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Construction of a counterselection-based in-frame deletion system for genetic studies of *Streptococcus mutans*

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Genetic studies of *Streptococcus mutans* have benefited greatly from the numerous techniques that have been successfully adapted for use in this organism. One notable exception is the lack of a negative selection system that can be employed for the easy isolation of markerless in-frame deletions. In this study, we report the development of a *galK*/galactose-based negative selection system in *S. mutans* for this purpose. This system consists of a recipient strain (IFD140) that contains a deletion in the *galKTE* operon and a suicide vector (pIFD-Sm) that carries the *S. mutans galK* open reading frame fused to the constitutive lactate dehydrogenase (*ldh*) promoter. Using this system we created a markerless in-frame deletion in the β-galactosidase (*lacG*) gene within the *S. mutans* lactose operon. After vector integration, plasmid excision after counterselection appeared to have occurred in 100% of the galactose-resistant colonies and resulted in in-frame deletions in 50% of the screened isolates. Based on the ratio of galactose-resistant cells to total cells, we determined that plasmid excision occurred at a frequency of approximately 1/3000 cells. Furthermore, the simplicity of this system should make it adaptable for use in numerous other gram-positive and gram-negative organisms.

Key words: galactose; galK; in-frame deletion; negative selection; *Streptococcus mutans*

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Streptococcus mutans is a gram-positive, biofilm-forming bacterium largely correlated with dental caries experience (5, 8). Within the past 15 years, great progress has been made in the characterization of this organism's biology as a result of the successful adaptation of a large number of genetic tools. For example, gene expression has been successfully assayed using various reporter systems such as chloramphenicol acetyltransferase (6, 15, 44, 45), firefly luciferase (12, 20, 29), green fluorescent protein (3, 20, 28, 47), monomeric red fluorescent protein (19), β-galactosidase (LacZ) (14, 36, 42) and β-glucuronidase (19). In addition, genetic screens have employed both various transposons (13, 22, 32) and random insertional mutagenesis (24, 39, 40). Also, defined genetic mutations are easily created in this organism with plasmid insertion duplication mutagenesis (single crossover) and allelic exchange with antibiotic cassettes (double crossover) (23). However, both of these mutagenesis techniques frequently create undesirable polar effects to downstream genes and are limited by the number of available selectable markers. Therefore, targeted mutagenesis strategies in *S. mutans* are subject to certain inherent limitations. In many other bacterial species, these issues have been addressed with the development of markerless in-frame deletion systems.

The in-frame deletion procedure usually involves two steps. First, polymerase chain reaction is used to amplify two similarly sized fragments homologous to sequences flanking the target mutation site. These fragments are ligated adjacent to each other in a suicide vector. This construct is then integrated onto the chromosome via a Campbell-type recombination and isolated using positive selection (antibiotic resistance). Next, recombinants are screened for a second recombination between the homologous regions created as a result of insertion duplication. Since

two similarly sized homologous fragments are incorporated onto the suicide vector, this second recombination occurs with equal frequency at either of the two sites. This results in the equal possibility of forming an in-frame deletion mutation or a reversion to the wild-type genotype.

Since the second recombination event occurs at a very low frequency, screening for these recombinants is potentially a laborious process. For example, in-frame deletions have been previously constructed in various streptococci by transforming either gene splicing by overlap extension polymerase chain reaction products or temperature-sensitive suicide vectors (4, 9, 10, 16). In both of these approaches, mutants have been reported to occur in 1–2.5% of the colonies screened (4, 16). For this reason, counterselection strategies are often incorporated to facilitate the screening process.

To employ a counterselection approach, the suicide vector requires both a positive and a negative selection mechanism. The negative selection pressure is only applied after the suicide vector has integrated onto the chromosome (i.e. counterselection) and therefore, only strains that have undergone the second recombination to excise the plasmid will be viable. In this case, about 50% of the resulting colonies will be in-frame deletions and 50% should be wild type. Various negative selection mechanisms have been utilized for counterselection in both gram-positive and gram-negative bacteria (11, 17, 21, 30, 35, 38, 46). For example, in the gram-negative organism *Myxococcus xanthus*, the *Escherichia coli galK* gene (galactokinase) is often introduced to phosphorylate galactose (41). In this species, galactose is not efficiently metabolized and therefore, this sugar can become a toxic metabolite when $galK^+$ cells are grown on media containing galactose (37). Alternatively, negative selection can be further enhanced in *M. xanthus* with the galactose analog, 2-deoxygalactose, which cannot be metabolized (41, 43).

In this study, we report the development of a similar *galK*-based in-frame deletion system in *S. mutans*. As a test of our system, a markerless in-frame deletion was constructed in the *S. mutans* β-galactosidase gene, *lacG*. Following counterselection, plasmid excision had occurred in every isolate tested, while 50% of the screened galactose-resistant isolates were in-frame deletion mutants. To our knowledge, this is the first report of a functional counterselection system in the genus *Streptococcus*.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains, plasmids, and their relevant characteristics are listed in Table 1. All *S. mutans* strains were grown in Todd–Hewitt medium (TH, Difco, Franklin Lakes, NJ) or on TH and casitone yeast extract (CYE, Difco) agar plates. For the selection of antibiotic-resistant colonies, TH plates were supplemented with 800 μg/ml spectinomycin (Sigma, St. Louis, MO). All the *S. mutans* strains were grown anaerobically (80% N_2, 10% CO_2 and 10% H_2) at 37°C. *E. coli* cells were grown in Luria–Bertani (LB, Fisher, Tustin, CA) medium with aeration at 37°C. The *E. coli* strains carrying plasmids were grown in LB medium containing 100 μg/ml ampicillin (Fisher, Tustin, CA), 250 μg/ml erythromycin, or 150 μg/ml spectinomycin.

Construction of a galactose-metabolism-deficient *S. mutans* strain

The genotype of the galactose-deficient *S. mutans* strain is illustrated in Fig. 1A. The sequence information for the galactose metabolism operon was extracted from the Los Alamos National Laboratories Oralgen *S. mutans* database (http://www.stdgen.lanl.gov/oralgen/bacteria/smut/index.html) as *galKTE* (Smu808–810) (2). Using this information, homologous fragments comprising approximately 1 kilobase (kb) upstream of *galK* and 1 kb downstream of the translational start of *galE* were polymerase chain reaction amplified using PfuUltra™ and the primers: Gal Up F (5′-GCTGAGTGGTCAATCTGGTC-3′), Gal Up R (5′-TGAGAAAGAAGGGTGATAATGG-3′), Gal Down F (5′-TTTGCTCTGTCGTTTACGGG-3′), and Gal Down R (5′-TGGATAGCTGGAATGCCATG-3′). These products were subsequently treated with *Taq* polymerase to generate A-overhangs and were cloned into pCR2.1™ (Invitrogen, Carlsbad, CA). The upstream and downstream homologous fragments were then digested from pCR2.1™ using *Not*I/*Spe*I and *Xho*I/*Hin*dIII respectively and ligated to compatible sites on either side of an erythromycin-resistance cassette in the vector pBsE (*ermFAM* cloned into the *Eco*RI site of pBluescript™; J. Merritt unpublished vector). After confirmation of the resulting plasmid, the insert was polymerase chain reaction amplified using PfuUltra™ and transformed into *S. mutans* as described by Li et al. (26). Erythromycin-resistant *S. mutans* clones were then confirmed by polymerase chain reaction for the expected genotype. A confirmed mutant isolate was renamed as IFD140.

Construction of the in-frame deletion cloning vector

The details for the construction of pIFD-Sm are summarized in Fig. 1B. The *S. mutans galK* open reading frame (ORF) was amplified using polymerase chain reaction with the primers Gal ORF F (5′-GATTATAAAATAGACTTGTAAAATCA-3′) and Gal ORF R (5′-CCGACCAAAGCAAGAATACG-3′). The corresponding fragment included the entire ORF and its presumed ribosome binding site just upstream of the translational start site. This fragment was cloned into pCR2.1™. The *S. mutans ldh* promoter region (27) was polymerase chain reaction amplified using the primers Ldhp F (5′-AAGAGCCCGAGCAACAATAAC-3′) and Ldhp R (5′-ACAGCACCATCACCAACAAG-3′). This fragment consisted of a small region upstream of the *ldh* translational start site (∼300 base pairs) and was also cloned into pCR2.1™. First, the *galK* fragment was excised using the enzymes *Xho*I and *Hin*dIII and was ligated to the streptococcal suicide vector pFW5 cut with the same enzymes. Next, the resulting vector was cut with *Eco*RV and *Stu*I and ligated with a Klenow-treated

Table 1. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics[1]	References
Strains		
UA140	*Streptococcus mutans* wild-type	(34)
E. coli DH5α	*Escherichia coli* cloning strain	
IFD140	UA140Δ*galKTE*; Em^r	This work
JMlg	IFD140Δ*lacG*; Em^r, Sp^r	This work
Plasmids		
pCR2.1	Cloning vector; Km^r and Ap^r	Invitrogen
pBluescript KS	Cloning vector; Ap^r	Stratagene
pFW5	Cloning vector; Sp^r	(31)
pIFD-Sm	Cloning vector; Sp^r; ldh_p::*galK*	This work
pJMlg	*lacG* insertion vector; Sp^r; ldh_p::*galK*	This work

[1]Em^r (erythromycin resistance), Km^r (kanamycin resistance), Ap^r (ampicillin resistance), Sp^r (spectinomycin resistance).

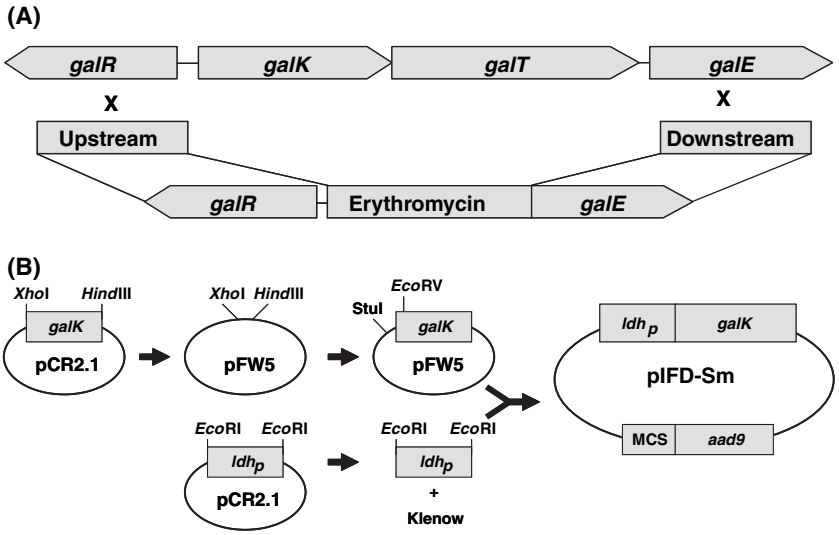

Fig. 1. Construction of the in-frame deletion system. (A) The *galKTE* operon was deleted by allelic replacement with an erythromycin cassette. Intergenic spaces are represented by single lines between genes. (B) Schematic representation of the cloning steps utilized to create the in-frame deletion cloning vector pIFD-Sm.

(NEB, Ipswich, MA) *ldh* promoter fragment generated by *Eco*RI digestion. The recombinant plasmid clones were screened for the proper orientation of the *ldh* promoter to generate pIFD-Sm.

Construction of the *lacG* in-frame deletion vector

Approximately 1 kb upstream and downstream fragments were polymerase chain reaction amplified with PfuUltra™ and cloned into pCR2.1™. Primer sequences for the *lacG* mutation are: LacG Up F (5′-GCGAGCTCACGCCTTGTTCTGGTTTGTC-3′), LacG Up R (5′-GCTCTAGACAACAGGACCCTTCCCATC-3′), LacG Down F (5′-GCTCTAGAGGTTATGAGAAGCGCTATGGT-3′), and LacG Down R (5′-GCACTAGTAAGGAAAATCTGCAAAATCCAA-3′). Upstream and downstream fragments for the *lacG* mutation were digested from pCR2.1™ with *Sac*I/*Xba*I and *Xba*I/*Xho*I, respectively, and ligated to compatible sites on the vector pBluescript™. The resulting insert was removed by digestion with *Bss*HII and ligated to pIFD-Sm cut with the same enzyme to create the plasmid pJMlg (Table 1). The resulting in-frame deletion vector was verified by polymerase chain reaction and restriction digestion.

Determination of plasmid excision frequency after counterselection

To determine the excision frequency of pJMlg following counterselection on galactose, serial dilutions of spectinomycin-resistant clones were plated on CYE and CYE + 4% galactose. Three independent insertion isolates were chosen. Clones were diluted 100-fold before plating 100 µl on galactose-supplemented CYE plates and 100,000-fold before plating 100 µl on non-selective CYE plates. Plates were incubated for 36–48 h under anaerobic conditions until colonies were visible. Colony-forming units resulting from each independent isolate were counted, multiplied by the appropriate dilution factor, and the ratio of galactose-resistant colony-forming units to total colony-forming units was calculated (Table 2).

Generation of in-frame deletion mutants

The plasmid pJMlg was transformed into IFD140 and selected on TH plates + 800 µg/ml spectinomycin. Antibiotic-resistant clones were purified and then grown in TH medium overnight under anaerobic conditions at 37°C. The following day, cultures were diluted 100-fold and 100 µl of the diluted culture was plated on CYE agar plates supplemented with 4% galactose. These plates were incubated for 36–48 h until galactose-resistant colonies appeared. Ten randomly chosen galactose-resistant isolates were restreaked on CYE + galactose plates and subsequently grown in TH medium. Each isolate was first confirmed to be spectinomycin-sensitive and later genomic DNA was extracted. Polymerase chain reaction was used to determine the genotype of each antibiotic-sensitive isolate using the primers LacG Up F and Down R (described previously).

S. mutans β-galactosidase phenotypes following counterselection

After confirming the genotypes of ten randomly selected galactose-resistant clones, a regenerated wild-type and a Δ*lacG* isolate were streaked onto a CYE plate containing 1% lactose and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). This plate was incubated in the presence of oxygen for 4 days at 37°C to allow for the oxidation of the X-gal cleavage products. The development of a blue color was indicative of β-galactosidase activity.

Table 2. Determination of plasmid excision frequency

Sample	Galactose	Total	Ratio	Average
lacG A	3.21×10^4	1.40×10^8	2.29×10^{-4}	3.61×10^{-4}
lacG B	2.74×10^4	1.037×10^8	2.64×10^{-4}	
lacG C	7.18×10^4	1.218×10^8	5.89×10^{-4}	

The values shown are the calculated number of colony-forming unit/100 µl of overnight culture. This experiment was repeated twice with similar results. Each sample is an independent isolate.

RNA extraction and real-time reverse transcription-polymerase chain reaction of *lacG*

Four samples were collected for the *lacG* isolates: wild-type, insertion (before counterselection), in-frame mutant (after counterselection), and wild-type (regenerated after counterselection). Each of these samples was grown in 40 ml TH medium to an optical density at 600 nm of approximately 0.6. The samples had 1% lactose added to the cultures to induce the *lac* operon. Cells were harvested, centrifuged (16, 000 **g**) and frozen at −80°C for later use. RNA extraction from the cell pellets was performed as has been described previously (28) and 3 μg RNA was used to make cDNA with Stratascript RT (Stratagene, LaJolla, CA). Fluorescence was detected with SYBR green (BioRad, Hercules, CA) and the iCycler polymerase chain reaction system. The primers used for the real-time polymerase chain reaction were as follows: Smu1690 RT F (5′-TTGGGCTTCTCGCACTTTAC-3′), Smu1690 RT R (5′-AGAACAGGGCCAACATCAAC-3′), LacG Down F (described previously), and LacG RT R (5′-TCAGTCAATTATTTGGGTCTCAGC-3′). Total cDNA abundance between samples was normalized based on the quantity of 16S cDNA and amplified with the primers 16s F (5′-GATAATTGATTGAAAGATGCA-3′) and 16s R (5′-ATTCCCTACTGCTGCCTCCC-3′).

Results

Galactose counterselection in *S. mutans*

Previous studies in *M. xanthus* demonstrated the utility of the *E. coli galK* gene for constructing in-frame deletions (41). In many organisms, GalK or galactokinase is responsible for phosphorylating galactose as the first step of galactose metabolism. *S. mutans* has its own version of this gene, which has been shown to be essential for its ability to metabolize galactose (1, 2). Since galactose is transported into *S. mutans*, we reasoned that galactose should easily accumulate in *S. mutans* cells and become toxic if phosphorylated, but not metabolized. As a test of this hypothesis, we created a polar insertional mutation into *galT* of the *S. mutans galKTE* operon to disrupt the transcription of *galT* and *E* without interrupting the transcription of *galK*. We then plated the insertion mutants on galactose-containing medium and discovered that a large percentage of the cells did not grow and the few colonies that did form had regained sensitivity to antibiotic selection (data not shown). This was indicative of a reversal of the original plasmid insertion and suggested the feasibility of using the galactose counterselection strategy in *S. mutans*.

Construction of IFD140, the galactose-sensitive strain of *S. mutans*

Since we had evidence to suggest that GalK activity in a galactose-metabolism-deficient strain was toxic, we decided to mutate the *galKTE* operon to create a phospho-galactose-sensitive strain of *S. mutans.* As shown in Fig. 1A, an allelic replacement strategy was used to replace *galK*, *T*, and part of *E* with an erythromycin cassette. This strain, renamed as IFD140, became our galactose-sensitive in-frame deletion recipient strain.

Construction of the in-frame deletion cloning vector

To produce our in-frame deletion cloning vector, pIFD-Sm, it was necessary to include an antibiotic marker for positive selection, a *galK* gene for negative selection, and a multiple cloning site for inserting homologous fragments. The strategy for constructing the in-frame deletion vector is shown in Fig. 1B. We chose to modify the streptococcal suicide vector pFW5 because this plasmid has two multiple cloning sites; one on either side of the spectinomycin-resistance gene, *aad9* (31). We cloned the coding region for the *S. mutans galK* in the upstream multiple cloning site of pFW5. We intentionally did not include the promoter region of this gene because previous experiments in our laboratory had determined that the normally repressed *galKTE* operon was only moderately inducible in the presence of galactose (Merritt, J. *et al.,* unpublished data). This potential limitation was addressed by adding the constitutive, highly expressed *S. mutans ldh* promoter to drive *galK* expression to ensure predictable negative selection (Fig. 1B).

Assessment of galactose-negative selection in IFD140

To test the utility of the counterselection system, we chose to mutate *lacG* because we had previously discovered that the *S. mutans* β-galactosidase was capable of hydrolysing X-gal to generate the characteristic blue precipitate (Merritt, J. *et al.,* unpublished data). Therefore, the phenotype of *lacG* mutants could be easily assessed with blue/white screening. The construct was made by cloning approximately 1-kb fragments homologous to the upstream and downstream regions of the intended mutation site. These two fragments were ligated adjacent to each other on pIFD-Sm and transformed into IFD140 (Fig. 2A). Plasmids could insert with either of the two homologous fragments via a Campbell-type recombination and therefore, insertion mutants could be either of two possible genotypes (Fig. 2B,C). To estimate the frequency of plasmid excision, three randomly selected spectinomycin-resistant recombinants were chosen for galactose-negative selection. Each isolate was incubated overnight in selective medium and plated the following day on medium ± galactose. As shown in Table 2, the galactose-negative selection only allowed a small fraction of the cells to survive. Based on the ratio of galactose-resistant cells to total cells, we calculated that approximately one in 3000 cells underwent a second recombination to excise the inserted plasmid (Table 2).

Genotype of *lacG* insertion mutants following counterselection

To screen for potential in-frame deletion mutants, 10 randomly selected galactose-resistant isolates were first confirmed for antibiotic sensitivity. Next, genomic DNA was extracted from each of these clones and tested for genotype by polymerase chain reaction using the primer pair illustrated in Fig. 3A. As shown in Fig. 3B, five of the 10 isolates generated amplicons of approximately 2.2 kb, which is indicative of an in-frame deletion, while the remaining five isolates generated the expected wild-type amplicons of approximately 3.4 kb. As a further confirmation, an in-frame mutant and a regenerated wild-type isolate were both assayed for β-galactosidase activity on CYE plates containing 1% lactose and X-gal. As shown in Fig. 4, only the regenerated wild-type exhibited the distinctive blue color from X-gal hydrolysis, while the *lacG* in-frame mutant was white. Finally, this mutant was also sequenced and found to exhibit the expected *lacG* in-frame deletion.

Transcription of downstream genes

One of the hallmarks of an in-frame deletion is a lack of polar effects to downstream genes. Therefore, real-time reverse transcription-polymerase chain reaction was used to compare the expression of *lacG* in the original wild-type, the

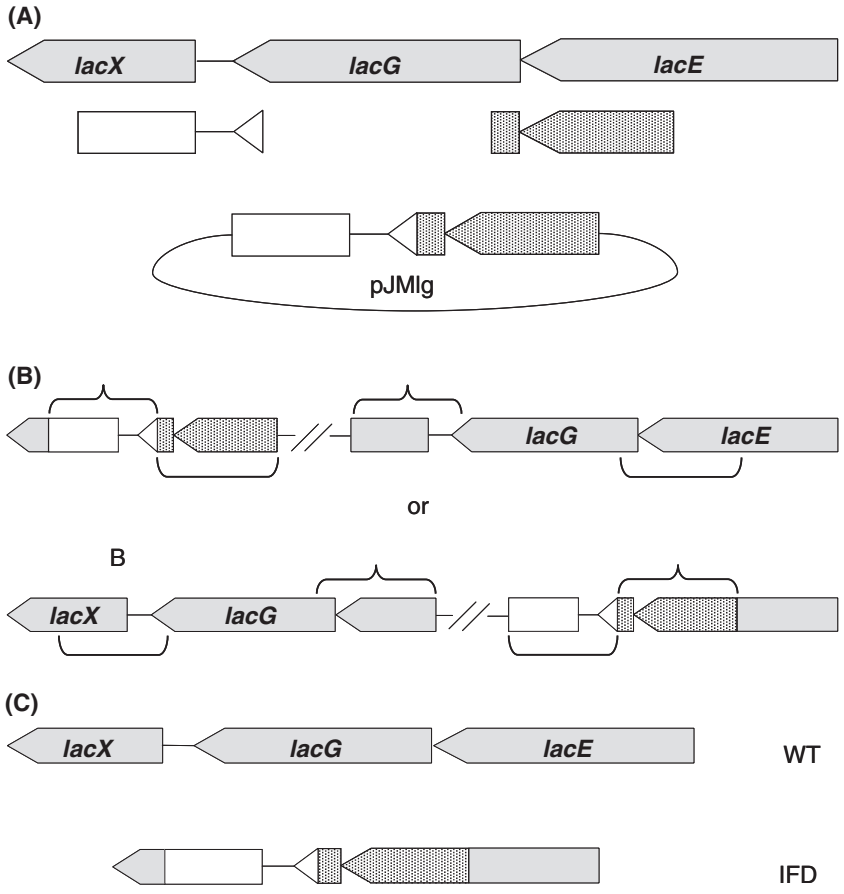

Fig. 2. Steps to produce an in-frame deletion. (A) The *lacG* region is shown. The intergenic space between *lacG* and *lacX* is represented by a single line. Approximately 1-kb fragments homologous to the upstream (shaded) and downstream (white) regions of *lacG* were ligated adjacently on the in-frame deletion vector (pIFD-Sm) to create the vector pJMlg. (B) Integration of pJMlg can occur with either of the two homologous fragments. Recombination with the downstream portion will produce the genotype illustrated in the first scenario, while recombination with the upstream fragment will generate the second scenario. Duplicated fragments are indicated by brackets. (C) Insertion duplication mutants plated on galactose will require plasmid excision to be viable. Recombination between the duplicated fragments will excise one of the duplicated fragments as well as the DNA in between, including the suicide vector. This results in either a wild-type genotype (top) or an in-frame deletion (bottom).

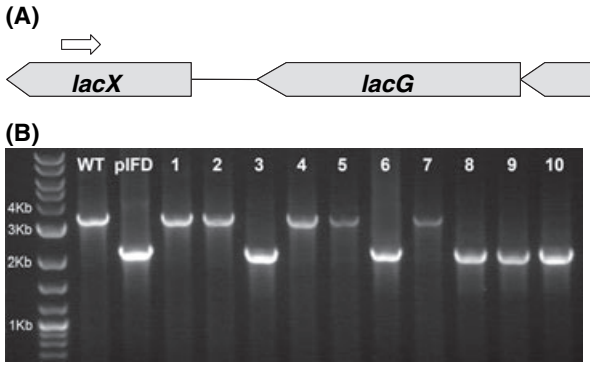

Fig. 3. Genotype of *lacG* isolates following galactose counterselection. (A) The arrows indicate the position of the primers used to determine the genotype of the *lacG* isolates. (B) Bands of approximately 2.2 kb indicate a *lacG* deletion, whereas bands of approximately 3.4 kb indicate a wild-type genotype. 'WT' represents the parent wild-type, UA140, and 'pIFD' represents the *lacG* plasmid insertion construct.

insertion duplication isolate, the in-frame mutant, and regenerated wild-type. Since *lacG* is the last gene of the *S. mutans lac* operon, we designed primers to amplify a section of the *lacG* gene downstream of the deletion site. Using these primers, we found that the *lacG* insertion strain had reduced expression of *lacG* compared to the wild-type (Fig. 5). This particular clone was also negative for X-gal hydrolysis (data not shown), which further indicated that the insertion had caused a polar effect upon *lacG*. However, both the *lacG* in-frame mutant (IFD) and the regenerated wild-type (IFD WT) exhibited an expression level that was consistent with the original wild-type (UA140) (Fig. 5). As expected, the transcriptional defect created in the insertion isolate was restored following plasmid excision.

Discussion

The data presented above describe a counterselection-based method to isolate in-frame deletion mutants in *S. mutans*. While in-frame deletions have been previously constructed in several species of *Streptococcus*, most studies relied upon enrichment strategies to identify the desired mutation. In our system, we have greatly simplified the screening process by including a mechanism for counterselection. Using this approach, we observed the desired in-frame mutation in 50% of the isolates we tested (five of 10). In addition, we tested a much larger number of galactose-resistant isolates for antibiotic resistance and each was found to be spectinomycin-sensitive, which suggested that the counterselection mechanism was 100% effective. Also, we have found in this study that the *S. mutans* β-galactosidase gene (*lacG*) is able to hydrolyse X-gal when incubated in the presence of oxygen. This suggested that a promoterless version of this gene could be adapted in *S. mutans* as an alternative to the *E. coli* LacZ reporter system.

To optimize the galactose counterselection, we first assayed with several concentrations of galactose. We found that 4% galactose provided the most reliable selection. Interestingly, 2% galactose worked well for obtaining *lacG* mutants, but some pinpoint-sized background colonies appeared when creating an in-frame deletion within a separate operon currently under investigation (data not shown). However, after increasing the galactose concentration to 4%, we no longer observed this phenomenon. This strong selective pressure also made the use of the expensive

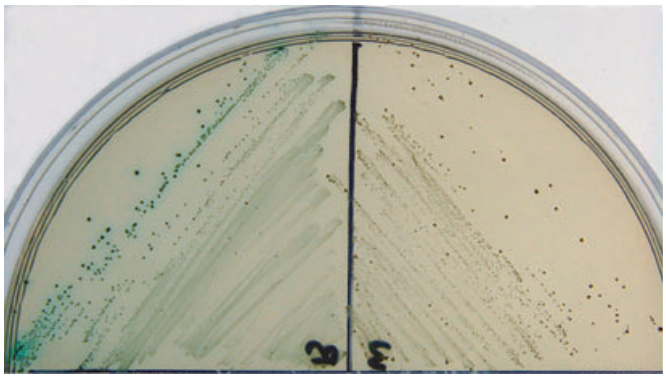

Fig. 4. LacG phenotypes following counterselection. Cells were streaked onto a CYE plate containing 1% lactose and X-gal. The left streak is an isolate determined by polymerase chain reaction to be a regenerated wild-type, while the right streak is an in-frame deletion of *lacG*.

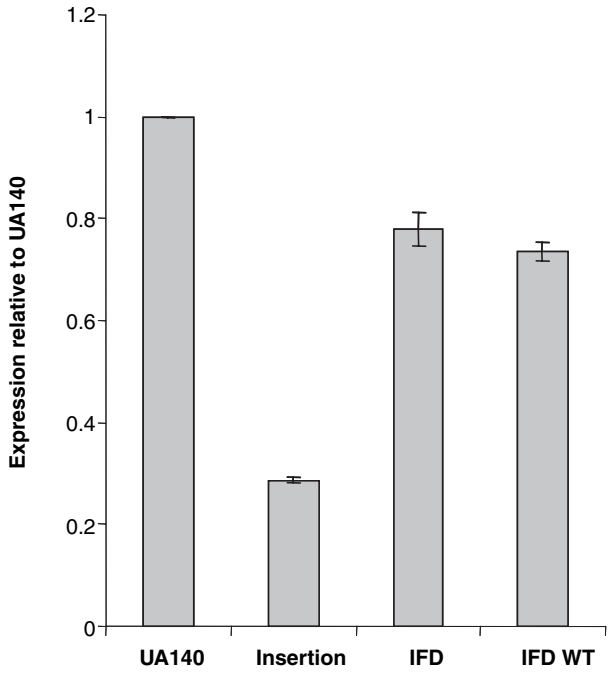

Fig. 5. Real-time reverse transcription-polymerase chain reaction downstream of the *lacG* mutation site. Expression downstream of the mutation site was compared between the parent wild-type (UA140), the plasmid insertion strain (Insertion), the in-frame deletion (IFD), and regenerated wild-type (IFD WT). Primers were designed for the region of *lacG* downstream of the deletion site. The cDNA abundance of each sample was normalized relative to UA140. The data are from one representative experiment and the error bars represent the variation within the experiment. This experiment was repeated twice with similar results.

galactose analog, 2-deoxygalactose, unnecessary and so the method was very cheap to perform as well.

The use of the *S. mutans ldh* promoter to drive *galK* expression provided efficient negative selection on galactose, but presented a potential concern. We had previously demonstrated the reliability of this promoter for strong, constitutive expression in reporter gene assays (27). However, we wanted to ensure that the homology of this promoter fragment to the native *ldh* locus would not affect the initial integration of our construct. Therefore, we kept this promoter fragment small (about 300 base pairs) to lower the chance of integration in this region. In addition, the two homologous fragments we cloned into pIFD-Sm were much larger than the *ldh* promoter fragment to create a strong bias for integration into either of the target homologous regions on the chromosome. This approach seemed to be successful because we were unable to detect any recombination at the *ldh* locus. However, the likelihood of integration at the *ldh* locus could increase if much smaller homologous fragments are used for integration. For example, if homologous fragments as small as the *ldh* promoter fragment (~300 bp) were utilized, it would be expected that about two-thirds of the total integrations would occur at either of the two intended sites with the other one-third occurring at the *ldh* locus. In this case, the percentage of in-frame mutations expected after galactose counterselection would likely drop from 50% to about 33%. Even at this frequency, screening for in-frame mutants would be relatively easy.

In current genetic studies of *S. mutans*, insertion duplication and allelic exchange mutagenesis techniques routinely create unwanted transcriptional artifacts (polar effects) to genes downstream of engineered mutations. Typically, polar mutations are more pronounced with insertion duplication mutagenesis because stem loop structures encoded on the inserted plasmid can cause premature termination of transcription within an operon (7). Various groups have been able to partially alleviate this problem by employing either additional promoters or terminator-less antibiotic cassettes; thereby assuring expression of downstream genes (18, 25, 33). However, this approach is limited by the possibility of downstream genes losing their typical pattern of gene regulation. This alteration of gene expression can have any number of unpredictable consequences in the cell, which makes phenotypic and transcriptional analysis of the intended mutation somewhat dubious. In-frame deletions are essentially free of these polar effects because the mutation does not introduce any genetic markers. Therefore, the phenotypes or transcriptional changes created in a mutant strain can be more directly attributed to the engineered mutation. In addition, the markerless mutations of in-frame mutants support an almost unlimited number of mutations within a strain; whereas other targeted mutation strategies are limited by the number of available selective markers.

In addition to markerless deletions, it is also possible to utilize this system for other purposes. For example, this approach is well suited for allelic replacement with point mutant versions of any gene of interest. This is easily accomplished by engineering a point mutation into one of the homologous fragments included on the suicide vector followed by the same integration/excision strategy for an in-frame

deletion. Alternatively, any exogenous genes can be inserted onto the chromosome simply by cloning a gene of interest between two homologous fragments flanking the target insertion site and using the in-frame integration/excision strategy. Therefore, a counterselection-based genetic system offers a wealth of possibilities beyond in-frame deletion analysis.

The data presented in this study support the utility of GalK activity for negative selection in *S. mutans*. This same approach is also theoretically feasible in a large number of other organisms that do not already possess an efficient negative selection system. First, it is necessary to render the organism sensitive to phospho-galactose. This is easily accomplished by deleting the galactose metabolism genes, if present. Next, a constitutive promoter fusion to the native *galK* can be introduced onto the suicide vector of choice. This vector can then serve as the cloning vector for all the future in-frame deletion experiments. Finally, the same two-step integration/excision strategy can be employed with galactose (or 2-deoxygalactose)-based counterselection.

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