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Research paper

The thioredoxin system in the dental caries pathogen *Streptococcus mutans* and the food-industry bacterium *Streptococcus thermophilus*

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ABSTRACT

The *Streptococcus* genus includes the pathogenic species *Streptococcus mutans*, the main responsible of dental caries, and the safe microorganism *Streptococcus thermophilus*, used for the manufacture of dairy products. These facultative anaerobes control the levels of reactive oxygen species (ROS) and indeed, both *S. mutans* and *S. thermophilus* possess a cambialistic superoxide dismutase, the key enzyme for a preventive action against ROS. To evaluate the properties of a crucial mechanism for repairing ROS damages, the molecular and functional characterization of the thioredoxin system in these streptococci was investigated. The putative genes encoding its protein components in *S. mutans* and *S. thermophilus* were analysed and the corresponding recombinant proteins were purified. A single thioredoxin reductase was obtained from either *S. mutans* (*Sm*TrxB) or *S. thermophilus* (*St*TrxB1), whereas two thioredoxins were prepared from either *S. mutans* (*Sm*TrxA and *Sm*TrxH1) or *S. thermophilus* (*St*TrxA1 and *St*TrxA2). Both *Sm*TrxB and *St*TrxB1 reduced the synthetic substrate DTNB in the presence of NADPH, whereas only *Sm*TrxA and *St*TrxA1 accelerated the insulin reduction in the presence of DTT. To reconstitute an *in vitro* streptococcal thioredoxin system, the combined activity of the thioredoxin components was tested through the insulin precipitation in the absence of DTT. The assay functions with a combination of *Sm*TrxB or *St*TrxB1 with either *Sm*TrxA or *St*TrxA1. These results suggest that the streptococcal members of the thioredoxin system display a direct functional interaction between them and that these protein components are interchangeable within the *Streptococcus* genus. In conclusion, our data prove the existence of a functioning thioredoxin system even in these microaerophiles.

1. Introduction

Streptococci belong to the group of gram-positive facultative anaerobic bacteria [1], whose energetic metabolism mainly depends on the glycolytic pathway, followed by the lactic fermentation [2]. Most of the *Streptococcus* species are known to be human pathogens, and some of them are highly virulent, such as *Streptococcus pneumoniae* or *Streptococcus agalactiae*, the causative agents of serious acute infections [3,4]. Other pathogens, such as *Streptococcus mutans* or *Streptococcus sobrinus*, are much less virulent, being part of the resident microflora of the human body. For instance, *S. mutans*, the main responsible in the aetiology of dental caries, colonizes the oral cavity from the early teeth eruption in infants [5,6]. Conversely, another species, *Streptococcus thermophilus*, is non pathogenic, being generally considered as a safe microorganism [7,8]. Indeed, *S. thermophilus* is vastly used for the manufacture of dairy products and therefore has a great importance in food industry [9–11].

Streptococci, such as *S. mutans* and *S. thermophilus*, are unable to use oxygen as an electron acceptor in aerobic respiration and their anaerobic metabolism is consistent with the absence of genes encoding heme-containing proteins, such as catalase and cytochrome oxidase, in the streptococcal genomes [12–14]. However,

Abbreviations: DTNB, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB); DTT, dithiothreitol; E_a, energy of activation; IPTG, isopropyl-β-thiogalactopyranoside; ROS, reactive oxygen species; *Sm*, *Streptococcus mutans*; *St*, *Streptococcus thermophilus*; SOD, superoxide dismutase; TrxA, thioredoxin; TrxB, thioredoxin reductase; Trx-S_2 and Trx-$(SH)_2$, oxidised and reduced form of TrxA, respectively.

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streptococci, usually growing in almost anaerobic ecological niches, tolerate moderate oxygen concentrations, being able to promote the induction of some specific scavengers of the reactive oxygen species (ROS) generated during the oxygen exposure [15–19]. For instance, streptococci eliminate the superoxide anion, the first ROS produced from the univalent reduction of oxygen, through the enzymatic scavenger action of superoxide dismutase (SOD) [20,21]. In particular, *S. mutans* and *S. thermophilus* possess a cambialistic SOD, which is adapted to the anaerobic or aerobic growth environment, thanks to the different properties exhibited by the Fe- and Mn-isoform of this metal enzyme [22,23].

Another key element for the cellular protection against ROS damages is the thioredoxin system, aimed at the removal of disulfide bridges eventually formed in intracellular proteins following an oxidative stress [24–26]. This system is composed by two protein components, thioredoxin (TrxA) and thioredoxin reductase (TrxB). TrxA is a small monomeric protein (10–15 kDa) containing a conserved CXXC sequence motif, in which the cysteine residues form a reversible disulfide bridge [24]. A similar sequence element is present also in bacterial TrxB, a NADPH-dependent flavoenzyme with a homodimeric organization and whose molecular mass is around 35 kDa/subunit [27,28]. The reparative mechanism of the thioredoxin system involves oscillation between oxidised and reduced form of both cysteine motifs of TrxA and TrxB; the role of NADPH as electron donor and the FAD/$FADH_2$ oscillation in the TrxB active site are also required for the activity [24,26]. Briefly, TrxB catalyses the NADPH-dependent electron transfer to the active disulfide of the oxidised form of TrxA, via an intermediate step involving the bound FAD molecule; in turn, the dithiolic form of TrxA reduces the disulfide bridge of the target proteins, thus contributing to the maintenance of the reducing intracellular environment. The thioredoxin system is often redundant in some eubacteria, because of the presence of more than one TrxA and/or TrxB [29]. This finding could be related to the necessity of specific thioredoxins for different groups of target proteins; furthermore, the different TrxAs could interact with specific TrxBs or with the same TrxB. Redundancy of the thioredoxin system also suggests the relevance of this reparative mechanism for the survival of the microorganism, a role becoming more crucial when the source is exposed to a prolonged oxidative stress.

This work reports a study on the thioredoxin system in *S. mutans* and *S. thermophilus*. Until now, the information on this antioxidant repairing system is limited to the annotation of the putative genes encoding TrxA and TrxB in the genomes of *S. mutans* and *S. thermophilus*, which indicated a gene redundancy in both sources [13,14]. Therefore, the functionality and the role of the corresponding protein components was assessed through a biochemical investigation on the purified recombinant forms of TrxA and TrxB obtained through the heterologous expression of the specific streptococcal genes. The study was focused on the identification of the actual components of the thioredoxin system in *S. mutans* and *S. thermophilus*, thus allowing the *in vitro* reconstitution of a crucial mechanism for repairing the damages produced during an oxidative stress suffered from these streptococci.

2. Materials and methods

2.1. Chemicals, enzymes, and buffers

FAD, β-NADPH, β-$NADP^+$, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), dithiothreitol (DTT), β-mercaptoethanol, isopropyl-β-thiogalactopyranoside (IPTG) and a solution of human insulin (10 mg mL^{-1}) were from Sigma–Aldrich. Restriction/modifying enzymes were from Promega or GE Healthcare. Plasmid pGEM-T Easy was Promega. Vectors pET-22b(+) and pET-28a(+) and the *Escherichia coli* BL21(DE3) strain were from Novagen. The chromatographic medium Ni-NTA agarose was from Qiagen. Oligonucleotide synthesis and nucleotide sequencing was carried out at CEINGE, Italy. Synthesis of pET derivatives was carried out by GenScript, USA. HPLC-grade solvents were obtained from Carlo Erba. All other chemicals were of analytical grade.

The following buffers were used: buffer A, 20 mM Tris·Cl, pH 7.8; buffer B, 100 mM potassium phosphate, pH 7.8, 10 mM EDTA; buffer C, 20 mM Tris·Cl, pH 7.8, 150 mM KCl.

2.2. Engineering of vectors for thioredoxin and thioredoxin reductase genes in S. mutans and S. thermophilus

Transformation of bacterial strains, preparation of plasmids and other details of DNA recombinant technology were as previously described [30]. For the preparation of vectors producing recombinant forms of the streptococcal thioredoxins and thioredoxin reductases, the corresponding genes annotated in the genome of *S. mutans* UA159 and *S. thermophilus* LMG 18311 were considered [13,14]. *S. mutans* UA159 contains two genes encoding putative thioredoxin reductases (*trxB* with ID: 1029596; *trxB2* with ID: 1029447) and three genes encoding putative thioredoxins (*trxA* with ID: 1029078; unnamed gene encoding a putative thioredoxin H1, hereafter called *trxH1*, with ID: 1029541; unnamed gene encoding a putative longer thioredoxin, hereafter called *trxL*, with I.D. 1028478) [13]. A somehow lower redundancy exists in the genome of *S. thermophilus* LMG 18311, as it contains two genes for putative thioredoxin reductases (*trxB1* with ID: 3164148; *trxB2* with ID: 3165597) and two genes for putative thioredoxins (*trxA1* with ID: 3163813; *trxA2* with ID: 3164959) [14]. The heterologous expression regarded all these genes, with the exclusion of the *trxB2* gene from either *S. mutans* or *S. thermophilus*, as explained in the Results section. The complete coding sequence of each gene was amplified by PCR, using the genomic DNA from *S. mutans* UA159 or *S. thermophilus* LMG 18311 as a template, and the couples of oligonucleotide primers reported in Table 1, annealing to the 5′- or 3′-untranslated region of the gene. Each PCR product was

Table 1
Primers for the amplification of genes encoding the putative protein components of the thioredoxin system in *S. mutans* and *S. thermophilus*.

Source	Gene	Primer[a]	
		Forward	Reverse
S. mutans	*trxB*	5′d-A$_{-14}$AGGAAAGTAACAT•*ATG*•TAC•GAT•ACA$_{12}$-3′	5′d-T$_{925}$TTCAAATCC•CTC•GAG•GTT•ATT•GAC•AAT•A$_{897}$-3′
	trxA	5′d-A$_{-15}$AGGAGAAATAACAT•*ATG*•ACA•AAA•GTA$_{12}$-3′	5′d-C$_{325}$CTGCTTATA•CTC•GAG•AAG•TTC•GGC•T$_{300}$-3′
	trxH1	5′d-A$_{-15}$TGAGGAGGATACAT•*ATG*•ATT•GTT•CCA•AAA$_{15}$-3′	5′d-T$_{345}$ATTATATGTAAAAA•CTC•GAG•TTT•CTC•CTC•TGT•TAA$_{310}$-3′
	trxL	5′d-T$_{-17}$TAAAAAGGAGATAGCC•*ATG*•GAA•AAG•GTT•ATT•T$_{16}$-3′	5′d-A$_{579}$GACGGGGAATTTAG•CTC•GAG•CAA•TTT•TTT•TAG$_{547}$-3′
S. thermophilus	*trxB1*	5′d-T$_{-13}$AAGGAGTTACAT•*ATG*•TAC•GAT•AC$_{11}$-3′	5′d-T$_{931}$CCATTTATG•CTC•GAG•ATC•ACC•AAG•TG$_{905}$-3′
	trxA1	5′d-A$_{-14}$GGAGGATAATCAT•*ATG*•ACA•AAA•GT$_{11}$-3′	5′d-C$_{325}$CATGATTAC•CTC•GAG•AAG•CTC•AGA$_{301}$-3′
	trxA2	5′d-G$_{-14}$AGGAGGAACACAT•*ATG*•ATT•ATT•CC$_{11}$-3′	5′d-T$_{325}$GGATTCTCC•CTC•GAG•TGT•TTC•CAA$_{301}$-3′

[a] Numbering begins from the starting codon shown in italics; underlined nucleotides indicate the mismatches introduced to create the cloning sites *Nde*I, *Nco*I or *Xho*I; dots in nucleotide sequence separate coding triplets.