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Cloning-Independent and Counterselectable Markerless Mutagenesis System in *Streptococcus mutans*▽

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Insertion duplication mutagenesis and allelic replacement mutagenesis are among the most commonly utilized approaches for targeted mutagenesis in bacteria. However, both techniques are limited by a variety of factors that can complicate mutant phenotypic studies. To circumvent these limitations, multiple markerless mutagenesis techniques have been developed that utilize either temperature-sensitive plasmids or counterselectable suicide vectors containing both positive- and negative-selection markers. For many species, these techniques are not especially useful due to difficulties of cloning with *Escherichia coli* and/or a lack of functional negative-selection markers. In this study, we describe the development of a novel approach for the creation of markerless mutations. This system employs a cloning-independent methodology and should be easily adaptable to a wide array of Gram-positive and Gram-negative bacterial species. The entire process of creating both the counterselection cassette and mutation constructs can be completed using overlapping PCR protocols, which allows extremely quick assembly and eliminates the requirement for either temperature-sensitive replicons or suicide vectors. As a proof of principle, we used *Streptococcus mutans* reference strain UA159 to create markerless in-frame deletions of 3 separate bacteriocin genes as well as triple mutants containing all 3 deletions. Using a panel of 5 separate wild-type *S. mutans* strains, we further demonstrated that the procedure is nearly 100% efficient at generating clones with the desired markerless mutation, which is a considerable improvement in yield compared to existing approaches.

Streptococcus mutans is a Gram-positive bacterial species that resides within multispecies oral biofilms formed on human tooth surfaces. It is also considered to be one of the principal species associated with dental caries initiation (6, 7, 34, 35, 44, 47, 52). *S. mutans* genetic research has benefited tremendously from the many genetic tools that have been adapted for use in studies of the organism (4, 5, 13, 15, 22, 25, 26, 29, 33, 45, 51). For genetic studies of *S. mutans*, defined mutations are usually engineered in either of two ways: insertion duplication mutagenesis via single-crossover homologous recombination or marked allelic replacement mutagenesis using double-crossover homologous recombination (25, 27, 41). Both approaches are highly reliable strategies for mutagenesis and are simple to engineer, but they also have the potential to create unwanted artifacts that could influence the outcome of a genetic study. For example, insertion duplication mutations often result in the production of truncated proteins. Rarely is it known with certainty whether the protein fragments actually influence mutant phenotypes. Furthermore, due to significant polar effects downstream of the mutation site, both insertion duplication mutagenesis and allelic replacement mutagenesis are of limited utility within operons. In some cases, these issues have been addressed by creating allelic replacement mutants by the use of antibiotic resistance cassettes that lack their endogenous terminators (1, 32, 53). In theory, such constructs permit efficient read-through from upstream promoters and are often referred to as nonpolar. However, since the antibiotic cassettes also contain their own promoters, downstream gene expression patterns are subject to read-through from both the upstream promoter and the antibiotic cassette promoter. This, too, creates a dubious situation for phenotypic analyses, as downstream genes are likely to be differentially regulated.

The preferred approach to circumvent these limitations is through the creation of markerless mutations. Typically, a markerless mutation is engineered using a two-step integration and excision strategy that utilizes either a conditionally replicating temperature-sensitive vector (3, 17, 43) or a suicide vector containing both positive- and negative-selection markers for counterselection (49). When available, counterselectable suicide vectors are often preferred to temperature-sensitive replicons, since the negative-selection marker eliminates clones that have not completed the excision step. This can significantly reduce the effort required to identify the markerless mutant clones. Depending on the construct design, counterselectable mutation constructs can yield a maximum of 50% of the recombinants with a mutant genotype, whereas the remaining clones contain a wild-type genotype (33). Consequently, it is still necessary to screen the resulting isolates to identify the mutant strains.

Currently, only a few negative-selection markers are available for use in bacteria, which greatly limits the utility of the technique for most species. For organisms that are unable to properly metabolize sucrose polymers or galactose, both the levansucrase (*sacB*) (49) and galactokinase (*galK*) (46) genes function well as selective markers. However, numerous species such as *S. mutans* already carry these genes. Alternatively, a

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recipient strain can be constructed in which an organism is rendered sensitive to a substrate through mutagenesis. Frequently, the *upp* mutation is used to create sensitivity to 5-fluorouracil (10, 12, 20, 24). Using *S. mutans*, we were able to create a galactose-sensitive recipient strain by mutagenizing the *galKTE* operon (33). This approach resulted in a strong negative selection in *S. mutans* that was used for the facile creation of a variety of unmarked mutations, such as in-frame deletions, truncations, reporter gene insertions, fusion proteins, and point mutations. The obvious drawback is the requirement for a mutant recipient strain. During our studies, we identified several instances in which the recipient background impacted the phenotypes of our markerless mutant strains (unpublished results). More recently, a Cre-*loxP* system has been adapted for use in *S. mutans* (5). The Cre-*loxP* approach has the advantage of circumventing the requirement for a mutant recipient strain and has been used to create unmarked gene deletions in a large variety of bacteria (5, 8, 21, 28, 30, 36, 39, 42). However, the resulting mutant strains also retain the *loxP* site after the excision step (5, 30, 36), which could be problematic for the creation of various types of mutations, such as truncations, point mutations, fusion proteins, etc.

Recently, a host strain-independent negative-selection marker developed in *Escherichia coli* (18) was adapted for use as a counterselectable marker in *Enterococcus faecalis* (23). This system utilizes a point mutant form of the *pheS* gene encoding the highly conserved phenylalanyl-tRNA synthetase alpha subunit. In a previous *E. coli* study, it was shown that a PheS protein containing an A294G substitution has the ability to aminoacylate phenylalanine analogs such as *p*-chloro-phenylalanine (*p*-Cl-Phe) (19). Thus, by incorporating this point mutant form of *pheS* into a vector, it was possible to perform negative selection in the presence of *p*-Cl-Phe (18, 23). Presumably, the toxicity is derived from the incorporation of *p*-Cl-Phe into cellular proteins. The immediate advantage of this system is its utility in a wild-type organism. In addition, it has been suggested that, since PheS is highly conserved in bacteria, this approach should be adaptable for use in other species (23). Thus, we began by examining the efficacy of *p*-Cl-Phe negative selection in *S. mutans*. Subsequently, we devised a novel cloning-independent counterselection methodology for the creation of markerless mutation constructs. This system completely abrogated the requirement for suicide vectors or temperature-sensitive replicons and greatly simplified the process of construct assembly. Using the cloning-independent approach, we easily created unmarked single, double, and triple in-frame deletion mutants. In addition, we tested the system using multiple wild-type backgrounds and demonstrated that the yield of isolates with the desired mutation was consistently nearly 100%.

MATERIALS AND METHODS

Primers, bacterial strains, and culture conditions. The primers used in this study are shown in Table 1. All *S. mutans* strains were grown in brain heart infusion broth (BHI; Difco) or on BHI agar plates (Table 2). *S. mutans* strains were grown anaerobically (in an atmosphere consisting of 85% N_2, 10% CO_2, and 5% H_2) at 37°C. DNA constructs were introduced into *S. mutans* by the use of natural transformation according to published protocols (38). For transformation experiments, cells were maintained in Todd-Hewitt medium (Difco) supplemented with 0.3% (wt/vol) yeast extract (THYE). For the selection of antibiotic-resistant colonies, BHI plates were supplemented with erythromycin (MP Biomedicals) (15 μg ml^{-1}), spectinomycin (Sigma) (1,000 μg ml^{-1}), or kanamycin (Sigma) (800 μg ml^{-1}). For counterselection, BHI plates were supplemented with 0.02 M *p*-chlorophenylalanine (*p*-Cl-Phe) (Sigma). *Escherichia coli* DH5α cells were grown in Luria-Bertani (LB; Difco) medium with aeration at 37°C. *E. coli* strains carrying plasmids were grown in LB medium containing ampicillin (Fluka) (100 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), or erythromycin (250 μg ml^{-1}).

General DNA manipulation. Phusion DNA polymerase, *Taq* DNA polymerase, restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were all purchased from New England BioLabs. Phusion DNA polymerase was used for overlapping PCR. *Taq* DNA polymerase was used for screening clones.

Construction of the IFDC1 counterselection cassette. In order to obtain the site-directed mutant of *pheS** (GCC314GGC), the wild-type open reading frame of *pheS* was first amplified with PCR using primer pair phesF and phesR. The PCR product was cloned into pGEM-T easy vector (Promega) to generate pWTphes. pWTphes was then used as a template for inverse PCR using the phosphorylated primer pair phesmF and phesmR. The PCR product was digested with DpnI followed by ligation of the PCR fragments and transformation of *E. coli*. Plasmids containing the expected mutant *pheS* (GCC314GGC) were confirmed by sequencing and designated pMTphes (Table 2).

The IFDC1 cassette (a *pheS*-ermAM* two-gene operon driven by the *ldh* promoter) was constructed by an overlapping PCR ligation strategy. First, the promoter region of the constitutive lactate dehydrogenase gene *ldh* was generated by PCR with primer pair ldhF-bamHI and ldhR. The *pheS** open reading frame was generated by PCR using pMTphes as a template and the primer pair pheSF-ldh and phesR. The *ermAM* open reading frame was generated by PCR using primer pair ermF-phes and ermR-HindIII. There are overlapping regions between the three amplicons, which allowed a subsequent overlapping PCR using primer pair ldhF-BamHI and ermR-HindIII. The resulting 2.2-kb amplicons were digested with BamHI and HindIII and ligated into the corresponding sites of pDL278 to obtain pIFDC1.

Construction of the IFDC2 counterselection cassette. In order to prevent mutagenesis of the wild-type, chromosomal copy of *pheS* through recombination, we introduced a series of silent mutations into the region of *pheS** after codon 314. The new in-frame-deletion cassette was named IFDC2. In order to generate IFDC2, three oligonucleotides (mphesF1, mphesF2, and mphesR) were synthesized. Each has 60 nucleotides; together, they spanned the entire 140-bp region of *pheS* that was targeted for mutagenesis. There is also a 20-bp overlapping region between mphesF2 and the other two primers, which allowed them to be ligated into a single 140-bp fragment by the use of overlapping PCR with primer pair mphesF1 and mphesR. The resulting 140-bp amplicon was cloned into the pGEM-T Easy vector to generate pMTmphes and was confirmed by sequencing. The IFDC2 cassette was also created using an overlapping PCR ligation strategy. Briefly, by the use of pIFDC1 as a template, a 1.1-kb region containing the *ldh* promoter and partial *pheS* was PCR amplified with primer pair ldhF-BamHI and phesR-mol. The same template was used to amplify *ermAM* with primer pair ermF-mol and ermR-hindIII. Using pMTmphes as a template, the 140-bp mutagenized region of *pheS** was PCR amplified with primer pair mphesF1 and mphesR. The three amplicons were mixed and used as a template for a subsequent PCR using primer pair ldhF-BamHI and ermR-HindIII. The resulting 2.2-kb amplicon was digested with BamHI and HindIII and ligated into the corresponding sites of pDL278 to create pIFDC2.

Generation of single-mutation in-frame deletion strains. *nlmA* (SMU.150) markerless in-frame deletion strain ZX-4IFD was constructed by a two-step transformation procedure. For the first step, a 1-kb region upstream of *nlmA* was PCR amplified with primer pair 150upF and 150upR-ldh, while a 1.2-kb region downstream of *nlmA* was PCR amplified with primer pair 150dnF-erm and 150dnR. The IFDC1 or IFDC2 cassette was PCR amplified with primer pair ldhF and ermR. The three amplicons contain overlapping regions, which allowed a subsequent overlapping PCR using primer pair 150upF and 150dnR. The resulting 4.4-kb amplicon was transformed into UA159, and transformants were selected on BHI plates containing erythromycin. The resulting strain was named ZX-4IFDC1 or ZX-4IFDC2. For the second transformation, 1-kb upstream and 1.2-kb downstream fragments surrounding *nlmA* were generated by PCR using primer pair 150upF and 150upR-IFD and primer pair 150dnF-IFD and 150dnR. Each amplicon had regions that overlap with regions of the other amplicon; those amplicons yielded a 2.2-kb amplicon when mixed and amplified using primers 150upF and 150dnR. The resulting amplicon was transformed into ZX-4IFDC1 or ZX-4IFDC2 and selected on BHI plates containing *p*-Cl-Phe.

nlmD (SMU.423) markerless in-frame deletion strain ZX-6IFD was constructed using the same strategy. For the first transformation, 1-kb upstream and downstream fragments flanking *nlmD* were generated by PCR using primer pair 423upF and 423upR-ldh and primer pair 423dnF-erm and 423dnR. Both amplicons have regions overlapping with the IFDC2 cassette, which was amplified with primer pair ldhF and ermR. For the second transformation, the 1-kb upstream

TABLE 1. Primers used in this study

Primer	Sequence (5′ → 3′)	Purpose
phesF	CTCGAGATGGATTTACAAGCACAATTAG	*pheS* amplification
phesR	TTAATTGAATTGTTCTGTAAAACGG	*pheS* amplification
phesmF	GCTTTGGCTTAGGCCA	Site-directed mutation of *pheS*
phesmR	CAAAACCAGAATATTCTTCAGAATTAACG	Site-directed mutation of *pheS*
ldhF-bamHI	GCCGGATCCCCGAGCAACAATAAC	*ldh* promoter amplification
ldhR	AACATCTCCTTATAATTTATTAAG	*ldh* promoter amplification
pheSF-ldh	CTTAATAAATTATAAGGAGATGTTCTCGAGATGGATTTACAAGCAC	IFDC1 construction
ermF-pheS	ACAGAACAATTCAATTAAGAAGGAGTGATTACATGAACAA	IFDC1 construction
ermR-hindIII	GCCAAGCTTGAAGCTGTCAGTAGTATACC	IFDC1 construction
mphesF1	TTAATTCTGAAGAATATTCTGGATTCGGGTTCGGGCTTGGACAGGAGAGA ATGGCAATGT	IFDC2 construction
mphesF2	ACAGGAGAGAATGGCAATGTTAAGATACGGTATAAACGACATAAGGGGA TTTTACCAGGG	IFDC2 construction
mphesR	CACTCCTTCCTAGTTAAACTGCTCAGTGAATCGACTATCTCCCTGGTAAAAT CCCCTTAT	IFDC2 construction
phesR-mol	CCCGAATCCAGAATATTCTTCAGAATTAACGC	IFDC2 construct
ermF-mol	ACTGAGCAGTTTAACTAGGAAGGAGTGATTACATGAACAA	IFDC2 construction
ldhF	CCGAGCAACAATAACACTC	IFDC2 amplification
ermR	GAAGCTGTCAGTAGTATACC	*ermAM* and IFDC2 amplification
150upF	TAAGATAGAAGTGGTTTCCC	*nlmA* deletion
150upR-ldh	GAGTGTTATTGTTGCTCGGTGCCTGTGTATTCATATGAT	*nlmA* deletion
150dnF-erm	TATACTACTGACAGCTTCGATCTGTAGTTTTTCCACAC	*nlmA* deletion
150dnR	CCACCACTGCTACTAACAAC	*nlmA* deletion
150upR-IFD	CATATGATAAGTACCCCTTT	*nlmA* deletion
150dnF-IFD	AGGGGTACTTATCATATGTAAATGGAATGGAGAATTAATAC	*nlmA* deletion
423upF	TCATCGTATTGAACGTGTTG	*nlmD* deletion
423upR-ldh	GAGTGTTATTGTTGCTCGGGCTTGTGTATTCATATGATAG	*nlmD* deletion
423dnF-erm	TATACTACTGACAGCTTCGTGCTGCTACTTTTTGTTGA	*nlmD* deletion
423dnR	TCATTTTGATGTCACCTCCA	*nlmD* deletion
423upR-IFD	TTCATGTTCACATATGATAGATACCTCTTTTC	*nlmD* deletion
423dnF-IFD	GAGGTATCTATCATATGTGAACATGAAAAATAGAAGATCCT	*nlmD* deletion
1914upF	CAATGTAGAAGTCTCAAGTA	*nlmC* deletion
1914upR-ldh	GAGTGTTATTGTTGCTCGGGTTCAAATGCTTGTGTATTC	*nlmC* deletion
1914dnF-erm	TATACTACTGACAGCTTCGCTCTTAATTCCTGTGGTTAG	*nlmC* deletion
1914dnR	CAATGATATTAGTTCTTATCCC	*nlmC* deletion
1914upR-IFD	AATTACTAAGATCCCATATGATAAATACCCCTTC	*nlmC* deletion
1914dnF-IFD	TTATCATATGGGATCTTAGTAATTTAATTTTTAAGGAGGCAG	*nlmC* deletion
150pF	GTGTAAAACTTCTATTAAACAG	*nlmA* mutant verification
423pF	TATGTTTGTAGTCAGTTGCG	*nlmD* mutant verification
1914pF	GAAAAAATCATGGATTTTCTTG	*nlmC* mutant verification

and downstream fragments were generated by PCR using primer pair 423upF and 423upR-IFD and primer pair 423dnF-IFD and 423dnR. Overlapping PCR was used to ligate the fragments using primer pair 423upF and 423dnR.

nlmC (SMU.1914c) markerless in-frame deletion strain ZX-5IFD was created as described above. For the first transformation, the 1-kb upstream and downstream fragments were generated by PCR using primer pair 1914upF and 1914upR-ldh and primer pair 1914dnF-erm and 1914dnR. Both amplicons have regions overlapping with the IFDC2 cassette. For the second transformation, the 1-kb upstream and downstream fragments were generated by PCR using primer pair 1914upF and 1914upR-IFD and primer pair 1914dnF-IFD and 1914dnR. Overlapping PCR was used to ligate the fragments using primer pair 1914upF and 1914dnR.

Generation of double in-frame deletion strains. *nlmA-nlmD* double in-frame deletion strain ZX-46IFD was constructed using the same protocol as described for ZX6IFD, except that ZX-4IFD was used as the starting strain. *nlmC-nlmD* double in-frame deletion strain ZX-56IFD was constructed using the same protocol as described for ZX-6IFD, except that ZX-5IFD was used as the starting strain. *nlmA-nlmC* double in-frame deletion strain ZX-45IFD was constructed using the same protocol as described for ZX-5IFD, except that ZX-4IFD was used as the starting strain.

Generation of triple in-frame deletion strain. *nlmA-nlmC-nlmD* triple in-frame deletion strain ZX-456IFD was constructed using the same protocol as described for ZX-6IFD, except that ZX-45IFD was used as the starting strain.

Verification of in-frame deletion strains. Genomic DNA was extracted from all of the putative in-frame deletion strains and used as a template for PCR verification. Wild-type genomic DNA was used as a control. To identify *nlmA* in-frame deletions, genomic DNA was PCR amplified using primer pair 150pF and 150dnR. The expected in-frame deletion yields a 1.4-kb amplicon, while the wild type yields a 1.6-kb amplicon. The *nlmD* in-frame deletions were confirmed using primer pair 423pF and 423dnR. The expected in-frame deletion yields a 1-kb amplicon, while the wild type yields a 1.3-kb amplicon. The *nlmC* in-frame deletions were confirmed using primer pair 1914pF and 1914dnR. The expected in-frame deletion yields a 1.1-kb amplicon, while the wild type yields a 1.3-kb amplicon. We randomly selected 2 triple-mutant isolates and sequenced each of the mutation sites using primer pair 150upF and dnR (*nlmA*), primer pair 423upF and dnR (*nlmD*), and primer pair 1914upF and dnR (*nlmC*).

Generation of *nlmA* (SMU.150) in-frame deletions in multiple *S. mutans* strains. In order to assess the efficiency of cloning-independent IFDC2 mutagenesis in *S. mutans*, UA159 and 4 additional *S. mutans* wild-type strains (UA140, 25175, L13, and CL1) were selected to create in-frame deletions of *nlmA*. Each of these strains was previously determined to possess *nlmA* loci nearly identical to those of UA159 (unpublished data). Therefore, it was possible to employ the same mutagenesis procedure as described for ZX-4IFD. For each strain, 33 colonies were randomly chosen after counterselection on *p*-Cl-Phe plates. Each colony was patched onto BHI plates containing erythromycin. Erythromycin-sensitive strains were scored as mutant clones, whereas antibiotic-resistant clones were scored as background. These results were also further verified by PCR using the primer pair 150pF and 150dnR. Three independent experiments were performed with all 5 strains, and the results were averaged.

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference
Strains		
E. coli DH5α	Cloning strain	
UA159	*Streptococcus mutans* reference strain	2
UA140	Wild-type *Streptococcus mutans*	40
ATCC 25175	Wild-type *Streptococcus mutans*	9
L13	Wild-type *Streptococcus mutans*	48
CL1	Wild-type *Streptococcus mutans*	50
ZX-4IFDC1	UA159 Δ*nlmA*, Em^r, *p*-Cl-Phe^s	This study
ZX-4IFDC2	UA159 Δ*nlmA*, Em^r, *p*-Cl-Phe^s	This study
ZX-4IFD	UA159 Δ*nlmA*, Em^s, *p*-Cl-Phe^r	This study
ZX-4IFD2	UA140 Δ*nlmA*, Em^s, *p*-Cl-Phe^r	This study
ZX-4IFD3	25175 Δ*nlmA*, Em^s, *p*-Cl-Phe^r	This study
ZX-4IFD4	L13 Δ*nlmA*, Em^s, *p*-Cl-Phe^r	This study
ZX-4IFD5	CL1 Δ*nlmA*, Em^s, *p*-Cl-Phe^r	This study
ZX-6IFD	UA159 Δ*nlmD*, Em^s, *p*-Cl-Phe^r	This study
ZX-5IFD	UA159 Δ*nlmC*, Em^s, *p*-Cl-Phe^r	This study
ZX-46IFD	UA159 Δ*nlmA*, Δ*nlmD*, Em^s, *p*-Cl-Phe^r	This study
ZX-45IFD	UA159 Δ*nlmA*, Δ*nlmC*, Em^s, *p*-Cl-Phe^r	This study
ZX-56IFD	UA159 Δ*nlmC*, Δ*nlmD*, Em^s, *p*-Cl-Phe^r	This study
ZX456IFD	UA159 Δ*nlmA*, Δ*nlmC*, Δ*nlmD*, Em^s, *p*-Cl-Phe^r	This study
Plasmids		
pDL278	*E. coli-S. mutans* shuttle vector	
pWTphes	pGEM::*pheS*, Ap^r	This study
pMTphes	pGEM::*pheS**, Ap^r	This study
pIFDC1	pDL278::ldh_p::*pheS**::*ermAM*, Sp^r, Em^r	This study
pMTmphes	pGEM::*mpheS**, Ap^r	This study
pIFDC2	pDL278::ldh_p::*mpheS**::*ermAM*, Sp^r, Em^r	This study

[a] Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Km^r, kanamycin resistance; Em^r, erythromycin resistance; *p*-Cl-Phe^r, *p*-chloro-phenylalanine resistance.

RESULTS

Creation of a hybrid positive- and negative-selection cassette. It was previously demonstrated that a point mutant *pheS** gene encoding an A294G substitution in *E. coli* PheS or an A312G substitution in *E. faecalis* PheS results in a pronounced sensitivity to the phenylalanine analog *p*-chloro-phenylalanine (*p*-Cl-Phe) (18, 23). Given the high sequence conservation of PheS among bacteria, we reasoned that the same mutation in the *S. mutans* PheS would likely result in a similar sensitivity to *p*-Cl-Phe. In order to identify the appropriate residue for mutagenesis, we used ClustalW to perform a multiple sequence alignment of the PheS proteins from a variety of distantly related Gram-positive and Gram-negative species. As shown in Fig. 1A, A312 in *Enterococcus faecalis* (23), A294 in *E. coli* (19), A317 in *Myxococcus xanthus*, A303 in *Bacteroides fragilis*, A322 in *Streptomyces coelicolor*, and A314 in *S. mutans* are all strictly conserved. Therefore, as described in Materials and Methods, we used inverse PCR to engineer a point mutation in codon 314 of *S. mutans pheS* to change it from GCC to GGC (Fig. 1B). This resulted in a mutant PheS protein containing an A314G substitution. In order to test whether this mutant *pheS** would also function as a negative-selection marker, we incorporated this gene into a hybrid IFDC1 cassette, which contained both positive- and negative-selective markers combined into one artificial operon (Fig. 1B). Transcription of the entire cassette was driven solely by the highly expressed *S. mutans* lactate dehydrogenase (*ldh*) promoter. The *pheS** open reading frame was engineered as a translation fusion to the *ldh* promoter followed by a promoterless erythromycin resistance *ermAM* cassette that still retained its original ribosome binding site. With this configuration, the acquisition of erythromycin resistance would be indicative of *pheS** transcription. Next, the IFDC1 cassette was cloned onto *E. coli-S. mutans* shuttle vector pDL278 and tested for its functionality for positive and negative selection in *S. mutans*. As shown in Fig. 2, this cassette provided stringent selection in the presence of either erythromycin or *p*-Cl-Phe.

Cloning-independent assembly of markerless mutation constructs. Given the success of the initial test of IFDC1, we

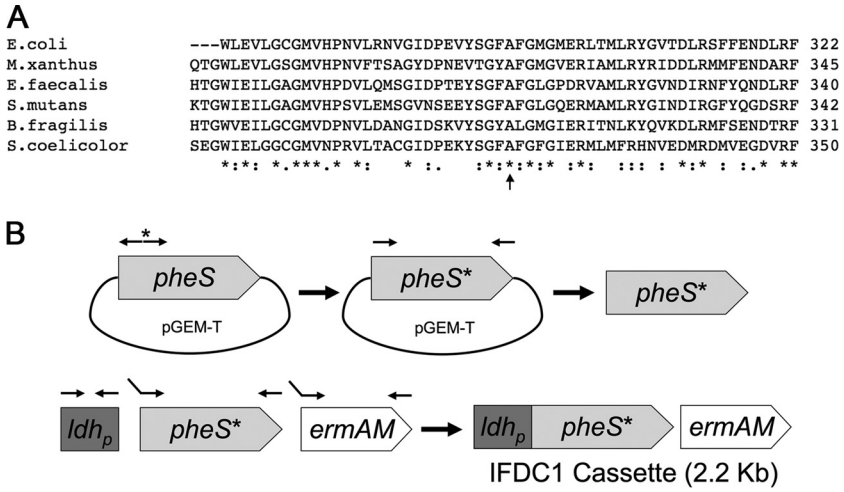

FIG. 1. Construction of a hybrid positive- and negative-selection cassette. (A) Results from a multiple sequence alignment of the C-terminal region of PheS determined using ClustalW. The arrow indicates the conserved alanine residue that can be mutagenized to create *p*-Cl-Phe sensitivity. (B) Graphic representation of the construction of the IFDC1 hybrid cassette. Primer binding sites are denoted by small arrows, whereas primers containing overlapping sequences are denoted by bent arrows. As described in Materials and Methods, the *S. mutans pheS* open reading frame was cloned into pGEM-T Easy vector and mutagenized using inverse PCR to introduce a GCC-to-GGC point mutation into codon 314. The *pheS** cassette was then amplified with PCR and mixed with PCR amplicons of the *ldh* promoter and promoterless *ermAM* cassette. Both the *pheS** amplicon and the *ermAM* amplicon were engineered to contain overlapping sequences that facilitated their assembly by PCR into a single cassette (IFDC1) controlled by the constitutive *ldh* promoter.

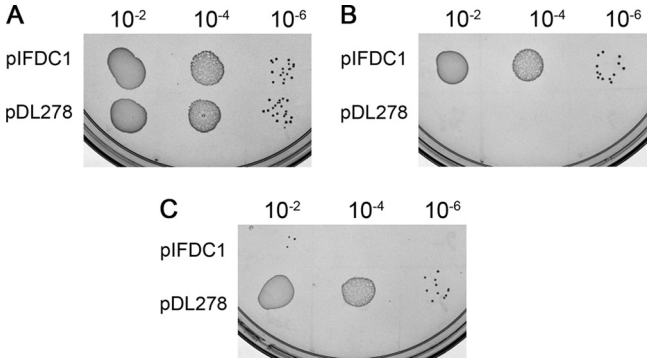

FIG. 2. Assessment of positive and negative selection with IFDC1. The empty pDL278 shuttle vector and the same vector containing the IFDC1 cassette (pIFDC1) were transformed into UA159 and selected with spectinomycin. Both vectors contain a spectinomycin resistance cassette. Cultures of confirmed transformants were spotted in successive dilutions onto BHI plates (A), BHI plates containing erythromycin (B), and BHI plates containing *p*-Cl-Phe (C). The dilution level is indicated in each image. This experiment was performed 3 times with similar results.

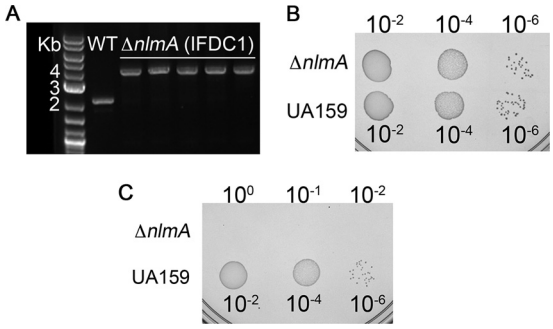

FIG. 4. Assessment of IFDC1 for markerless mutagenesis. IFDC1 was used to assemble a construct for the in-frame deletion of *nlmA*. (A) Five erythromycin-resistant clones were PCR amplified to confirm the insertion of the IFDC1 cassette. The expected PCR amplicon from the wild type is approximately 2.2 kb, while the expected amplicon from the mutant is approximately 4 kb. (B and C) Mutant strains confirmed to contain the IFDC1 cassette were subjected to a second transformation reaction to remove the IFDC1 cassette and create the markerless in-frame deletion mutation. The results of successive dilutions of that second transformation reaction mixture after it was spotted onto BHI plates (B) and BHI plates containing *p*-Cl-Phe (C) are shown. The UA159 parent strain was included for comparison. The dilution level is indicated in each image. This experiment was performed 3 times with similar results. WT, wild type.

decided to use this cassette for a novel markerless mutagenesis strategy. In the classic approach, 2 homologous DNA fragments flanking the intended mutation site are ligated together onto a suicide vector containing both positive- and negative-selection markers (17, 33, 49). In our case, those markers would be supplied by the IFDC1 cassette. However, we reasoned that it should be possible to forgo the cloning steps altogether by adopting an allelic replacement strategy (Fig. 3). In order to test this approach, we targeted the gene encoding the bacteriocin mutacin IV (SMU.150 [*nlmA*]) for an in-frame deletion. After the first transformation, we were able to easily isolate erythromycin-resistant clones that had replaced the *nlmA* open reading frame with IFDC1 (Fig. 4A). However, we were surprised to discover that the second transformation yielded no clones on the *p*-Cl-Phe plates (Fig. 4B and C). Subsequent analyses determined that the wild-type copy of *pheS* had recombined with the mutant *pheS** of IFDC1 (data not shown). Therefore, it was necessary to reconstruct IFDC1 by the use of a *pheS** cassette with lower homology to the wild-type locus.

Creation of the next-generation cassette IFDC2. Initially, we replaced the *S. mutans pheS** in IFDC1 with several other *pheS** open reading frames we had created using the genomic DNA from closely related species. We found that this approach offered no selection in the presence of *p*-Cl-Phe (data not shown). Consequently, it was necessary to continue using the *S. mutans pheS** cassette, albeit we deemed it necessary to further alter the sequence of *pheS** to reduce its homology to the wild-type gene. By engineering a series of silent mutations in the remaining codons after the *pheS314AG* mutation site, we created a new *pheS** cassette (*mpheS*) that still retained the amino acid sequence of the original cassette but exhibited much lower homology at the nucleotide level downstream of *pheS314AG* (Fig. 5A and B). The new *mpheS* cassette was then used in place of *pheS** for the second-generation IFDC2 counterselection cassette. Using this cassette, we repeated the selection procedure described for Fig. 3 and once again targeted *nlmA* for in-frame deletion. With IFDC2, the second transformation step yielded dramatically different results in the presence of *p*-Cl-Phe selection. We observed *p*-Cl-Phe-resistant colonies at a frequency that was highly suggestive of a successful transformation (Fig. 5C and D). To further confirm the utility of this system, we repeated the *nlmA* mutagenesis and

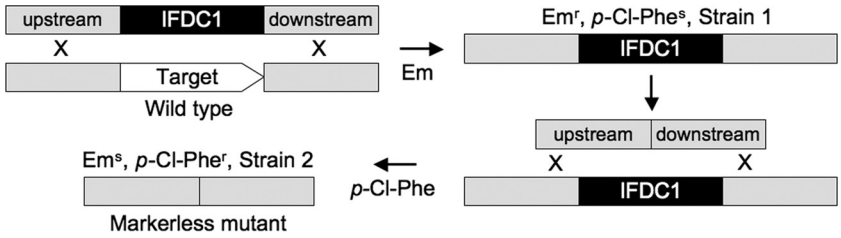

FIG. 3. Cloning-independent markerless mutagenesis strategy. The positive- and negative-selection cassette is ligated between 2 homologous fragments flanking the intended mutation site. The linear construct is transformed, and mutants are selected using erythromycin. The erythromycin-resistant mutants are then transformed with a second linear construct containing the two homologous fragments directly ligated together without the intervening selection cassette. The transformation reaction is then selected on plates containing *p*-Cl-Phe. The resulting markerless mutants are erythromycin sensitive and *p*-Cl-Phe resistant.

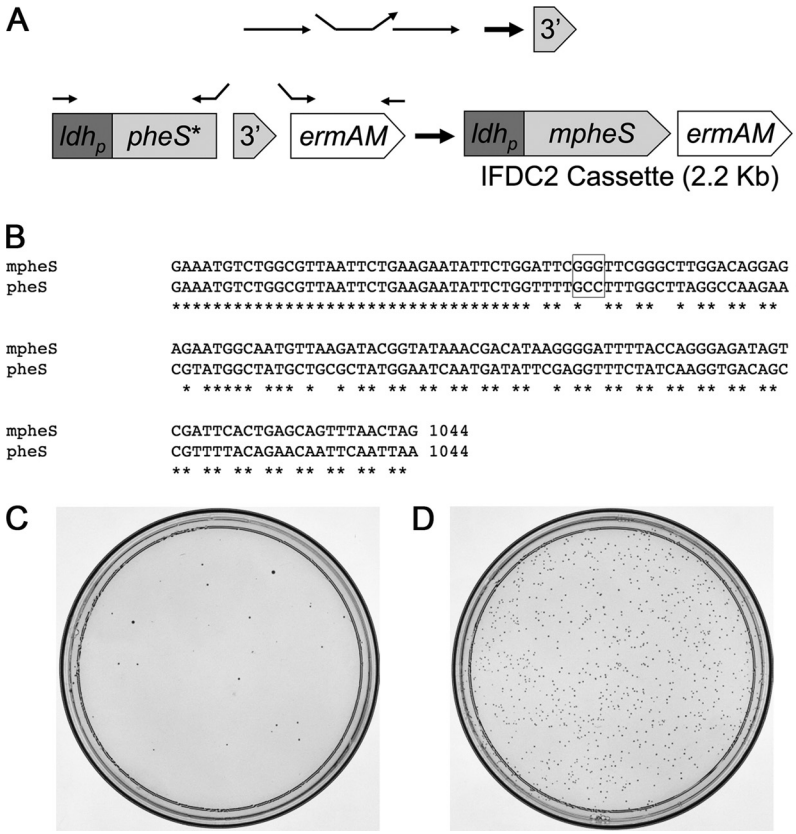

FIG. 5. Creation of the IFDC2 second-generation cassette. (A) As described in Materials and Methods, a series of silent mutations were engineered in the region downstream of codon 314 to create the new *mpheS* negative-selection cassette. Three overlapping oligonucleotides containing the desired *pheS* silent mutations were synthesized. The oligonucleotides were mixed and subjected to overlapping PCR to produce an amplicon carrying the 3′ portion of *pheS*. The remaining portion of *pheS* and the *ermAM* cassette were both amplified from IFDC1. All 3 amplicons were mixed in a single reaction and subjected to overlapping PCR to generate the final IFDC2 product. Primer binding sites are indicated by small arrows, whereas bent arrows indicate primers containing overlapping sequences. (B) The nucleotide sequence of the 3′ region of *mpheS* was aligned to wild-type *pheS* by the use of ClustalW. Codon 314 is enclosed within a box. Next, *nlmA* was targeted for in-frame deletion with the IFDC2 cassette. After transforming UA159 with the IFDC2 mutagenesis construct and selection on erythromycin, confirmed mutants were subjected to a second transformation to remove the IFDC2 cassette and selected using *p*-Cl-Phe. The results from the negative-control reaction receiving no DNA are shown in panel C, while results from the transformation reaction are shown in panel D. This experiment was performed 3 times with similar results.

targeted 2 additional bacteriocin genes, *nlmD* (SMU.423) (50) and *nlmC* (SMU.1914c; also referred to as *cipB*), for in-frame deletion (14, 37). For all 3 mutations, we obtained similar results. After the second transformation and negative selection on *p*-Cl-Phe, all of the randomly screened colonies exhibited the expected deletion (Fig. 6A to C). Lastly, we chose several confirmed *nlmA* in-frame deletion clones and used the same approach to create unmarked triple deletion strains of *nlmA*, *nlmD*, and *nlmC* (Fig. 6D). As a final confirmation, we selected 2 of the resulting triple mutants and sequenced each of the mutation sites to verify the expected deletions (data not shown).

IFDC2 is highly efficient for counterselection in multiple *S. mutans* wild-type backgrounds. Given our success with mutagenesis of *S. mutans* reference strain UA159, we were next curious to determine whether our mutagenesis system would be broadly applicable for use with other *S. mutans* strains as well. Consequently, we used the same approach to engineer markerless *nlmA* deletions in 4 additional strains that we had previously determined to carry *nlmA*. The performance of the system was evaluated by assaying the proportion of colonies that had excised the IFDC2 cassette after the counterselection step. For all 5 strains, we consistently detected from 93 to 100% of the clones with the expected genotypes and few, if any, background clones (Table 3). As expected, counterselection also resulted in the markerless deletion of *nlmA* in all 5 strains (Fig. 6E). Thus, the results with respect to the performance of the system were quite similar for all of the strains, with nearly every tested clone exhibiting the desired mutation.

DISCUSSION

In the current report, we describe a highly efficient cloning-independent counterselection approach for creating markerless mutations. This system is based upon the proven utility of the *pheS** gene as a negative-selection marker in the presence of *p*-Cl-Phe (23). For this approach, we incorporated the *pheS** open reading frame into a hybrid positive- and negative-selection cassette, which facilitated the allelic replacement strategy we used to introduce the constructs. In addition, by eliminating

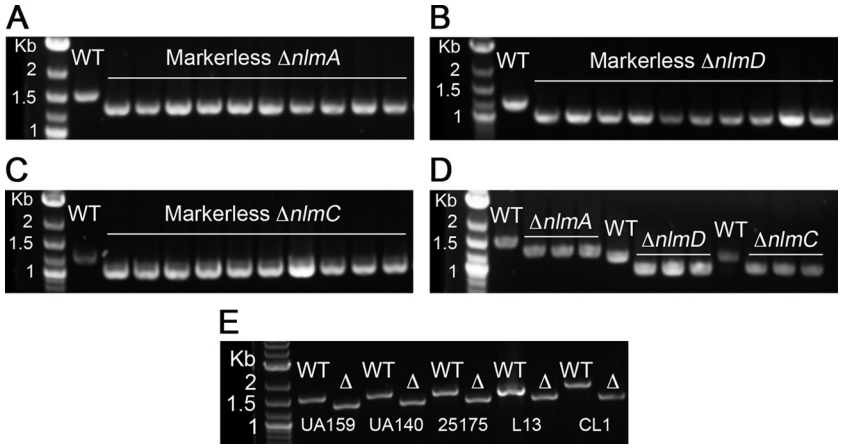

FIG. 6. Generation of markerless in-frame deletion mutants. The bacteriocin-encoding genes *nlmA*, *nlmD*, and *nlmC* were all targeted for unmarked in-frame deletions by the use of IFDC2. After the second transformation, 10 randomly selected *p*-Cl-Phe-resistant clones were PCR amplified using primers flanking the targeted gene. (A) For *nlmA*, the expected wild-type amplicon is approximately 1.6 kb, while an in-frame deletion mutant should be approximately 1.4 kb. (B) For *nlmD*, the expected wild-type amplicon is approximately 1.3 kb, while an in-frame deletion mutant should be approximately 1 kb. (C) For *nlmC*, the expected wild-type amplicon is approximately 1.3 kb, while an in-frame deletion mutant should be approximately 1.1 kb. These experiments were performed 3 times with similar results. (D) Three markerless in-frame *nlmA*, *nlmD*, and *nlmC* deletion triple-mutant strains were independently constructed. Each of these strains was tested with PCR to confirm the presence of all 3 mutations. The expected amplicons are identical to the single mutations described above. (E) A total of 5 wild-type *S. mutans* strains were used for the construction of *nlmA* in-frame deletions. The identities of the parent strains are listed below the corresponding amplicons.

the cloning requirement for construction, we were able to reliably assemble all of the mutation constructs via overlapping PCR within 1 to 2 days, which is a considerable reduction in time and effort compared to cloning-based approaches. Another benefit of the cloning-independent approach is that it circumvents the issues of toxicity associated with introducing heterologous DNA fragments into *E. coli*. For example, *E. coli* routinely transcribes from any *S. mutans* promoters located within the cloned fragments of mutagenesis constructs. As a result, we have encountered instances where, due to the foreign gene products produced, particular constructs were unstable in *E. coli* (unpublished results). Likewise, cloned heterologous DNA from extremely AT-rich organisms is also known to be exceptionally unstable in *E. coli*, due to frequent deletions and rearrangements (11). Thus, a cloning-independent methodology should be particularly useful for constructing markerless mutations in these species. Furthermore, we consistently obtained nearly 100% of the *p*-Cl-Phe-resistant transformants with the expected excision of IFDC2 (Table 3). To the best of our knowledge, our approach represents the first markerless mutagenesis system capable of generating such a high proportion of unmarked mutants in a wild-type background. In contrast to plasmid-based approaches, it was largely unnecessary to screen for the mutant clones after the final selection step, since the vast majority contained the desired mutation. Furthermore, if a further confirmation of a mutant genotype is desired, one need only to patch clones onto antibiotic plates and verify their sensitivity. PCR screening is unnecessary. As noted by Kristich et al., the high conservation of PheS in bacteria also suggests that the *pheS** gene should be adaptable for use in a wide range of species (23). Indeed, we found that the particular alanine residue required for creating PheS* is strictly conserved in a diverse array of both Gram-positive and Gram-negative organisms (Fig. 1A). Thus, our system is likely to be widely applicable for the creation of unmarked mutations in bacteria.

TABLE 3. Comparison of mutagenesis efficiencies in different strains

Strain	Mutant CFU[a]	Background CFU[a]	% mutant ± SE	Source or reference
UA159	30.7	2.3	93.0 ± 5.5	2
UA140	30.7	2.3	93.0 ± 2.0	40
25175	33.0	0.0	100	ATCC (9)
L13	32.3	0.7	97.9 ± 1.8	48
CL1	33.0	0.0	100	Clinical isolate (50)

[a] Values are presented as the averages of 33 randomly selected CFU determinations from 3 independent experiments.

While we now have the markerless mutagenesis system fully optimized, the system went through several iterations before reaching its current state, due to the need for improvements upon the *pheS** negative-selection cassette. In our case, we found that *p*-Cl-Phe selection was unacceptably inefficient at suppressing background growth unless the *pheS** cassette was very highly expressed. Presumably, this is because the A314G mutant PheS* must compete with the endogenous wild-type PheS to form complexes with PheT (31). Initially, we had fused *pheS** to the synthetic lactococcal CP25 promoter (16). Previously, we found CP25 to be a suitable promoter for ensuring strong, constitutive gene expression in *S. mutans*. However, for negative selection with *pheS**, it did not function nearly as well as the *S. mutans* lactate dehydrogenase (*ldh*) promoter (data not shown). In our experience, the *S. mutans ldh* promoter gives even higher gene expression than CP25 (unpublished results). Given that we expressed *pheS** as a translation fusion to the *ldh* promoter, it is also certainly possible that differences in translation efficiency could have further contributed to the greater success of the experiments performed with *ldh* versus

CP25. Another hurdle we encountered was due to an apparent high rate of recombination between the *pheS** cassette and the chromosomal copy of *pheS*. Initially, we thought that this could be prevented simply by substituting a *pheS** cassette created from another closely related organism. However, we tested several other *pheS** cassettes and found that strategy to be unsuccessful. The problem was finally solved by introducing a series of silent mutations downstream of the *pheS** point mutation site to reduce the overall homology of the cassette with the wild-type *pheS* (Fig. 5A and B). We were also mindful of the engineered silent mutations to avoid inadvertently impeding the translation of the cassette due to the introduction of multiple rare codons. Currently, it is unknown whether *pheS* silent mutations are required for the successful utilization of *p*-Cl-Phe counterselection in organisms other than *S. mutans*. Such an approach was apparently unnecessary for use in *E. faecalis* (23). However, if this system is to be adapted for use in other species, it is advisable to start by synthesizing or constructing a synthetic *pheS** with reduced homology to the wild-type gene. In that way, any potential issues with *pheS* recombination can be avoided altogether.

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