A new dual-function regulator modulating GlnR regulon expression and biofilm formation by *Streptococcus mutans* GS5

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Abstract

Background:

Streptococcus mutans is the etiologic agent for dental caries, and an opportunistic pathogen of infective endocarditis. Studies have shown that GlnR-mediated repression on the GlnR regulon at acidic pH is essential for the acid tolerance response in *S. mutans* GS5. Our recent study identified an ORF (SMUGS5_RS05810) that shares 33% identity with TnrA. The result of electrophoretic mobility shift assay indicates that SMUGS5_RS05810 also recognizes the binding consensus of GlnR, suggesting that both proteins regulate the expression of the GlnR regulon. This study aimed to investigate the regulatory activity of SMUGS5_RS05810 in response to nitrogen nutrients and the impact of its regulation on *S. mutans* GS5 biofilm formation.

Material/methods:

All isogenic mutant strains were generated by allelic exchange. To evaluate whether the activity of SMUGS5_RS05810 is modulated by nitrogen nutrients, wild-type and mutant strains were cultivated in the defined medium, FMC, containing different nitrogen nutrients. The expression of the GlnR regulon in these cells was evaluated by using various promoter-chloramphenicol acetyltransferase gene (*cat*) fusions. The regulation of SMUGS5_RS05810 on SMUGS5_RS05805 was measured by quantitative real-time PCR. Biofilm formation in wild-type GS5 and the recombinant strains was examined in static cultures and the flow cell biofilm system by the standard methods. The structure of the biofilm was examined by confocal laser scanning microscope (CLSM).

Results:

The expression of the GlnR regulon in wild-type GS5 was mainly repressed by GlnR under nitrogen-excess condition, and the CAT activities in GlnR mutant and the GlnR/RS05805 double knockout host were higher than that in wild-type GS5 regardless the nitrogen contents, suggesting that a basal level of repression was mediated by GlnR under nitrogen-limiting. Although SMUGS5_RS05810 interacts with the GlnR consensus, the activity of SMUGS5_RS05810 was insensitive to nitrogen nutrients. Furthermore, SMUGS5_RS05810 activated the *glnQ* promoter and repressed the *gdhA* and *citB* promoters. SMUGS5_RS05810 also repressed the expression of SMUGS5_RS05805, which is not regulated by GlnR. Finally, inactivation of SMUGS5_RS05810 (ΔRS05810) led to an enhanced biofilm formation; however the enhancement was not related to the expression of glucosyltransferases.

Introduction

Streptococcus mutans is the etiologic agent for dental caries, and an opportunistic cause of bacteremia and infective endocarditis. The major pathogenic factors associated with the cariogenicity of *S. mutans* are the acidogenicity and the aciduricity. Moreover, the ability to form biofilm on the tooth surface also participates in its cariogenicity.

In *Bacillus subtilis*, Nitrogen-dependent regulation is mainly governed by GlnR and TnrA. Both proteins are members of the MerR family of transcription factors, and recognize a similar target sequence (5'-ATGTNAN7TNACA), the GlnR box. However, GlnR represses gene expression during growth under nitrogen excess, while TnrA exerts its regulation when cells are grown under nitrogen limitation. Althouhg oral streptococci generally harbotrs GlnR only, our recnet study identified an ORF, SMUGS5_RS05810, that also recognizes the binding consensus of GlnR, suggesting that both proteins regulate the expression of GlnR regulon. As the regulation of the GlnR regulon expression by GlnR in response to growth pH is critical an optimal acid tolerance response of *S. mutans*, this study aimed to investigate the regulatory activity of SMUGS5_RS05810 on the GlnR regulon and the impact of its regulation on biofilm formation in *S. mutans* GS5.

Results

1. Alignment of the GlnR box of the *S. mutans* GS5 GlnR regulon genes

A conserved GlnR box sequence was found in the 5' flanking regions of the GlnR regulon genes. A GlnR-like box was also identified 5' to the translation start site of SMUGS5_RS05810 (Fig. 1).

Gene		Sequence	Distance
SMUGS5_RS01600	*glnR*	TTTATGTCAGGAAATATGACATATA	- 54 nt - ATG
SMUGS5_RS06830	*glnQ*	ATAATGTTAGAAAAGCTAACAATAA	- 25 nt - ATG
SMUGS5_RS04110	*gdhA*	TGAATGTTATTTTTTATAACATGAA	- 108 nt - ATG
SMUGS5_RS03040	*citBZC*	TTTATGTTATAAAACATAACATAAA	- 75 nt - ATG
SMUGS5_RS07470	*nrgA*-1	ATAATGTTATATTATATAACATAAA	- 17 nt - ATG
SMUGS5_RS07470	*nrgA*-2	GAAATGTTAAAAAACATAACATATA	- 182 nt - ATG
SMUGS5_RS03640	*glnP*	TAGATGTTATATTTCCTTACATAAC	- 84 nt - ATG
SMUGS5_RS05810		TTTATGT.AGAAAAACT.ACATTTA	- 97 nt - ATG
Consensus		ATGT-A-------T-ACA	

Fig.1. Alignment of the GlnR box of the *S. mutans* GS5 GlnR regulon genes. The distance between the GlnR box and the translational start sites are listed

2. The regulation of the GlnR regulon by GlnR and SMUGS5_RS05810 in response to nitrogen nutrients

To evaluate the impact of the nutrient conditions on the activities of GlnR and SMUGS5_RS05810, the wild-type and the mutant strains were cultivated in FMC-Q, and FMC-E (Fig. 2). By using various promoter-*cat* fusions, the result determined that the expression of the GlnR regulon in wild-type GS5 was upregulated under the FMC-E compared to that the FMC-Q. However, the expression of the newly identified SMU_RS05810, and its flanking ORF, SMU_RS05805, is insensitive to nitrogen nutrients. Furthermore, the expression of *glnQ* was downregulated in the RS05810-deficnet strain (ΔRS05805), whereas *gdhA* and *citB* promoters were upregulated under FMC-Q. Moreover, SMUGS5_RS05810 also repressed the expression of SMUGS5_RS05805, which is not regulated by GlnR.

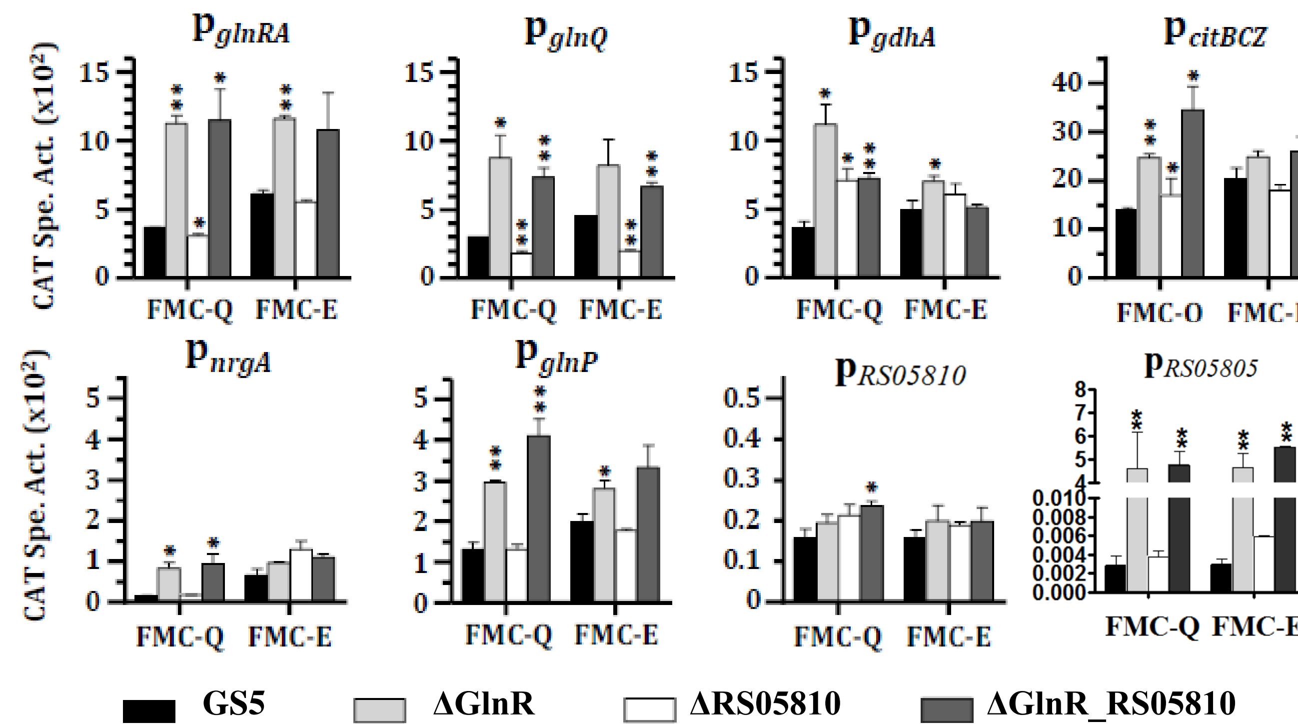

Fig.2. **The promoter activity of the GlnR regulon in various host backgrounds under nitrogen limiting and excess conditions.** Wild-type GS5, ΔGlnR, ΔRS05810, and the double knockout strain (ΔGlnR_RS05810) harboring various promoter-*cat* fusions grown in FMC containing an excess amount of glutamine (FMC-Q) and limited nitrogen nutrients (FMC-E). The significant difference between the wild-type and mutant strains under each growth condition were calculated by the student *t*-test, **, $P < 0.001$; *, $P < 0.01$.

3. The effect of GlnR and SMU_RS05810 on biofilm formation in *S. mutans* GS5

To evaluate the impact of GlnR and SMU_RS05810 on biofilm formation, all strains were cultivated in BMG medium over 24 hours (Fig. 3A). A time-dependent biofilm formation was seen in all strains. The strucure of the biofilm in the flow cell system was exmined by CLSM (Fig. 3B). An enhaced biofilm formation was seen in strains ΔRS05810 and ΔGlnR. The chain length of strains ΔRS05810 and ΔGlnR is longer than that of the wild-type GS5 in BMG (Fig. 4), suggesting that the elongated chains may facilitate biofilm development. The competition assay shown that the two mutant strains survived equally well within the biofilm (Fig. 5).

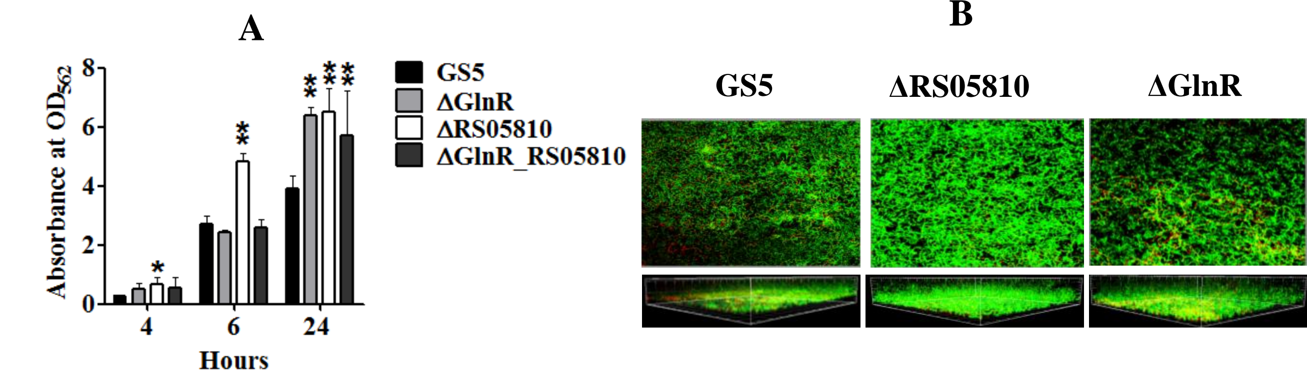

Fig. 3. The biofilm formation of *S. mutans* GS5 and its derivatives. (A) The mass of the biofilm of strains grown in BM containing 40 mM glucose (BMG) was determined by crstyal violet staining. The statistic differences between the mutant and wild-type strains were anlyzed by Student's *t*-test. *, $p < 0.05$; **, $p < 0.001$, (B) Structures of the biofilms formed by wild-type *S. mutans* GS5 and ΔRS05810 in the flow cell system. The biofilms were grown in BHI for 16 h. The biofilm was stained with the SYTO 9/PI fluorochrome reagents and visualized by CLSM. The live cells (green fluoresce) and dead cells (red fluoresce) are shown. The side view of the biofilm was shown below.

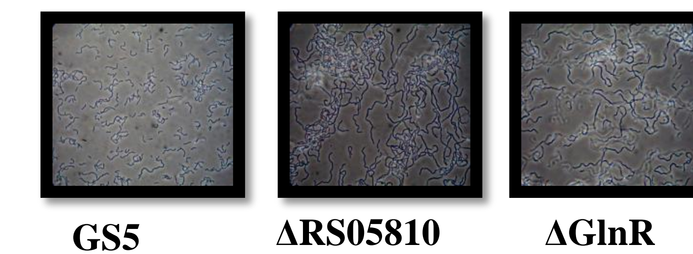

Fig. 4. The phenotypic characteristic of *S. mutans* GS5 and its derivatives. All strains were grown in BMG. The cells were observed at 1000X magnification.

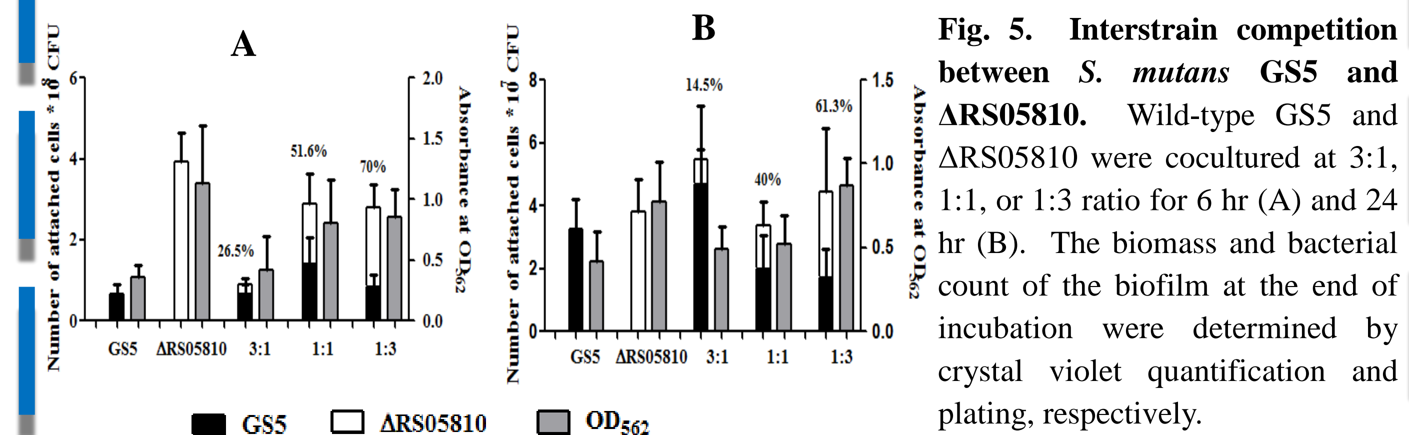

Fig. 5. Interstrain competition between *S. mutans* GS5 and ΔRS05810. Wild-type GS5 and ΔRS05810 were cocultured at 3:1, 1:1, or 1:3 ratio for 6 hr (A) and 24 hr (B). The biomass and bacterial count of the biofilm at the end of incubation were determined by crystal violet quantification and plating, respectively.

4. The *gtfD* is regulated by GlnR and SMUGS5_RS05810

To elevated biofilm formation in ΔGlnR and ΔRS05810 were caused by enhanced glucan production via the activity of glucosyltransferases (Gtf). A significant decrease of expression level of *gtfD* was obeserved in ΔGlnR and ΔRS05810(Fig. 6).It possibly that additional targets are regulated by SMUGS5_RS05810 and GlnR.

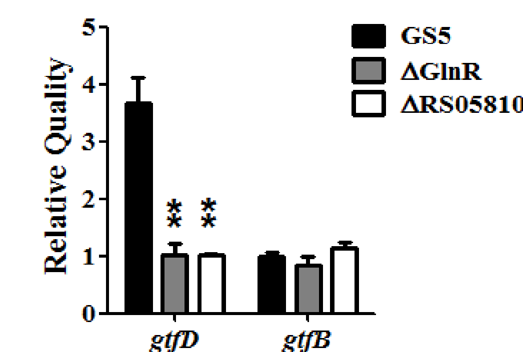

Fig.6. Expression levels of *gtfD* and *gtfB* in wild-type *S. mutans* GS5 and its derivatives grown in BMG. The relative quantities of mRNA of *gtfD* and *gtfB* were measured by q-PCR. The statistic differences between the mutant and wild-type strains are indicated with stars: **, $p < 0.001$, Student's *t*-test.

Conclusions

1. **SMUGS5_RS05810 appears to be a dual regulator in *S. mutans* GS5.**
2. **The regulatory activity of SMUGS5_RS05810 was relatively insensitive to nitrogen nutrients, and SMUGS5_RS05810 regulates the expression of genes that are not identified as part of the GlnR regulon.**
3. **SMUGS5_RS05810 and GlnR modulate indirectly biofilm formation.**