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**The *rggC* locus, with a frameshift mutation, is involved in oxidative
stress response by *Streptococcus thermophilus***

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SUMMARY

In *Streptococcus thermophilus*, the locus *rggC* contains a frameshift mutation and thus consists of two ORFs, *rggC₁* and *rggC₂* which encode proteins exhibiting similarity with the Rgg transcriptional regulator family. In this work, mutants showing a partial deletion of *rggC₁* and *rggC₂* were constructed and their response to menadione, a superoxide generating compound, was analysed. These mutants exhibited different behaviour to this oxidative stress compared to the wild-type strain. Analysis of this locus among 21 strains of *S. thermophilus* showed a polythymine tract length variability and a strain dependant adenine residue could be found upstream of this repeat. This inter-strain polymorphism supports evidence for the hypothesis that the *rggC* locus is phase variable.

Key words: *Streptococcus thermophilus*, oxidative stress response, transcriptional regulator, Rgg, genetic variability, frameshift mutation

INTRODUCTION

The genus *Streptococcus* is composed of numerous pathogenic species and the food grade species *S. thermophilus* which is used as a starter of fermentation in yogurt and cheese production. During the biotransformation process, the formation of reactive oxygen species (ROS) may occur and be deleterious to the cells. *S. thermophilus* is an anaerobic aerotolerant organism that can grow in presence of oxygen and survive in low concentrations of superoxide and hydroxyl radicals (Thibessard et al. 2001). This suggests that it has developed strategies for cell detoxication and damage repair caused by ROS.

Recently, the genome sequences of two *S. thermophilus* strains LMG18311 and CNRZ1066 were completed (Bolotin et al. 2004) and putative genes involved in oxidative stress defence were identified by genomic analysis (Hols et al. 2005). Genes in the disulfide-reducing pathway like glutathione reductase, thioredoxin or thioredoxin reductase have been detected. These genes products help to maintain the redox state of the cell and allow reducing intracellular disulfide. *S. thermophilus* antioxidant defence also comprises genes encoding enzymes that detoxify oxygen or ROS. Thus, the *S. thermophilus* genome encodes an H₂O-forming NADH oxidase, an essential enzyme for the NAD⁺regeneration during aerobic growth (Higuchi et al. 1999). In *Streptococcus mutans*, induction of H₂O-forming NADH oxidase protects cells against oxygen toxicity (Higuchi 1992). *S. thermophilus* also encodes a superoxide dismutase thought to catalyze superoxide radicals dismutation in O₂ and H₂O₂. Although deprived of H₂O₂-detoxifying enzymes like catalase, NADH-peroxidase or alkyl hydroperoxidase, *S. thermophilus* possess an H₂O₂ defence system to reduce damage induced by this compound. This system could include *psaD* and *dpr* genes. *PsaD* is encoding a thiol-peroxidase and Dpr is an iron-binding protein required for oxygen tolerance in *S. mutans* (Yamamoto et al. 2004). Dpr iron sequestration activity is thought to prevent the formation of toxic hydroxyl radicals via the Fenton reaction (Niven and Ekins 2001; Yamamoto et al. 2002; Pulliainen et al. 2003; Yamamoto et al. 2004; Pulliainen et al. 2005). Valuable information on *S. thermophilus* oxidative stress processes have also acquired by genetic approach using random IS mutagenesis.

Inactivation of several genes involved either in cell wall metabolism, exopolysaccharide translocation, tRNA modification, purine metabolism, or iron metabolism have been implicated in cell survival during oxidative stress (Thibessard et al. 2004).

Among the well characterised oxidative stress-regulators in other organisms such as OxyR, PerR, sigma(B) and OhrR, controlling the peroxide response (for reviews: Hecker and Volker 1998; Mongkolsuk and Helmann 2002) and the superoxide stress response system SoxRS (Dempfle 1996) only *perR*, is present in the *S. thermophilus* genome.

In pathogenic streptococci, several regulators involved in oxidative stress have been described. Among these regulatory proteins is RopA, a key regulator of acid and oxidative stress tolerance, genetic competence, biofilm formation and virulence in *S. mutans* (Wen et al. 2005). In *Streptococcus pneumoniae*, the perception of O₂ deprivation is associated with DNA uptake and controlled by CiaRH and MicAB two-component systems (Echenique et al. 2000; Echenique and Trombe 2001). Both *ropA* and *micAB* genes have been identified in the *S. thermophilus* genome (Bolotin et al. 2004). Additional to this list are the regulatory factors belonging to the Rgg family. These proteins are only found in *Streptococcus* and few other low-GC Gram-positive genera, such as *Listeria*, *Lactobacillus* and *Lactococcus*. In *S. pyogenes*, Rgg interacts with regulatory networks to co-regulate virulence factor expression (Chaussee et al. 2001) and has also been implicated in oxidative and thermic stress responses as well as in the synthesis or stability of growth phase-regulated proteins associated with amino-acid metabolism (Chaussee et al. 2004).

In order to characterise *S. thermophilus* oxidative stress tolerance molecular basis, we previously used a random IS mutagenesis approach to produce mutants and screened them for their potential impaired tolerance to menadione (a superoxide generating compound). This strategy led us to identify 18 *S. thermophilus* CNRZ368 mutants (Thibessard et al. 2004). One of which is *rggC*. This gene is known to contain a frameshift mutation from the *S. thermophilus* genome sequence. This work is focused on *rggC* and attempts to determine how such a locus inactivated by a frameshift mutation is involved in oxidative stress tolerance. The *rggC* locus

analysis was performed by engineering partial deletions of this locus and examining the ability of mutants to survive in the presence of menadione. Our results support an active role for RggC in *S. thermophilus* oxidative stress response.

MATERIALS AND METHODS

1. Bacterial strains growth conditions

The bacterial strains used in this study are listed in Table 1. Streptococcal strains including wild-type and mutant strains were cultured either in M17 (Terzaghy and Sandine 1975), or TPPY (a Tryptose, Proteose Peptone, Yeast extract containing medium) (Bracquart 1981) or milk mediums, at 30°C or 42°C, depending on the experiments requirement. When appropriate, these media were supplemented with antibiotics at the following concentrations: erythromycin at 2 µg.ml⁻¹, and chloramphenicol at 3 µg.ml⁻¹.

E. coli EC101 was used for cloning experiments and transformed by electroporation with various plasmids as indicated below. *E. coli* strains were grown on Luria-Bertani (LB) medium (Sambrook et al. 1989) and the following selective antibiotics were added, when needed: erythromycin (150 µg.ml⁻¹), chloramphenicol (15 µg.ml⁻¹) or ampicillin (100 µg.ml⁻¹).

2. DNA manipulations

Isolation of genomic or plasmid DNA, agarose gel electrophoresis, restriction enzyme digestions, Southern hybridisation, PCR, DNA ligation and *E. coli* transformation were performed as described in Sambrook et al. (1989). Sequencing was carried out with the DNA sequencing kit (Applied Biosystems) on an ABI Prism 377 Genetic Analyser (Applied Biosystems). Sequence data were analysed with Blast, ProDom and TIGRfam Model software. The *rggC1* and *rggC2* DNA sequences have previously been deposited in GenBank under accession numbers from AY598750. The *orf2* and *rsp* DNA sequences obtained in this work were deposited under accession number AM087201.

3. RNA manipulations

RNA was extracted from *S. thermophilus* CNRZ368 overnight ($OD_{600} = 1.6$) cultures with a single guanidinium thiocyanate step and phenol-chloroform extraction by using the Tri-Reagent (Sigma). Northern blot hybridisation analysis, templates of RNA samples (30 μ g) were electrophoresed into a denaturing (i.e. formaldehyde) 1.1 % agarose gel and transferred to a HybondN⁺ (Amersham pharmacia biotech) according to the manufacturer's instructions. Digoxigenin-labelled transcripts complementary to *rggC*₂ were used as a probe. These *rggC*₂ transcripts were synthesised in vitro using the T3 promoter RNA polymerase system carried by the pBKS-*rggC*₂ vector. The latter was constructed by cloning a 533 bp internal fragment of *rggC*₂ (nucleotides 932 to 1464) in the pBKS vector. Hybridisation and detection procedures were performed according to manufacturer's instructions (Roche Molecular Biochemicals). RT-PCR experiments were carried out as previously described (Fernandez et al. 2004). Primers used in PCRs were purchased from MWG Biotech AG (Ebersberg) and their sequences were 5' -AGGTGACTAGAATGAAATATGG-3' for P₁, 5' -TCCATTTGGCTAATTAATAGC-3' for P₂, 5' -TATTGCTGCCACGAAATTGGT-3' for P₃ and 5' -CACCGCCACAAGAATGACTT-3' for P₄. Primers used in *rggC* locus sequencing were 5' -AGGAGGTGACTAGAATGAAATATGG-3' for V1 and 5' -TCCATTTGGCTAATTAATAGC-3' for V2.

4. Constructions of *ΔrggC*₁, *ΔrggC*₂, *orf2* and *rggC*_{8T} mutants

The *ΔrggC*₁ gene contained a deletion of 43 % of the CDS. A stop codon (TAA) was inserted between the potential RBS of *rggC*₁ and the start codon of *rggC*₂ to prevent using this RBS sequence for *RggC*₂ translation. The 5' and 3' ends of the *rggC*₁ gene were independently amplified with the following sets of primers: 5' -CTTGCTTTGAATTCAGGCT-3' (named I₁) and 5' -TAAAGATAAAGCTTAAGATTCTC-3' for the 5' region; 5' -GGCTGATTTAAGCTTCAGCAAATT-3' and 5' -ATCATATGAATTCCTTAGCAT-3',

(named I₂), for the 3' region (engineered restriction sites are underlined, stop codon is in boldface type). Amplified fragments were digested with *Hind*III and ligated to each other. The resulting fragment was amplified, digested with *Eco*RI and cloned into pGh9 vector to produce pGh9 Δ *rggC*₁ plasmid. The same procedure was used to produce pGh9 Δ *rggC*₂ and pGh9 Δ *orf2*_{Z1,2} plasmids. pGh9 Δ *rggC*₂ carried a 44 % deletion of *rggC*₂ and was constructed using the primers: 5' -TTATAAGAATTCAGGAGGTG-3' and 5' -TGATTTAAGCTTCTATTGTAT-3' to amplify the 5' region; 5' -AAATGTAAAGCTTCCACGAAA-3' and 5' -AAATCCAGAATTCGATTATCC-3' for the 3' region.

The construction of the pGh9 Δ *orf2*_{Z1,2} plasmid containing 2 stop codons (TAA, TAA) in the 2 first codons of *orf2* was carried out using the following set of primers: 5' -TCGAAGAATTCGTTTACG-3' and 5' -TCACCTGAAGCTTATTACATGGT-3' for the 5' region; 5' -AACCATGTAATAAGCTTCAGGTGAT-3' and 5' -TTTGATGAATTCTAGTATATGT-3' for the 3' region.

The pGh9 Δ *rggC*_{8T} plasmid, carrying a thymine point deletion allowing a *RggC*₁-*C*₂ fusion, was constructed. To generate this mutation, the 5' and 3' regions of *rgg* were amplified with the following sets of primers: I₁ and 5' -CTAACGCTAAAAAAATTTGCTG-3' for the 5' region; 5' -CAAATTTTTTTTAGCGTTAGATGC-3' and I₂ for the 3' region. These 2 products were mixed together, used as templates in a PCR reaction to generate a full length fragment and cloned into pGh9 vector to produce the pGh9 Δ *rggC*_{8T} plasmid. This recombinant plasmid DNA was extracted and used to transform *S. thermophilus* CNRZ368 cells as previously described by (O'Sullivan and Fitzgerald 1999). Allelic gene replacements were done as previously described (Biswas et al. 1993) with the following modifications: the medium used for *S. thermophilus* growth was M17, 3 cycles of growth were carried out with two steps, one at 30°C (permissive temperature) and another one at 42°C (restrictive temperature). The chromosomal structure of mutant alleles was checked by PCR, southern blot analysis and sequencing.

6. Menadione assay

S. thermophilus and its mutant derivatives were grown overnight in milk medium and diluted 100 times into TPPY liquid medium. Log-phase cultures ($OD_{600} = 0.6$) were exposed for 3h to menadione concentrations (0 to 72 mM). Aliquots of the culture were then diluted in TPPY and appropriate dilutions plated on TPPY agar plates. The plates were stocked in jars containing a GENbox anaer system (bioMérieux) providing hypoxic conditions. Colony-forming units (CFU) were counted after 20 h of incubation and the percentage of surviving CFUs was determined by comparing viability with or without treatment. All experiments were performed independently at least three times.

RESULTS

1. Characterisation of the disrupted loci in *osrC* genome

In a previous study, the *osrC* mutant of *S. thermophilus* CNRZ368 was characterised in its resistance to menadione, a superoxide-generating agent (Fernandez et al. 2004). In this mutant, pGhost9::ISS1 was inserted in *rggC2* region. The *rggC1* ORF encodes a predicted 65 aa protein exhibiting 77% identity to the C-terminal region of *S. mutans* Rgg. The second ORF, *rggC2* encodes a putative 232 aa protein sharing 54 % identity with the N-terminal region of *S. mutans* Rgg. These two ORFs could initially have constituted a single ORF, having undergone a frameshift mutation (Fig.1A). This mutational event would have occurred between positions 886 to 894, where a 9-mer (TTT TTT TTT) known to be genetically unstable is observed. Ribosome-binding site (RBS) consensus sequence (AAAGGAGG in Gram-positive bacteria) scan revealed a potential RBS (AGGAGG) located 8 bp upstream *rggC1*. No canonical RBS sequence-preceding *rggC2* was detected upstream of the first putative start codon or within the CDS of *rggC1*. These results suggest that only RggC1 or a full fusion protein RggC1-2, could be produced, indicating a translational or transcriptional frameshifting.

Since Rgg proteins often regulate adjacent genes (Sulavik and Clewell 1996; Lyon et al. 1998; Chaussee et al. 1999; Qi et al. 1999; Neely et al. 2003), the ORFs localised downstream of

the *rggC* locus were analysed. *RggC*₂ is followed by a small ORF, *orf*₂ that encodes a putative 35 aa protein with no significant homology to any database sequences. *orf*₂ is preceded by a canonical RBS with the sequence, AAAGGAGG suggesting it was expressed. Downstream *orf*₂, there is an *rsp* gene (for radical SAM protein) that encodes a putative protein sharing 29 % identity (46% similarity) with a possible Fe-S oxidoreductase of *Thermoanaerobacter tengcongensis* belonging to the radical SAM protein family (Sofia et al. 2001). A RBS sequence (GGAGG) was also found upstream *rsp*.

The transcriptional signals scan analysis allowed us to identify a potential promoter (TATGATATAAT), located 61 bp upstream of *rggC*₁, and constitute an extended –10 region (Voskuil and Chambliss 1998). Moreover, a putative rho-independant terminator consisting of a perfect inverted repeat of 10 bp followed by a stretch of T's, was visualized 9 bp downstream of the *orf*₂ stop codon. A potential promoter located upstream *rsp* was identified including a –10 (TGTAAT) and a –35 (TTTAGA) region with a 15 bp spacer.

2. Transcriptional analysis of the *rggC* locus

Northern blot analysis using *rggC*₂ antisense-RNA (nucleotides 932 to 1464) as a probe was performed on RNA extracted from stationary phase cells. One transcript of approximately 1200 nucleotides was detected (Fig.1B). Since the *rggC*₂ gene size is 0.7 kb, this transcript observation is compatible with the polycistronic transcription of *rggC*₂ with the neighbouring ORFs.

To better characterise this transcript, RT-PCR analyses were carried out. cDNA generated from *S. thermophilus* CNRZ368 RNA were used in PCRs experiments using primers P₁/P₂ and P₃/P₄ localized in *rggC*₁/*rggC*₂ and *rggC*₂/*orf*₂ respectively (Fig. 1C). In both cases, a PCR product of the expected size (310 bp and 265 bp respectively) was amplified suggesting the existence of at least one transcript including *rggC*₁, *rggC*₂ and *orf*₂. The transcript size was in agreement with the transcription signals visualized on the sequence.

3. Involvement of the *rggC* locus in the oxidative stress defence

Since *rggC* locus exhibits operon structure, the resistance phenotype could be either the result of the inactivation of *rggC₂* gene or/and the result of polar effects on the *rggC₁* and *orf2* neighbouring genes. In this study, non-polar Δ *rggC₁*, Δ *rggC₂* and *orf2* mutants were constructed via allelic replacement and their survival in presence of menadione was studied (see materials and methods). Compared to the reference strain, the Δ *rggC₁* mutant displayed a 2-fold higher resistance following exposure to menadione at concentrations of 27 mM and 36 mM (Fig. 2A). The Δ *rggC₂* mutant exhibited a 2-fold decreased of surviving cells at a menadione concentration of 9 mM, 18 mM and 27 mM (Fig. 2B). Thus, a decreased survival was observed for the Δ *rggC₂* mutant, while the survival of the Δ *rggC₁* strain was enhanced indicating the involvement of both of these ORFs in *S. thermophilus* oxidative stress response. In contrast, *orf2* mutant survival was the same as the reference strain (data not shown) suggesting that *orf2* was not involved in this pathway.

4. Intraspecific polymorphism of *rggC* potential shift site

The analysis of the DNA sequence of the *rggC* locus predicted 2 ORFs: *rggC₁* and *rggC₂*. A sequence of 9 T's was observed at the 3' end of *rggC₁*. Since such mononucleotide repeats are known to exhibit high frequencies of nucleotide deletion or insertion events, the potential polymorphism of the *rggC* shift site was investigated among 21 strains *S. thermophilus*. The *rggC₁* gene was shown to be present in all 21 studied strains, using PCR amplification with primers V1 and V2 designed to bind the 5' part of *rggC₁* and the central part of *rggC₂*, respectively. The frameshift mutation of *rggC₁* was analysed by sequencing the PCR products with primer V1. Four different sequences were found varying from 7 to 9 T's (Table 2). A deletion of one A upstream of the T tract in *rggC* from CNRZ1402, CNRZ455 and LMD9 strains was also observed. The strain ATCC1958 is characterised by the presence of 8 instead of 9 Ts.

This mutation would result in formation of an in-frame ORF, allowing potentially full-length RggC protein translation whereas in all the other cases a RggC₁ protein was expected.

5. Involvement of RggC in oxidative stress defence of *S. thermophilus* CNRZ368

To determine the involvement in oxidative stress defence of a full-length RggC protein, an additional *rggC_{8T}* mutant was constructed via allelic replacement. This mutant from CNRZ368 contains an eight T chromosomal sequence. Its survival in the presence of different concentrations of menadione was determined and showed to be reduced at a menadione concentration of 18 mM, 27 mM and 36 mM compared to wild-type confirming a role for *rggC* in *S. thermophilus* CNRZ368 oxidative stress response (Fig. 3).

DISCUSSION

Among the fully sequenced streptococcal genomes, that of *S. thermophilus* exhibits the highest proportion of pseudogenes. Indeed, 10% of the *S. thermophilus* genes are characterised by frameshift, nonsense mutation, deletion or truncation and are therefore considered as non functional (Bolotin et al. 2004). Here, we examined *rggC*, considered in *S. thermophilus* as a pseudogene, and we demonstrated its role in *S. thermophilus* oxidative stress response.

In *osrC* mutant, the disruption of *rggC₂*, with pGh9:ISS1, confers to the cells a resistance to menadione. This insertion mutant shows a polar mutation affecting the transcript stability of the *rggC* locus and of the neighbouring *rsp* gene.

To understand this phenomenon, deletions of *rggC₁* and *rggC₂* were undertaken and demonstrated that both ORFs were involved in oxidative stress response. However, these 2 mutants displayed 2 distinct phenotypes: one being resistant and the other sensitive to oxidative stress. Two alternative hypotheses can explain this. Firstly, the 2 proteins RggC₁ and RggC₂ could be synthesised and would be characterised by different activities. Nevertheless, the lack of a RBS sequence-preceding *rggC₂* is a strong argument in disfavour of this hypothesis. Secondly,

the $\Delta rggC_2$ phenotype could result from the synthesis of a full RggC protein by frameshifting as observed in translational or transcriptional slippage (for review : Baranov et al. 2005).

Taking all these data into account, both RggC₁ and RggC proteins are likely to be synthesised in the wild type strain.

In CNRZ368 strain, a stretch of 9 T residues could allow a transcriptional slippage, and consequently lead to the RggC fusion protein by translation of a subpopulation of *rggC* transcript in the alternate reading frame. Alternatively, since RNA-DNA hybrids of 9 are described as genetically unstable they could provide RNA polymerase slippage by re-alignment of the nascent RNA chain and its DNA template (Wagner et al. 1990). Few examples of transcriptional slippage are identified in prokaryotes. In *Thermus thermophilus*, τ and γ subunit proteins of DNA polymerase III are synthesized from *dnaX* gene by this mechanism. This ORF shows 9 adenine residues allowing the slippage of RNA polymerase and producing many mRNAs with additional or subtractive adenine residues (Larsen et al. 2000). In *Shigella flexneri*, the *mixE* gene, encoding a transcription activator, consists of 2 overlapping ORFs. A track of 9 thymine residues is observed at the site of overlapping. Transcriptional incorporation of one additional nucleotide allows synthesis of the functional MixE protein (Penno et al. 2005).

The putative RggC protein belongs to the family of Rgg-like transcriptional regulators. RggC₁ displayed the typical N-terminal helix-turn-helix motif suggestive of a DNA-binding function. Among characterised Rgg-like proteins, *rgg* from *Streptococcus gordonii*, *rgg* and *ropB* from *S. pyogenes* are located immediately adjacent to their regulatory target sequence (Sulavik and Clewell 1996; Lyon et al. 1998; Chaussee et al. 1999; Qi et al. 1999; Neely et al. 2003). Thus, the transcriptional expression of *orf2* and *rsp* ORFs from *S. thermophilus* CNRZ368 was analysed by quantitative RT-PCR in $\Delta rggC_1$, $\Delta rggC_2$ and wild-type genetic contexts (data not shown). Partial deletion of *rggC* locus did not affect the expression of *orf2* and *rsp* ORFs compared with the level seen in the wild type CNRZ368 strain. However, *S. thermophilus* genome contains 7 *rgg* genes (Bolotin et al. 2004). It is therefore possible that one of the Rgg proteins replace RggC function in the defective strain. Moreover, it still is possible that RggC

directly or indirectly regulates the transcription of other genes since deletion of *rgg* from *S. pyogenes* has been demonstrated to have genome wide effects on virulence-associated transcription (Chaussee et al. 2001; Chaussee et al. 2002).

Analysis of 21 *S. thermophilus* *rgg* sequences revealed a polymorphism of the 9 T residue region. Repeats of a single or several nucleotides, named microsatellite DNA, are involved in numerous phase variation events that could be defined as a high-frequency and reversible mechanisms of gene-switching (for review: (Hallet 2001). This inter-strain length polymorphism supports evidence for the phase variability of the *rggC* locus. Some cases of phase variation have been identified in the *Streptococcus* genus, allowing the control of transcriptional or translational expression (Lukomski et al. 2001; Puopolo and Madoff 2003). A phase variation event at the *rggC* locus could influence a potential programmed frameshifting by inactivation.

In 20/21 cases, a +1 or -1 frameshift mutation was observed resulting in a 'gene off' configuration. If the full-length *rggC* ORF was an ancestral allele, it is possible that alleles showing this frameshift mutation may have been selected for a growth advantage associated with the loss of *rggC* function. The menadione sensitivity phenotype observed for the *rggC_{8T}* mutant (whose *rggC* phase is restored) argued in the favour of that hypothesis.

The results of this study indicate the involvement of the *rggC* locus in *S. thermophilus* CNRZ368 oxidative stress response. Accordingly, the Rgg transcriptional regulatory protein from *S. pyogenes* that coordinates virulence factor synthesis and catabolic activity (Chaussee et al. 2003) was also demonstrated to be involved in thermal and in oxidative stress response (Chaussee et al. 2004). The *S. pyogenes* *rgg* mutant strain was more susceptible than wild-type to paraquat (a hydroxyl ion radicals generating compound) and was characterised by an elevated level of the oxidoreductases AhpC and Nox1 (Chaussee et al. 2004). All these data suggest a role for the Rgg family members in oxidative stress response, and more generally that these proteins may be important in the *streptococcus* adaptation to environmental changes.

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Strains and plasmids	Relevant feature(s)	Source and/or citation
<i>S. thermophilus</i>		
CNRZ007	Wild-type strain	CNRZ, INRA strain collection
CNRZ302	Wild-type strain	CNRZ, INRA strain collection
CNRZ307	Wild-type strain	CNRZ, INRA strain collection
CNRZ308	Wild-type strain	CNRZ, INRA strain collection
CNRZ368	Wild-type strain	CNRZ, INRA strain collection
CNRZ385	Wild-type strain	CNRZ, INRA strain collection
CNRZ388	Wild-type strain	CNRZ, INRA strain collection
CNRZ391	Wild-type strain	CNRZ, INRA strain collection
CNRZ445	Wild-type strain	CNRZ, INRA strain collection
CNRZ455	Wild-type strain	CNRZ, INRA strain collection
CNRZ702	Wild-type strain	CNRZ, INRA strain collection
CNRZ1066	Wild-type strain	CNRZ, INRA strain collection
CNRZ1068	Wild-type strain	CNRZ, INRA strain collection
CNRZ1402	Wild-type strain	CNRZ, INRA strain collection
ATCC19258	Wild-type strain	American Type Culture Collection
ATCC19987	Wild-type strain	American Type Culture Collection
LMG18311	Wild-type strain	BCCM/LMG strain collection
A054	Wild-type strain	Lab collection
IP6756	Wild-type strain	Institut Pasteur strain collection
IP6757	Wild-type strain	Institut Pasteur strain collection
<i>osrC</i>	Em ^R <i>S. thermophilus</i> CNRZ368 containing integrated pGh9::ISS1 into <i>rggC</i> ₂	((Fernandez et al. 2004)
<i>ΔrggC</i> ₁	CNRZ368 strain carrying a deletion from nt 50 to 127 of <i>rggC</i> ₁	This study
<i>ΔrggC</i> ₂	CNRZ368 strain carrying a deletion from nt 318 to 619 of <i>rggC</i> ₂	This study
<i>orf2</i>	CNRZ368 strain carrying 2 stop codons in the first codon of <i>orf2</i>	This study
<i>rggC</i> _{8T}	CNRZ368 strain presenting an in-frame <i>rggC</i> gene	This study
<i>E. coli</i>		
EC101	TG1 derived strain [<i>supE hsd-5 thi Δ(lac-proAB)</i> F' (<i>traD6proAB⁺ lacI^q lacZΔM15</i>)] containing a chromosomal copy of the pWV01 <i>repA</i> gene	(Leenhouts 1995)
Plasmid		
pBKS	Amp ^R	Stratagene
pGhost9	Em ^R , thermosensitive replication origin from pVE6002	(Maguin et al. 1996)
pGh9 <i>ΔrggC</i> ₁	pGh9 plasmid carrying <i>rggC</i> ₁ deleted from position 50 to 127	This study
pGh9 <i>ΔrggC</i> ₂	pGh9 plasmid carrying <i>rggC</i> ₂ deleted from position 318 to 619	This study
pGh9 <i>orf2</i> _{21,2}	pGh9 plasmid carrying <i>orf2</i> with 2 stop codons inserted in the first and the second codon	This study
pGh9 <i>rggC</i> _{8T}	pGh9 plasmid carrying an in-frame <i>rggC</i> gene	This study

427 **Table 2. Analysis of the *rggC* locus in 21 strains of *S. thermophilus***

Sequence	Strain name	Mutation
AGC AAA TTT TTT TTT AGC	A054, ATCC19987, CNRZ007, CNRZ302, CNRZ307, CNRZ368, CNRZ385, CNRZ388, CNRZ391, CNRZ702, LMG18311, IP6756	+1 FS
AGC AAA _TT TTT TTT AGC	ATCC19258	/
AGC AA _TT TTT TTT AGC	CNRZ308, CNRZ445, CNRZ1066, CNRZ1068, IP6757	-1 FS
AGC AA _ _T TTT TTT AGC	CNRZ455, CNRZ1402, LMD9 ^a	+1 FS

428

429 ^a according to sequence available <http://www.jgi.doe.gov>.

430

FIGURE LEGENDS

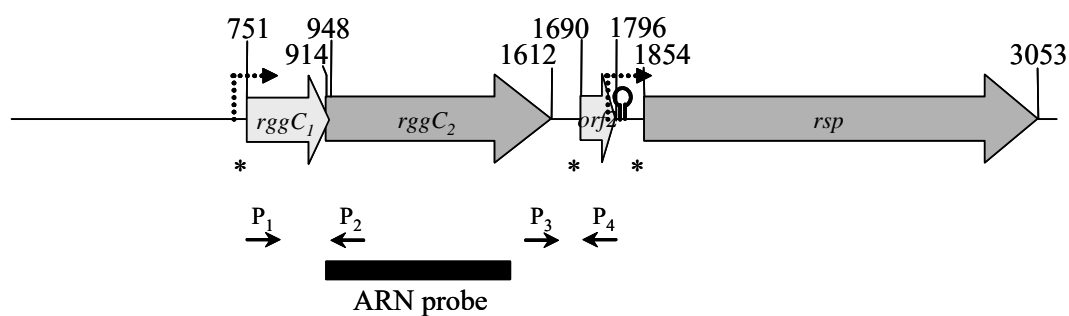
Fig. 1 Organisation of the *rggC* locus of *S. thermophilus* CNRZ368. (A) The solid line represents the *S. thermophilus* genomic DNA. ORFs are indicated as open arrows. The broken arrow and the hairpin loop indicate the putative promoter and rho-independent terminator respectively. Putative RBS localisation is indicated by asterisks. Primers used for RT-PCR experiments are represented by small arrows under ORFs. The expected size of the P₁-P₂ and P₃-P₄ PCR fragments is 310 bp and 265 bp, respectively. ARN probe localisation is indicated by the black box. (B) Northern blot analysis of *rggC*₂ transcripts. Left: gel electrophoresis staining with ethidium bromide: lane 1 is RNA size marker and lane 2 is RNA (30µg) total extracted from *S. thermophilus* CNRZ368. 16S and 23S indicate the migrating positions of 16S and 23S rRNA, respectively. Right: result of the hybridisation with the *rggC*₂ RNA probe. (C) Gel electrophoresis showing the RT-PCR products obtained using the primers P₁-P₂ and P₃-P₄. *Pst*I-digested λ DNA used as a DNA fragment size marker is indicated by λ *Pst*I.

Fig. 2 Survival of *ΔrggC*₁ and *ΔrggC*₂ mutants and the reference strain with increasing menadione concentrations. The graphs point the average values obtained from experiments performed at least in triplicate. The error bars represent standard deviations.

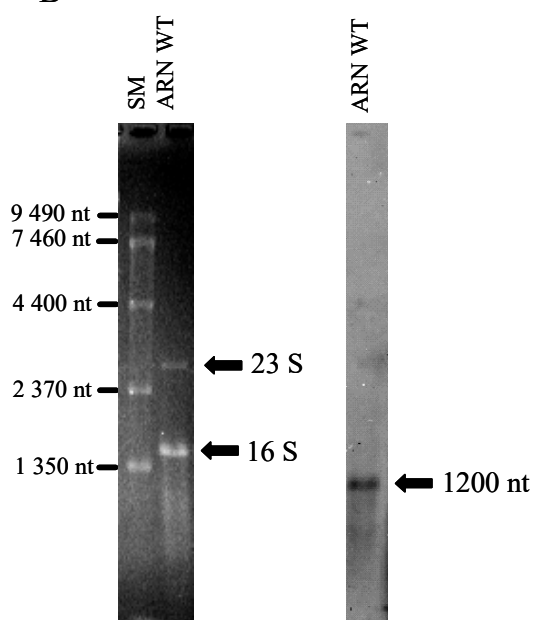
Fig. 3 Survival of *rggC*₈₇ mutant and the reference strain with increasing menadione concentrations. The graphs point the average values obtained from experiments performed at least in triplicate. The error bars represent standard deviations

Fig. 1

A



B



C

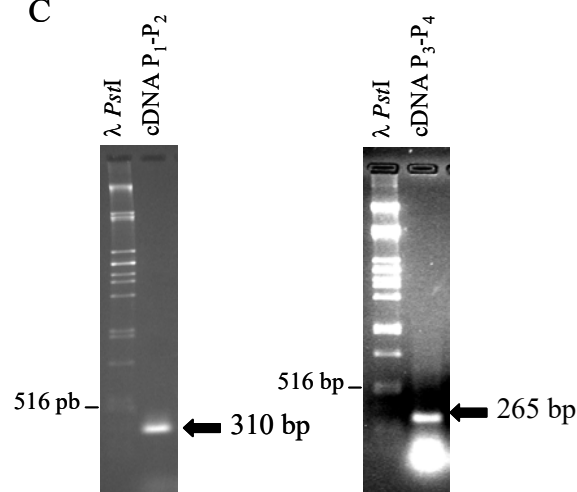
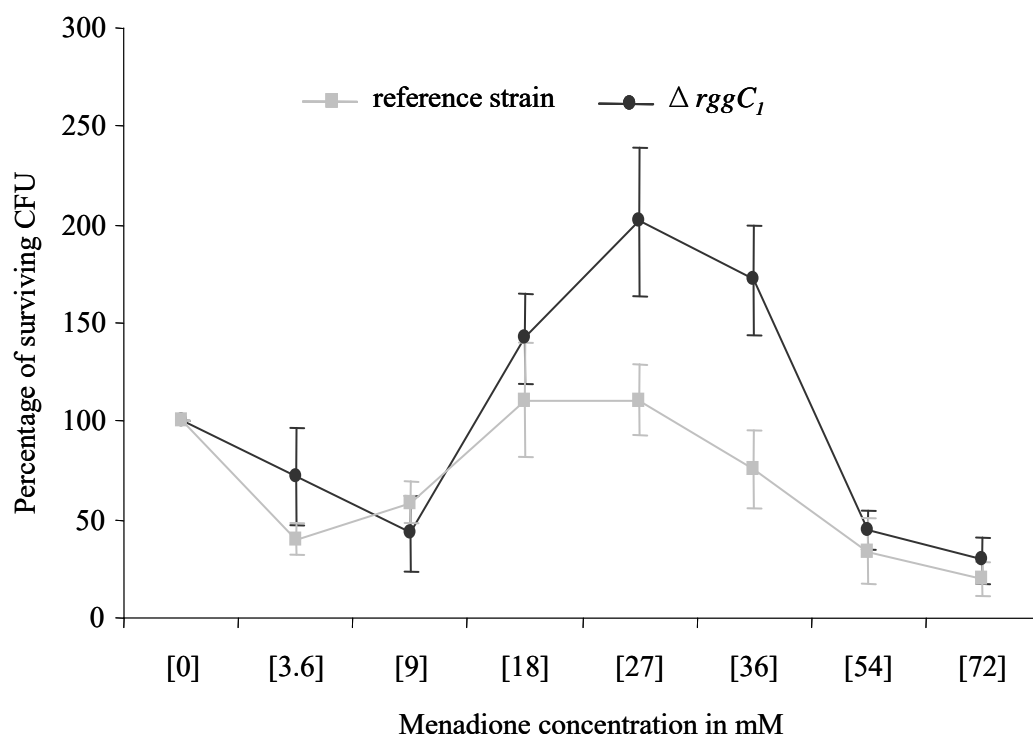


Fig. 2

A



B

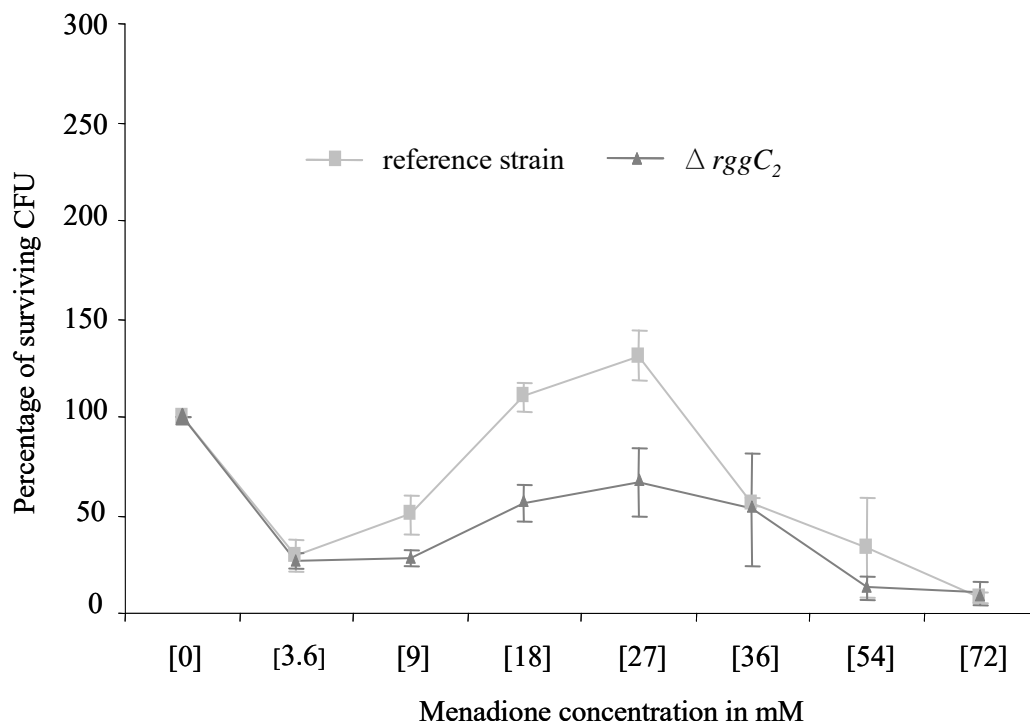


Fig. 3

