Video Article

Generation of a Gene-disrupted *Streptococcus mutans* Strain Without Gene Cloning

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Abstract

Typical methods for the elucidation of the function of a particular gene involve comparative phenotypic analyses of the wild-type strain and a strain in which the gene of interest has been disrupted. A gene-disruption DNA construct containing a suitable antibiotic resistance marker gene is useful for the generation of gene-disrupted strains in bacteria. However, conventional construction methods, which require gene cloning steps, involve complex and time-consuming protocols. Here, a relatively facile, rapid, and cost-effective method for targeted gene disruption in *Streptococcus mutans* is described. The method utilizes a 2-step fusion polymerase chain reaction (PCR) to generate the disruption construct and electroporation for genetic transformation. This method does not require an enzymatic reaction, other than PCR, and additionally offers greater flexibility in terms of the design of the disruption construct. Employment of electroporation facilitates the preparation of competent cells and improves the transformation efficiency. The present method may be adapted for the generation of gene-disrupted strains of various species.

Video Link

The video component of this article can be found at https://www.jove.com/video/56319/

Introduction

Gene function analysis typically involves a phenotypic comparison between a wild-type strain and a strain in which a particular gene of interest has been disrupted. This gene-disruption technique has been utilized for gene function analyses in a wide range of taxa, from bacteria to mammals. A gene-disruption construct is necessary for the generation of the gene-disrupted strain; this deoxyribonucleic acid (DNA) construct consists of the antibiotic resistance marker gene fused to the upstream and downstream flanking regions of the target gene, and is incorporated into the genome via homologous recombination. The gene-disrupted strain may be isolated using selective media containing the antibiotic. The conventional method used for the construction of the gene-disrupted strain requires complex steps, such as the amplification of the target gene by polymerase chain reaction (PCR), ligation of the gene into a suitable plasmid, digestion with restriction enzymes, and religation to insert the antibiotic-resistance marker gene. This process is time-consuming, taking several days to several weeks, depending on the success of each step. In addition, the design of disruption constructs is dependent on the restriction enzyme sites of the target gene. To resolve these issues, PCR-based DNA splicing methods[1,2] for the design of gene-disrupted mutants of *Dictyostelium discoideum*[3] and *Synechocystis* sp. PCC6803[4] have been reported. In the present study, a method was developed to generate a gene-disrupted streptococcal strain in a relatively rapid, facile, and inexpensive manner, utilizing a modified PCR-based method (designated 2-step fusion PCR) for the construction of the disruption construct and electroporation for genetic transformation.

The 2-step fusion PCR requires genomic DNA, the antibiotic resistance marker gene as a PCR template, and four pairs of appropriately designed oligonucleotide primers. In the first step (1st PCR), a 1-kb upstream flanking region of the target gene, a 1-kb downstream flanking region of the target gene, and the antibiotic-resistance marker gene are amplified by PCR. The 5' regions of both the reverse primer and the forward primer, for amplification of the upstream and downstream flanking regions, respectively, include 15 bases complementary to the ends of the marker gene. The 5' regions of both forward and reverse primers for marker gene amplification similarly include 15 bases complementary to the lower end of the upstream flanking region of the target gene and the upper end of the downstream flanking region of the target gene, respectively. Therefore, the three amplified fragments contain overlapping 30-base pair regions at the fusion site. In the second step (2nd PCR), PCR is performed with nested PCR primers using the three fragments amplified by the 1st PCR as templates. The complementary regions of the templates are additionally linked to each other via the 2nd PCR. Finally, the construct for homologous recombination is introduced to the wild-type strain by electroporation. Successful gene disruption may be verified by PCR using specific primers. Although the disruption construct is amplified using high-fidelity DNA polymerase, additional enzymatic reactions, such as DNA ligation or DNA digestion, are not necessary to generate the construct. In addition, a deleted or preserved region on the genome may be flexibly chosen according to primer design.

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In the present work, deletion of the entire coding region of the *gtfC* gene, which encodes glucosyltransferase in *Streptococcus mutans*, was performed to demonstrate the rapid, easy gene disruption method in a streptococcal species. Additionally, a *gtfB*-disrupted strain was generated in the same manner. The glucosyltransferases encoded by both *gtfC* and *gtfB* contribute to cariogenic dental biofilm development[5,6]. The biofilm-forming abilities of the wild-type strain (*S. mutans* WT), *gtfC*-disrupted strain (*S. mutans* Δ*gtfC*), and *gtfB*-disrupted strain (*S. mutans* Δ*gtfB*) were evaluated for comparative phenotypic analyses. This protocol extends the application of a two-step method for gene disruption to an additional species and may be modified for studies of gene function in a wider range of taxa.

Protocol

1. Primer Design

1. **Prepare primers for 2-step fusion PCR and verification of gene disruption.**
   NOTE: **Table 1** shows the primer sequences used in this protocol. **Figure 1** shows a schematic illustration of the 2-step fusion PCR method used to generate *S. mutans* Δ*gtfC*.
   1. Design primers for the replacement of the target region in the genome with the spectinomycin-resistance gene (*spc*^{r})[7]. This protocol replaces the entire coding region of the *gtfC* gene with *spc*^{r}.
   2. Include 15 bases complementary to the upper and lower ends of *spc*^{r} at the 5' regions of up-reverse and down-forward primers, respectively. Similarly, include 15 bases complementary to the lower end of the upstream flanking regions of *gtfC* and the upper end of the downstream flanking regions of *gtfC* at the 5' regions of *spc*^{r}-forward and *spc*^{r}-reverse primers, respectively (**Figure 1A**).
      NOTE: Sequences of up-reverse, down-forward, *spc*^{r}-forward, and *spc*^{r}-reverse primers are automatically determined depending on the site of incorporation of the disruption construct.
   3. Design the up-forward and down-reverse primers to comprise >1-kb upstream and downstream flanking regions of the *gtfC* gene, respectively. Set the melting temperature (T_m) of up-forward and down-reverse primers in accordance with the melting temperature of up-reverse and down-forward primers, respectively (**Figure 1A**).
      NOTE: Refer to the following formula to determine T_m: T_m (°C) = 2(*NA + *NT) + 4(*NC + *NG) - 5, where *N represents the number of primer nucleotides with the specified identity (A, T, C, or G).
   4. Design nested primers (N_up-forward and N_up-reverse) for the 2nd PCR (**Figure 1B**).
      NOTE: Although the outermost primer pair (up-forward and down-reverse) may be used as primers for the 2nd PCR, nested primers (N_up-forward and N_up-reverse) were designed in this protocol. Nested primers are frequently required for the 2nd PCR, as detailed in the **Representative Results**.
   5. Design primers specific for *spc*^{r}. The primers are used for colony PCR to screen for *gtfC* disruption.
   6. Design primers for the verification of *gtfC* disruption.
      NOTE: The PCR products amplified using these primers straddle the border between *gtfC* or *spc*^{r} and upstream or downstream flanking regions of *gtfC*. The primer sets '*gtfC* up-forward and *gtfC* up-reverse' and '*gtfC* down-forward and *gtfC* down-reverse' are specific for *gtfC*, and '*gtfC* up-forward and *spc*^{r}2-reverse' and '*spc*^{r}2-forward and *gtfC* down-reverse' are specific for *spc*^{r}.

2. Genomic DNA Extraction from *S. mutans*

1. Streak stock *S. mutans* UA159 onto a brain heart infusion (BHI) agar plate. Incubate the plate overnight at 37 °C under anaerobic conditions.
2. Pick up a single colony using a sterilized toothpick and inoculate it into 5 mL of BHI broth. Incubate the *S. mutans* culture overnight at 37 °C under anaerobic conditions.
3. Centrifuge the bacterial cell culture for 15 min at 2,000 × g. Resuspend the cell pellet in 5 mL of phosphate-buffered saline (PBS).
4. Centrifuge for 15 min at 2,000 × g. Resuspend the cell pellet in 200 µL of PBS.
5. **Extract genomic DNA from the suspension using a bead-beating-base genomic DNA extraction kit according to the instructions provided by the manufacturer.**
   1. Transfer the suspension to a 2.0-mL sample tube containing glass beads (included in the kit). Add 750 µL of lysis solution (included in the kit) to the cell suspension.
   2. Cap tightly and place the tubes symmetrically in the tube holder of the bead-beating disruption apparatus. Process at the maximum speed for 5 min.Centrifuge the bead-beaten samples for 1 min at 10,000 × g. Transfer the supernatant to the spin filter (included in the kit) in a 2.0-mL collection tube and centrifuge for 1 min at 7,000 × g.
   3. Add 1,200 µL of DNA binding buffer (included in the kit) to the filtrate. Transfer 800 µL of the mixture to the spin column (included in the kit) to a 2.0-mL collection tube and centrifuge for 1 min at 10,000 ×g.
   4. Discard the flow-through, add 200 µL of pre-wash buffer (included in the kit) to the spin column to wash the column matrix, and centrifuge for 1 min at 10,000 × g. Discard the flow-through, add 500 µL of wash buffer (included in the kit) to the spin column to wash the column matrix, and centrifuge for 1 min at 10,000 × g.
   5. Place the spin column into a new 1.5-mL microcentrifuge tube and add 100 µL of elution buffer (included in the kit) to the column matrix.
   6. Centrifuge for 30 s at 10,000 × *g* to elute the genomic DNA. Estimate the DNA concentration and purity by measuring the absorbance at 260 nm and 280 nmusing a spectrophotometer and confirm that the A_{260}/A_{280} ratio is greater than 1.8.

3. PCR Amplification

1. Perform the 1st PCR using the *S. mutans* WT genome and the synthetic *spc*^{r} gene as PCR templates (see **Table 1**). Amplify the upstream flanking region of the *gtfC* gene, the downstream flanking region of the *gtfC* gene, and the *spc*^{r} gene using the three sets of primers described above (**Figure 1A**), as per **Table 2** and **Table 3**.

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NOTE: PCR primers, reagents, and amplification cycles are summarized in **Table 1**, **Table 2**, and **Table 3**, respectively.

2. Fractionate each PCR product on a 1% agarose gel and excise the corresponding bands using a gel band cutter.
3. **Purify each amplified DNA product from the gels using a spin column- and silica membrane-based gel extraction kit.**
    1. Transfer the gel slices to a clean microcentrifuge tube and mix with 500 µL of binding buffer (included in the kit). Incubate the mixture at 55 °C for 15 min until the gel slices are completely dissolved.
    2. Place the spin column (included in the kit) into a collection tube (included in the kit), load the sample onto the spin column, and centrifuge for 30 s at 11,000 × *g*. Discard the flow-through, add the 600 µL of Wash Buffer (included in the kit) to the Spin Column to wash the silica membrane, and centrifuge for 30 s at 11,000 × *g*.
    3. Discard the flow-through and centrifuge for 2 min at 11,000 × *g* to dry the silica membrane.
    4. Place the spin column into a new 1.5-mL microcentrifuge tube, add 50 µL Elution Buffer (included in the kit), and incubate at room temperature for 2 min.
    5. Centrifuge for 2 min at 11,000 × *g* to elute the amplified DNA. Estimate the DNA concentration and purity by measuring the absorbance at 260 nm and 280 nmusing a spectrophotometer and confirm that the A_{260}/A_{280} ratio is greater than 1.8.
4. Perform the 2nd PCR of 2-step fusion PCR using the three approximately equimolar fragments as PCR templates, as per **Table 2** and **Table 3**.
   NOTE: These fragments were amplified and spliced using the nested primers 'N_up-forward and N_down-reverse' or the outermost primers 'up-forward and down-reverse' (**Figure 1B**). PCR primers, reagents, and amplification cycles are summarized in **Table 1**, **Table 2**, and **Table 3**, respectively.
5. Load one-tenth of the PCR reaction mixture (5 µL) on a 0.8% agarose gel and compare the amplified band size with the expected band size to confirm the generation of the appropriate amplicon (**Figure 1C**).
6. **Concentrate the remaining final PCR product by ethanol precipitation without purification.**
    1. Add 55 µL of ultra-pure water to the remaining PCR mixture to adjust the total volume to 100 µL.Add a one-tenth volume of 3 M sodium acetate (10 µL), pH 5.2, followed by 2.5 volumes of 100% ethanol (250 µL). Mix and freeze for 30 min at -80 °C.
    2. Centrifuge for 20 min at 15,000 × *g* at 4 °C, check for the pellet on the bottom of tube, and discard the supernatant.Wash the pellet with 1 mL of 70% ethanol, centrifuge for 5 min at 15,000 × *g* at 4 °C, and discard the supernatant. Air-dry the pellet for approximately 30 min and dissolve with 10 µL of ultra-pure water.

4. Competent Cell Preparation and Cell Transformation

1. Streak stock *S. mutan*s UA159 onto a BHI agar plate. Incubate the plate overnight at 37 °C under anaerobic conditions.
2. Pick up a single colony using a sterilized toothpick and inoculate it into 5 mL of BHI broth. Incubate the *S. mutan*s culture overnight at 37 °C under anaerobic conditions.
3. Transfer 2 mL of the *S. mutan*s culture to 50 mL of BHI broth and incubate for 6-8 h at 37 °C to an optical density at 600 nm (OD_{600}) of 0.2-0.4 under anaerobic conditions.
4. Centrifuge the cells for 15 min at 2,000 × *g* at 4 °C, discard the supernatant, and wash the cells twice with 40 mL of ice-cold water followed by 40 mL of 10% glycerol.
   NOTE: Residual substances affect subsequent electroporation.
5. Aspirate the supernatant carefully with a Pasteur pipette as the adherence of the cell pellet in 10% glycerol is reduced. Resuspend the cells in 1 mL of fresh 10% glycerol, dispense 50 µL of the suspension into each 1.5-mL microcentrifuge tube, and store at -80 °C until just before use.
6. Mix the 50 µL aliquot of ice-cold competent cells with 5 µL of the disruption construct described above in section 3. Add the mixture to electroporation cuvettes (with a 0.2 cm distance between electrodes). Employ the *Staphylococcus aureus* mode (1.8 kV, 600 Ω, 10 µF, 1 pulse) of the electroporation apparatus to electroporate.
7. Suspend the cells in 500 µL of BHI broth immediately after electroporation and add 50 µL of the suspension to spectinomycin-containing BHI-agar plates.
   NOTE: Extra incubation time after electroporation is not required. However, incubation for 1-2 h after electroporation may improve transformation efficiency.
8. Incubate the plate for 2-6 days at 37 °C under anaerobic conditions.
   NOTE: *S. mutans* Δ*gtfB* is constructed as described above. The entire coding region of the *gtfB* gene is replaced with the erythromycin resistance gene[8] in *S. mutans* Δ*gtfB*. Each *S. mutans* strain is cultured in BHI broth at 37 °C under anaerobic conditions.

5. Verification of Gene Disruption and Storage

1. Pick random colonies and inoculate them into the PCR mixture to perform colony PCR as per **Table 2** and **Table 3**. PCR primers, reagents, and amplification cycles are summarized in **Table 1**, **Table 2**, and **Table 3**, respectively.
2. Load the PCR reaction mixture on a 1% agarose gel to confirm the *spc*^{r}-specific DNA band.
3. Pick the positive colony using a sterilized toothpick and subculture cells in 10 mL of spectinomycin-containing BHI broth for 2 days at 37 °C under anaerobic conditions.
4. **Verify the generation of *S. mutans* Δ*gtfC*.**
    1. Divide the cell suspension equally. Extract genomic DNA from the cells described above (steps 2.3. to 2.5.5.). Perform PCR with genomic DNA as the PCR template, using primers for the verification of *gtfC* disruption (**Table 1**) as per **Table 2** and **Table 3**.
       NOTE: The PCR primers, reagents, and amplification cycles are summarized in **Table 1**, **Table 2**, and **Table 3**, respectively.
    2. Load the PCR mixture on a 2% agarose gel and compare the amplified band size with the expected band size to confirm the generation of the appropriate amplicon.
    3. Centrifuge the remaining cell suspension for 15 min at 2,000 × *g* at 4 °C. Resuspend the cell pellet in 5 mL of PBS.

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4. Centrifuge for 15 min at 2,000 × *g* at 4 °C. Resuspend the cell pellet in 1.5 mL of BHI broth containing 25% glycerol and store in -80°Cor -20 °C.

6. Phenotypic Analysis

1. Streak each *S. mutans* strain onto a BHI agar plate. Incubate the plate overnight at 37 °C under anaerobic conditions.
2. Pick up a single colony using a sterilized toothpick and inoculate it into 2 mL of BHI broth with or without an appropriate antibiotic. Incubate overnight at 37 °C under anaerobic conditions.
3. Inoculate 20 µL of the overnight culture suspension into 2 mL of BHI broth containing 1% or 5% sucrose without antibiotics in a glass test tube; culture the cells with the test tube in an inclined position overnight at 37 °C under anaerobic conditions.
4. Vortex the glass test tubes for 10 s and decant culture suspensions. Wash the test tubes three times with distilled water. Add 1 mL of 0.25% Coomassie brilliant blue (CBB) to stain the biofilms on the tube wall, and then incubate for 1 min.
5. Decant the staining solution, wash the test tubes three times with distilled water, and dry the test tubes.
6. Evaluate both color density and extension of the CBB stained-biofilm visually to determine the biofilm-forming ability in a phenotypic analysis.

Representative Results

Figure 2 shows that the size of each product from the 1st PCR, as observed on the 1% agarose gel, was in good agreement with the predicted size of approximately 1 kb. **Figure 3** shows the gel electrophoresis analysis of the products of the 2nd PCR. **Figure 4** shows *S. mutans* colonies transformed with the disruption construct, and a gel electrophoresis analysis of the colony PCR products. The amplicons from all colonies were of the predicted size. **Figure 5** shows the primer-binding sites for the verification of *gtfC* disruption and gel electrophoresis of the PCR products. PCR products obtained by amplification using the *gtfC*-specific primer set were confirmed in *S. mutans* WT, but not in *S. mutans* Δ*gtfC*, whereas PCR products obtained using the *spc*^{r}-specific primer set were confirmed in *S. mutans* Δ*gtfC,* but not in *S. mutans* WT. **Figure 6** shows the sucrose-derived biofilm-forming ability of each *S. mutans* strain. An adherent biofilm was formed by *S. mutans* WT in the presence of sucrose; the adherent biofilm-forming ability of *S. mutans* Δ*gtfB* was lower than that of *S. mutans* WT. *S. mutans* Δ*gtfC* exhibited very low adherent biofilm formation in the presence of sucrose.

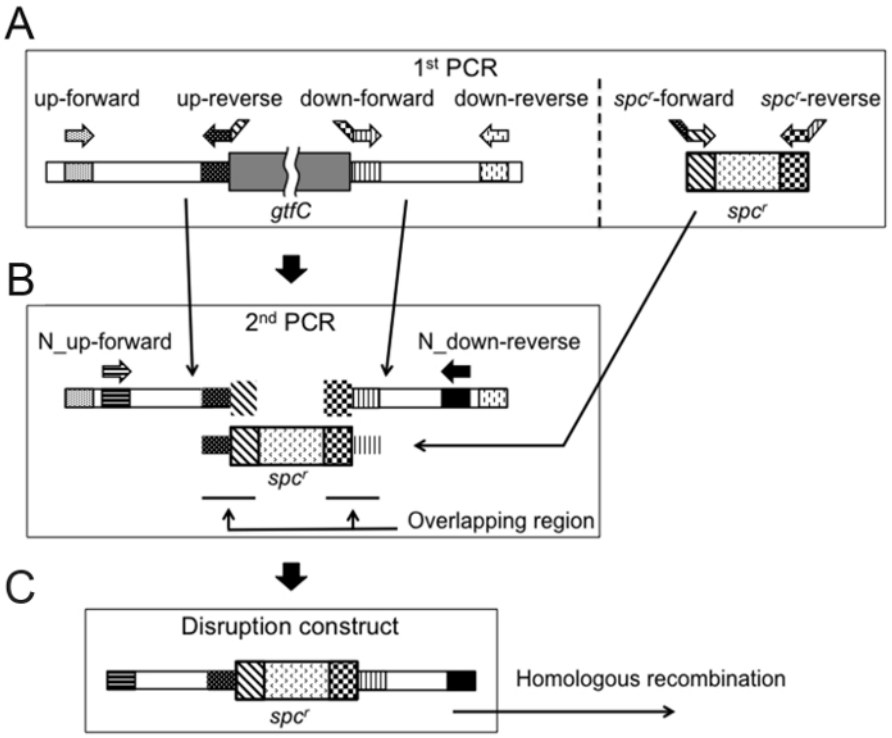

Figure 1:Strategy for 2-step fusion PCR. A schematic illustration of *S. mutans* Δ*gtfC* construction is shown. The gene lengths are not to scale. **(A)** Upstream and downstream flanking regions of the *gtfC* and *spc*^{r} loci were amplified by PCR. The primer binding sites in the template are indicated by identical patterns. **(B)** The second PCR step was performed using (as templates) the 3 fragments that were amplified in the first PCR step with nested primers. **(C)** The disruption construct was obtained for homologous recombination in the bacterial strains. Please click here to view a larger version of this figure.

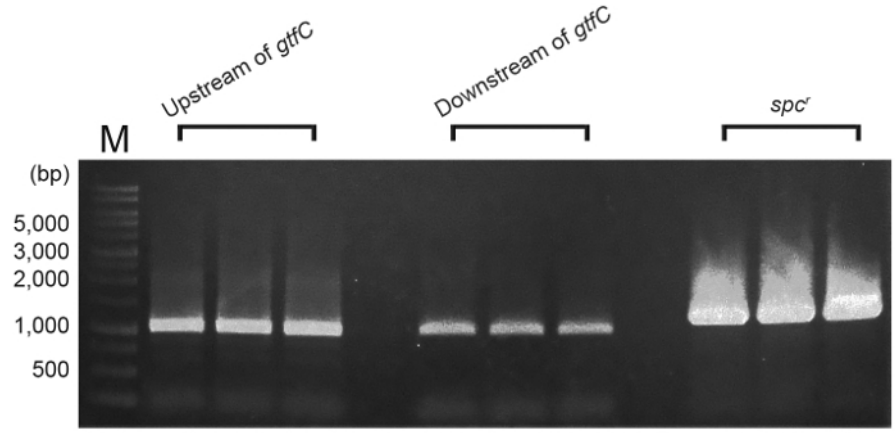

Figure 2: Representative gel electrophoresis of products from the 1st PCR. Amplicons of the upstream flanking region of *gtfC* and downstream flanking region of *gtfC* and *spc*^{r} are shown. M: molecular marker. Please click here to view a larger version of this figure.

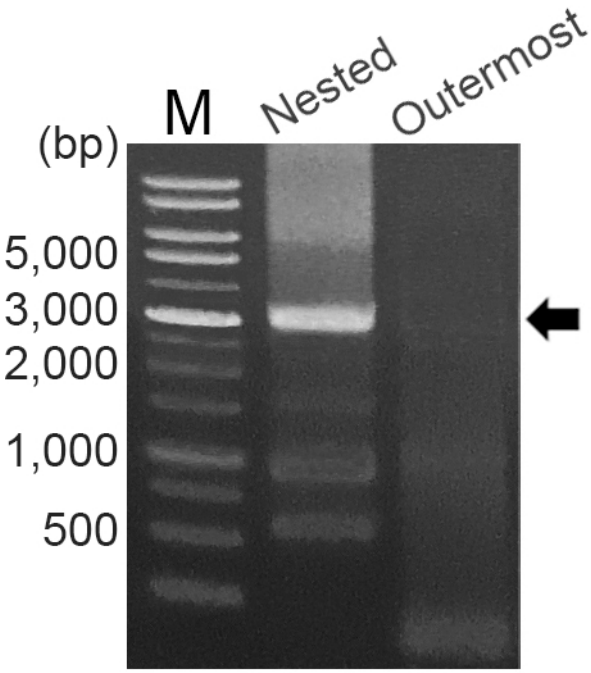

Figure 3: Representative gel electrophoresis of products from the 2nd PCR. Products amplified with the nested primers and the outermost primers are shown. The solid arrow indicates the predicted size of the disruption construct. M: molecular marker.

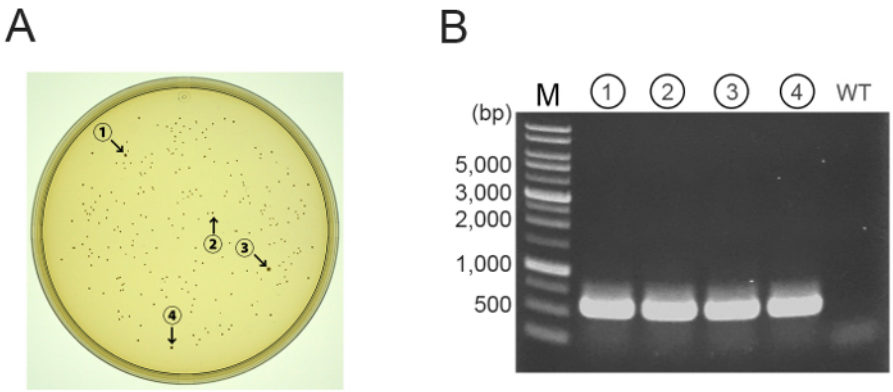

Figure 4: Screening for *gtfC* disruption by colony PCR (**A**)**.** *S. mutans* colonies on the spectinomycin-containing BHI-agar plate; each circled number indicates colony ID. **(B)** Representative gel electrophoresis of colony PCR products; each circled lane number indicates the corresponding colony ID in **Figure 4A**. M: molecular marker, WT: Wild-type genomic DNA used as template. Please click here to view a larger version of this figure.

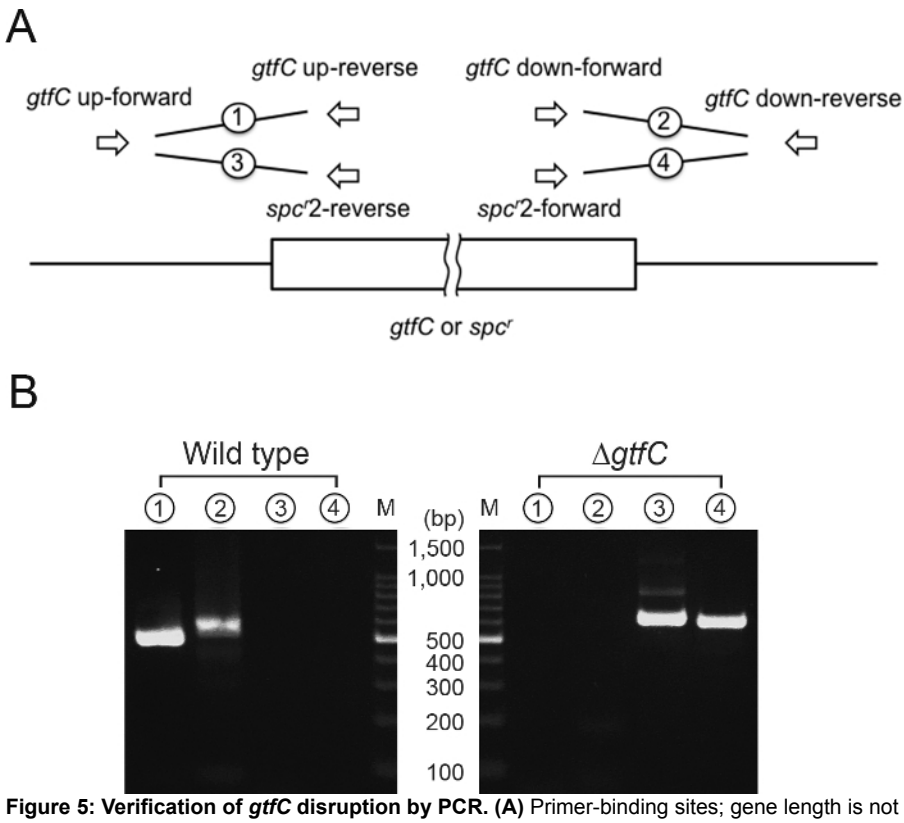

Figure 5: Verification of *gtfC* disruption by PCR. (A) Primer-binding sites; gene length is not to scale. PCR was performed using *S. mutans* genomic DNA as a template. Primer sets: *gtfC* up-forward and *gtfC* up-reverse (specific for *gtfC*); *gtfC* down-forward and *gtfC* down-reverse (specific for *gtfC*); *gtfC* up-forward and *spc*^{r}2-reverse (specific for *spc*^{r}); *spc*^{r}2-forward and *gtfC* down-reverse (specific for *spc*^{r}). **(B)** Representative gel electrophoresis of the PCR products; each circled lane number indicates the corresponding primer set in **Figure 5A**. A single electrophoretic image is divided to label the marker bands. M: molecular marker (100-base pair ladder). Please click here to view a larger version of this figure.

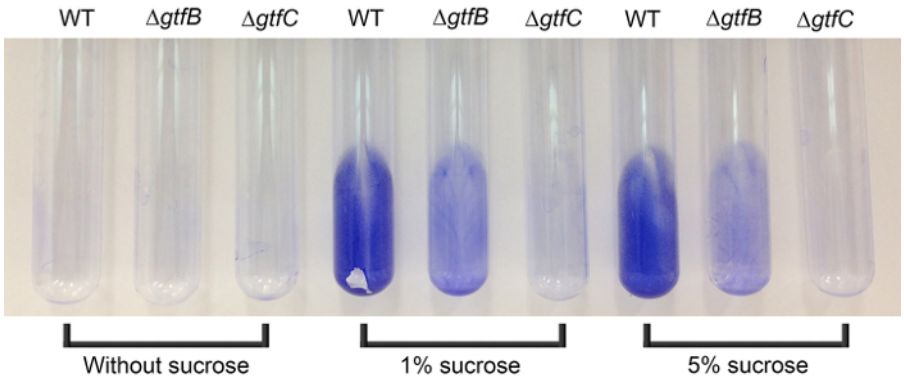

Figure 6: Phenotypic analysis based on biofilm development. Sucrose-derived biofilms formed by each *S. mutans* strain were stained with Coomassie brilliant blue. WT: *S. mutans* WT, Δ*gtfB*: *S. mutans* Δ*gtfB*, Δ*gtfC*: *S. mutans* Δ*gtfC*. Please click here to view a larger version of this figure.

Primer pair	Sequence (5' to 3')	Expected band size (bp)
2-step PCR for *gtfC* disruption		
1st PCR		
up-forward up-reverse	AAGATATTTTGATAAGCAATCTGGGAACATGTATC CGAACGAAAATCGA**TATTTCCTCCAAAAAT**AGTTA	1,036
spc^r-forward *spc*^{r}-reverse	**ATTTTTGGAGGAAATA**TCGATTTTCGTTCGTGAAT CTTCTAAGATAAGTA**CATATGCAAGGGTTT**ATTGT	1,188
down-forward down-reverse	**AAACCCTTGCATATG**TACTTATCTTAGAAGAATAG TTAAGAGCAAGTTTAAGATAGAACATGTTACTCAC	1,059
2nd PCR		
N_up-forward N_down-reverse	TTTTATTGAAAACGAAGAAGGTAAATGGCTGTATC GCCATACTTAGAGAAATTTCTTTGCTAAATTCTTG	3,132
Verification of *gtfC* disruption		
Colony PCR for screening		
S_*spc*^{r}-forward S_*spc*^{r}-reverse	GGATCAGGAGTTGAGAGTGGACTAAAACCAAATAG CAGCCACTGCATTTCCCGCAATATCTTTTGGTATG	535
Final verification		
gtfC up-forward *gtfC* up-reverse	TACGGCCGTATCAGTTATTACGATGCTAACTCTGG GGTTGGTTGAGATGTTGCTGAAGTTGCTGTACTTG	498
gtfC down-forward *gtfC* down-reverse	GCCTTAATTGGTTGGCATGTTGTTGAAGGAAGACG TTGTCCACTTTGAAGTCAACGTCTTGCAAGGCATG	557
gtfC up-forward *spc*^{r}2-reverse	Described above CCACTCTCAACTCCTGATCCAAACATGTAAGTACC	641
spc^{r}2-forward *gtfC* down-reverse	GTGGCTGAATCTTCTCCATTAGAACATAGGGAGAG Described above	623

Table 1: Primers used in this protocol. The underlined sequences of 'up-reverse and *spc*^{r}-forward' and '*spc*^{r}-reverse and down-forward' are complementary. The bold-typed sequences of 'up-reverse and *spc*^{r}-forward' and '*spc*^{r}-r and down-forward' are complementary.

Reagent	Concentration of stock solutions	Volume	Final concentration
DNA polymerase premix	2×	25 µL	1×
Forward primer	5 µM	2 µL	0.2 µM
Reverse primer	5 µM	2 µL	0.2 µM
Template DNA	Variable	Variable	Variable * ** ***
Deionized water	-	up to 50 µL	-

Table 2: PCR reagents. *For 1st step PCR; 100 ng. **For 2nd PCR; each 30-50 ng of 1st step PCR amplicon. ***For colony PCR; direct addition of bacterial cells to the reaction mixture.

Cycle step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	2 min	1
Denaturation Annealing Extension	98 °C 55 °C 72 °C	10 s 5 s Amplicon dependent (1 min/1 kbp)	35
Final Extension	72 °C	Amplicon dependent (1 min/1 kbp)	1

Table 3: PCR amplification cycles.

Discussion

In the present protocol, the primers for the 1st PCR must be designed to amplify approximately 1 kb upstream and downstream flanking regions of the target region in the genome. Such long flanking sequences are necessary to improve the efficiency of homologous recombination.

The sequences of the primers (up-reverse, down-forward, *spc*^{r}-forward, and *spc*^{r}-reverse) in this protocol were automatically determined based on the site of incorporation of the disruption construct. Each primer includes 15 bases complementary to the end of the 2nd PCR template at the 5' region. A longer binding site enables more efficient production of the disruption construct. Inclusion of at least 10 complementary bases at the 5' region of each primer is recommended[9].

Nested primers (N_up-forward and N_up-reverse) were used for the 2nd PCR, rather than the outermost primers (up-forward and down-reverse). The use of the outermost primers for the 2nd PCR is beneficial in some cases; successful amplification of the disruption construct for *S. mutans* Δ*gtfB* was achieved using the outermost primers (data not shown). However, the use of the outermost primers for the 2nd PCR step frequently results in insufficient PCR amplification, as shown in **Figure 3**. The use of nested primers was found to resolve this problem[9]. When the disruption construct is not substantially amplified even using the nested primers, however, redesign of the primers used in the 1st PCR may be required.

Colony PCR enables convenient screening of the gene-disrupted strain. Primers of known sequences may be applied for the generation of the gene-disrupted strain using *spc*^{r}. Electroporation was used for the introduction of disruption constructs into *S. mutans* WT, as the transformation efficiency achieved using electroporation is generally higher than that enabled by other procedures. Transformation using horse serum, or horse serum with competence-stimulating peptides, is widely accepted in *Streptococcus*[10,11,12]. Although an electroporation apparatus is required, the procedures involved, such as competent cell preparation, are much simpler than those in alternative methods[10,11]. In addition, electroporation is recommended from the point of view of animal welfare.

The present protocol is applicable to multiple gene disruption, depending on the number ofantibiotic markers. To generate both *gtfC*- and *gtfB*-disrupted *S. mutans* strains, for example, the DNA construct for *gtfC*-disruption constructed by 2-step PCR may be transformed into *S. mutans* Δ*gtfB*. Because *gtfC* and *gtfB* are adjacent in this case, *S. mutans* Δ*gtfC* and *S. mutans* Δ*gtfB* genomes must be used as templates for 2-step PCR to maintain the homologous sequences for homologous recombination. In fact, double gene-disrupted *S. mutans* strain was previously constructed by this protocol[9].

The present protocol for gene disruption in *S. mutans* may be adapted for the generation of gene-disrupted strains of various species. This protocol does not require the gene cloning step involved in conventional protocols. In addition, PCR is the only required enzymatic reaction; therefore, plasmid vectors, *Escherichia coli*, ligase, and restriction enzymes are not necessary. Furthermore, the present protocol offers greater flexibility in terms of design of the disruption construct, as the construct design is independent of restriction enzyme sites of the target gene. Researchers may determine regions that are deleted or preserved in the genome to design the reverse primer for amplification of the upstream flanking regions and the forward primer for amplification of the downstream flanking regions. These flanking regions are immediately upstream and immediately downstream of the region of DNA desired for deletion, respectively. Such flexibility may be used to decrease external influences, such as polar effects, on the expression of proximate genes.

Disclosures

The authors have nothing to disclose.

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