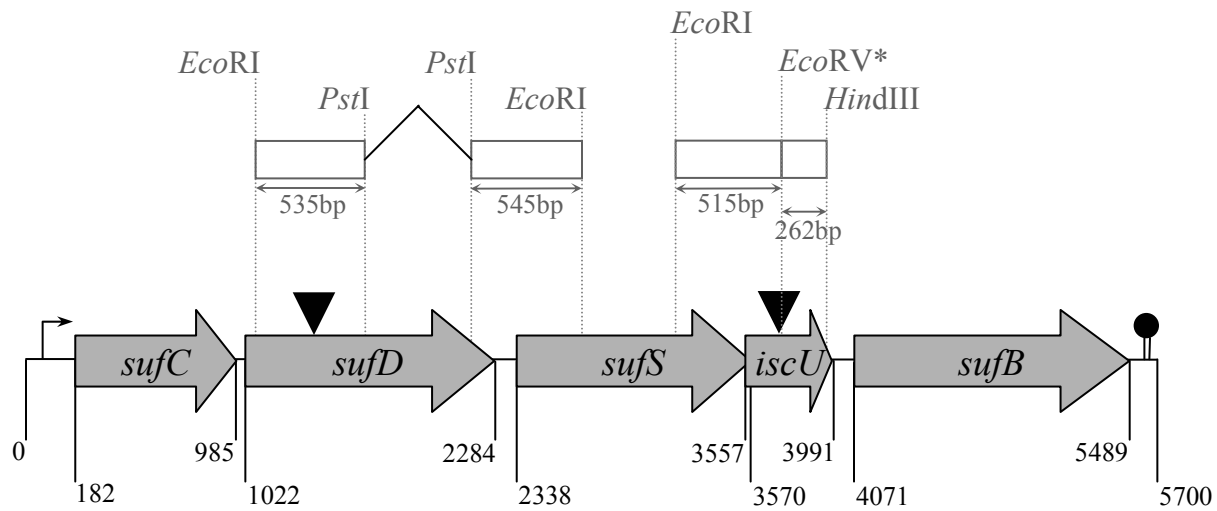
755 ^a Cells were grown in milk medium for 15^oh at 42°C and diluted in FeSO₄-deficient medium
756 (TPPYC medium supplemented with MgCl₂ (1 mM) and CaCl₂ (1 mM)). After one night
757 growth, cells were washed twice and resuspend either in FeSO₄-deficient medium or
758 supplemented with 100 μM FeSO₄. The growth of the cells was then followed by measuring
759 the OD₆₀₀. The OD₆₀₀ were measured after 24h incubation at 42°C. This experiment was done
760 in triplicate.

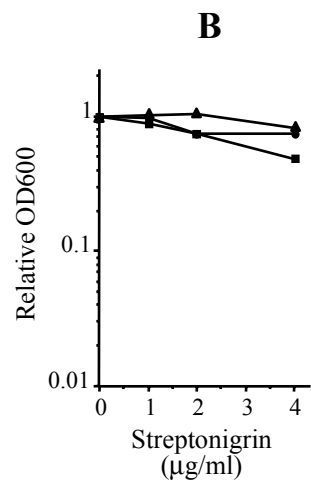
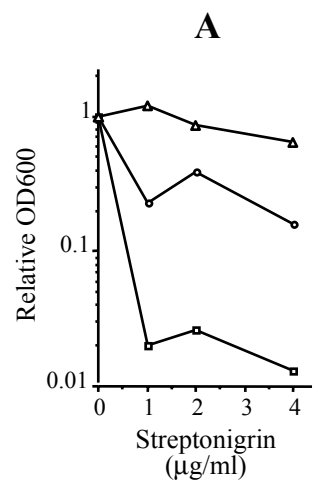
761











ABSTRACT

19

20

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

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

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

INTRODUCTION

39 Oxygen is an essential element for the viability of most living organisms through its involvement
40 in oxidative phosphorylation that takes place during the **aerobic** respiration process. However,
41 oxygen can also be harmful, due to the production, especially during respiration, of toxic
42 derivatives namely ROS (reactive oxygen species) such as hydrogen peroxide, hydroxyl and
43 superoxide radicals (for a review, (25)). Accumulation of these molecules, triggers lipid, protein
44 and nucleic acid oxidation, causing lethal cellular damage (for reviews (7, 25, 53)). In response
45 to such attack, aerobic micro-organisms have evolved anti-oxidant mechanisms (eg catalase,
46 peroxidase and superoxide dismutase activities) to protect and adapt cells against ROS excess (6,
47 18, 24, 65, 68).

48 Although **they do not achieve aerobic respiration**, anaerobic micro-organisms can be subject to
49 ROS. The strict anaerobes *Bacteroides fragilis* and *Clostridium perfringens* are able to survive in
50 the presence of a low amount of oxygen, due to the induction of an adaptative oxidative stress
51 response that is activated under aerobic conditions (13, 46-49). The peroxide response of
52 *B. fragilis* shares some characteristics with that of *E. coli* such as the involvement of the redox-
53 sensitive transcriptional activator OxyR (46) and some antioxidant activities under its control
54 (47, 49).

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

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

64 ROS defense system and its inducibility by hydrogen peroxide are established in
65 *S. thermophilus* CNRZ368 (58). This organism possesses a single manganese-containing
66 superoxide dismutase (19) and a glutathione reductase (43). However, no catalase activity was
67 found. In other streptococci, antioxidant enzymes were reported to play a role in the defense
68 against oxidative stress (27, 33, 44, 66) and in pathogenesis (45, 67, 69).
69 The aim of this work was to identify genes involved in the oxidative stress defense of
70 *S. thermophilus* CNRZ368 without presuming their function. For this purpose a collection of
71 mutants was created by random insertional mutagenesis and the mutants were screened for
72 their sensitivity to menadione (a superoxide generating agent). The locus disrupted in 10
73 *S. thermophilus* menadione sensitive clones was identified and the potential physiological role
74 of the mutated genes in oxidative stress defense is discussed. The implication of these 10 loci
75 during heat shock was also evaluated to assess their specificity to oxidative stress defense.
76 Moreover, the characterization of two of these mutants, disrupted in *sufD* and *iscU* genes
77 which may participate in Fe-S clusters assembly or repair, is more detailed. The results
78 presented in this paper indicate that the inactivation of *sufD* and *iscU* genes caused a higher
79 sensitivity to superoxide stress and suggest that Fe-S cluster repair or assembly could play an
80 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⁻¹, when required. Streptonigrin sensitivity was monitored using a TPPYC
88 medium which contained a reduced concentration of metallic dications (including Fe²⁺).
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 MgCl₂ and 1 mM CaCl₂.

92 Recombinant plasmids were transformed into *E. coli* EC101, a TG1 strain [*supE hsd-5 thi Δ(lac-*
93 *proAB)* F' (*traD6 proAB⁺ lacI^f 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^R 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

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

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

123

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

132

133 **Construction of the *ΔsufD* and *iscU₉₇* mutants.** Oligonucleotides used to construct the
134 *ΔsufD* and *iscU₉₇* mutants were purchased from MWG Biotech AG (Ebersberg, Germany). To
135 construct non-polar truncated *ΔsufD* mutant, the 5' and 3' ends of the *sufD* gene were
136 independently amplified by PCR. The 5' end was amplified by using the primers
137 GAGCGTGTTGAATTCCACCG containing an *EcoRI* site, symbolized in bold characters
138 and AACTTAACTGCAGCACCGTCT containing a *PstI* site. The 3' end was amplified by
139 using the primers GGTGTTCCAGAATTCAAATAACC containing an *EcoRI* site and
140 TGGTCTGTCATCAGGTCG. This last primer does not contain any *PstI* site, but the PCR
141 product generated does contain a *PstI* restriction site. These two PCR products were digested

142 by *Pst*I and ligated with each other. The resulting fragment was amplified by PCR, then
143 digested by *Eco*RI and cloned into pGh9. The final construct (pGh9 Δ *scfD*) was used to
144 transform *S. thermophilus* CNRZ368. For IscU protein inactivation, the strategy consisted in
145 replacing the TGT codon encoding the conserved Cys₉₇ by a TAT codon encoding a Tyr. This
146 was achieved by replacing, in oligonucleotides, the 3 **underlined** nucleotides of the sequence
147 (**AGG TGT ACA**) generating an *Eco*RV restriction site (**AGA TAT CCA**). Thus, the three
148 amino-acids Gly₉₆ Cys₉₇ Tyr₉₈ were replaced by Arg₉₆ Tyr₉₇ Thr₉₈ in the mutated protein. Two
149 internal and complementary primers containing the mutation
150 (**GCTGGTGATGATATCACCATTTCACA** and
151 **GAAATGGTGATATCATCACCAGCAAAGG**) were used with two additional external
152 primers (**AAGCGGAATTCAAGCATCCACTCAGGC** containing an *Eco*RI site and
153 **TGTGACAAGCTTGTAGAGGCAATTCTT** containing an *Hind*III site), respectively, to
154 amplify 5' and 3' end of *iscU*. The two PCR products of 515 bp and 262 bp were digested by
155 *Eco*RI and *Eco*RV and by *Hind*III and *Eco*RV respectively before being ligated to each other.
156 The resulting fragment was cloned into pGh9 digested by *Eco*RI and *Hind*III. The final
157 construct, (pGh9 Δ *iscU*₉₇) was used to transform *S. thermophilus* CNRZ368. Preparation of
158 *S. thermophilus* competent cells was performed as previously described by O'sullivan and
159 Fitzgerald (41). The following gene replacements were done as previously described (8) but
160 in TPPY medium and at 30°C/42°C as permissive and restrictive temperature, respectively.

161

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

163 **Exposure to methyl viologen.** For “short” time exposure, cultures of CNRZ368 and its
164 derivative strains were grown in TPPY medium. At an OD₆₀₀ of 0.6, cultures were divided into
165 two subcultures (in a ratio of liquid to air space of approximately 1/1). Methyl viologen
166 (paraquat; Sigma-Aldrich) was added to one of them with a final concentration of 50 mM. After
167 3 h incubation at 42°C, cultures were diluted to appropriate concentrations and spread onto

168 TPPY agar plates. For “long” time methyl viologen exposure, strains were **grown** in TPPY
169 medium until OD₆₀₀ of 0.6. Cultures were then diluted to a final theoretical OD₆₀₀ of 0.001 in
170 TPPY medium **(in a ratio of liquid to air space of approximately 5/6)** supplemented with 1.7 mM
171 methyl viologen, or not for control experiment. After 18h incubation at 42°C, cultures were
172 diluted in TPPY to appropriate concentrations and spread onto TPPY agar plates. In both
173 procedures, methyl viologen sensitivity was estimated by comparing CFU/ml obtained with
174 treated cultures, with respect to non treated cultures.

175

176 **Heat shock.** The ability of *S. thermophilus* and its derivatives to respond to a heat stress was
177 assessed as follow. Each strain is grown at 42°C in TPPY medium until early log phase
178 (OD₆₀₀ = 0.3). Then the culture was divided into two. One part was kept at 42°C (control
179 experiment); the other one was immediately transferred to 60°C for 15 min. Appropriate
180 dilutions were spread on TPPY agar plates. The fraction of surviving cells was calculated by
181 dividing the surviving CFU/ml by the count for the control experiment.

182

183 **Streptonigrin sensitivity.** Streptonigrin sensitivity was monitored on cells grown overnight
184 in TPPYC medium at 42°C. Cells were harvested by centrifugation, washed twice in TPPYC
185 medium, resuspended in TPPYC, diluted to OD₆₀₀ = 0.01 and cultivated in TPPYC for 9 h at
186 42°C. Then, the cultures were diluted again to OD₆₀₀ = 0.005 and divided into 4 parts which
187 were all incubated for 21 h at 42°C. One part was supplemented with 0.4% (v/v) dimethyl
188 formamide (control experiment), the 3 other parts were supplemented with different
189 concentrations of streptonigrin (1 µg/ml, 2 µg/ml and 4 µg/ml dissolved in dimethyl
190 formamide). As a control experiment, cultures were also carried out in TPPYC supplemented
191 with 1 mM of 2'2-dipyridyl (an Fe chelator). In each experiment, OD₆₀₀ value was used to
192 estimate the cellular density after 21h incubation. Relative optic density was determined by

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

195

196

RESULTS

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

198 A collection of 2112 mutants of *S. thermophilus* CNRZ368 was generated by insertional
199 mutagenesis using the plasmid pGh9:ISS1 (35, 56). This plasmid carries an erythromycin
200 resistance gene for its selection and the insertional element ISS1 that allows random
201 integration of the plasmid into *S. thermophilus* genome. The 2112 mutants obtained were
202 screened to select clones sensitive to menadione (a superoxide-generating compound). Two
203 distinct selection procedures were used to identify mutants susceptible to menadione (see
204 materials and methods). The characterization of 10 mutants, disrupted in loci originally named
205 *ossA* to *ossJ* (for oxidative stress sensitive locus) is described in this paper.

206

207 **2. Sensitivity of *S. thermophilus* CNRZ368 mutants to methyl viologen.** Previously, we

208 demonstrated that *ossB* and *ossC* mutants (previously named 6G4 and 18C3, respectively) are
209 compromised in their ability to survive superoxide stress (57). To confirm that the other 8
210 mutants selected were sensitive to superoxide stress, their sensitivity to methyl viologen was
211 examined (Fig. 1). In this experiment, we used the non-polar mutants *ΔsufD* and *iscU₉₇*
212 constructed in this work (see further) rather than the potential polar clones *ossI* and *ossJ*.
213 Short-term methyl viologen exposure revealed the extreme sensitivity of *ossG*, as well as the
214 4 to 5-fold increased sensitivity of *ossD*, *ossH* and *iscU₉₇* mutants (Fig. 1A). Although less
215 severe the *ΔsufD* mutant exhibited about 2-fold less survival following such stress. Under
216 these conditions, the other 3 mutants (*ossA*, *ossE* and *ossF*) did not exhibit any significant
217 sensitivity, however long term methyl viologen exposure confirmed their sensitivity. Indeed,
218 long term methyl viologen exposure (Fig. 1B) showed that the *ossA* mutant strain displayed a

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

221

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

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

233

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

241

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

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

245 *E. coli*, conferring the cell its characteristic rod shape (5, 28, 29). The *ossA* mutant was
246 defective in a gene encoding a protein, with 30% identity with MreD of *Streptococcus*
247 *mutans*. Sequence analysis of the surrounding region indicated that *S. thermophilus mreD*
248 gene (*ossA*) was clustered with a gene encoding a putative protein similar to MreC. Moreover,
249 hydrophopathy profiles of MreD (OssA) determined by using TMHMM and HMMTOP
250 showed 5 regions of sufficient length and hydrophobicity to be membrane-spanning segments
251 (data not shown), suggesting that this protein is the counterpart of MreD. The relatively low
252 identity (30%) observed between *S. thermophilus* and *S. mutans* MreD was in agreement with
253 the general idea that the structure of this protein is more highly conserved than its primary
254 sequence (17). To study the effect of *mreD* disruption in *S. thermophilus*, TPPY cultures of
255 the wild type and *mreD* mutant strains were observed under light microscopy. In contrast to
256 wild type cells, *mreD* mutant cells formed very long chains, up to 180 cells per chain (Fig. 3).
257 Moreover, a reduction of about 25% of the cell size was observed in the mutant. Growth of
258 the *mreD* mutant and of the wild type in TPPY medium were monitored by measuring the
259 OD₆₀₀. Both strains displayed a similar doubling time of 30 min, but the maximal OD₆₀₀ of the
260 *mreD* mutant (OD_{max} = 1.7 ± 0.1) was lower than that of the wild type (OD_{max} = 2.4 ± 0.2)
261 showing that the *mreD* mutant entered stationary phase earlier than wild type cells. The
262 characterization of *rodA* and *pbp2b* mutants of *S. thermophilus* CNRZ368 is described in a
263 previous paper (57).

264

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

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

276

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

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

290

291 **Class IV: Gene of unknown function.** The *ossG* **product shows 67% identity with the**
292 **product of** a gene of unknown function (*smu.1428c*) of *S. mutans* (Table 1). Although *ossG*
293 function is not known yet, the mutation of this gene is now associated with a sensitivity
294 phenotype to superoxide stress. The TMHMM and HMMTOP software predictions suggest
295 the existence of two transmembrane domains in the N-terminal region of OssG, making of
296 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 Fe³⁺ 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

323 neighborhood of *sufD* and *iscU* revealed that they both belong to a cluster of 5 genes all
324 orientated in the same direction. This cluster, named *sufCDS iscU sufB* cluster, is shown in
325 Fig. 4. The highest **sequence identities** of these gene products are found with putative proteins
326 from *S. agalactiae* (Table 2). They also share sequence similarity with proteins encoded by
327 *E. coli* and *Er. chrysanthemi suf* gene clusters. The putative SufC, SufD, SufS and SufB
328 proteins encoded by the *S. thermophilus* cluster present respectively 72%, 48%, 65%, and
329 57% similarity to their *E. coli* **counterparts**. Moreover, IscU presents 47% similarity with IscU
330 from *E. coli*.

331 Sequences corresponding to promoter regions were searched by inspection of the total
332 sequence of the *sufCDS iscU sufB* cluster. A unique extended -10 sequence
333 (5'-TTTGGTATAAT-3') is found upstream *sufC*, the first ORF of the cluster. A potential
334 rho-independent transcriptional terminator is identified 63 bp downstream *sufB*, the last ORF
335 of the cluster ($\Delta G = -10$ kcal/mol). These features, together with the short or missing
336 intergenic regions and the same orientation of the genes, suggest that these 5 ORFs form an
337 operon, as it is the case for the *Er. chrysanthemi suf* locus.

338

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

340 **5.1. Construction of Δ *sufD* and *iscU*₉₇ mutants.** Since *S. thermophilus suf* genes are
341 potentially clustered in an operon, non polar Δ *sufD* and *iscU*₉₇ mutants were constructed and
342 analysed. The *sufD* mutation consists of an internally in frame deletion leading to the loss of
343 43% of the whole protein (see materials and methods and Fig. 4). Sequence comparison of
344 NifU family proteins from various organisms indicates that *S. thermophilus* IscU protein
345 contains the three highly conserved cystein residues that have been shown to be the assembly
346 site of a "transitory" [2Fe-2S] center within NifU (70). Thus, the construction of a null *iscU*
347 mutant was achieved by replacing one of these conserved residues, the Cys₉₇, by a Tyr (see

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

350

351 **5.2. Role of *sufD* and *iscU* in iron metabolism.**

352 To investigate the possibility that Δ *sufD* and *iscU*₉₇ mutations impair growth due to the lack
353 of functional [Fe-S] cluster biosynthesis, the mutants were grown in TPPY medium and
354 compared to wild type. Growth of the Δ *sufD* and *iscU*₉₇ mutants showed no significant
355 difference compared to the WT strain. Assuming that the high iron concentration of the TPPY
356 medium could allow self assembling of the [Fe-S] clusters (71), and consequently could be
357 responsible for the absence of difference in growth between strains, the same experiment was
358 conducted in TPPYC, a divalent cation depleted medium. Under these conditions, the mutants
359 displayed a growth rate similar of that of the WT strain but their maximal OD₆₀₀ was lower
360 than that of the wild type, showing their premature entry in stationary phase (Table 3).
361 Moreover, this difference disappeared when the cultures were grown on TPPYC
362 supplemented by 100 μ M of FeSO₄. Taken together, these results suggest that (i) *iscU* and
363 *sufD* genes of *S. thermophilus* are involved in iron metabolism and (ii) their presence would
364 be of particular importance under iron-limiting conditions.

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

373

Discussion

374

375 To investigate the oxidative stress defense system of *S. thermophilus*, a collection of random
376 insertion mutants was screened to select clones with high sensitivity to menadione. Using this
377 approach, we aimed to identify genes involved in superoxide stress defense without
378 presuming their function. It can be noted that no gene encoding a protein considered, in other
379 organisms, as major antioxidant enzyme were found using this approach. This can be due to
380 the fact that (i) the collection of mutants was non-exhaustive (ii) the corresponding mutant
381 could have a weak phenotype (iii) such mutations could be lethal (iv) except from SOD, most
382 anti-oxidant enzymes are protective against H₂O₂ and not O₂⁻. The genes identified in the
383 present work were classified into 5 groups according to their function: genes potentially
384 involved in cell wall metabolism, exopolysaccharide translocation, tRNA modification, and
385 iron metabolism and gene of unknown function.

386 Perhaps the most striking observation of this study is that 5 out of the 10 sensitive mutants
387 selected are disrupted in a gene encoding an membrane protein (OssH, MreD, RodA, PBP2b
388 and CpsX). This could reflect that the cell envelope is the main target of the stress applied.
389 Among these mutants, three (*mreD*, *rodA* and *pbp2b*) are impaired in genes potentially
390 encoding proteins described in *E. coli* as playing a role in peptidoglycan biosynthesis during
391 cell elongation (28, 52, 63). Phenotypic analysis of these three *S. thermophilus* mutants
392 showed that they all present a reduced cell size associated with a modification of the cell
393 shape, as observed in *rodA*, *pbp2* and *mre* mutants of *E. coli* or *Salmonella typhimurium* (5,
394 20, 28, 29, 63, 64). Moreover, the 3 *S. thermophilus* mutants also presented an increased
395 sensitivity to heat shock compared to the wild type strain suggesting that the protective role of
396 MreD, RodA and PBP2b is not specific to oxidative stress but could be enlarged to a more
397 global stress response. In support of this hypothesis is the existence in *Lactococcus lactis* of
398 mutations in *pbp* genes that negatively affect acid tolerance of a *guaA* mutant (16).

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

405

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

424

425 Iron is essential for cellular metabolism, due to its requirement as a cofactor for a large number
426 of enzymes. However, an excess of iron is toxic because of its involvement in the Fenton
427 reaction that enhances the formation of ROS. In this work, a gene homologous to *fatD*, encoding
428 a putative Fe³⁺ ABC-transporter was found to be involved in *S. thermophilus* oxidative stress
429 response. To test whether or not *S. thermophilus* FatD is indeed an iron transporter, the
430 sensitivity of the *fatD* mutant to streptonigrin (an iron activated-antibiotic) was assessed. No
431 significant difference between the mutant and the wild type strains was detected (data not
432 shown) suggesting that disruption of *fatD* would have no effect on intracellular iron
433 concentration. Thus, either FatD is not a major iron transporter under the experimental procedure
434 we used or *S. thermophilus fatD* encodes an ABC transporter for another metallic cation. A
435 possible cation could be Mn²⁺ known to have a protective function against oxidative damage
436 either via direct dismutase activity, such as reported in other organisms (4, 26, 60), or via its role
437 as a cofactor of the only SOD reported into *S. thermophilus* (19). Thus, inactivation of a Mn²⁺
438 ABC transporter could trigger sensitivity to oxidative stress.

439

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

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

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

482

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

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488

489

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494

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698

699

LEGENDS FIGURES

700

701 **FIG. 1. Relative methyl viologen sensitivity.** (A). Results of short term methyl viologen
702 exposure. (B) Results of long term methyl viologen exposure (see materials and methods).

703 The relative methyl viologen sensitivity was determined by comparing viability with and
704 without treatment. Levels are shown as values relative to the survival level of the wild type
705 strain. All experiments were performed at least in duplicate. Error bars show two standard
706 deviations of the data.

707

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

714

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

718

719 **FIG. 4. Schematic map of the *S. thermophilus* *sufCDS iscU sufB* cluster.** Genes are
720 indicated by grey arrows, and the positions of putative start and stop codons are given (in bp)
721 below the map. The site of insertion of the pGh9:ISSI plasmid in *sufD* (*ossI*) and *iscU* (*ossJ*)
722 disrupted mutants is indicated by the solid black triangles. The broken arrow indicates the
723 position of the *suf* cluster putative promoter. Putative rho-independent terminator is
724 represented as hairpin loop. Fragments used for generation of the non-polar mutants are
725 indicated as open boxes. The star indicates the position of the *EcoRV* restriction site causing
726 the Cys₉₇ replacement by a Tyr in the *iscU*₉₇ mutant.

727

728 **FIG. 5. Streptonigrin tolerance.** *S. thermophilus* strain tolerance to streptonigrin was
729 assessed by measuring OD₆₀₀ after growth in TPPYC medium containing various
730 concentrations of streptonigrin without (A) or supplemented with 1 mM of 2,2'-dipyridyl (B)
731 (see material and methods for details). Relative OD_{600nm} was determined by comparing the
732 OD₆₀₀ obtained with or without streptonigrin. Wild type strain (○ and ●); *iscU*₉₇ (□ and ■);
733 Δ *sufD* (Δ and ▲).

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

735

Gene name ^a	Accession number	Gene encoding the most similar product ^c	amino acid identity ^d	Insertion site ^e	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>) ^b	AF399833	<i>S. thermophilus Sfi6 pbp2b</i> (704)	98% (641-704)	640	Peptidoglycan elongation	
<i>ossD</i> (<i>cpsX</i>) ^b	AY386241	<i>S. thermophilus cpsX</i> (378)	98% (212-378)	264	Membrane translocation of exopolysaccharide	II
<i>ossE</i> (<i>tgt</i>) ^b	AY386243	<i>S. pneumoniae tgt</i> (380)	92% (1-333)	99	tRNA-guanine transglycosylase	III
<i>ossF</i> ^b	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	

736

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

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

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

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

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

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

747

Gene product	Closest similar protein (organism, accession number) ^a	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

748

749 a. Accession number of the protein giving the best alignment score. In brackets, the name of the

750 organism is indicated.

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

753

Strain	Final OD ₆₀₀ resulting from growth ^a in:	
	TPPYC	TPPYC + FeSO ₄
WT	0.96	1.32
<i>ΔsufD</i>	0.58	1.48
<i>iscU₉₇</i>	0.58	1.15

754

755 ^a Cells were grown in milk medium for 15^oh at 42°C and diluted in FeSO₄-deficient medium
756 (TPPYC medium supplemented with MgCl₂ (1 mM) and CaCl₂ (1 mM)). After one night
757 growth, cells were washed twice and resuspend either in FeSO₄-deficient medium or
758 supplemented with 100 μM FeSO₄. The growth of the cells was then followed by measuring
759 the OD₆₀₀. The OD₆₀₀ were measured after 24h incubation at 42°C. This experiment was done
760 in triplicate.

761