Role of Biosynthetic Gene Cluster BGC3 in the Cariogenic Virulence of *Streptococcus mutans*

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Objective: *To investigate the role of the biosynthetic gene cluster BGC3 of Streptococcus mutans (S. mutans) in the process of dental caries.*

Methods: *BGC3 and ΔBGC3 S. mutans strains were constructed and their growth curves were evaluated. Acid production capacity was assessed by evaluating pH reduction levels over identical culture periods. The survival of bacteria in phosphate citrate buffer solution (pH 3.0) was quantified. The expression levels of virulence genes (atpF, gtfC, gtfD, spaP, vicR and ftf) were analysed using the qPCR. Co-culture experiments were conducted to evaluate bacterial adaptability. Bacterial viability was determined by microscopical examination of live/dead staining.*

Results: *Deletion of BGC3 did not significantly impact S. mutans growth or acid production in biofilms. The ΔBGC3 strain exhibited enhanced acid resistance and higher expression levels of virulence genes compared to the wild type. In addition, ΔBGC3 exhibited superior bacterial viability in the co-culture system.*

Conclusion: *BGC3 affected the acid resistance and expression of caries-related genes in S. mutans. The BGC3 knockout strain exhibited a more robust survival capability than the wild-type strain.*

Keywords: *biofilm, biosynthetic gene clusters, dental caries, Streptococcus mutans, virulence genes*

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Dental caries is one of the most common chronic diseases globally and a major public health challenge. It is a multifactorial disease and one in which bacterial biofilm, commonly called dental plaque, is thought to play a role in the occurrence and progression.[1] *Streptococcus mutans* (*S. mutans*), a prominent member of the Streptococcus genera in the natural oral microbiota, is prevalent in various oral habitats, including saliva, dental plaque, gingival crevicular fluid and carious teeth.[2] By the 1960s, clinical and animal research had established *S. mutans* as a significant pathogen in dental caries.[3] Subsequent studies have reinforced its crucial role in caries development with its acid production, acid tolerance, adhesion and biofilm formation abilities.[4,5]

Biosynthetic gene clusters (BGCs) are sequences of nucleotides containing specific genetic information. The small-molecule products encoded by these clusters have been recognised for their unique biochemical potential.[6] These molecules are critical mediators in microbial communities, facilitating microbe-microbe

Table 1 Strains and plasmids used in this study.

Strain/Plasmid		Characteristics	Sources
Strain	*S. mutans* CIM3001	Wild type	This study
	S. mutans CIM3001ΔBGC3	BGC3 in-frame deletion mutant	This study
Plasmid	pIFDC2	Amp^r, IFDC2 cassette	Baker et al[5]

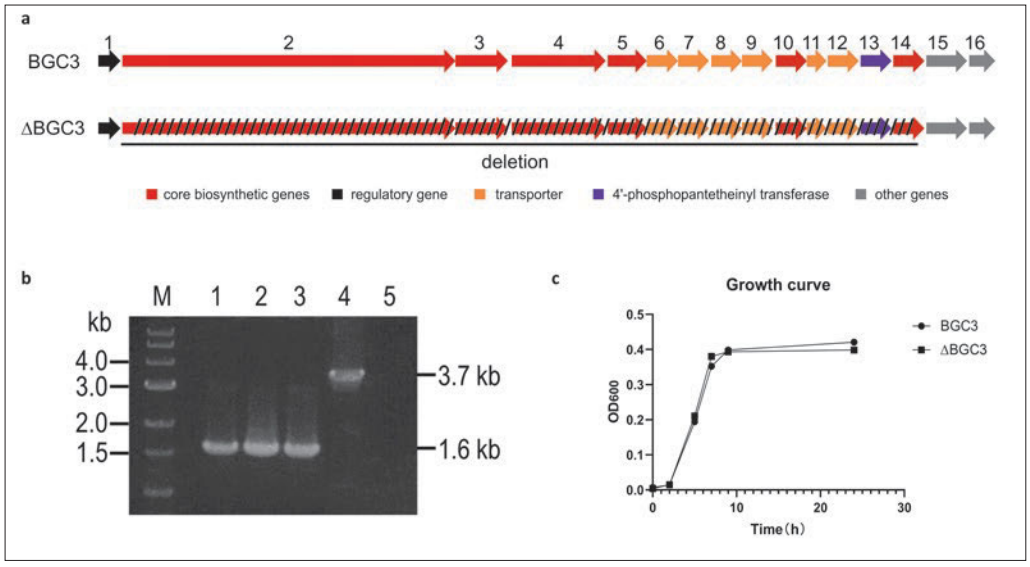

Fig 1a to c Construction, PCR verification and growth curves of *S. mutans* CIM3001ΔBGC3 and CIM3001BGC3 strains. The genes are sequentially encoded as 1 to 16 from left to right, and their functional predictions and proteins are shown in Table 3. The slash represents the in-frame deletion part. The genes are indicated by arrows with different colours **(a)**. Lane M shows the DNA marker; lanes 1 to 3 present the PCR verifications of *S. mutans* CIM3001ΔBGC3 strains; lane 4 illustrates the PCR verification of a BGC3 gene cluster disrupted mutant with an IFDC2 cassette; and lane 5 is the negative control using the genomic DNA of *S. mutans* CIM3001 **(b)**. Growth curves of BGC3 and ΔBGC3 strains in brain heart infusion (BHI) media over 24 hours under anaerobic conditions **(c)**.

and host-microbe interactions. Their functions span antimicrobial activity, signalling activation, immune regulation and biofilm formation.[7]

Recent investigations have revealed that the genome of *S. mutans* encompassed numerous BGCs associated with microbial secondary metabolism. These include non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS) and hybrid PKS/NRPS gene clusters.[6] Notably, previous studies have identified specific hybrid PKS/NRPS gene clusters in *S. mutans*, namely *mub*, muc and *muf*, that contribute to various physiological activities. These activities encompass interspecies competition, oxidative stress response and biofilm formation, offering new avenues for understanding the cariogenic mechanisms of *S. mutans* and strategies for caries prevention.[8-10]

In a prior investigation, 169 *S. mutans* genomes were analysed, leading to the identification 136 of hybrid PKS/NRPS gene clusters ranging from 12.6 to 36.2 kb in length. These gene clusters were divided into six groups based on their gene composition and organisation.[6] Group III, referred to as BGC3 in this study, consists of 29 hybrid PKS/NRPS gene clusters. BGC3 encodes two PKS modules, three NRPS modules, six transporter proteins, etc. It can release thioester-tethered product reductively, and may also hydrolyse incorrectly loaded elongation units in thiolation domains.[6] However, its biological functional attributes remain unexplored.

Expanding on this research, the current study analysed 413 *S. mutans* genomes from the NCBI database (as of 20 March 2023). Of these, 97 were found to contain BGC3 (NCBI accession number NZ_JAPMUF000000000.1). This finding ranks BGC3 as the second most prevalent gene cluster responsible for mutanobactin production, following Group I which was found to be associated with survival of *S. mutans* in a biofilm environment.[6]

To investigate the impact of BGC3 on the cariogenic properties of *S. mutans*, we isolated the *S. mutans* CIM3001 strain, containing BGC3, from the oral cavity and created a mutant strain, *S. mutans* CIM3001ΔBGC3 (hereafter called ΔBGC3, with the characteristic of being susceptible to gene editing), for experimental purposes.

Table 2 Primers used in this study.

Primers	Sequence (5' to 3')	Description
ldhF-BsaI	CAGGTCTCTGAGCAACAATAACACTCATAGC	IFDC2 cassette PCR
ermR-BsaI	GTGGTCTCAGAAGCTGTCAGTAGTATACCTAA	
BGC3ko-upF	ATCGTTTGCCCCATATAATC	BGC3 in-frame deletion
BGC3ko-upR	GTGGTCTCAGCTCTGTTCTTTTGCTGAGGCTGT	
BGC3ko-upR2	GTGGTCTCATGTTCTTTTGCTGAGGCTGT	
BGC3ko-dnF	CAGGTCTCTCTTCCACTTTATCCACCAACT	
BGC3ko-dnF2	CAGGTCTCTAACACACTTTATCCACCAACT	
BGC3ko-dnR	TAGTTCCGTTATTGTTTCCA	
BGC3-vF	TAAGCTCAAAAGTAAAATGGC	mutant verification
BGC3-vR	TTCATGTCATAGTTATCCGTT	
atpF-F	TTGATAACGCTAAGGAAACTGGTA	H^+-translocating F-ATPase
atpF-R	AACGCTTGATAGGGCTTCTG	
gtfC-F	TCAGACAACACCTTCCTTCCTA	Glucosyltransferase SI (GTFC)
gtfC-R	GAGCACCAGTGACCATATAACC	
gtfD-F	GCCTTTTTACGCTTGTTTGT	Glucosyltransferase I (GTFD)
gtfD-R	CCATATTCATATTCTCCGCC	
spaP-F	TCCGTGCCGATAATCCAAGA	Surface-associated protein P1
spaP-R	CGCCTGTTTGTCCCATTTGT	
vicR-F	GCATCACTTAGCGACACACA	Two-component regulatory system regulator
vicR-R	CAGACGACGAACAGTAACATCAA	
ftf-F	CGAACGGCGACTTACTCTTAT	Fructosyltransferase (FTF)
ftf-R	TTACCTGCGACTTCATTACGATT	
BGC3-F	GTCTTGATAGCAAAGGTGAA	Specific DNA primers
BGC3-R	TATCAAAATAAAACGGCAACC	
BGC3ko-F	TAAAGTCAACAGAGTTTCTC	Specific DNA primers
BGC3ko-R	TAATTCTTGTTTGGTGAAAG	

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids utilised in this study are enumerated in Table 1. *S. mutans* strains were cultured anaerobically on brain-heart infusion (BHI) agar plates or in BHI broth (Oxoid, Basingstoke, England) at 37°C. Antibiotic-resistant colonies were selected on BHI plates supplemented with erythromycin (12.5 μg/ml), whereas p-Cl-Phe-sensitive colonies were selected on BHI plates containing 4 mg/ml p-Cl-Phe.

General DNA manipulations

DNA extraction and genome sequencing of *S. mutans* CIM3001 were conducted by Novogene (Beijing, China). All polymerase chain reaction (PCR) primers used were synthesised by Azenta Life Sciences (Beijing, China) and are detailed in Table 2. The PCRs were performed using Taq DNA polymerase or Phanta DNA polymerase (Vazyme, Nanjing, China), following the protocols provided by the manufacturer. Golden Gate cloning was executed as previously described.[11] Genomic DNAs from various *S. mutans* strains were prepared as outlined in the literature.[12] For natural transformation experiments, cells were incubated in Todd-Hewitt medium (Becton-Dickinson, Sparks, MD, USA) enriched with 0.3% yeast extract, with the comprehensive natural transformation procedure described in an earlier report.[13]

Construction of the gene cluster in-frame deletion mutant

The BGC3 and ΔBGC3 genes are shown in Fig 1a. The in-frame deletion mutant of the BGC3 gene cluster, *S. mutans* CIM3001ΔBGC3, was constructed through a two-stage homologous recombination process. Initially, two DNA fragments flanking the BGC3 gene cluster, measuring 0.5 and 0.8 kb, were PCR-amplified using the primers BGC3ko-upF/R and BGC3ko-dnF/R, with genomic DNA from *S. mutans* CIM3001 as the template. Concur-

Table 3 Genes of BGC3 and their function predictions and proteins.

Gene	Function prediction	Protein (NCBI Reference Sequence)
1	Helix-turn-helix domain-containing protein	WP_002277533.1
2	T-KS-AT(Malonyl-CoA)-T-C-A(Phe)-T-C-A(Glu)-T	WP_195426493.1
3	KS-T	WP_002285336.1
4	C-A(Ser)-T-R	WP_195426492.1
5	Class I SAM-dependent methyltransferase	WP_002277529.1
6	Macrolide transporter	WP_002269643.1
7	ABC transporter ATP-binding protein	WP_002283253.1
8	ABC transporter permease	WP_002278315.1
9	ABC transporter permease	WP_002264134.1
10	acyl-CoA ligase	WP_002285333.1
11	ABC transporter ATP-binding protein	WP_002264137.1
12	ABC transporter permease	WP_002277525.1
13	4'-phosphopantetheinyl transferase superfamily protein	WP_195426491.1
14	Thioesterase	WP_002264142.1
15	Methionine adenosyltransferase	WP_002283366.1
16	SDR family oxidoreductase	WP_002278149.1

rently, a 2.1 kb IFDC2 cassette was PCR-amplified using the primer pair ldhF-BsaI/ermR-BsaI with pIFDC2 as the template. These flanking fragments and the IFDC2 cassette were then joined through Golden Gate cloning and introduced into *S. mutans* CIM3001. Erythromycin-resistant colonies on BHI plates were selected and PCR-validated as BGC3 cluster-disrupted mutants using the primer pair BGC3-vF/R. Subsequently, the IFDC2 cassette was removed via a second homologous recombination round. Here, fragments flanking the IFDC2 cassette (0.5 and 0.8 kb) were PCR-amplified using primers BGC3ko-upF/R2 and BGC3ko-dnF2/R, ligated and transformed into the BGC3-disrupted mutant. Colonies capable of growing on BHI plates supplemented with p-Cl-Phe were selected and PCR-confirmed as *S. mutans* CIM3001ΔBGC3 using the primer pair BGC3-vF/R, with final validation by sequencing.

Growth conditions and growth curves

BGC3 and ΔBGC3 strains were cultured anaerobically in BHI broth (Difco) or on BHI agar plates (pH 7.0) at 37°C (85% N_2, 10% CO_2, 5% H_2). Overnight cultures of *S. mutans* were diluted in fresh BHI to an optical density at 600 nm (OD_{600}) of 0.5, representing log-phase bacteria, and further diluted 100-fold in BHI medium containing 2% (w/v) sucrose for biofilm formation. The bacterial concentration was adjusted to OD_{600} = 1.00, and 5% of the inoculum was added to the new BHI broth medium for cultivation. Growth curve was determined by measuring the OD_{600} values was measured at 2, 5, 8, 10 and 24 hours, respectively.

Differences in acid production, acid tolerance and expression of adhesion-related genes

BGC3 and ΔBGC3 strains were cultivated in BHI broth (pH 7.0) and BHI broth with 2% (w/v) sucrose (pH 7.0), respectively. After overnight cultivation, the supernatants were collected for measuring pH value by using a PHS-3C precision pH meter (INESA Scientific Instrument Co, Shanghai, China). The acid production capacity of the indicated strains was determined by the changes in pH.

The harvested bacteria at the mid-logarithmic growth phase were resuspended to an optical density at 600 nm (OD_{600}) of 0.2 in phosphate-citrate buffer solution (pH 3.0) and incubated at 37°C for 90 minutes. Viable cell counts were performed on the samples after 24 hours of incubation.

To assess the differences in acid resistance and adhesion gene expression levels between the BGC3 and ΔBGC3 strains, SYBR reverse transcription-quantitative PCR was employed, targeting the acid resistance-related gene *atpF* and the adhesion-related genes *gtfC*, *gtfD*, *spaP*, *vicR* and *ftf*. Bacterial RNA extraction was conducted using an RNAprep Pure Cell/Bacteria Kit. The extracted total RNA was then reverse-transcribed with the targeted primers listed in Table 2. qPCR reactions were carried out on an ABI 7500 Real Time PCR system using the GoTaq qPCR Master Mix kit (SYBR Green Reagent), in a total reaction volume of 20 µl. The qPCR protocol included an initial 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression levels of the candidate genes were quantified using the $2^{-\Delta\Delta Ct}$ method.

Microscopic examination of live/dead staining

The bacterial viability was assessed using a SYTO9-PI Live and Dead Bacterial Stain Kit. This kit comprises SYTO9, a green fluorescent dye that permeates both live and dead cells, and propidium iodide, a red fluorescent dye that only enters dead or dying cells with compromised cell membranes. As previously mentioned, biofilms cultured for 2 hours and 16 hours on cover glasses were stained with a 1:1 (volume ratio) mixture of SYTO9 and propidium iodide solution for 15 minutes, adhering to the guidelines provided by the manufacturer.[14] The biofilms were washed with deionised water to remove residual dye.

Images were obtained using a con-focal laser scanning microscope (CLSM) (OLYMPUS, Tokyo, Japan) with 63× oil immersion objectives. Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) was employed to analyse the images, based on three duplicate samples from each group.

Co-culture assay

The same concentration of BGC3 and ΔBGC3 bacterial fluids (OD_{600} = 1.00) were inoculated into BHI medium in 6-well plates at a ratio of 100 μl: 100 μl for cultivation at indicated time points. At 3, 24 and 47 hours after co-culture, bacteria were harvested and subjected to extract DNA using the TIANamp Bacterial DNA Kit. The qPCR was performed on an ABI 7500 Real Time PCR machine. qPCR reactions were set as described above, except using BGC3 and ΔBGC3 specific primers (Table 2).

Statistical analysis

All experiments were conducted with a minimum of three technical and three biological replicates. Statistical analyses for growth curve and expression levels of targeted genes were performed using a Student t test. Statistical analysis for co-culture was carried out using a two-way analysis of variance. The data were analysed using GraphPad Prism (version 8.0.2) software (GraphPad Software, San Diego, CA, USA), with the level of statistical significance set at $P < 0.05$.

Results

Construction of ΔBGC3 strain and investigating the growth curves of BGC3 and ΔBGC3 strains

We mapped the structure of the BGC3 gene cluster based on the published reference (Fig 1a).[6] To determine the impact of BGC3 deletion on the growth of *S. mutans*, the *S. mutans* CIM3001 ΔBGC3 strain was successfully generated after screening by PCR (Fig 1b). The ΔBGC3 mutant strain exhibited a growth profile similar to the wild-type strain (Fig 1c), suggesting that the absence of BGC3 did not significantly affect the growth of *S. mutans*.

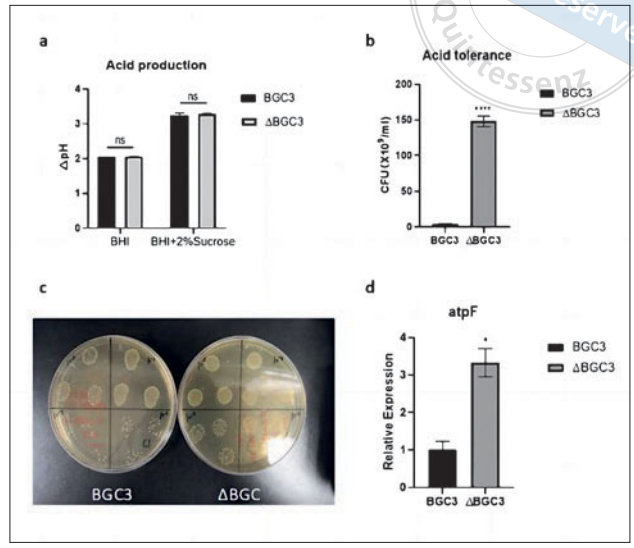

Fig 2a to d Acid production and tolerance comparison between BGC3 and ΔBGC3. Difference in pH values of BGC3 and ΔBGC3 over 16 hours in both BHI and BHI supplemented with 2% sucrose **(a)**. Colony-forming units per millilitre for ΔBGC3 and BGC3 after treatment with acidic solution (pH 3.0) **(b)**. Growth of ΔBGC3 and BGC3 on the culture medium after treatment with acidic solution (pH 3.0). The dilution times for the upper right, upper left, lower right and lower left quadrants are 10^4,10^5,10^6 and 10^7 respectively **(c)**. Expression level of *atpF* in BGC3 and ΔBGC3 **(d)**. Data are expressed as mean ± standard deviation (SD). NS, not significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

Virulence assessment of BGC3 and ΔBGC3 strains

Bacterial virulence was evaluated in both the BGC3 and ΔBGC3 strains. No significant difference in acid production was revealed between the two strains (Fig 2a); however, a marked increase in acid tolerance was noted in the absence of BGC3 (Fig 2b). This increase was evidenced by the higher number of bacterial colonies formed by the ΔBGC3 group in an acidic environment compared to the control group (Fig 2c). Additionally, the expression of the acid resistance gene atpF was upregulated in the ΔBGC3 group relative to the wild type (Fig 2d).

Further investigation into gene regulation potentially influenced by BGC3 was conducted using reverse transcription-qPCR. This analysis showed significant upregulation of key biofilm formation-associated genes such as *gtfC*, *gtfD*, *spaP*, *vicR* and *ftf* in the ΔBGC3

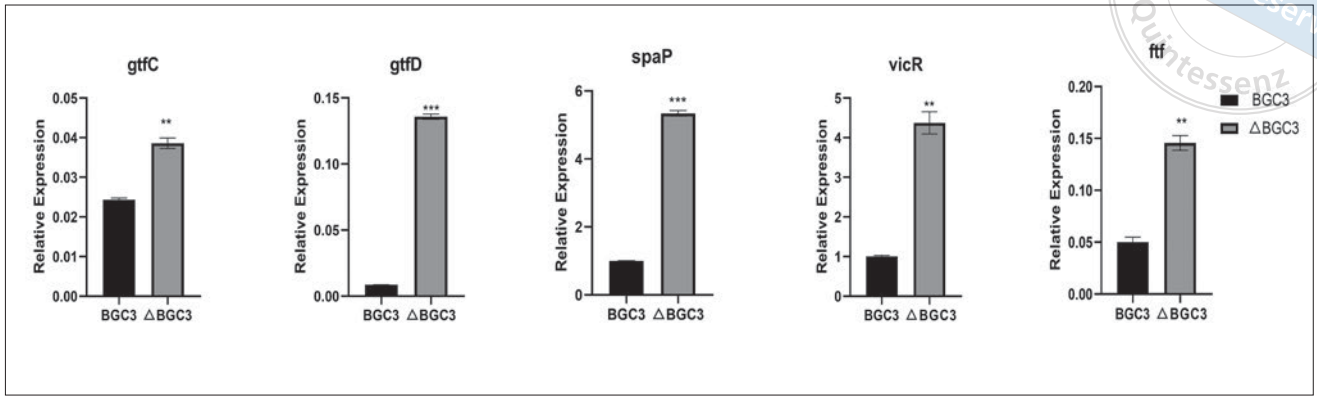

Fig 3 Expression of adhesion-related genes by reverse transcription-quantitative PCR. Effects of BGC3 on *gtfC/D*, *spaP*, *vicR* and *ftf* gene expression levels in *S. mutans*. Data are expressed as mean ± SD. ***P* < 0.01, ****P* < 0.001.

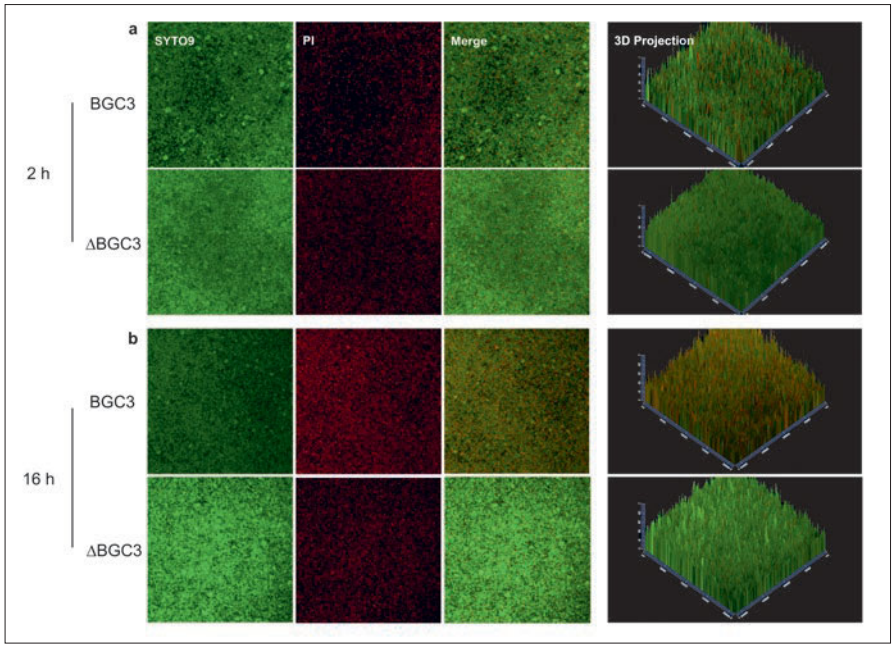

Fig 4a and b Confocal microscope images of biofilms at 2 and 16 hours. Green indicates living/active cells (SYTO9) and red shows inactive/dead cells (PI). Confocal microscope images of biofilms cultured for 2 hours **(a)**. Confocal microscope images of biofilms cultured for 16 hours **(b)**.

mutant compared to the BGC3 strain (*P* < 0.05), as indicated in Fig 3.

Bacterial viability and dominance of ΔBGC3 in co-culture

SYTO9-PI live/dead staining enabled clear differentiation between viable (green) and dead (red) bacteria. At 2 hours (Fig 4a) and particularly at 16 hours (Fig 4b), the BGC3 group exhibited more dead bacteria.

In co-culture assays, BGC3 displayed significantly lower viability when competing with ΔBGC3. A growth competition assay was conducted to assess any potential growth benefits of BGC3. This process involved mixing the two bacterial cultures in a ratio of approximately 50:50 and culturing them together in fresh medium. This assay, conducted over various hours, indicated superior growth in ΔBGC3 compared to the wild-type strain, as shown in Fig 5.

Discussion

Six types of hybrid PKS/NRPS gene clusters have been identified in *S. mutans*. BGC1, with the highest detection rate, has been shown to confer protection against oxygen and H_2O_2 stress, thereby enabling *S. mutans* to counteract the defence mechanisms of pioneer colonisers.[8] Our analysis of 413 *S. mutans* genomes revealed that the frequency of BGC3, the focus of this study, ranked second. Notably, *S. mutans* strains both with and without

the BGC3 gene clusters exist naturally.[8] However, the specific functions of BGC3 have not been explored previously.

S. mutans, as the primary pathogen in human dental caries, manifests its cariogenic toxicity predominantly in three aspects: strong adhesion ability, generation of substantial amounts of organic acids leading to the corrosion of dental hard tissues, and the ability to withstand environmental stress, particularly under low pH conditions.[1,15] Therefore, we sought to compare the impacts of BGC3 and ΔBGC3 on the virulence of *S. mutans* in these respects. Considering that the adhesion of *S. mutans* to teeth surface relies on sucrose-dependent and independent mechanisms, we measured the expression levels of five related genes in total. Among them, the sucrose-independent adhesin gene *SpaP* represents adhesion in a sucrose free environment.[16] In the sucrose-dependent mechanism, *gtfC*, *gtfD* can synthesise glucans, while *ftf* is related to fructans synthesis.[17,18] The response regulator *VicR* positively regulated the expression of exopolysaccharide synthesis and adhesion related genes *gtfC*, *gtfD* and *ftf*.[19] The glucosyltransferase genes (*gtfC*, *gtfD*) and a single fructosyltransferase gene (*ftf*) encode enzymes which are important in formation of exopolysaccharides, and these genes are also more frequently detected in research. The expressions of gtf and *ftf* genes were crucial for the initial adhesion of *S. mutans* to the tooth surfaces.[20] However, the sucrose-independent mechanism cannot be ignored, and in order to be as comprehensive as possible, we have selected these genes. In this study, compared to the wild type, the knockout strain showed significantly higher expression of the aforementioned genes, indicating that the impact of BGC3 on the two adhesion mechanisms of *S. mutans* is consistent. *VicRK* is a signalling system of *S. mutans* in response to environmental changes. The response regulator *VicR* positively regulated the expression of exopolysaccharide synthesis and adhesion related genes *gtfC*, D and *ftf*.[19,21] The trend of the expression of these genes was the same in this study. By measuring the expression of these genes, we found that the presence of BGC3 reduced the expression of virulence genes related to adhesion of *S. mutans*; however, the mechanisms require further study.

The ΔBGC3 strain exhibits greater acid tolerance and significantly higher expression of the acid tolerance-related gene *atpF* than the wild-type strain. The *atpF* gene encodes the H^+-translocating ATPase on the cell membrane. It hydrolyses ATP and expels protons to maintain a transmembrane pH gradient, making a relatively alkaline environment inside the cell.[22] The presence of BGC3 may affect bacterial tolerance to acidic environments by downregulating the expression of atpF. We found no significant difference in acid production between the BGC3 and ΔBGC3 *S. mutans* strains. An article reported that the presence of BL-BGCs significantly reduced the biofilm pH in *S. mutans*, while other virulence and fitness properties remained unchanged compared to the knockout strain.[9] Thus it can be seen that different BGC gene clusters may have different effects on the acid production and acid resistance of *S. mutans*.

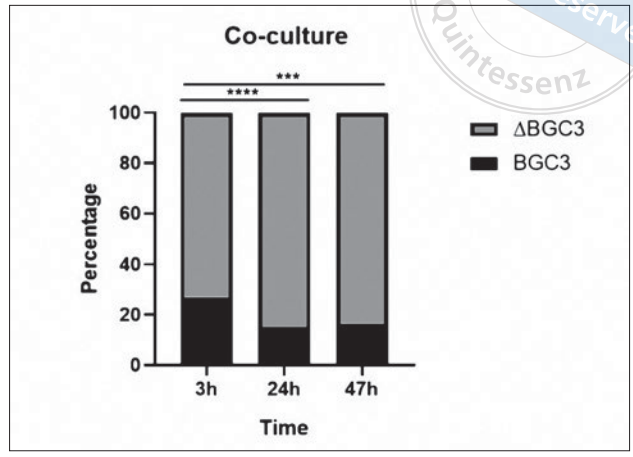

Fig 5 Co-culture of BGC3 and ΔBGC3 in a 50:50 ratio over different durations. The vertical axis represents the percentages of BGC3 and ΔBGC3 strains. ***P < 0.001, ****P < 0.0001.

In this study, the ΔBGC3 strain exhibited lower mortality and dominated in co-culture experiments compared to the wild type, while the BGC3 strain presented worse adaptability to harsh environments, which indicates the presence of BGC3 negatively affected the expression of certain virulence genes. This outcome is understandable, secondary metabolite synthesis demanded substantial energy which imposes an additional metabolic burden on *S. mutans*. Therefore, gene deletion may reduce the energy consumption associated, offering a survival advantage.[23] In addition, *S. mutans* has also been detected in a caries-free group,[24] which provides evidence to suggest that the cariogenic ability of different *S. mutans* strains varies. Moreover, previous studies suggest that the numerous BGCs identified in *S. mutans* may affect its cariogenic virulence from different perspectives, raising questions about potential interactions among them.[6,9,10]

For *S. mutans* strains that do not carry BGC3, it is easier to withstand the screening of acidic environment and survive, which may be conducive to the expression of other cariogenic gene clusters and provide a good basis for cariogenesis. However, it is necessary to con-

sider the limitations of in vitro experiments. Further studies should examine the effect of BGC3 on plaque biofilms containing a variety of bacteria. Furthermore, given the complex bacteria-host interactions, animal models could reflect the role of BGC3 more intuitively.

Conclusion

Overall, the BGC3 gene cluster in *S. mutans* was associated with bacterial acid tolerance and expression of adhesion-related genes, and the knockout strain exhibited greater adaptability, highlighting the potential influence of this gene cluster on bacterial survival and virulence characteristics.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Jing Yi YANG, Yi Xin ZHANG and Yu Wei ZHANG contributed to the acquisition and analysis of data and the drafting, revision and editing of the manuscript; Drs Ying CHEN, Min Di XU and Dan Dan WANG contributed to the acquisition and analysis of data; Drs Yi Hua CHEN, Bin XIA and Yi Xiang WANG contributed to the conception and study design; Drs Bin XIA and Yi Xiang WANG also contributed to the critical revision of the manuscript.

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