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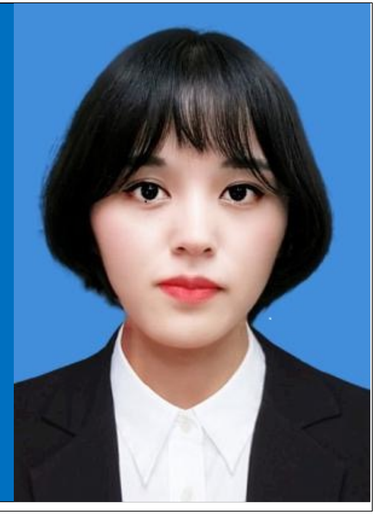
OrtR regulates the oxidative stress adaptation of *Streptococcus mutans*

Shuxing Yu[1,2], Qizhao Ma[1,2], Hu jun[1,2], Yuqing Li[1], Jing Zou[1,2]

1. State key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Sichuan University, Chengdu 610041, China
2. Department of Pediatric Dentistry, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China



Introduction

Caries is one of the most common chronic bacterial infectious diseases. *Streptococcus mutans* is considered to be the major caries-causing bacteria, and its virulence depends on its ability to form biofilms and withstand extreme environmental changes, including fluctuations in oxygenation, shear stress, and nutrient sources and availability. For example, when reactive oxygen species (ROS) are present in the environment, *S. mutans* must be able to rapidly alter the expression of certain transporters and metabolic enzymes to decompose multiple host-derived ROS with greater vigor. ROS can cause cell damage by damaging lipids, proteins and nucleic acids. This is of great significance to maintain the balance of bacterial plaque in plaque microecology and to reduce caries caused by *S. mutans*. The combination of reactive oxygen inducers and commercial drugs shows great promise in the selective killing of bacteria, and its inhibition potential is greatly enhanced.

S. mutans is a bacterium that lacks catalase and a complete electron transport chain, and is unable to undergo oxidative phosphorylation. Therefore, *S. mutans* mainly uses some clearing and protection systems to prevent the accumulation of toxic ROS, including superoxide dismutase (Sod), nicotinamide-adenine dinucleotide oxidase (Nox), Dps-like peroxide resistance protein (Dpr), alkyl hydroperoxidase (AhpCF), thioredoxin (Trx) and glutathione Reduction System.

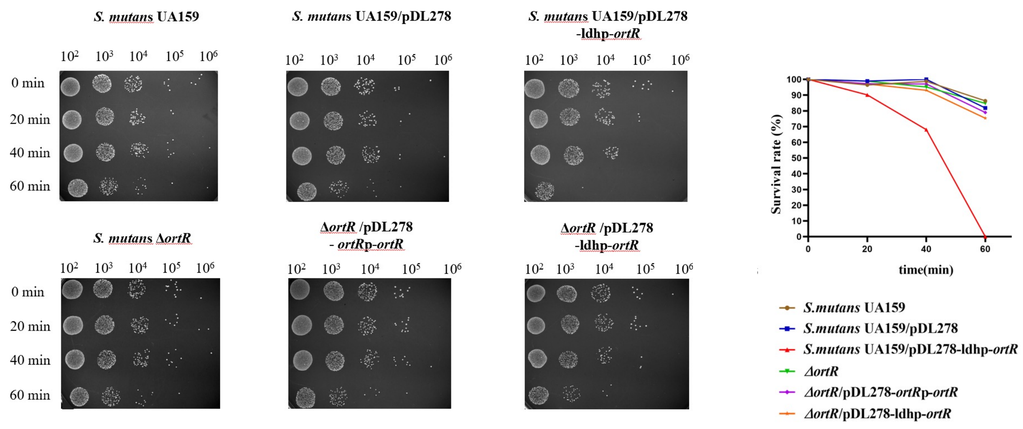
In *S. mutans*, transcriptional regulators are gene products with transcriptional regulation activity, and they play an important role in the expression regulation of genes related to environmental stress response. At present, studies have found transcription factors regulating *sod*, *nox*, *drp*, *ahp*CF and other related genes of *S. mutans*, including PerR, SloR, Spx and Rex. However, many transcription factors that regulate these genes remain undiscovered and need further investigation.

Methods

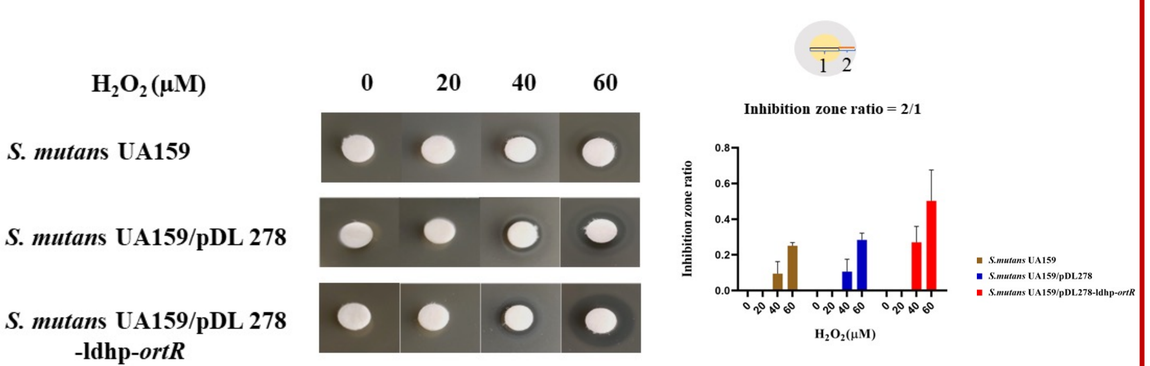
Overexpression of the *S. mutans ortR* gene results in decreased cell viability in the planktonic state of *S. mutans* and under the three species biofilms. RNA sequencing using the *ortR* mutant showed that downstream genes directly regulated genes related to oxidative stress adaptation such as *ahpCF* and *smu.140*. The OrtR protein was purified and the conserved DNA binding motifs were determined by electrophoretic migration and DNA enzyme I footprint analysis.

Results

Planktonic *S. mutans* UA159/Pdl279-ldhp-*ortR* Strain Shows Increased Hydrogen Peroxide Sensitivity



Planktonic bacteria of different *S. mutans* strains were treated with a 0.02% v/v H_2O_2 for 20, 40, and 60 min, respectively. When the treatment time reached 60 min, the overexpression strain was completely colony-free at dilution 10,000, while all other strains had some number of colonies present at this concentration. By calculating the survival rate of different strains at dilution 10,000, it was found that the survival curve of *ortR* overexpressed strain was steeper than that of other strains.

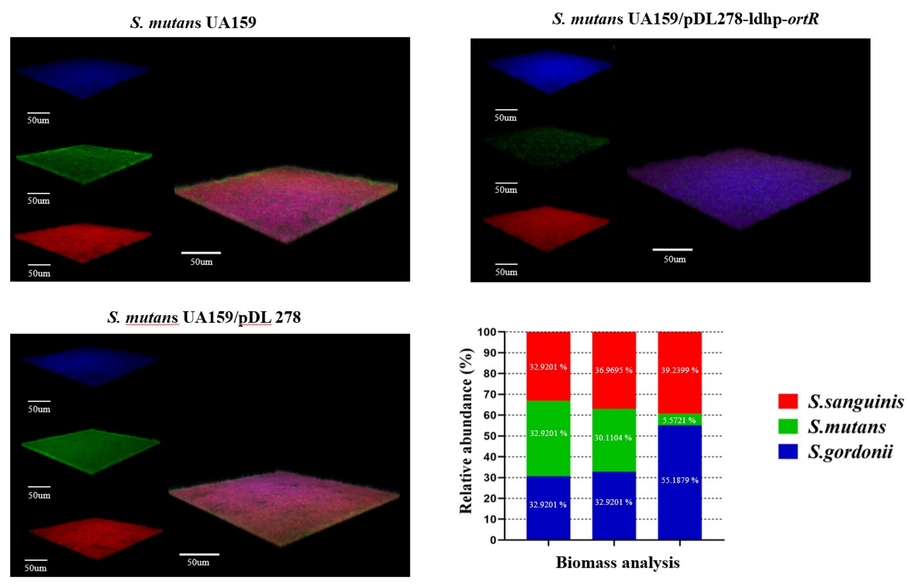


Inhibition was observed when filter discs were injected with H_2O_2 at concentrations ranging from 20 to 60 μM. The appearance of inhibition rings on the plates of each strain was seen when the H_2O_2 concentration reached 40 μM, and when the concentration reached 60 μM, the inhibition rings of the overexpressing strains were relatively more pronounced, which was analyzed by measuring the ratio of the longest diameter of each inhibition ring to the filter disc and plotted as a line graph.

Conclusions

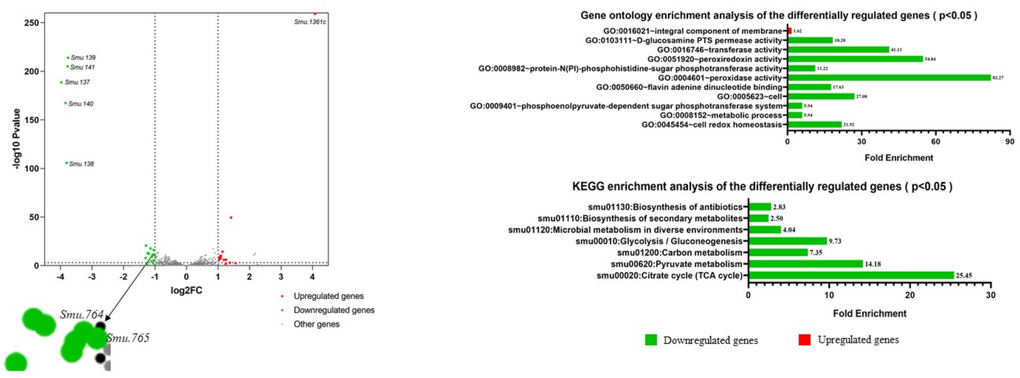
S. mutans coordinates the transport and metabolism of ROS through a variety of pathways under complex environmental conditions. Our results indicate that OrtR is a transcriptional activator and has pleiotropy effect on bacterial oxidative stress adaptation formation and growth metabolism, which are important virulence traits of *S. mutans*. On this basis, it is important to identify specific signals that regulate oxidative stress adaptation gene-related regulatory pathways. Notably, this study deciphered the physiological functions of OrtR as a potential target for controlling bacterial survival and oxidative stress adaptation, thus better preventing dental caries.

OrtR overexpressing strain was at a disadvantage when co-cultured with strains producing H_2O_2.



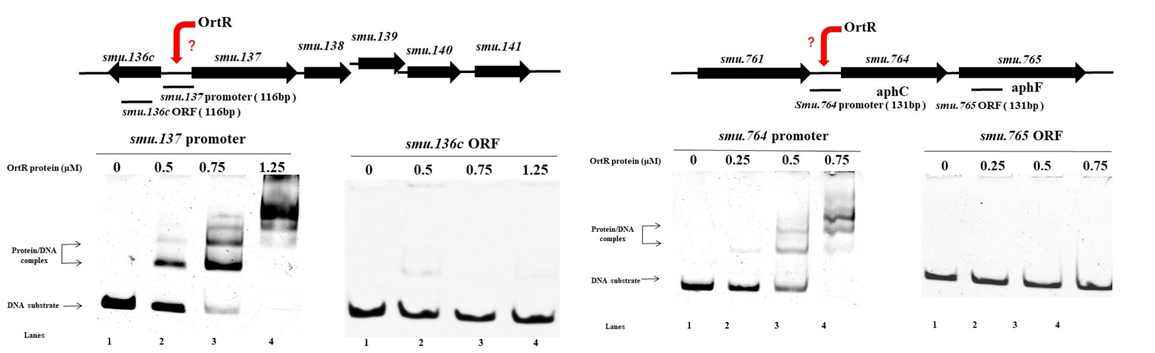
We used FISH to study the microbial composition and interactions of three biofilm endomorphic *S. mutans*, *S. sanguis* and *S. gordonii*. Visualization by fluorescence confocal microscopy of 3D scans reveals the abundance of *S. mutans* UA159/pDL278-ldhp-*ortR* mutant strains was significantly reduced compared to *S. mutans* UA159 and *S. mutans* UA159/pDL278, whereas the abundance of *S. sanguis* and *S. gordonii* was increased. Biomass analysis of the three bacteria showed that the abundance of *S. mutans* decreased to approximately 5.5721% of *ortR* overexpression strains, whereas the abundance of *S. sanguis* and *S. gordonii* decreased respectively.

Transcriptome analysis of the *ortR* overexpressing srain.



Since we found that overexpression of *ortR* significantly affected planktonic growth and interspecies competition in *S. mutans* under H_2O_2, we analyzed the transcriptome of overexpression strains of *ortR* to determine changes in gene expression across the board. 22 genes were significantly down-regulated and 13 genes were significantly up-regulated in the *S. mutans* UA159/pDL278-ldhp-*ortR* strain compared to *S. mutans* UA159/pDL278. Gene enrichment and functional annotation clustering analysis was further performed for differentially expressed genes (DEGs). As shown, DEGs were enriched in all seven Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. DEGs are mainly involved in antibiotic biosynthesis, metabolite synthesis, glucolysis/gluconegenesis activity, carbon metabolism, pyruvate cycle, and citric acid cycle. Gene ontology (GO) term enrichment analysis revealed that DEGs are mainly involved in peroxidase activity, intergral composition of membrane, regulation of cellular redox homeostasis, D-glucosamine PTS permease activity, transferase activity, and flavin adenine dinucleotide binding.

OrtR directly binds to the antioxidant adaptation promoters.



Transcriptome data showed that transcription of *smu.140*, *aphC* and *ahpF* was controlled by OrtR. To investigate whether the targeted promoter is directly regulated by the protein, we used electrophoretic mobility shift assay (EMSA) to study the binding activity and specificity of OrtR to DNA. The speculated promoter sequence is divided into two fragments: *smu. 137* promoter (116 bp) and *smu.764* promoter (131 bp). After the recombinant His marker protein OrtR was incubated with two DNA fragments, OrtR specifically bound to the two fragments. In the negative control group, no changes in mobility were observed during the incubation of OrtR with *smu*.136c ORF and *smu.765* ORF. OrtR therefore binds specifically to promoter regions of oxidative stress adaptation *smu.140*, *ahpC* and *ahpF* inhibit their expression.

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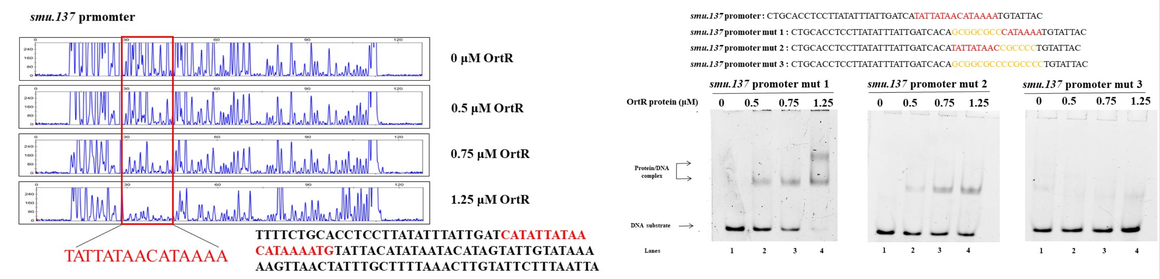

The *Smu. 137* promoter fragment labeled with 5-carboxyfluorescein (5-FAM) was further analyzed to identify the potential binding motifs of OrtR by the DNase I footprinting assay. As the protein concentration increased, we found the footprint region protected by OrtR, which covered a DNA motif 5 '-TATTATAACATAAAA-3', a 15 bp short palindrome. In order to further confirm that DNA motifs are necessary for OrtR recognition, a pair of DNA substrates were designed, *smu.137* promoter mut 1, *smu.137* promoter mut 2 and s*mu.137* promoter mut 3. All substrates were labeled with FAM. These results clearly indicate that the DNA motif 5 '-TATTATAACATAAAA-3' is the binding site recognized by OrtR.