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Short communication

Unmarked gene deletion mutagenesis of *gtfB* and *gtfC* in *Streptococcus mutans* using a targeted hit-and-run strategy with a thermosensitive plasmid

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Unmarked gene deletion of the *Streptococcus mutans gtfB–gtfC* locus was achieved using a thermosensitive plasmid. DNA fragments flanking the locus were amplified by polymerase chain reaction and jointly ligated into pG+host5, which was transformed into *S. mutans* at 37°C to facilitate integration. A transformant was then grown at 28°C for 60 generations without antibiotics to facilitate excision. Antibiotic sensitive clones appeared at a frequency of about 99% and were analyzed for deletions of *gtfB*, *gtfC* and a part of *mbrA* by the lack of insoluble glucan synthesis, sensitivity to bacitracin, and polymerase chain reaction. Targeted gene deletions occurred at a frequency of 2.5%.

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Streptococcus mutans is considered the primary causative agent of dental caries (11). Its ability to metabolize sucrose is directly related to its cariogenicity. *S. mutans'* sucrose metabolism involves at least five extracellular enzymes: GtfB, GtfC, GtfD, Ftf, and FruA (2, 9) and three transport systems: *scr, tre,* and *msm* (10, 14, 15). To assess the role of individual systems in the overall *S. mutans* sucrose transport and metabolism, it would be beneficial to construct mutants defective in genes encoding all the other sucrose systems except the one under study.

To date, various methods have been developed to generate genetic mutations in *S. mutans*, including transposons (5) and plasmids (17) tagged with up to four different antibiotic resistance markers. Because each antibiotic resistance marker can only be used to create one mutation, the total number of mutations in a bacterial strain is limited by the number of available antibiotic resistance markers. This limitation has hampered the progress of our genetic studies involving multiple genes in the *S. mutans* sucrose transport and/or metabolism systems. As a result, the development of a method for the introduction of unmarked gene deletions became a high priority in our work.

In this study, we developed a procedure for generating site-specific gene deletions without leaving an antibiotic marker. We chose the genes encoding water-insoluble glucan-synthesizing enzymes, *gtfB* and *gtfC,* as our initial targets because of their association with *S. mutans* virulence, their tandem genetic loci, and their uniquely detectable phenotype. The procedure included the following: polymerase chain reaction (PCR) amplification of two DNA sequences flanking the target genes, joint-cloning of the two PCR amplicons into a thermosensitive plasmid suitable for use in gram-positive bacteria, selection of the gene-deletion mutant after plasmid excision at a lower temperature, and confirmation of the gene deletion with genotypic and phenotypic assays. Although *gtfB* and *gtfC* deletion mutants have been previously constructed in *S. mutans* (13, 21), these mutants were constructed by insertion of an antibiotic resistance marker. An unmarked deletion mutant of *gtfB* and *gtfC* would be desirable for future studies of *S. mutans*

sucrose metabolism involving multiple gene deletions.

The hit-and-run gene deletion strategy

As shown in Fig. 1, the left and right flanking DNA fragments were first amplified by PCR with *S. mutans* LT11 DNA as the template. The PCR primers were derived from *S. mutans* UA159 genomic sequence (AE014133). The left flanking DNA fragment of 1870 bp at 1086 bases upstream of *gtfB* was amplified with primers gtfBu-F1(5′-CGATTCTGATAA-GACCAAAG-3′) and gtfBu-R1(5′-CCTCAATAGGCATGCAACCT-3′). The right flanking DNA fragment of 1978 bp at 321 bases downstream of *gtfC* was amplified with primers gtfCd-F2(5′-GA-AGAGACGCATGCCTTGCA-3′) and gtfCd-R2(5′-AGACCGGAAATCATCTT-GCG-3′). The two PCR amplicons were purified from an agarose gel and individually cloned into a PCR cloning vector, pTrueBlue-*rop* (GenomicsOne, Quebec, Canada), digested out with restriction enzymes to release the fragments, and ligated at the *Sph*I site in tandem into the gram-positive thermosensitive plasmid pG+host5 (1). The resultant plasmid, pDA3, was used to transform *S. mutans* LT11 (18) selecting for erythromycin resistance (Em^R, 10 μg/ml) at 37°C, the temperature at which pDA3 could not propagate unless it was integrated into the *S. mutans* chromosome via insertion duplication. One of the transformants was chosen to grow in Todd-Hewitt broth (BBL) at 28°C without antibiotic selection for about 60 generations. At this lowered temperature, the temperature-sensitive plasmid replication was activated and thereby increased the frequency of segregation of the integrated plasmid by homologous recombination. Because the plasmid integration introduced two duplicated sequences in the insertion site, excisions of the plasmid could occur in two ways. One way, excision occurs by a recombination between the two nearby duplicated sequences, causing reversion to the wild type. The other way, excision occurs between the two distant duplications, resulting in deletion the target genes. The gene deletion could be readily detected by phenotypic and genotypic analyses.

Phenotypic analysis

Because the recombinant plasmid pDA3 includes both the left and right flanking DNA fragments, the plasmid could insert into the *S. mutans* chromosome at either 1086 bases upstream of *gtfB* or 321 bases downstream of *gtfC*. The insertion of the plasmid was outside the *gtfB–gtfC* locus, and thus it should not affect the expression of *gtfB* and *gtfC*. Therefore, no glucosyltransferase-defective mutants were expected after the first round of genetic transformation and insertion-duplication event. One of the transformants was selected and grown at 28°C in Todd-Hewitt broth with several transfers for about 60 generations without antibiotic selection to allow segregation of the integrated plasmid. The culture was streaked on Todd-Hewitt agar plates to obtain single colonies, which were in turn replica-plated onto the same agar with or without erythromycin. About 99% of

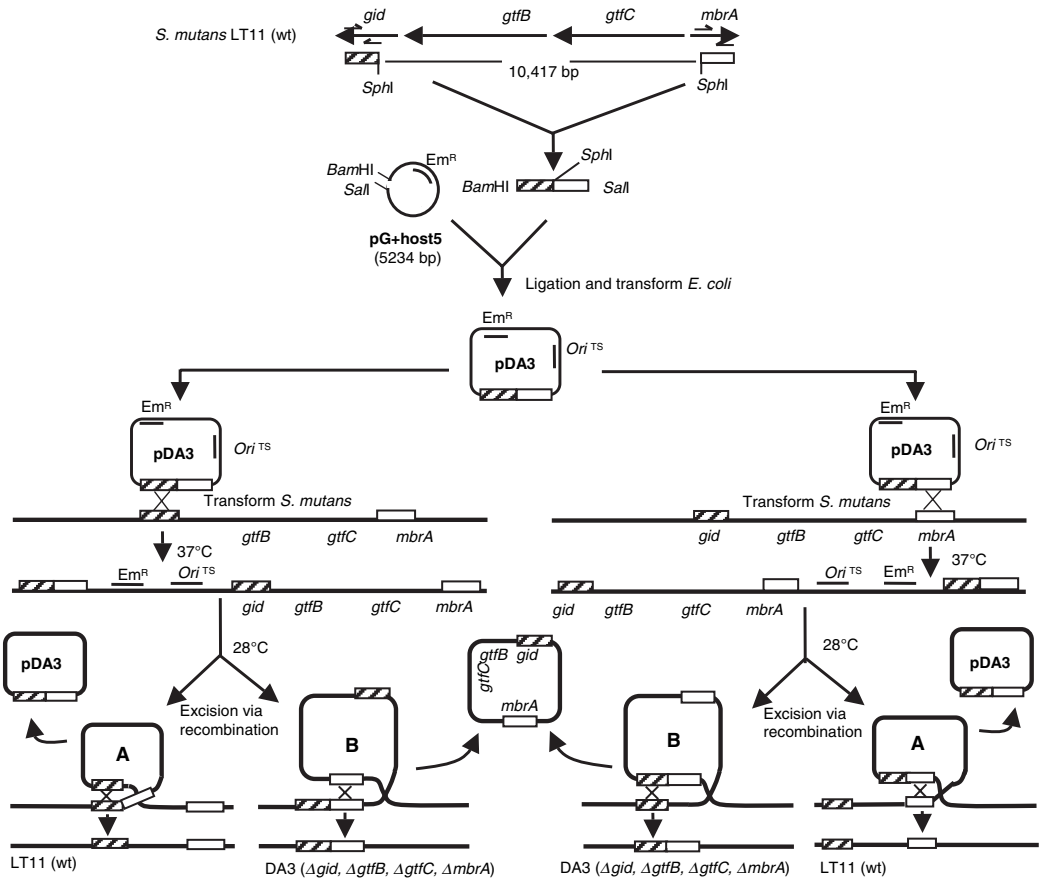

Fig. 1. Unmarked, site-specific gene deletion by the hit-and-run strategy. The plasmid integration may occur in either upstream or downstream of the targeted genes. A) Recombination occurred between the closer duplications. B) Recombination occurred between the distant duplications.

transformants (403/408 colonies) became erythromycin sensitive, indicating segregation of the plasmid. Because the *mbrA* gene downstream of *gtfC* encodes bacitracin resistance (19), deletion of a part of this gene could make the mutant sensitive to bacitracin. Erythromycin sensitive colonies were thus replica plated onto bacitricin-containing agar (1 μg/ml) and incubated overnight at 37°C. Of 403 colonies, 10 were bacitricin sensitive. Jordan medium (8) supplemented with 5% sucrose was used to grow the 10 mutant clones for testing adhesion to a glass rod in a test tube. All the 10 bacitracin-sensitive strains were negative in sucrose-dependent adhesion (Fig. 2). One strain was selected and named DA3. It grew on the Mitis-Salivarius agar, a selective agar for oral streptococci, and fermented melibiose, sorbitol, and mannitol, the three sugars fermented only by *S. mutans* among various oral streptococcal species (3), indicating that DA3 is an *S. mutans*-derived mutant.

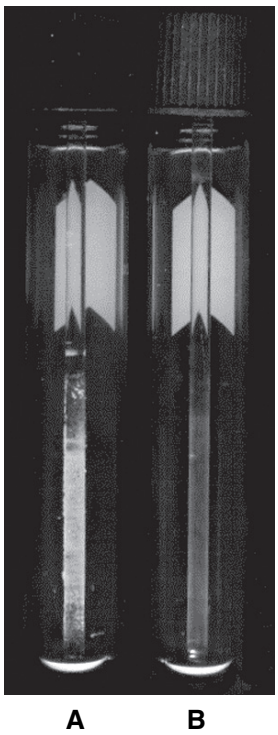

Fig. 2. Sucrose-dependent adhesion to the glass rod. A) LT11. B) DA3. The bacterial strains were grown in 5 ml Jordan medium supplemented with 5% sucrose at 37°C. The glass rods were transferred daily to new test tubes containing the same medium for 5 days. The rods were then photographed in blank tubes without growth medium.

Genotypic analysis

To facilitate positive selection and/or confirmation of an unmarked gene-deletion mutant, two primers (gtfBu-F3, 5′-TGACCGTAGCGGC TATGCTC-3′ and gtfCd-R3, 5′-ATTTCGGAGCAAGGCG-ATAC-3′) were designed with a short distance flanking the deletion site and used to perform PCR with DNA isolated from LT11 and DA3. If the deletion of the *gtfB–gtfC* locus occurred in DA3, the distance between the two primers would be 704 bp, while in the wild-type strain the distance is 11,121 bp. The PCR with a 2-min extension time (Fig. 3) showed only a DNA fragment slightly smaller than the 750-bp marker with the template DNA from the deletion mutant DA3, but not from LT11 (the 2-min extension time was not sufficient for the PCR to produce a long DNA of 11,121 bp). The PCR product was submitted for automated sequence determination. The sequence result confirmed the fusion between the left and right flanking DNA fragments with the *Sph*I site in the center. Additionally, two pairs of internal primers within *gtfB* and *gtfC* were designed: GtfB-F1(5′-TTCTAATGGT-GAAAAGCTTC-3′), GtfB-R1(5′-AT-CTTTATTTACAGTGTC-3′), GtfC-F2(5′-CCATCAAAGCGTTACACAG-3′) and GtfC-R2(5′-TAATCTTAACAGA AG-GATCC-3′). These primers produced PCR products only with the template DNA from LT11, but not from DA3 (Fig. 3). These data further confirmed the deletion of the *gtfB–gtfC* locus in *S. mutans* DA3.

A portion of *gid* was also deleted (Fig. 1). *gid* encodes a hypothetical glucose-inhibited division protein. The *gid* deletion in *S. mutans* did not appear to have an obvious phenotype, because no difference in cell morphology between the wild-type strain and the deletion mutant was observed when grown in a medium with glucose as the sole carbon source (data not shown).

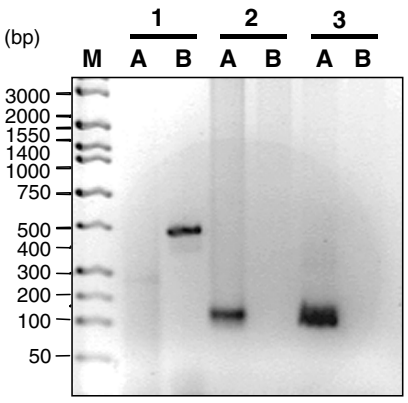

Fig. 3. Confirmation of gene deletion by polymerase chain reaction. M) DNA size marker. 1) Amplification with primers outside the deletion site. 2) Amplification with *gtfB*-specific primers. 3) Amplification with *gtfC*-specific primers. A), LT11. B) DA3.

Several unmarked gene deletion mutagenesis methods have been developed for construction of mutants in other bacterial species. Overlapping extension PCR has been recently used to generate gene-deletion mutants in the highly transformable *Streptococcus pneumoniae* (6). Counter-selectable markers, such as *sacB* (*Bacillus subtilis* levansucrase) and *rpsL* (streptomycin resistance mutation in the gene *rpsL* that causes a lysine replacement in protein S12 of the small ribosomal subunit), have been used to construct mutations in *Rhodococcus erythropolis* (20), *Neisseria gonorrhoeae* (7) and *Streptococcus pneumoniae* (16). Unfortunately, the *sacB* counter-selection can only be used in gram-negative bacteria because they cannot secrete the fructosyltransferase. The *rpsL* strategy (16) and the regular streptococcal integration vectors (17) did not work for us in the construction of unmarked gene deletion mutants in *S. mutans* (data not shown). The major problems were low competence compared with *S. pneumoniae* and/or low rate of excision of the integrated plasmid. This prompted us to develop a hit-and-run strategy using a thermosensitive plasmid.

Although a similar method for gene deletion in *Streptococcus pyogenes* (4) used the thermosensitive plasmid pG+host9 (12), we selected to use pG+host5 (1) because in addition to a thermosensitive replicon derived from *Lactococcus*, it also contains a pBR322 replicon for propagation in *Escherichia coli* at 37°C. We noticed that pG+host5 was unstable, e.g. there were frequent deletions, during propagation in *E. coli* (data not shown). The recombinant plasmid pDA3, however, was highly stable. The stability could be due to interruption of a gene or a promoter-like sequence on pG+host5 by the cloning of a DNA insert. As a result, for later cloning experiments we were able to obtain stable pG+host5 by cutting out the cloned DNA insert from pDA3. To our knowledge, this is the first unmarked, site-specific gene-deletion method successfully applied with a thermosensitive plasmid in the mutans group of streptococci.

Previously a plasmid, pTV1-OK, with the same pWV01-derived thermosensitive

origin of replication as pG+host5 was used to deliver the transposon Tn*917* for random mutagenesis in *S. mutans* (5). With this method, *S. mutans* is first transformed by the plasmid at a lower temperature to establish extrachromosomal replication of the plasmid and then shifted to a higher temperature to facilitate transposon integration into the chromosome. For the site-specific hit-and-run mutagenesis, the sequence of the temperature shifting is reversed. The bacterium is incubated first at a higher temperature for plasmid integration into the host bacterial chromosome and then shifted to a lower temperature to facilitate its excision. Because the excision rate of the integrated thermosensitive plasmid can reach about 99% at 28°C, a counter-selective marker is unnecessary.

Compared with the antibiotic resistance gene-marked *gtfB–gtfC* deletion mutant (21), the unmarked *gtfB–gtfC* deletion mutant is more advantageous. First, it does not carry a foreign gene, such as an antibiotic resistance marker or a plasmid promoter, which might have an unknown effect on the bacterial physiology. Second, it offers a clean host background for further genetic modification. For example, at least three additional genes can be deleted with currently available antibiotic resistance markers in conjunction with the unmarked *gtfB–gtfC* deletion. The bacitracin-sensitivity can be used as a readily identifiable trait for the *gtfB–gtfC* deletion mutant.

Although this method was effective for the deletion of two relatively large genes, it may be also useful for deletion of smaller genes or generation of site-specific in-frame deletions. Because the PCR method can detect gene deletions effectively, this method can even be used to generate deletion mutants for genes without apparently detectable phenotypes. With the availability of the unmarked gene-deletion method, we can delete a large number of genes in *S. mutans* for analysis of specific functions of individual genes and/or assessment of their roles in virulence.

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