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REVIEW ARTICLE

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Oxidative stress response: a critical factor affecting the ecological competitiveness of *Streptococcus mutans*

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ABSTRACT

Oral microecological balance is closely associated with the development of dental caries. Oxidative stress is one of the important factors regulating the composition and structure of the oral microbial community. *Streptococcus mutans* is linked to the occurrence and development of dental caries. The ability of *S. mutans* to withstand oxidative stress affects its survival competitiveness in biofilms. The oxidative stress regulatory mechanisms of *S. mutans* include synthesis of reductase, regulation of metal ions uptake, regulator PerR, transcription regulator Spx, extracellular uptake of glutathione, and other related signal transduction systems. Here, we provide an overview of how *S. mutans* adapts to oxidative stress and its influence on oral microecology, which may offer novel options to investigate the cariogenic mechanisms of *S. mutans* in the oral microenvironment, and new targets for the ecological prevention and treatment of dental caries.

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Introduction

Dental caries, with high incidence and prevalence, affects oral and general health of both children and adults worldwide, resulting in a major public health concern. The occurrence and development of dental caries is associated with the homeostasis of dental plaque biofilm, and microbial synergistic and antagonistic interactions in the biofilm play a significant role in maintaining the oral microecological balance [1]. Dental plaque biofilms are frequently stimulated by various environmental factors in the oral cavity. Oxidative stress contributes to the spatial and temporal development of oral biofilms, playing important roles in biofilm homeostasis maintenance.

Oxidative stress refers to an imbalanced state between the oxidation and antioxidant systems, which normally regulate cellular metabolism and activate a series of signaling pathways [2]. Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), are critical origins of oxidative stress. ROS mainly exert toxicity in oxidative inactivation of iron-containing enzymes, including both iron/sulfur-containing dehydratases and mononuclear iron enzymes. H_2O_2 reacts with ferrous iron through Fenton chemistry, and the resulting highly reactive hydroxyl radical can impair metabolism through DNA and protein damage, inevitably leading to cell death [3]. Oxidative stress in oral biofilms can be triggered by diatomic environmental oxygen present in the oral cavity, human physiological activities (e.g. the use of oral care products containing H_2O_2), as well as bacteria-derived H_2O_2. H_2O_2 generated by commensal bacteria such as *Streptococcus sanguinis* and *Streptococcus gordonii* can damage DNA and thwart the growth of competing biofilm members, especially the cariogenic species *Streptococcus mutans*. Therefore, H_2O_2 is able to mediate microbial interactions and shape the homeostasis of dental plaque, playing crucial roles in the pathogenesis of dental caries [4,5].

S. mutans is a major etiological agent of human dental caries. *S. mutans* can rapidly metabolize a wide variety of carbohydrates to produce acid, and survive in low pH environments; it can also produce extracellular matrix contributing to the biofilm formation and adapt to frequent environmental challenges encountered in oral biofilms [6]. *S. mutans* typically lacks catalase and relies on other mechanisms to respond to the oxidative stress, such as synthesis of reductases and regulation of metal homeostasis [7,8]. *S. mutans* with reduced oxidative stress tolerance exhibits inhibited virulence factor expression and weakened competitiveness in oral biofilms [9].

Therefore, investigating and getting more information about how *S. mutans* adapts to the complex oxidative stress may facilitate designing targeted small

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molecular compounds to weaken the virulence of *S. mutans* and modulate the oral microecology. Here, we provide an overview about the oxidative stress regulatory mechanisms of *S. mutans*, including synthesis of reductases, regulation of metal ions uptake, PerR and Spx regulators, glutathione (GSH) pathway, and other related signal transduction systems. We also highlight gaps in the field, and discuss the way forward. This review may help provide novel options for investigating the cariogenic mechanisms of *S. mutans* and guide the development and identification of new targets for the ecological management of dental caries.

Synthesis of reductases

S. mutans is a facultative anaerobic bacterium that can resist oxidative stress by synthesizing various reductases such as superoxide dismutase (SOD) and NADH oxidase (Nox). SODs are metalloenzymes that use metal ions such as Cu, Zn, Fe, and Mn as co-factors in bacteria. Mn and Fe SODs are generally intracellular while Cu and Zn SODs are extracellular. In *S. mutans*, SOD is active with either Mn or Fe as a co-factor depending on which metal is available in the culture medium [10]. Kono et al. [7] demonstrated that SOD mutant strains of *S. mutans* showed slowed growth and reduced tolerance to H_2O_2. MnSOD or FeSOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen, playing important roles in the protection of *S. mutans* against toxic O_2^- [10].

The NADH oxidase Nox is a flavin-containing oxidoreductase. *S. mutans* reduces O_2 to H_2O via Nox, preventing the formation of damaging ROS. Moreover, Nox oxidizes NADH to NAD^+, which is a carbon cycle metabolite. A previous study has demonstrated that Nox is a major oxygen-metabolizing enzyme used by *S. mutans*, responsible for approximately 40% reduction of the dissolved oxygen encountered in dental biofilm [11]. Nox lies at the intersection of multiple regulatory pathways. Nox is involved in the activity of global transcription factors Spx and Rex, being a key contributor to the growth of *S. mutans* under conditions of oxidative stress [12]. Poole et al. [13] reported that *S. mutans* Nox-1 was homologous with alkyl hydroperoxide reductase (AhpC), both of which are cysteine-based peroxidase systems that catalyze the reduction of organic hydroperoxides/H_2O_2 to alcohol or H_2O [13]. However, knocking out the genes encoding these two enzymes did not significantly affect the anti-oxidative stress ability of *S. mutans*, indicating that there are other antioxidant systems in *S. mutans* involved in oxidative stress resistance [13].

Roles of metal ions and regulation of their uptake

Bacterial metal regulatory proteins are the 'monitors' for maintaining the stable state of intracellular metal ions in bacteria and are essential for the physiological activity and pathogenicity of *S. mutans*. Metal ions such as Fe^{2+} and Mn^{2+} are important coenzyme factors in bacterial cells, and the accumulation of excess Fe ions in bacteria causes the Fenton reaction, which produces toxic hydroxyl radicals, resulting in reduced cell activity or cell death [8]. Unlike Fe ions, Mn ions do not trigger the Fenton chemistry. Mn itself acts as an antioxidant, and it is also a cofactor of superoxide dismutase that converts harmful hydroxyl radicals into harmless by-products, which play a key role in bacterial defense against oxidative stress [14].

Dpr is a member of the Dps family with iron-binding ability. Unlike SOD, Dpr does not react with oxygen and reactive oxygen species. Dpr inhibits the Fenton reaction and protects cells from oxidative damage by sequestering iron ions and preventing the interaction between iron and H_2O_2 to generate toxic hydroxyl radicals [15]. Tight regulation of intercellular free iron concentration is an important factor for bacterial survival under aerobic conditions. Yamamoto et al. [15] found that Dpr enabled bacteria to acquire oxygen tolerance by regulating intracellular-free iron ion concentration and played a major regulatory role in bacterial anti-oxidative stress responses. The *dpr* gene can be positively regulated by *S. mutans* SpxA1 and SpxA2 regulators [16]. The iron transporter operon is encoded by *smu995-smu998*. The *S. mutans dpr* mutant exhibits increased oxidative stress sensitivity due to the inability to sequester iron ions; however, the sensitivity phenotype can be reversed upon deletion of *smu995* or the entire *smu995-smu998* operon [17]. On the contrary, knockout of the gene encoding ferrous ion transport system (FeoABC) could not reverse the oxidative stress-sensitive phenotype of *dpr* mutant [17]. These findings suggest that Dpr works along with other metal transport systems of *S. mutans*, i.e. FeoABC and Smu995-Smu998 system, to coordinate iron uptake.

SloR is a metal-regulatory protein of *S. mutans* which belongs to the DtxR metalloregulator family. The *sloR* gene is located at the downstream of *sloABC* operon, which encodes a dual iron/manganese ABC transport system. SloR is transcribed from its own promoter as well as via transcriptional readthrough from the *sloABC* promoter. SloR modulates the expression of the *sloABC* operon and plays a critical role in bacterial Mn^{2+} homeostasis to defend against oxidative stress [18]. Expression of *sod*, *tpx* and *sloC* genes was upregulated in *S. mutans sloR* mutant strain compared with the wild-type and *sloC* mutant strains, suggesting that the effect of oxidative stress on *S. mutans* is more pronounced in the absence

of SloR [18]. Rolerson et al. [19] found through gel migration experiments that SloR could bind to the *sloABC, sloR, comDE, ropA, sod* and *spaP* promoter regions to regulate the *S. mutans* oxidative stress response. A recent study has identified small non-coding RNAs (sRNAs) that are mediators of *S. mutans* SloR and manganese regulons. Two sRNAs can bind SloR directly in their promoter regions and are involved in intracellular metal ion homeostasis and oxidative stress tolerance in *S. mutans* [20].

It should be noted that although Zn does not undergo redox-cycling, Zn homeostasis is also important in *S. mutans* oxidative stress tolerance. AdcABC is the main transporter responsible for zinc uptake in *S. mutans*. The bacterial growth was revealed impaired in the Δ*adcA* and Δ*adcCB* strains of *S. mutans* when exposed to H_2O_2 or high concentrations of manganese. However, $ZnSO_4$ supplementation abolished the increased H_2O_2/Mn^{2+} sensitivity of both mutants [21]. It suggests that Zn^{2+} plays a critical role in metal homeostasis and oxidative stress response in *S. mutans*. More recently, Ganguly et al. have identified a multi-metal translocating P-type ATPase transporter, named ZccE, which is unique to *S. mutans* [22]. The *zccE* gene is highly induced when *S. mutans* is exposed to excess $ZnSO_4$, and the *in vitro* Zn^{2+} stress can modify the expressions of oxidative stress genes, including *dpr*, *gor, ahpCF*, *nox*, *sodA*, and *tpx*. ZccE works synergistically with CopA (Cu-translocating P-type ATPase) to protect *S. mutans* against Cu intoxication in oxidizing environments. Inactivation of *zccE* drastically alters Zn/Mn ratios in *S. mutans*, resulting in greater sensitivity to H_2O_2 and attenuated competition ability against *S. gordonii* [22]. All these findings indicate that disruption of Zn homeostasis diminishes oxidative stress tolerance of *S. mutans*.

Regulators perR and spx

PerR, a member of the Fur family, encodes transcriptional repressors associated with peroxide resistance. While closely related microorganisms encode the two proteins PerR and Fur, *S. mutans* is one of the bacteria encoding only PerR, not Fur. Although metal co-factors are required for the function of the PerR protein, PerR responds directly to a wide variety of redox stresses (O_2, H_2O_2, HOCl, NO), not to metals; this is a major distinction between Fur and PerR [23]. PerR senses and responds to oxidative stress, negatively regulates *dpr* gene expression, and the *perR* mutant strain of *S. mutans* exihibits enhanced oxidative stress tolerance [24]. Ruxin et al. [25] found that the expression of PerR protein was upregulated upon exposure to H_2O_2. By directly binding to the Fur protein or the PerR homologous sequence on the *sloR* operon, SloR transcription was inhibited. When the *perR* gene was knocked out, *S. mutans* increased its tolerance to H_2O_2, and a *sloR-perR* double-knockout mutant upregulated *tpx* and *dpr* antioxidant genes [25]. The above results imply that PerR and SloR proteins interact in the *S. mutans* oxidative stress response and synergistically exert an important regulatory effect.

Spx serves as a transcriptional activator of genes involved in stress response and detoxification in *Firmicutes*. The N-terminal end of Spx contains a Cys-X-X-Cys (CXXC) motif, which is a latent target for redox-sensitive control. Spx lacks a DNA-binding domain and its function depends on direct interactions with the RNA polymerase α-subunit to impact gene transcription [26]. Spx was initially identified in *Bacillus subtilis* and was subsequently found to be ubiquitous in low-GC Gram-positive bacteria [27]. While the model organism *B. subtilis* encodes a single Spx protein, many streptococcus species (as well as *Listeria* and *Bacillus*) encode two Spx paralogs. SpxA1 and SpxA2 are important mediators of oxidative stress responses in *S. mutans*, with SpxA1 playing a dominant role [28]. SpxA1 and SpxA2 are mainly involved in activating the expression of oxidative stress response genes such as *dpr*, *nox*, *sodA*, and *tpx* [29]. It was found that *spxA1* mutant strains exhibited impaired growth under oxidative stress conditions, decreased competitiveness in biofilms co-cultured with peroxigenic oral streptococci, attenuated ability to colonize rat teeth, and reduced cariogenic virulence [30]. Transcription of *nox*, *sodA*, *ahpC*, *ahpF*, *dpr*, *gor*, *tpx* and *trxB* is downregulated in the Δ*spxA1* strain [28]. Additionally, Spx performs an important role in iron homeostasis by regulating the expression of genes involved in iron transportation, including genes encoding the Fe-S cluster assembly system (*smu247-smu251*), iron-binding protein (*dpr*), and iron transport system (*feoABC*) [16]. Therefore, Spx functions as a starting point for the characterization of novel genes and pathways that allow for *S. mutans* to cope with oxidative stress.

A comparison of SpxA1 and SpxA2 protein sequences in *S. mutans* revealed a difference in the C-terminal end, with SpxA1 containing an abnormal number of acidic residues [31]. Transcription of SpxA1 is co-inhibited by the metal regulators PerR and SloR, while SpxA2 transcription relies primarily on the envelope stress regulator LiaFSR. As part of the LiaR regulator, SpxA2 is critical for the growth of *S. mutans* under envelope pressure conditions. Furthermore, redox sensing is important for the activation of SpxA1-dependent oxidative stress and is indispensable in the SpxA2-mediated envelope stress response [31].

Glutathione pathway

GSH is a tripeptide that contains an unusual peptide linkage between the amine group of cysteine, which is attached by normal peptide linkage to a glycine, and the carboxyl group of the glutamate side chain. GSH acts directly as an essential non-enzymatic antioxidant to protect cells against ROS such as free radicals and peroxides, and as a cofactor for antioxidant and detoxification enzymes such as glutathione peroxidases, glutathione S-transferases, and glyoxalases. GSH also regulates redox signaling through reversible oxidation of critical protein cysteine residues by S-glutathionylation [32]. GSH uptake from the extracellular environment plays an important role in antioxidant stress response and regulating the functions of associated proteins in *S. mutans* [33].

GSH reductase is a critical enzyme that recycles oxidized glutathione back to the reduced form. The reduced glutathione plays key roles in the cellular control of ROS. GSH reductase determines the most suitable condition for redox control within a cell [34]. The GSH reductase gene *gor* mutant of *S. mutans* could grow under aerobic conditions but stopped growing in the presence of 2 mM diamide (A thiol-oxidizing agent for the intracellular oxidation of glutathione to the disulfide). The growth of wild-type strain was unaffected by 2 mM diamide while its GSH reductase activity increased 2.2-fold. In addition, GSH reductase activity in the wild-type strain was increased 3.6-fold upon air exposure, suggesting that GSH reductase is involved in protecting *S. mutans* from excessive oxidative stress [33]. The GSH dual-functional enzyme encoding gene *gshAB* encodes proteins with functional domains of glutamylcysteine synthetase and GSH synthetase, which play an important role in *S. mutans* oxidative stress and competition with H_2O_2-producing bacteria. Zheng et al. [35] found that *gshAB*-deficient *S. mutans* strains were more sensitive to H_2O_2 than wild-type strains. Furthermore, the competitiveness of *gshAB*-deficient *S. mutans* was significantly reduced when co-cultured with *S. sanguinis*. Additionally, Vergauwen et al. [36] showed that the *S. mutans* GSH transporters GshT and cysteine ABC input protein TcyBC were critical in preventing oxidative damage from bacteria and regulating protein function.

S-glutathionylation is an essential post-translational modification pathway that targets protein cysteine thiols by the addition of glutathione. This modification process can prevent proteolysis caused by excessive oxidation of protein cysteine residues under oxidative stress conditions. Recent studies have identified the S-glutathionylated proteins in *S. mutans* and found that the glutathionylation on Cys41 residue of thioredoxin-like protein (Tlp) played a crucial role in *S. mutans* antioxidant stress and competition with *S. sanguinis* and *S. gordonii*. Additionally, a rat carious model proved that loss of S-glutathionylation reduced the cariogenicity of *S. mutans* [37]. These data provide insights into the role of S-glutathionylation in the oxidative stress resistance and interspecies competition of *S. mutans*.

Conclusions and outlook

In the oral cavity, *S. mutans* colonizes the tooth surface in the form of dental plaque biofilm. *S. mutans* is resistant to changes in the oral environment, such as pH and temperature fluctuations, changes in carbohydrate availability and oxidative stress. *S. mutans* is capable of counteracting changes in oxidative stress that origins from human physiological activity (use of hygiene products) and bacteria-derived H_2O_2 through various pathways,

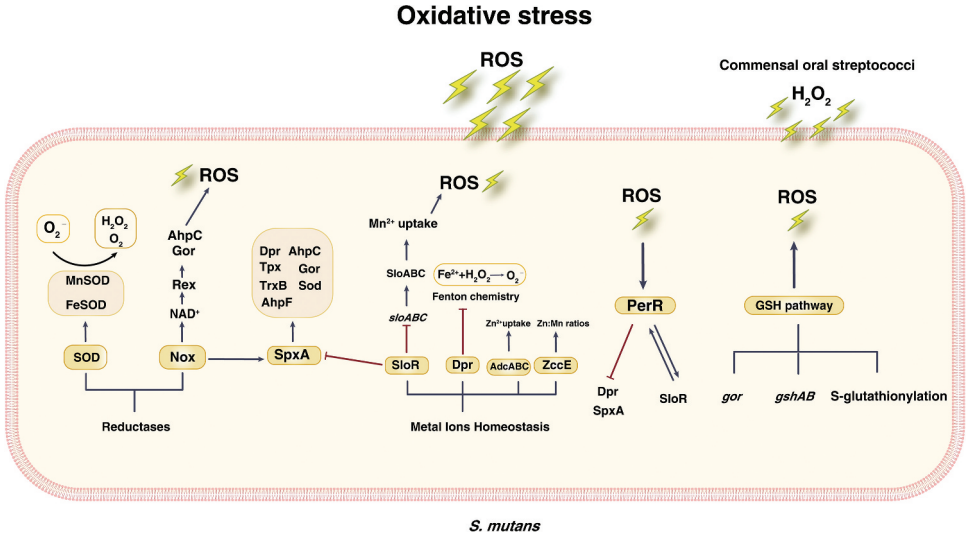

Figure 1. *S. mutans* is capable of counteracting changes in oxidative stress through various pathways, including reductase synthesis, regulation of metal ion uptake, regulator PerR, transcription regulator Spx, and glutathione (GSH) pathway.

including reductase synthesis, regulation of metal ion and GSH uptake, and regulators PerR and Spx (Figure 1).

Exogenous oxygen stimulation or bacteria-derived H_2O_2 damages DNA and inhibits the growth of *S. mutans*, with its acid production and tolerance, extracellular polysaccharide synthesis, and biofilm formation subsequently being influenced. Studies have demonstrated that the oxidative stress tolerance ability of *S. mutans* could affect its competitiveness in dental plaque biofilm and plays an important role in cariogenicity. Approximately 7% of the *S. mutans* UA159 genome shows altered transcription with 100 genes upregulated and 39 genes downregulated after H_2O_2 stress. The differentially expressed genes are enriched in amino acid biosynthesis, carbon metabolism, DNA metabolism, protein fate, stress tolerance, uptake and transport, and other hypothetical proteins [38]. Further studies are needed to investigate how exposure to oxidative stress impacts the pathogenic virulence of *S. mutans*. The underlying mechanisms by which *S. mutans* adapts to oxidative stress and its influence on oral microecology and development of dental caries still require further exploration.

Studies have suggested that two-component signal transduction systems such as LytST, Cid/Lrg, and VicRK might play important roles in the oxidative stress response of *S. mutans* [39–41]. The *mub* gene cluster is able to synthesize mutanobactin to protect *S. mutans* against oxidative stress [42,43]. Additionally, studies have found that cyclic diadenosine monophosphate, a bacterial secondary messenger, is also involved in regulating the oxidative stress response of *S. mutans* [44,45]. It is also noteworthy that *S. mutans* is able to evolve some other mechanisms to adapt to oxidative stress triggered by oral care products/commensal pressure. Kajfasz et al. accidentally found that *perR* gene had occurred spontaneous mutation in laboratory stocks of UA159, and the *perR*-inactive strain exhibited increased oxidative stress tolerance [24]. TnSmu2 is a mobile genetic element commonly acquired through horizontal gene transfer events. Many genes encoded within the TnSmu2 regions are affected in the deletion of *fstH*, *cidB*, or *covR*, which are involved in oxidative stress resistance of *S. mutans* (https://doi.org/10.1101/2023.07.10.548324). These studies suggest that routine conditions may facilitate the emergence of spontaneous mutation or horizontal gene transfer, which may impact the bacterial oxidative stress resistance. These pathways are potentially promising research directions worthy of future efforts.

Understanding the oxidative stress regulatory mechanisms of *S. mutans* may provide novel options for investigating the cariogenic mechanisms of *S. mutans* in the oral microenvironment, and new methods and targets for the ecological management of dental caries. In future, targeted small molecular compounds can be designed for key signal pathways to inhibit oxidative stress and weaken *S. mutans* virulence, which is important for oral microecological modulation and prevention and treatment of dental caries.

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Disclosure statement

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Author contributions

XC and JN drafted the manuscript. XX and XZ edited and added valuable insights to the manuscript. All authors approved the final manuscript and agreed to be accountable for all aspects of the work.

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