Synonymous point mutation of \textit{gtfB} gene caused by therapeutic X-rays exposure reduced the biofilm formation and cariogenic abilities of \textit{Streptococcus mutans}

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Abstract

\textbf{Background:} The shift of oral microbiota is a critical factor of radiation caries in head and neck cancer patients after the radiotherapy. However, the direct effects of irradiation on the genome and virulence of cariogenic bacteria are poorly described. Here we investigated the genomic mutations and virulence change of \textit{Streptococcus mutans} (\textit{S. mutans}), the major cariogenic bacteria, exposed to the therapeutic doses of X-rays.

\textbf{Results:} X-ray reduced the survival fraction of \textit{S. mutans} and impacted its biofilm formation. We isolated a biofilm formation-deficient mutant #858 whose genome only possessed three synonymous mutations (c.2043 T > C, c.2100C > T, c.2109A > G) in \textit{gtfB} gene. The "silent mutation" of c.2043 T > C in \textit{gtfB} gene can cause the down-regulation of all of the \textit{gtfs} genes’ expression and decrease the GtfB enzyme secretion without the effect on the growth due to the codon bias. #858 and synonymous point mutation strain \textit{gtfB}\textsuperscript{2043 T>C}, similar to the \textit{gtfB} gene null mutant \textit{ΔgtfB}, can significantly decrease the extracellular polysaccharide production, biofilm formation and cariogenic capabilities both in vitro and in vivo compared with wild type.

\textbf{Conclusion:} The direct exposure of X-ray radiation can affect the genome and virulence of oral bacteria even at therapeutic doses. The synonymous mutations of genome are negligible factors for gene expression and related protein translation due to the codon usage frequency.

Background

Radiotherapy (RT) plays an essential role in the treatment of head and neck cancer (HNC), alone or in combination with surgery and chemotherapy [1, 2]. However, due to the complex anatomical characteristics of the head and neck, radiotherapy can adversely affect surrounding tissues include salivary glands, oral mucosa and dentition, leading to a series of side effects, such as hyposalivation, radiation caries and mucositis [3]. Radiation caries (RC) is a typical clinical symptoms response to radiotherapy, which can lead to aggressive tooth destruction [4, 5], loss of masticatory efficiency, persistent chronic oral infections, and osteoradionecrosis (ORN) [6]. The changes of oral microbes after the radiation treatment have been considered as one of the etiological factors in the development of radiation caries [7, 8]. Brown et. al. found that the cariogenic microbial species including \textit{Streptococcus mutans}, \textit{Lactobacillus} spp., and \textit{Candida} spp. were significantly increased, while the other oral bacteria, such as

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S. sanguinis, Neisseria spp., and Fusobacterium spp. were significantly decreased [9]. Eliasson found an increased number of lactobacilli and Candida species in irradiation patients compared with Sjögren's syndrome group [10]. In addition to these data obtained by traditional cultural method, pyrosequencing was also used to detect microecological changes during radiotherapy. Hu et al. have revealed that there was a negative correlation between the number of operational taxonomic units (OTUs) and radiation dose, and five genera (Actinomyces, Veillonella, Prevotella, Streptococcus, Campylobacter) were found in all supragingival plaque samples which distributed differently in different time points [11, 12]. To our knowledge, current studies only focused on the species composition and abundance change after radiotherapy, the functional properties of oral flora are not quite investigated. In addition, it is widely accepted that the shifts of microbial population of the radiation caries patients are mainly due to the decreased amount of saliva flow and the changes of the saliva properties [13], however, the direct role of irradiation on oral flora is still unclear.

High-energy X-rays are used to kill cancer cells frequently due to their capability of substantial DNA damage including base modifications, apurinic and apyrimidinic sites and strand breaks [14, 15]. X-rays can induce both the various-sized deletions and the base substitutions in the genome such as synonymous and non-synonymous mutation [16–18]. The total dose for treatment of head and neck cancer is around 70 Gy [19]. However, this dosage can not only cause DNA damage from tumor cells, but also potentially lead to DNA mutations of the microbes around the tumor tissues. Numerous studies have found that X-rays radiation (0–90 Gy) can not only cause microecology changes [20] but also induce genetic mutations in yeast and Escherichia coli [21, 22]. Therefore, the genetic mutations induced by X-ray may also be occurred in the oral microbes during the head and neck cancer radiotherapy.

S. mutans, the main pathogen of dental caries, can adhere to the tooth surface and form a stable biofilm (dental plaque) [23, 24]. The major virulence factors known as glucosyltransferases (Gtfs), which synthesize adhesive extracellular polysaccharides (EPS), can promote the formation of dental plaque [25]. Gtfs in S. mutans is encoded by three gtf genes (gtfB, gtfC and gtfD). Among these genes, gtfB and gtfC can mediate synthesis of water-insoluble glucan, while glucosyltransferase encoded by gtfD can synthesize water-soluble glucan. GtfB and GtfC share a high degree of nucleotide and amino acid sequence similarity, and these two genes are highly homologous and arranged in tandem in the chromosome, while GtfD is dependent on the acceptor for glucan synthesis [26, 27]. The expression level of the gtf genes can interfere with the synthesis of glucan, which then affects the adhesion of bacteria as well as biofilm formation [28].

The abundance of S. mutans was significantly increased in dental plaque after head and neck radiation [10, 29] indicating its major contributions on radiation caries. However, the effects of the irradiation on the genome and virulence of S. mutans are still not described. In this study, we subjected S. mutans cells to X-rays irradiation, and then analyzed the changes in the genome and its carcinogenic pathogenicity to investigate how X-rays directly affect oral bacteria.

Results

X-rays irradiation impacted the biofilm formation ability of S. mutans

First, we tested the sensibility of S. mutans in response to X-rays. The survival fraction of S. mutans was decreased along with the increase of X-ray dosages (Fig. 1a). When the irradiation dosage reached to 80 Gy, about half of S. mutans were dead (LD50), while only 11.7% S. mutans cells remained alive when the irradiation dose reached 300 Gy (Additional file 1: Figure S1). Then we investigated the effect of X-rays irradiation on the biofilm formation ability of S. mutans. The irradiated S. mutans suspension had a higher biofilm formation ability than control group (p < 0.05), but there were no significant differences among irradiated groups (Fig. 1b). To explore the individual phenotype changes of S. mutans cells, we had randomly isolated 147 S. mutans single colonies from irradiation samples. As shown in Fig. 1c, different biofilm formation abilities were observed in these isolates, specifically, 12 strains had stronger biofilm formation activities compared to WT, while 31 strains reduced the abilities significantly including the most reduced isolate #858 (p < 0.05). These results indicated that therapeutic dose of X-rays can have an impact on virulence characteristics of S. mutans.

Strain #858 showed decreased EPS productions and abnormal biofilm structure

From these 147 isolated strains, the most biofilm formation-deficient strain #858 was isolated from 80 Gy group. Its biofilm formation ability (Fig. 2a) was obviously decreased compared to the WT strain (p < 0.05). In addition, the biofilm of #858 was easily drop off during crystal violet stain process (Fig. 2b) indicating that #858 lacked the normal adhesive and biofilm formation abilities. The growth rates between #858 and WT showed no significant differences (Fig. 2c) indicating that the biofilm formation deficiency of #858 was independent of cells growth ability.
Next, we examined the biofilm structure and EPS production of #858. From the SEM analysis, the biofilm of #858 was much sparser compared to the WT, and no obvious extracellular matrix was observed between bacterial cells (Fig. 2d). According to the confocal laser scanning, #858 showed fewer and thinner biofilm structure, with little EPS distributed in the biofilm compared with WT (Fig. 2e). Moreover, the biofilms of WT had a higher EPS: bacteria ratio than #858 (p < 0.05; Fig. 2f). The water-insoluble EPS responsible for biofilm formation produced by #858 was also significantly lower than WT (p < 0.05, Fig. 2g), in line with the results of confocal laser scanning.

We then measured the expressions of EPS-related genes since #858 failed to produce EPS. The EPS biosynthesis genes (gtfB, gtfC, gtfD) of #858 were all significantly down-regulated compared to WT (Fig. 2h), particularly, the expression of gtfB and gtfD were reduced by 50% and 70% respectively (p < 0.05). Interestingly, there was almost no expression of gtfC in #858.
X-ray caused the synonymous mutations in gtfB gene

In order to investigate whether the biofilm formation deficiency of #858 was result from the X-rays caused genome mutations, we sequenced the whole genome of #858. The whole-genome resequencing (WGRS) information for the WT and #858 were shown in Additional file 3: Table S2. Compared with WT, #858 only had 3 synonymous mutation sites at gtfB gene without any nonsynonymous mutation (Table 1). The 3 synonymous mutations of gtfB included c.2043 T > C, c.2100C > T, c.2109A > G, coding isoleucine, arginine and arginine respectively.

Thus, we hypothesized that the 3 synonymous mutations may cause the abnormal biofilm formation of #858 due to the codon usage bias. Based on the codon usage frequency of S. mutans UA159 from Codon Usage Database (http://www.kazusa.or.jp/codon/) (Additional file 3: Table S3), we found that the codon usage frequency of first mutation c.2043 T > C, which encodes isoleucine changed from AUU into AUC on transcriptional level, decreased from 53.4 to 15.9 per thousand. The codon usage frequency of the other two mutations c.2100C > T and c.2109A > G, changed from CGC into CGU, and CGA into CGG respectively, had no obvious variation, indicating that the synonymous mutation...
c.2043 T>C of gtfB gene may be responsible for the incapacity of biofilm formation of #858.

**Synonymous point mutant strain gtfB 2043 T>C decreased the EPS production and biofilm formation**

To investigate the role of first mutation c.2043 T>C in the pathogenicity of *S. mutans*, we constructed a point mutant strain gtfB 2043 T>C by homologous recombination (Figure S2) and we also knockout the gtfB gene (ΔgtfB) as control. As shown in Fig. 3a, biofilm formation of gtfB 2043 T>C, same as strain #858, was significantly reduced by 35% compared to WT, while ΔgtfB reduced by 46% (p < 0.05). We can easily observe incomplete biofilm of #858 and gtfB 2043 T>C under microscope, and the biofilm of ΔgtfB almost completely detached compared with WT (Fig. 3b). Similarly, there were no obvious growth differences between four groups (Fig. 3c). According to the SEM observation, there were large amounts of extracellular matrices between bacterial cells in WT group, while #858, gtfB 2043 T>C and ΔgtfB showed very few extracellular matrices with abnormal biofilm structure (Fig. 3d). The biofilm thickness of #858, gtfB 2043 T>C and ΔgtfB decreased significantly (Fig. 3e), and biofilms of WT had the highest EPS: bacteria ratio than other three groups (p < 0.05; Fig. 3f). In addition, #858, gtfB 2043 T>C and ΔgtfB significantly reduced the water-insoluble EPS production compared to WT (p < 0.05; Fig. 3g), and the expressions of gtfB in these three strains were significantly down-regulated compared to WT (Fig. 3h). Interestingly, both of the gtfB “silent mutation” and gtfB knockout can further influenced the expression levels of gtfC and gtfD.

To investigate whether the changes in gtf genes transcription would affect enzyme production, we then checked the Gtfs protein patterns. Our results indicated that the GtfB/D band were separated from GtfC in all
groups, among them, the GtfB/D of #858, gtfB^2043 T>C and ΔgtfB were significantly reduced compared to WT, which was consistent with the expression of gtfB gene. Moreover, down-regulation of gtfC in mutant strain groups also decreased the bottom GtfC band (Fig. 3i,j). The protein expressions of Gtf enzymes indicated that the “silent mutation” of gtfB not only reduced the transcription of gtf genes but also decreased the protein levels.

**Synonymous mutant c.2043 T > C reduced the tooth demineralization in vitro**

To validate whether the decrease of GtfB caused by the synonymous point mutant c.2043 T>C would affect demineralization ability, we used transverse microradiography (TMR) to test the demineralization effect of S. mutans strains, respectively. After 72 h treatment, all of the S. mutans strains have caused obvious enamel demineralization (Fig. 4a, b). However, the mineral loss of the three mutant strains #858, gtfB^2043 T>C and ΔgtfB were significantly lower than WT (Fig. 4c, p < 0.05). The lesion depths of the #858, gtfB^2043 T>C and ΔgtfB groups were also shallower than WT (Fig. 4d). There were no significant differences among #858, gtfB^2043 T>C and ΔgtfB in both mineral loss and lesion depth.

**Synonymous mutant c.2043 T > C reduced the cariogenic abilities in vivo**

We then compared the cariogenic ability of S. mutans strains in vivo. As shown in Fig. 5a, the WT infected mice had a significantly higher level of S. mutans colonization than other three groups, #858, gtfB^2043 T>C and ΔgtfB (p < 0.05), indicating that the mutation of gtfB gene can inhibit the colonization of S. mutans in oral environment. There was a significant decrease in the incidence and severity of sulcal-surface caries when infected with #858, gtfB^2043 T>C and ΔgtfB compared with WT (Fig. 5b, c and Table 2), indicating that synonymous mutation c.2043 T > C of gtfB gene can reduce the cariogenic abilities of S. mutans in vivo.

**Discussion**

After the exposure of irradiation, we found that S. mutans suspension showed enhanced biofilm formation ability, but different single isolates had different biofilm formation abilities. These findings demonstrate that X-rays radiation markedly alters the virulence of individual cells and then affects overall bacterial population. Similarly, some in vitro studies also showed that the direct effect of radiation on oral Candida albicans cells lead to a rapid proliferation ability, increase of virulent factors and resistance to drugs [30]. Moreover, irradiated Klebsiella oxytoca strains of oral origin were more virulent than nonirradiated ones [31]. All of these results indicated that X-ray irradiation can affect the virulence of different microbes.

Our results also indicated that the radiotherapy therapeutic dosage can cause the genomic mutations of S. mutans and affect its cariogenic abilities. Among all
of the isolated single colonies, we found a biofilm formation-deficient mutant strain named #858 from LD50 group. #858 showed reduced biofilm formation capacity, which was controlled by the down-regulated expression of gtfS and decreased secretion of GtfS, suggesting that the biofilm-inhibiting activity after radiation might depend on the gtfS related gene mutation. The WGRS results indicated that there were 3 synonymous mutations located at gtfB gene of #858 when compared with WT. Based on the codon usage frequency of S. mutans UA159 from Codon Usage Database (http://www.kazusa.or.jp/codon/), we found the corresponding codon of first mutation c.2043 T > C, which encodes isoleucine changed from AUU into AUC, have decreased twofold.

It’s well known that synonymous mutations could also influence biological phenotype from gene and protein levels [32]. Chava et al. found that C3435T, a synonymous SNP in the Multidrug Resistance 1 (MDR1) gene could alter function of its product P-glycoprotein (P-gp) [33]. Another report indicated that the polymorphism C3435T had an effect on mRNA secondary structure, causing decreases mRNA stability and reduced levels of mRNA expression [34]. This phenomenon may result from codon usage bias (CUB), but the detail mechanisms were still not clear [35]. The prevailing view is that the infrequently used codons arise by deficiency of tRNAs to decode them, which may depend on mutational biases, genomic GC content, or through the optimization of fundamental cellular processes [36].

![Fig. 5](image-url)  
**Fig. 5** Synonymous mutation of gtfB gene reduced the cariogenic abilities of S. mutans. a The S. mutans population colonized in rats (n = 4). b Stereo microscopy images of caries lesions. c Statistical analysis of the Keyes scores (n = 4). The error bars represent the standard deviation (SD). (* p < 0.05, ** p < 0.01)

| Group        | Incidence of sulcal caries | Severity of sulcal caries |
|--------------|---------------------------|---------------------------|
|              | Ds                        | Dm                        | Dx                        |
| WT S. mutans | 24.75 ± 1.1               | 18.25 ± 1.92              | 14.25 ± 1.1               |
| #858         | 19.75 ± 0.8               | 14.5 ± 1.5                | 9.75 ± 0.8                |
| gtfB C2043T>C| 21 ± 0.7                  | 15.5 ± 0.5                | 10.5 ± 0.5                |
| ΔgtfB        | 17.75 ± 1.5               | 13 ± 2.5                  | 9 ± 0.7                   |

Ds, the involvement of 1/4 of the dentin between the enamel and the pulp chamber  
Dm, the involvement of 1/4 ~ 3/4 of the dentin region  
Dx, caries progression beyond 3/4 of the dentin region
of synonymous mutations on related genes may be a key evolutionary driver for different subtypes in the natural evolution of microorganisms. Our results demonstrate that synonymous mutations can bring significant changes in codon usage frequency, while the use of rare codons appears to influence the transcription and translation rates of different genes, which then in turn affects protein synthesis [37].

The expression of gfts can alter the amount of EPS [38]. In our study, the synonymous mutations of gtfB can dramatically affected the expression of the genes associated with EPS production, including gtfB, gtfC and gtfD. The deletion of gtfB significantly down-regulated the expression of gtfC and gtfD [39], and this phenomenon was confirmed by our results. The tandem arrangement of gtfB (SMU-1004) and gtfC (SMU-1005) is mere 198 bp between coding sequences, which strongly suggested coordinated regulation between them [40]. There was a common promoter at the upstream of gtfB (445 bp), which create a polycistronic message that included the gtfC coding sequence [41, 42]. Meanwhile there was another possible promoter in the intergenic space (195 bp) between gtfB and gtfC [43], which might allow the independent expression of gtfC. In our study, the mutation site c.2043 T>C is approximately 2.5 kb away from the two promoters, which may affect the binding of transcriptional factors to form transcription initiation complex. In addition, since the base changed from “T” to “C”, the number of hydrogen bonds between the base pairs increased, which may affect the uncoiling of the DNA double-strand during transcription and lead to the down-regulation of gtfC. The gtfD (SMU-910) gene does not share much homology with the other two gtf genes, which is independent from the gtfBC expression [44, 45]. In present study, the expression of gtfD was not decreased at the same level as gtfC, since the spacer was about 86 kb between gtfB and gtfD. During the substrate metabolism, the expression of gtfB and gtfD was more pronounced at the early bacterial exponential growth phase and also increased the expression at late exponential growth phase under some carbohydrates such as sucrose, while gtfD was mainly activated in the late exponential growth phase [46]. Since #858 lost the ability to form mature biofilms, the abnormal biofilm structure cannot maintain the accumulation of substrates and metabolites, which may repress the gtfD expression.

From transverse microradiography (TMR) analysis, we can see that all samples at different groups caused enamel demineralization, because there were no obvious differences in acid production among four S. mutans strains (Additional file 5: Figure S3). However, without EPS, #858, gtfB 2043 T>C and ΔgtfB failed to form stable biofilm, the acid may not accumulate on enamel surface for an enough time to demineralize the teeth as WT. To further investigate the cariogenic capacities of these S. mutans strains, we established caries model in vivo. Similarly, we found that the caries incidence and severity in the three mutant strains groups were lower than in the WT group. This might be due to the abnormal adhesive ability of mutant strains in rats.

To our knowledge, this is the first study showed that a synonymous mutation in gtfB gene could impact the cariogenic abilities of S. mutans without killing the bacteria, indicating that this site could be a good anti-caries target. The application genome-editing techniques to modify the nucleotide of this site can significantly reduce the caries risks instead of the destroying of the oral microecology since the interactions of S. mutans and other species could play an essential part in microbial balance [47]. Moreover, the synonymous mutation strain with decreased cariogenic ability is expected to be a probiotic candidate to replace the cariogenic isolates from clinical dental caries patients.

Currently, we have not found a isolate with extremely enhanced biofilm formation ability, but we randomly selected 10 isolates with enhanced biofilm formation and detected the gfts genes’ expression according to the qRT-PCR assay (Additional file 6: Figure S4). We found that the expressions of gtfB from 5 isolates (#412, #415, #416, #442, #818) were significantly increased compared with wild type, while the others (#205, #219, #220, #223, #838) showed no significant difference. The expressions of gtfC from 8 isolates (#205, #220, #223, #412, #415, #442, #818, #838) were significantly increased, the isolate #416 significantly decreased the gtfC expression, and #219 showed no significant difference. For gtfD, 3 isolates (#220, #442, #818) increased the expression, while the others (#205, #219, #223, #412, #415, #416, #838) showed no significant difference (Additional file 6: Figure S4). We then sequenced the gtfB gene of the 10 isolates and found that there was no mutation on gtfB gene (Additional file 7: Figure S5) indicating that the enhanced biofilm formation of these isolates was not relied on the mutation of gtfB gene and there may be some new mechanisms to elevate the biofilm formation of these isolates. We are continuing to isolate more strains with enhanced biofilm formation and planning to sequence the whole genomes of more strains to investigate the mechanisms.

Our results indicated that synonymous mutations of codons significantly affected the virulence of S. mutans especially on biofilm formation and cariogenic capacity. This may be a reason that some S. mutans isolates showed different virulence. Previous studies found that among S. mutans strains isolated from high-severity caries and caries-free patients, there were some silent point mutations in vicR gene, without any nucleotide sequence
insertions or deletions [48]. Similar results were found in other virulence-related gene such as scrA [49], suggesting the potential relationship between post-radiotherapy disease and synonymous mutation of oral microbes. According to Additional file 3:Table S3, we can find that mutations in the frequently codons such as UAU, AUU and GUU may also change the gene expression of S. mutans. Thus, our results indicated that not only the hyposalivation of patients under radiotherapy, but also some synonymous hotspot mutations in cariogenic bacteria can be the reasons for radiation caries and we may monitor those hotspots to check the cariogenic status of oral bacteria. Our research also provided valuable information that radiation can directly affect phenotype of oral bacteria and induce virulence gene mutation, and patients with undergoing radiotherapy should be alert to the virulence variation of oral pathogens besides the radiation-induced tissue damage.

Conclusion
In conclusion, we investigated the genomic mutations and virulence change of Streptococcus mutans, the major cariogenic bacteria, exposed to the therapeutic doses of X-rays for the first time. We found the radiation at therapeutic doses can directly affect the phenotype of Streptococcus mutans, and we isolated a biofilm formation-deficient mutant #858 which showed decreased extracellular polysaccharide production, biofilm formation and cariogenic capabilities both in vitro and in vivo. Whole-genome resequencing indicating that #858 only possessed three synonymous mutations (c.2043 T > C, c.2100C > T, c.2109A > G) in gtfB gene. The "silent mutation" of c.2043 T > C in gtfB gene can cause the down-regulation of all of the gtf5 genes’ expression and decrease the GtfB enzyme secretion without the effect on growth due to the codon bias. The synonymous mutations of genome are negligible factors for gene expression and related protein translation due to the codon usage frequency. Our results provide valuable information that radiation can directly affect phenotype of S. mutans and induce virulence gene mutation, and suggest that patients undergoing radiotherapy should be alert to the virulence variation of oral pathogens besides radiation-induced tissue damage.

Methods
Bacterial strains and culture conditions
Streptococcus mutans wild type UA159 (WT), provided by the State Key Laboratory of Oral Diseases (Sichuan University, Chengdu, China), were routinely anaerobically grown at 37 °C (90% N2, 5% CO2, 5% H2) in a brain–heart infusion broth (BHI; Difco, Sparks, MD). For biofilm formation, bacteria were inoculated at a concentration of 10^6 colony-forming units [CFUs]/mL in BHI with 1% sucrose, the bacteria culture medium was changed every 24 h.

Irradiation treatment by X-rays
S. mutans cells were harvested at mid-logarithmic phase by centrifugation (4000 g, 4 °C, 10 min), washed twice with PBS and re-suspended (optical density at 600 nm, OD600 nm = 0.5) in the same solution. The bacteria were seeded in 35-mm tissue culture plastic plates sealed with parafilm and irradiated with 6MV X-rays generated by the irradiation equipment Varian Unique at the Sichuan Cancer Hospital in Chengdu, with a dose rate of 1 Gy/min. The radiation doses were respectively 0, 20, 40 and 80 Gy, which were similar to the cumulative dose used clinically for the treatment of head and neck cancer [50]. In addition, the radiation dose was further increased to 300 Gy to test the lethal dose. The irradiated S. mutans cell suspension was diluted and spread on solid BHI medium to determine the viable counts after 48 h of incubation at 37 °C. To calculate the survival fractions, following equation was used: survival fraction = (average number of colonies on treatment plates/average number of colonies on control plates) × 100%.

The crystal violet assay
The irradiated S. mutans cell suspensions were cultivated overnight and diluted at a concentration of 10^6 CFUs/mL in 200 μL of BHI with 1% sucrose in 96-well plates. After 24 h incubation, the supernatant was removed and the biofilms were washed with PBS, fixed with methanol and stained with 0.1% crystal violet solution. The crystal violet stained biofilms were solubilized in 95% ethanol with shaking and the ethanol was transferred to a new 96-well-plates. The absorbance of the solution was measured by Thermo Scientific Multiskan GO reader (Thermo Fisher Scientific Inc, Waltham, MA, USA) at 595 nm [51].

For single colonies screening, cell suspensions from irradiation groups were diluted to 10^-6, and 200 μL of the dilution was spread on solid BHI medium and incubated at 37 °C for 48 h. Then the crystal violet assay method was performed with the isolated strains.

Growth curve assay
The growth of test bacteria was studied using a technique described by Shao [52], the overnight grown bacteria were harvested and resuspended (OD600 nm = 0.1) in fresh BHI medium, then the cultures were incubated at 37 °C. The OD600 values were recorded at 1 h interval during the 20-h cell growth period with the spectrophotometric (microquant microplate spectrophotometer; BioTek Instruments Inc, Winooski, Vt., U.S.A.).
Quantitative determination of water-insoluble EPS

The quantitative determination of water-insoluble EPS was studied using an anthrone-sulfuric acid colorimetric assay described by a previous study [38]. After 48 h of biofilm incubation, the supernatant was removed and the biofilms were washed twice with PBS. Then the S. mutans cells and biofilm were resuspended in PBS and transferred to a sterile 1.5-ml centrifuge tube. The pellets were harvested by centrifugation (6000 g, 4 °C, 10 min) and washed 3 times with sterile PBS to remove the water-soluble EPS. NaOH was added to react with Water-insoluble EPS in a final concentration of 0.4 M for 2 h at 37 °C. Then the alkali-soluble carbohydrate solution was mixed with three volumes of anthrone-sulfuric acid reagent and was heated in a water bath at 95 °C for 5 min. After reaction, the absorbance was measured by Thermo Scientific Multiskan GO reader (Thermo Fisher Scientific Inc, Waltham, MA, USA) at 625 nm.

Quantitative real-time PCR (qRT-PCR)

For quantitative real-time PCR, the primers used in this part are shown in Additional file 2: Table S1. Bacterial RNA isolation, purification, cDNA reverse transcription, and PCR reactions were performed as previously described [28]. Amplification specificity was assessed using melting curve analysis. The expressions of EPS-related genes gtfB, gtfC and gtfD were quantified, with 16S rRNA as an internal control. The data were analyzed by Bio-Rad CFX Manager software (Bio-Rad Laboratories, Hercules, CA, USA) according to the 2^(−ΔΔCT) method.

Biofilm imaging

Biofilm imaging were studied according to methods described in previous studies [53]. For scanning electron microscopy (SEM) imaging, biofilms at 24 h were washed twice with PBS, fixed with 2.5% glutaraldehyde overnight, and serial dehydrated with ethanol (50%, 60%, 70%, 80%, 90%, 95%, and 100%). Then the samples were putter coated with gold for SEM imaging (FEI, Hillsboro, OR, USA).

For EPS staining, the EPS was labeled with Alexa Fluor 647-labeled dextran conjugate (Molecular Probes) at the beginning of biofilm formation, the bacterial cells of the 24 h S. mutans biofilm were labeled with SYTO 9 (Molecular Probes, Invitrogen Corp., Carlsbad, CA) for 15 min. Biofilm images were captured with a Leica DMIRE2 confocal laser scanning microscope (Leica, Wetzlar, Germany) equipped with a 60 × oil immersion objective lens. Three-dimensional reconstruction of the biofilms were performed with IMARIS 7.0.0 (Bitplane, Zurich, Switzerland). COMSTAT image-processing software was used to calculate the biomass (covering percentage) of EPS and bacterial cells according to the fluorescence value.

Whole-genome resequencing

The DNA of #858 and WT was extracted using a TIANamp bacteria DNA kit (Tiangen, Beijing, China) according to the manufacturer’s instructions, the whole-genome resequencing (WGRS) was performed on the Illumina Hiseq sequencing platform (Paired-end, 2 × 150 bp) and the entire data was analyzed by Shanghai Personalbio Biotechnology (Shanghai, China). The variant analysis procedure was list in Additional file 3. The sequence data files have been deposited in the National Center for Biotechnology Information’s Sequence Read Archive: #858 (SRR11905797, https://dataview.ncbi.nlm.nih.gov/object/SRR11905797) and WT (SRR11905798, https://dataview.ncbi.nlm.nih.gov/object/SRR11905798).

Synonymous point mutant construction

In this study, we construct gtfB-gene point mutant strain of S. mutans UA159 with IFDC2 cassette through overlapping polymerase chain reaction (PCR) and allelic homologous recombination [54, 55]. The mutant codon of gtfB gene was mutated from ATT to ATC, and the primers used in this study are shown in Additional file 4: Table S4. The mutation method was list in Additional file 4, and the erythromycin-resistant mutant was named ΔgtfB after first transformation, the resulting p-Cl-Phe resistant mutant was named gtfB 2043 T>C.

Gtf5 isolation and SDS-PAGE

The protein amount and activity of Gtf5 analysis was studied using a technique described by Peng [56] with modifications. 50 mL overnight cultures of various S. mutans strains were centrifuged 13,000 rpm at 4 °C for 10 min, the supernatants were saved and concentrated 50-fold by using 30 KD Amicon® Ultra Centrifugal Filters. Then 1/3 volume ethanol was added into concentrated culture supernatants and save it in −80 °C for 30 min. The pellets were harvested by centrifugation (25000 g, 4 °C, 15 min), mixed with loading buffer and run SDS-PAGE. The quantification of Gtf5 was performed with COMSTAT.

Demineralization effect on bovine enamel

The enamel lesions of demineralization were observed by transverse microradiography (TMR) technique described by Han [57]. After 72 h of biofilm demineralization treatment with BHI containing 1% sucrose, all bovine tooth specimens polished to a thickness ranging from 100 to 150 nm. The specimens were exposed to CuKa X-rays source (Philips B.V., Amsterdam, The Netherlands) operating at 25 kV and 10 mA for 15 min. Then the fixed film was analyzed using a transmitted light microscope (Axioplan; Zeiss, Oberkochen, Germany) and calculated by
Rat model of caries

Animal protocols were conducted in accordance with the Declaration of Helsinki, the policy of Sichuan University and West China School of Stomatology, and the protocol was approved by the Ethical Committee of West China School of Stomatology, Sichuan University (Chengdu, China) (Project identification code: WCHSIRB-D-2019–184, approval date: 07/08/2019).

The cariogenic effect of S. mutans strains was assessed on 20 specific pathogen-free Sprague Dawley rats of male sex using a modified method of a previously study [58]. 17-d-old rats were randomly assigned into 4 groups based on S. mutans strains: WT, #858, gtfB2043T>C and ΔgtfB. For the first 3 days, all animals were provided ampicillin (0.1% w/v), streptomycin (0.1% w/v) and carbenicillin (0.1% w/v) in their drinking water to suppress endogenous flora and then the animals were screened for indigenous S. mutans by an oral swab streaked on mitis salivarius agar (Difco) plus bacitracin. After washed with deionized water for 3 days, animals were then infected with S. mutans strains (1.0 × 10⁷ CFU/ml) for 4 consecutive days and fed a cariogenic diet (Keyes 2000) and sterile drinking water containing 5% sucrose. Colonization of S. mutans strains were confirmed by plating. The animals were weighed weekly and sacrificed after 5 weeks. The bilateral mandibles were aseptically excised and sonicated in sterile PBS. The suspensions were plated on mitis salivarius agar (Difco) plus bacitracin (Sigma) to calculate the S. mutans population colonized in rats. Then the teeth were stained and the caries status was scored using the Keyes method with a stereo microscope.

Statistical analysis

All the experiments were repeated at least 3 times independently. Statistical analysis was performed with the SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). For the in vitro studies, One-way analysis of variance and Student–Newman–Keuls test were used for all pairwise comparison. Independent t-tests were used for the in vivo study. Significant differences were considered when p < 0.05.

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Authors’ contributions

ZW and YZ performed experiments, collected and analyzed the data, writing and editing the manuscript. XY, YY, YS, YL and JZ obtained, analyzed and interpreted the data. GQ provided radiation equipment. QH performed the transverse microangiography. XZ, LC and BR supervision, designed the study, writing and editing the manuscript, funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Animal study was approved by the Ethical Committee of West China School of Stomatology, Sichuan University (Chengdu, China) (Project identification code: WCHSIRB-D-2019–184, approval date: 07/08/2019).

Consent for publication

All participants have given consent for publication.

Abbreviations

RT: Radiotherapy; HNC: Head and neck cancer; RC: Radiation caries; OTUs: Operational taxonomic units; EPS: Extracellular polysaccharides; WT: Wild type; SEM: Scanning electron microscope; WGRS: Whole-genome resequencing; BHI: Brain heart infusion broth; RT-PCR: Real-time polymerase chain reaction; TMR: Transverse microradiography; CUB: Codon usage bias; CFUs: Colony-forming units.

Supplementary Information

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Competing interests
The authors declare that they have no competing interests.

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