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Original article

Characterisation of *Streptococcus thermophilus* CNRZ368 oxidative stress-resistant mutants: involvement of a potential Rgg-like transcriptional regulator

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Abstract – Eight mutants of *Streptococcus thermophilus* CNRZ368 presenting a menadione-resistant phenotype were selected and the locus mutated in each mutant was identified. Among these clones, 5 were disrupted in a gene of unknown function, 2 were impaired in genes involved in cellular metabolism and the last one (the mutant 15H7) was disrupted in *rgg* encoding a putative transcriptional regulator. To determine if *rgg* could be a key regulator of the superoxide defence of *S. thermophilus*, the 15H7 mutant was further characterised. The results from our work indicate that the *rgg* gene, mutated in the 15H7 genome, is a pseudogene composed of 2 ORFs (*rggA* and *rggB*) that are potentially translated in two different frames. Moreover, transcription analysis demonstrated the existence of a transcript containing *rggA*, *rggB* and *orf2*. Construction of *ΔrggA*, *ΔrggB* and *orf2* mutants and their phenotypic analysis confirmed the involvement of *rggA* and *rggB* in the oxidative stress response. The question of the role of the pseudogene *rgg* is still open.

***Streptococcus thermophilus* / oxidative stress / Rgg-like transcriptional regulator**

Résumé – Caractérisation de mutants résistants au stress oxydant chez *Streptococcus thermophilus* CNZR368 : implication d'un régulateur transcriptionnel putatif de type Rgg. Huit mutants de *Streptococcus thermophilus* CNRZ368, présentant un phénotype de résistance à la ménadione ont été sélectionnés et le locus muté a été identifié pour chacun d'eux. Parmi ces clones, 5 sont interrompus dans un gène de fonction inconnue, 2 sont mutés dans des gènes impliqués dans le métabolisme cellulaire et un autre (15H7) est interrompu dans le gène *rgg* codant potentiellement un régulateur transcriptionnel. Afin de déterminer si *rgg* est un régulateur majeur de la défense contre le stress superoxyde le mutant 15H7 a été plus amplement étudié. Les résultats de notre travail indiquent que le gène *rgg*, muté dans le génome de 15H7, est un pseudogène composé de deux ORF (*rggA* and *rggB*) potentiellement traduites dans deux phases de lecture différentes. De plus, l'analyse transcriptionelle démontre l'existence d'un transcrit englobant *rggA*, *rggB* et *orf2*. La construction des mutants *ΔrggA*, *ΔrggB* et *orf2* ainsi que l'analyse de leur phénotype confirme l'implication de *rggA* et *rggB* dans la réponse contre le stress oxydant. La question concernant le rôle du pseudogène *rgg* est toujours ouverte.

***Streptococcus thermophilus* / stress oxydant / régulateur transcriptionnel de type Rgg**

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1. INTRODUCTION

S. thermophilus is a Gram-positive bacterium with low G+C content that belongs to the group of lactic acid bacteria (LAB). Due to their ability to metabolise sugar in lactic acid, these microorganisms are commonly used in dairy factories as starters of fermentation of yoghurt and are involved in the process of ripening of cheeses.

S. thermophilus CNRZ368 is considered to be an anaerobic aerotolerant microorganism. Although this bacterium grows more slowly in the presence of oxygen (growth rate = 40 min) than in its absence (growth rate = 27 min), it can survive in the presence of oxygen [20]. The ability of *S. thermophilus* to survive in the presence of oxygen presumably results from the existence of a defence mechanism that helps cells to eliminate reactive oxygen species (ROS) and to repair damage [21]. Data concerning the ability of lactic acid bacteria to survive in the presence of oxidative stress are scarce. In the literature, the existence of antioxidant enzymes is reported in *Streptococcus* genera [1, 7, 9, 14, 25]. Moreover, the existence of PerR, an oxidative stress-responsive repressor is reported in *Streptococcus pyogenes* [9, 15]; nevertheless, the global network of oxidative stress regulation in streptococci remains to be elucidated.

On the contrary, the oxidative stress response of aerobic organisms such as *E. coli* is well documented. This response necessitates antioxidant enzymes involved in the detoxification of cells and repair of enzymes necessary to eliminate damage. In *E. coli*, two networks of proteins can be induced depending on the reactive oxygen species: superoxide radicals induce the SoxR/S regulon, whereas the OxyR/S regulon is induced in the presence of hydrogen peroxide (for a review see [17]). These two regulons are partially overlapping. Although the presence of genes homologous to *oxyR* and *soxR* is a characteristic shared by many bacteria, other regulatory proteins have also emerged through evolution. Among them are PerR [15], sigma(B) and OhrR [8] and σ^R/RsrA that are involved in the response against H_2O_2 and superoxide radicals, respectively [12, 13].

To identify the genes involved in the oxidative stress response of *S. thermophilus* CNRZ368, a collection of mutants was generated and screened for the menadione-resistant phenotype. This work allowed the identification of eight loci potentially involved in the mechanism of superoxide tolerance: among them is the locus *rgg*, described in the literature as a transcriptional regulator [2, 3, 18, 19].

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

S. thermophilus CNRZ368 and *E. coli* TG1 were purchased from the INRA-CNRZ strain collection (Jouy-en-Josas, France) and from Stratagene (Amsterdam, The Netherlands), respectively. The thermosensitive plasmids pGhost9 and pGhost9:IS*S1* were used [11]. They carry an erythromycin resistance gene and a thermosensitive origin of replication. Depending on the experiment, *S. thermophilus* CNRZ368 was cultivated in TPPY or M17 media. For selection of menadione-resistant clones, plates containing TPPY medium supplemented with menadione (concentration going from 1 to 120 $\mu g \cdot mL^{-1}$) were used. *E. coli* TG1 cultures were grown on Luria-Bertani medium.

2.2. Cloning of the fragments flanking the chromosomal insertion site

Cloning of the fragments flanking the pGhost9:IS*S1* insertion locus was done as previously described by Thibessard et al. [23] and allowed the identification of the mutagenised site of each mutant.

2.3. General DNA techniques, DNA sequencing and analysis

Isolation of genomic or plasmidic DNA, agarose gel electrophoresis, restriction enzyme digests, Southern hybridisation,

PCR, DNA ligation and *E. coli* transformation were carried out as described in [16]. Sequencing was done as previously described in [22].

2.4. Construction of *S. thermophilus* mutants

S. thermophilus mutants were constructed via allelic replacement. The *ΔrggA* and *ΔrggB* mutants presented a chromosomal deletion of the residues 14 to 41 of RggA and of the residues 106 to 207 of RggB, respectively. The mutant *orf2* presented 2 stop mutations affecting the first two residues of the protein.

2.5. RNA preparation, RT-PCR and Northern analysis

Total RNA was extracted from *S. thermophilus* with a single guanidinium thiocyanate step and phenol-chloroform extraction by using Tri-Reagent (Sigma, Saint-Quentin-Fallavier, France). In the RNA preparation, possible contaminating DNA was eliminated by the action of DNaseI. cDNA was generated from 2.5 μg of RNA reverse transcribed with MMLV-reverse transcriptase and hexamer oligonucleotide random primers (Amersham Pharmacia Biotech, Freiburg, Germany). For Northern hybridisation analysis, template RNA samples (30 μg) were electrophoresed into a denaturing (i.e. formaldehyde) agarose gel and transferred to a HybondN$^+$ (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Digoxigenin-labelled transcripts complementary to *rggB* were used as a probe. These *rggB* transcripts were synthesised in vitro using the T3 promoter RNA polymerase system carried by the pBKS-*rggB* vector. The latter was constructed by cloning a 533 bp internal fragment of *rggB* in the pBKS vector. Hybridisation and detection procedures were done according to the manufacturer's instructions (Roche Molecular Biochemicals, Meylan, France).

2.6. Sensitivity to oxidising agent

The sensitivity of CNRZ368 derivatives to oxidative stress was checked on exponentially growing cells ($OD_{600} = 0.6$) by the addition of menadione (1 to 20 mg·mL^{-1}). The cells were left in contact with menadione for 3 h. Aliquots of the culture were diluted in TPPY, and appropriate dilutions plated on TPPY agar plates to determine cell viability. The plates were stocked in jars containing a GENbox anaer system (bioMérieux, Marcy l'Étoile, France) providing hypoxic conditions, and colonies were counted after 20 h of incubation. The percentage of surviving CFU was determined by comparing viability with or without treatment. All experiments were performed in triplicate.

3. RESULTS

3.1. Identification of genes disrupted in *S. thermophilus* menadione-resistant mutants

To identify the genes implicated in the oxidative stress response of *S. thermophilus*, a collection of mutants, constructed by insertional mutagenesis using pGhost9:IS*S1,* was screened for the tolerance of the clones to menadione (a superoxide radical generating molecule). Among the 2112 clones, eight were selected for their increased resistance to menadione and were further characterised. For each mutant, the disrupted locus was cloned and sequenced. Sequence analysis allowed the grouping of these 8 mutants into 3 classes (Fig. 1). The first class assembled the mutants 7H3, 13B12, 14H11, 15H8 and 11C1, that were disrupted in genes of unknown function. For the last clone 11C1, the disrupted gene was specific to the genome of *S. thermophilus*. The second class brought together the mutants 6H7 and 16C10, that were mutagenised in genes involved in cellular metabolism. The mutant 6H7 was impaired in a gene encoding DeoB, a protein

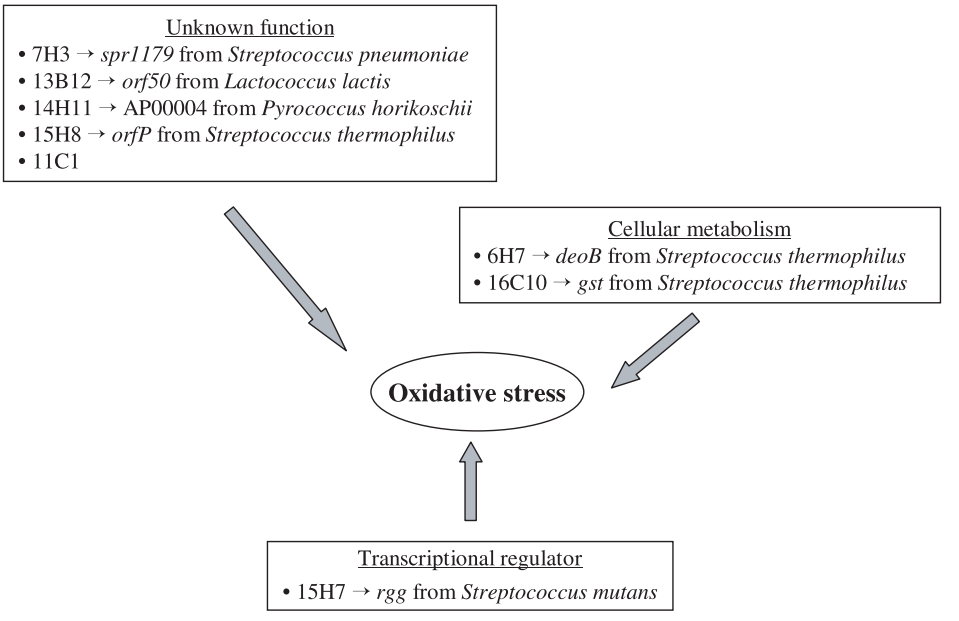

Figure 1. Classification of the menadione-resistant mutants according to their putative function. The best homologous gene for ORF disrupted in CNRZ368 mutants is indicated for each clone after the arrows.

involved in purine metabolism. The mutant 16C10 was affected in *gst,* that encodes a putative proton-glutamate symport protein.

The third class included the mutant 15H7, that was disrupted in a gene, homologous to a putative transcriptional regulator of *Streptococcus mutans*, encoding a protein belonging to the Rgg-like family. To determine the role of the *rgg* locus in menadione tolerance and to understand the mechanism of regulation of *S. thermophilus* oxidative stress response, we decided to focus our research on the mutant 15H7.

3.2. Characterisation of the locus disrupted in the mutant 15H7

3.2.1. Identification of the rgg *locus*

Figure 2A shows a scheme of the three ORFs, named *rggA*, *rggB* and *orf2*, identified within the nucleotide sequence flanking the pGhost9:IS*S1* insertion locus of the genome of 15H7. The *rggA* ORF potentially encoded a protein of 65 residues that showed 77% identity with the putative transcriptional regulator Rgg of *S. mutans*. The 232 amino acid residues potentially encoded by *rggB* presented 56% identity with the same putative transcriptional regulator Rgg of *S. mutans*. Thus, the homology of the Rgg protein from *S. mutans* began with the protein RggA and continued with the protein RggB of *S. thermophilus* in the frame +1. These data indicate that the *rgg* gene disrupted in the mutant 15H7 is a pseudogene. Finally, *orf2* potentially encoded a protein of 35 residues presenting no homology with sequences present in the databases.

The search for transcriptional signals allowed the identification of a potential promoter composed of an extended –10 sequence located 61 pb upstream of the start codon of *rggA*. Moreover, a putative

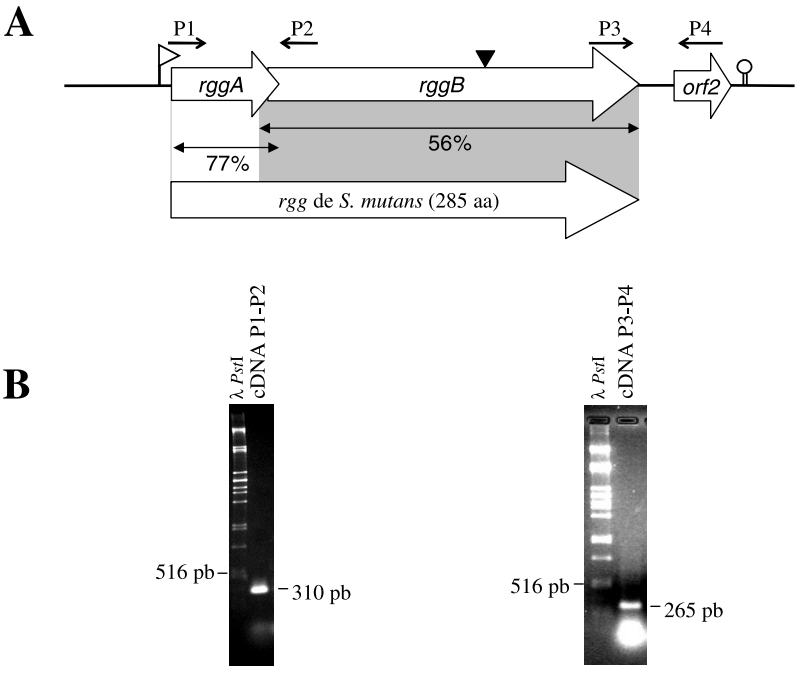

Figure 2. Organisation of the *rgg* locus of *S. thermophilus* CNRZ368. (**A**) The solid line symbolises the *S. thermophilus* genomic DNA. ORFs are indicated as open arrows. The black arrowhead indicates the insertion site of pGh9:IS*S1* within the genome of the 15H7 mutant. The open flag indicates the putative promoter and the putative rho-independent terminator is represented as a hairpin loop. Grey boxes symbolise the homologous region between *rgg* from *S. thermophilus* and from *S. mutans*: the percentage of amino acid identity is indicated below the double arrows. Primers used for RT-PCR experiments are indicated. The expected size of the P1-P2 and P3-P4 PCR fragments is 310 pb and 265 pb, respectively. (**B**) Gel electrophoresis showing the RT-PCR products obtained using the primers P1-P2 and P3-P4.

rho-independent terminator consisting of a 10 pb stem and a 3 pb loop was seen downstream of the stop codon of *orf2*. Located 8 pb upstream of *rggA* was a potential Shine-Dalgarno sequence, suggesting that this gene is likely to be translated. On the contrary, no Shine-Dalgarno sequence preceded *rggB*, thus rendering improbable the synthesis of RggB alone.

3.2.2. Transcriptional organisation of the rgg *locus*

Northern blot analysis performed with a RNA probe complementary to *rggB* revealed a strong band of approximately 1200 nucleotides (data not shown). To determine which ORFs were included in this transcript, RT-PCR analyses were carried out. cDNA generated from *S. thermophilus* CNRZ368 RNA were used in PCR experiments (Fig. 2B). Primers P1 and P2 were designed to verify the presence of a transcript going from *rggA* to *rggB* and P3 and P4 to determine the existence of a transcript going from *rggB* to *orf2*. In both cases, a PCR product of the expected size (310 pb and 265 pb, respectively) was amplified. These data suggested the existence of one transcript including *rggA*, *rggB* and *orf2*. Thus, the *rgg* locus showed an operonic structure.

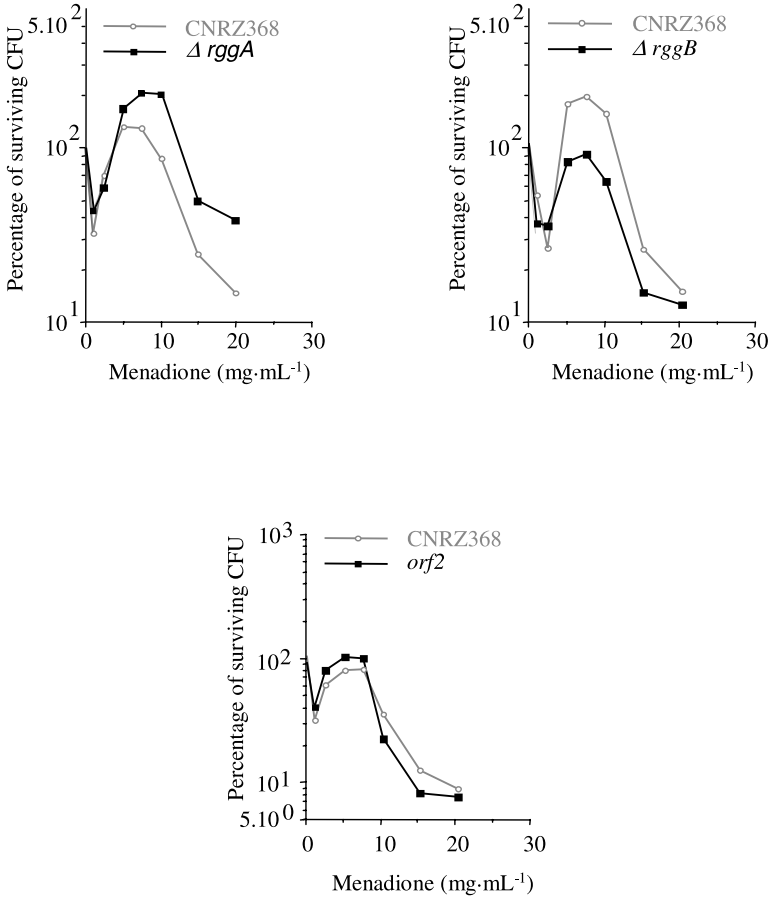

Figure 3. Survival of the *ΔrggA*, *ΔrggB* and *orf2* mutants in the presence of increasing concentrations of menadione. Cells grown overnight in milk medium were diluted a hundred-fold into fresh TPPY medium. At OD_{600} = 0.6, samples of the culture were treated with menadione for 3 h. Cells were diluted and plated on TPPY solid medium and then incubated for 20 h in jars. Colony forming units (CFUs) were then counted and the percentages of surviving CFUs were estimated relative to non-treated samples. All these experiments were done in triplicate. Results of typical experiments are presented here.

3.2.3. Involvement of the rgg *locus in* S. thermophilus *oxidative stress response*

The results from our analysis indicated that impairment of a pseudogene, composed of *rggA* and *rggB,* resulted in an increased tolerance of *S. thermophilus* to menadione. To gain insight into the involvement of the *rgg* locus in the oxidative stress defence, a series of mutants (*ΔrggA*, *ΔrggB* and *orf2)* was constructed via allelic replacement. The *ΔrggA* and *ΔrggB* mutants presented a deletion of 43% and of 44% of the total protein, respectively. The mutant *orf2* carried 2 stop mutations.

The ability of these 3 mutants to survive in the presence of increasing concentrations

of menadione was tested and compared with that of the wild-type strain. As shown in Figure 3, the *ΔrggA* mutant showed an increased ability to survive in the presence of menadione compared with the wild-type whereas the ability of the *ΔrggB* clone to tolerate menadione was reduced. Moreover, no difference in behaviour was seen for the mutant *orf2* and the wild-type strain. These data suggest that *rggA* and *rggB* are both involved in *S. thermophilus* response to oxidative stress. However, their exact role in this mechanism remains to be solved. In contrast, *orf2* does not seem to play a role in the survival of *S. thermophilus* submitted to oxidative stress.

4. DISCUSSION

The dairy organism, *S. thermophilus*, is continuously exposed to stress conditions generated during industrial processes. To identify the mechanisms that confer tolerance to ROS, we isolated menadione-resistant strains by insertional mutagenesis. This study reports the characterisation of 8 genes involved in *S. thermophilus* CNRZ368 oxidative stress defence. According to their putative function, these genes were classified into 3 groups. The first class, which contains 5/8 genes, brings together genes of unknown function. Thus, the strategy used in this work allowed the identification of new genes involved in oxidative stress: a phenotype of superoxide resistance can now be assigned to their disruption. The second class groups genes implicated in cellular metabolism: *deoB* encodes a phosphopentomutase that is involved in the purine salvage pathway, whereas *gst* is a potential glutamate-proton transporter. Both pathways are known to be connected with stress responses. In *Lactococcus lactis*, the *deoB* mutation confers multiple stress resistance on the cell [5, 6]. In *S. mutans*, genes encoding enzymes responsible for glutamate synthesis are repressed in acid and thermal stress [4]. All these results reveal that cellular metabolic pathways are intimately related to stress response and that the flux of particular metabolites are likely to be implicated in stress response in LAB. The last class contains a transcriptional regulator that belongs to the family of Rgg-like proteins. These are DNA-binding proteins including a helix-turn-helix (HTH) motif at their N-terminal end. Rgg-like proteins are only found in Gram-positive bacteria belonging to the *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Listeria* genera. These regulators are reported to stimulate positively or negatively the transcription of numerous genes of different functions [3].

Our work describes for the first time the involvement of a Rgg-like protein in oxidative stress response. Moreover, it asks the question of how the pseudogene *rgg*, disrupted in the mutant 15H7, can influence the tolerance of ROS. To address the question of whether RggA or RggB or both were involved in stress response: both *ΔrggA* and *ΔrggB* mutants were constructed and their ability to respond to menadione was checked. The results of these analyses indicated that both RggA and RggB are implicated in oxidative stress defence. Taking into account the lack of visible RBS sites downstream of *rggB*, the independent synthesis of RggB seems improbable. An alternative hypothesis concerns the synthesis of a RggAB protein that would be generated by frameshifting. Sequence analysis of the region overlapping the *rggA* and *rggB* ORFs revealed the presence of a frameshift mutation within the sequence TTT TTT TTT. One T-deletion would abolish the frameshift mutation and restore the proper *rgg* ORF. In the literature, frameshift mutations (and particularly transcriptional frameshifting) are known to arise in sequences composed of multiple thymine or adenine [10, 24]. Such events can result either from DNA polymerase slippage during replication, or may require RNA polymerase slippage (transcriptional frameshift) or ribosome slippage (translational frameshift). It is now important to know if such a frameshifting

event could appear in *S. thermophilus*, and if so, if it could be stimulated by environmental conditions (for instance, by the presence of oxidative molecules). To fully understand the role of Rgg in the oxidative stress response, the characterisation of the genes regulated by this protein and implicated in stress defence would be essential.

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