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Markerless Multiple-Gene-Deletion System for *Streptococcus mutans*▽

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Inactivation or selective modification is essential to elucidate the putative function of a gene. The present study describes an improved Cre-*loxP*-based method for markerless multiple gene deletion in *Streptococcus mutans*, the principal etiological agent of dental caries. This modified method uses two mutant *loxP* sites, which after recombination creates a double-mutant *loxP* site that is poorly recognized by Cre recombinase, facilitating multiple gene deletions in a single genetic background. The effectiveness of this modified strategy was demonstrated by the construction of both single and double gene deletions at the *htrA* and *clpP* loci on the chromosome of *Streptococcus mutans*. HtrA and ClpP play key roles in the processing and maturation of several important proteins, including many virulence factors. Deletion of these genes resulted in reducing the organism's ability to withstand exposure to low pH and oxidative agents. The method described here is simple and efficient and can be easily implemented for multiple gene deletions with *S. mutans* and other streptococci.

The availability of ever-increasing numbers of genome sequences from pathogenic microorganisms has greatly facilitated the genetic dissection of the factors that determine their fitness and pathogenic potential. Genetic analysis of many oral streptococci, including *Streptococcus mutans*, which is considered the principal etiological agent of dental caries (29), has thus far been limited due to a lack of advanced genetic tools. Typically, the function of a gene is assessed by either inactivation or selective modification. Classic strategies used to obtain gene deletions from *S. mutans* include single-crossover insertion duplication mutagenesis (45) or allelic exchange with antibiotic cassettes via double-crossover recombination (22, 27). In addition, various transposon (16, 23, 39) and random insertional mutagenesis (25, 46, 47) strategies are employed for obtaining gene deletions in *S. mutans*. Generally, these strategies result in the introduction of a suitable marker into the genome, facilitating the selection of mutants. However, when multiple gene deletions are required, the limited number of convenient, selectable markers makes the adoption of these strategies less feasible.

The use of the Cre-*lox* recombination system to remove selectable markers from the genome is an efficient way of circumventing this problem. The versatility of Cre recombinase makes it ideal for use in various gene manipulation strategies involving plants (15), *Saccharomyces cerevisiae* (40), mice (41, 49), human cell lines (32), and various microorganisms (12, 37, 44). The Cre recombinase of bacteriophage P1 is a 38-kDa protein that belongs to the integrase family of site-specific recombinases (42). It catalyzes cofactor-independent recombination between two of its recognition sites, known as *loxP*, which also originates from *Escherichia coli* phage P1 (1). The 34-bp consensus sequence for the *loxP* site consists of an asymmetrical core spacer of 8 bp, defining the orientation of the *loxP* site, and two 13-bp palindromic flanking sequences (19). A DNA sequence flanked by the *loxP* sites is excised when the *loxP* sites are convergently oriented, whereas the sequence is inverted when the *loxP* sites are divergently oriented (33). Cre recombinase is able to act on both inter- and intramolecular *loxP* sites, although recombination of the intramolecular *lox* sites is kinetically favorable (26). However, wild-type *loxP* sites may cause problems during multiple gene deletions. This is because the integration of multiple *loxP* sites into the genome can cause genomic instability, due to potential recombination between *loxP* sites from different cassettes.

In this study, we demonstrate the use of an improved Cre-*loxP*-based system for the simultaneous inactivation of multiple genes in *S. mutans*. HtrA and ClpP, two important serine proteases, were selected for analysis, since they are associated with the virulence of this pathogen and because of their uniquely detectable phenotypes. Using the modified Cre-*loxP* method, we successfully deleted *htrA* and *clpP* from *S. mutans*. Mutant strains lacking *clpP* and/or *htrA* exhibited a wide range of stress-sensitive phenotypes, such as reduced tolerance to low pH and to oxidative stress-inducing agents.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strain DH5α was grown in Luria-Bertani medium supplemented, when necessary, with ampicillin (100 $\mu g \cdot ml^{-1}$), kanamycin (Km; 50 $\mu g \cdot ml^{-1}$), and spectinomycin (Sp; 100 $\mu g \cdot ml^{-1}$). *S. mutans* strains were routinely grown in Todd-Hewitt medium (BBL; Becton Dickinson) supplemented with 0.2% yeast extract (THY) and, when necessary, Km (300 $\mu g \cdot ml^{-1}$), Sp (300 $\mu g \cdot ml^{-1}$), or erythromycin (Em; 10 $\mu g \cdot ml^{-1}$). The strains used in this study are available upon request.

Construction of Cre-*loxP*-mediated gene replacement mutants. Based on an analysis of the *S. mutans* strain UA159 genome (accession no. AE015037) (3), 1.9- and 1.7-kb DNA fragments containing *htrA* and *clpP*, respectively, were PCR amplified using the primer pairs Smu-HtrA-F1/Smu-HtrA-R1 and Smu-ClpP-F1/Smu-ClpP-R1 (Table 1). The PCR products were cloned into the pGEM-T-Easy TA cloning vector (Promega) (pIB102 and pIB143 for *htrA* and *clpP*, respectively) and were confirmed by using restriction digestion. A Km resistance cassette amplified from pUC4ΩKm2 (34), using two different sets of primers (*loxP*-Km-F/*loxP*-Km-R and *lox71*-Km-F/*lox66*-Km-R), was cloned into BaeI-PflMI-digested/T4 DNA polymerase-blunted *htrA* in pIB102, yielding pIB501, containing wild-type *loxP* sites (*htrA*::*loxP*-Km-*loxP*), and pIB506, containing mutant *loxP* sites (*htrA*::*lox71*-Km-*lox66*); both products were verified by restriction digestion. An Sp resistance cassette was amplified from pUC-Spec (20),

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TABLE 1. List of oligonucleotides[a]

Primer name	Sequence (5′ to 3′)	Purpose
Smu-HtrA-F1	gactagcattatttggaattttctcatcgg	Amplification of *htrA* and construction of pIB101
Smu-HtrA-R1	gaggtgagatatgaagacaacttcgttgac	
Smu-HtrA-F2	aataacgaaggtcaaatcagaa	Verification of the *htrA* gene deletion
Smu-HtrA-R2	atatctttagtattaagatatt	
Smu-ClpP-F1	gttccaggtcttggggatgcaggcgatcgcttg	Amplification of *clpP* and construction of pIB143
Smu-ClpP-R1	ggtggtttatgcggtgccagcggtaaacccg	
Smu-ClpP-F2	ctgaagatattgaccaacgtca	Verification of the *clpP* gene deletion
Smu-ClpP-R2	cgtatcaatataggatggaaga	
loxP-Km-F	**CGATAACTTCGTATAATGTATGCTATAGCAAGTTAT** gaggatgaagaggatgaggaggcag	Amplification of Km^r marker with flanking native *loxP* sequences
loxP-Km-R	**CGATAACTTTGTATAGCATACATTATACGAAGTTAT** gctttttagacatctaaatctagg	
lox71-Km-F	**CGTACCGTTCGTATAGCATACATTATACGAAGTTAT** gaggatgaagaggatgaggaggcag	Amplification of Km^r marker with flanking mutant *loxP* sequences
lox66-Km-R	**CGTACCGTTCGTATAATGTATGCTATACGAAGTTA T**gctttttagacatctaaatctagg	
lox71-Sp-F	**CGTACCGTTCGTATAGCATACATTATACGAAGTTAT** ctagataaaaaatttagaagcccaatg	Amplification of Sp^r marker with flanking mutant *loxP* sequences
lox66-Sp-R	**CGTACCGTTCGTATAATGTATGCTATACGAAGTTA T**ctgttatttaaatagtttatagttaaatttac	

[a] *loxP* sites are presented in boldface, and mutations created to generate *lox66* and *lox71* sites are underlined. DNA sequences homologous to template DNA are presented in lowercase letters.

using the primers *lox71*-Sp-F and *lox66*-Sp-R (Table 1), and inserted into the BbsI-ClaI-digested and blunt-ended *clpP* gene in pIB143, yielding pIB507. The plasmids pIB501, pIB506, and pIB507 were linearized with NotI and used for the transformation of *S. mutans* UA159 as previously described (9), with transformants selected on THY plates containing Km or Sp. For the simultaneous inactivation of *htrA* and *clpP*, *S. mutans* UA159 was cotransformed with NotI-linearized pIB506 and pIB507, and transformants were selected on THY plates containing Km and Sp. Deletion of the *htrA* (IBS501 and IBS508) and *clpP* (IBS509) genes by double crossover was verified by PCR using the primer pairs Smu-HtrA-F2/Smu-HtrA-R2 and Smu-ClpP-F2/Smu-ClpP-R2, respectively.

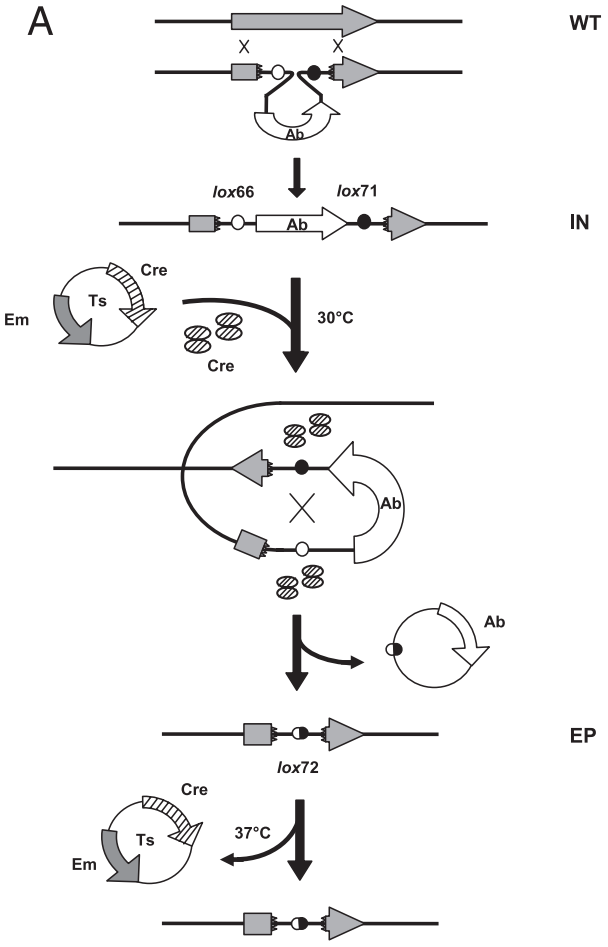
Cre-mediated mutant locus resolution. The pCrePA plasmid (kindly provided by S. H. Leppla, NIH) contains the *cre* gene under the promoter of *Bacillus anthracis* protective antigen (*pagA*) gene isolated from pAE5 (35, 36), an Em^r gene as a selectable marker, and a temperature-sensitive replicon (pWV01) from pHY304 (30, 38). To excise the *loxP*/*P**-Km^r (where *P** indicates the mutant *loxP* sites) resistance cassette or the *loxP**-Sp^r resistance cassette from the chromosome, IBS501, IBS508, IBS509, and IBS510 were transformed with pCrePA. Transformants were grown at 30°C on THY-Em plates, and selected colonies were then grown at 30°C in THY-Em broth and plated on THY-Em plates. Approximately 500 colonies from THY-Em plates were patched onto THY plates containing either Km or Sp. Colonies that were Em resistant and Km sensitive (Em^r Km^s), Em resistant and Sp sensitive (Em^r Sp^s), or Em resistant and Km and Sp sensitive (Em^r Km^s Sp^s) were transferred into antibiotic-free THY plates by patching. The plates were incubated overnight at 37°C to cure pCrePA (30, 38), generating Em^s cells, which were selected for further analysis. Deletions of the *htrA* and *clpP* genes and the proper resolution of *loxP* (both native and mutant) sites were verified by PCR analysis and DNA sequencing.

Evaluation of stress-sensitive phenotypes. Cultures grown overnight were washed and resuspended in 0.85% saline to an optical density at 600 nm of 5.0. The cultures were serially diluted 10-fold in 0.85% saline, and 7.5 μl of each dilution was spotted onto THY plates containing puromycin (4.0 to 6.0 μg/ml; Sigma-Aldrich) or methyl viologen (MV; 5.0 to 10.0 mM; Sigma-Aldrich). Plates were incubated at 37°C under microaerophilic conditions, and the bacterial growth was evaluated as described previously (8).

For experiments involving pH changes of the growth medium, the initial pH of the THY broth was adjusted to pH 5.5 or pH 7.0 with HCl prior to sterilization. A citrate-phosphate buffer (50 mM) with the desired pH was then added to the medium after sterilization. The buffered THY medium was inoculated with *S. mutans* cultures (3% [vol/vol]) grown overnight and incubated at 37°C. Growth of the various cultures was monitored as described previously (8).

RESULTS

A strategy for markerless gene deletion using the Cre-*loxP* system. The genetic events involved in Cre-*loxP*-based gene deletion are presented in Fig. 1A. Although this method has been used with many microorganisms, it has never been applied to streptococci. Using this technique, we attempted to delete the *htrA* gene in *S. mutans*. The *htrA* gene was first cloned into a plasmid vector (pIB102) and then inactivated via the insertion of a Km^r cassette flanked by wild-type *loxP* sequences (*loxP*-Km^r-*loxP*, pIB501). Following the transformation of pIB501 in *S. mutans*, double-crossover events were selected on the basis of Km resistance. The double-crossover recombination frequency was found to be 2.3×10^{-3} with respect to the number of viable cells, and the allelic exchange event was verified by PCR (Fig. 2). One such mutant, designated IBS501, was transformed with pCrePA. The chromosomally integrated *loxP*-Km^r-*loxP* cassette was then excised by the transient expression of Cre recombinase. After overnight growth at 30°C, approximately 13% of the colonies were Km^s, indicating the loss of the Km^r marker, which was also confirmed by PCR (Fig. 2). Longer periods of incubation at 30°C in THY-Em increased the yield of colonies with the successful excision of the *loxP*-Km^r-*loxP* cassette. Em^r Km^s colonies were selected and grown in THY broth at 37°C. As pCrePA has a temperature-sensitive replicon (30, 38), the elevation of the growth temperature to 37°C without the selection pressure (Em) resulted in rapid curing of the plasmid. After colonies were incubated overnight, 99.9% of the *S. mutans* colonies (500 colonies tested) were cured of pCrePA, resulting in a mutant strain containing a deletion of the *htrA* gene (IBS502). This result is similar to the gene deletion results with *B. anthracis*, where a single passage at 37°C completely cured the



B

	LE	Spacer	RE
*lox*P	ATAACTTCGTATA	ATGTATGC	TATACGAAGTTAT
lox71	**TACCG**TTCGTATA	ATGTATGC	TATACGAAGTTAT
lox66	ATAACTTCGTATA	ATGTATGC	TATACGAA**CGGTA**
lox72	**TACCG**TTCGTATA	ATGTATGC	TATACGAA**CGGTA**

FIG. 1. (A) Schematic diagram of the Cre-*loxP** system for gene deletion with *S. mutans*. The wild-type (WT) gene targeted for inactivation is cloned into a vector and interrupted by the insertion of a *lox66*-Ab-*lox71* cassette. (i) The construct is transformed into *S. mutans*, and the successful allelic exchange event (IN, integrated product) is selected using appropriate antibiotic-containing plates. (ii) Removal of the *lox66*-Ab-*lox71* cassette from the chromosome is achieved by Cre-mediated excision (EP, excised product) after the strain is transformed with pCrePA and grown at permissive temperature (30°C). (iii) Elevation of the growth temperature to 37°C results in the loss of the temperature-sensitive (Ts) pCrePA plasmid, resulting in a mutant strain carrying a deletion in the target gene without a selectable marker. (B) Schematic representation of the *loxP** sites. The *loxP* site consists of two 13-bp inverted repeats surrounding an 8-bp asymmetric core sequence (spacer). *lox71* and *lox66* have 5 bp mutated in the left and right 13-bp repeats, respectively. Mutated sequences are in boldface letters and underlined. Cre-mediated recombination between *lox66* and *lox71* results in the formation of *lox72*, which has mutations in both 13-bp repeat sequences.

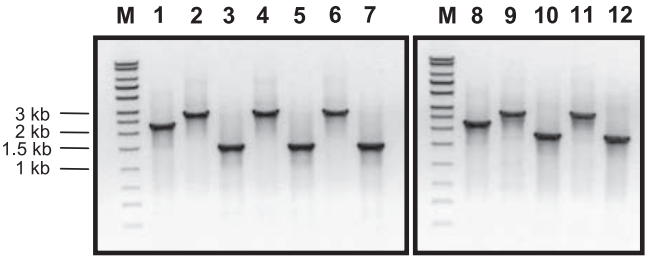


FIG. 2. PCR analysis of the modified *S. mutans* strains. The *htrA* gene amplified with the Smu-HtrA-F2/Smu-HtrA-R2 primers from UA159 (lane 1), strain IBS501 (lane 2), strain IBS502 (lane 3), strain IBS508 (lane 4), strain IBS511 (lane 5), strain IBS510 (lane 6), and strain IBS511 (lane 7). The *clpP* gene amplified with the Smu-ClpP-F2/Smu-ClpP-R2 primers from UA159 (lane 8), strain IBS509 (lane 9), strain IBS512 (lane 10), strain IBS510 (lane 11), and strain IBS513 (lane 12).

cells of pCrePA (37). These results clearly demonstrate that the Cre-*loxP* system can be efficiently employed for gene deletions in *S. mutans*; the entire protocol takes less than a week to obtain a particular mutant.

Single and multiple deletions using the Cre-*loxP* mutant. As described above, the use of wild-type *loxP* sites is not ideal for multiple gene deletions due to potential Cre-mediated recombination between two *loxP* sites from different *loxP*-Ab^r-*loxP* cassettes. This limitation can be circumvented by employing *loxP** sites, as described by Araki et al. (5). Two mutant sites, *lox71* and *lox66*, were selected based on the following properties. The *loxP* site is composed of an 8-bp spacer flanked by 13-bp inverted repeats (19). In *lox71*, the left 13-bp repeat element has five mutated bases, whereas in *lox66*, the right 13-bp repeat element has five mutated bases. Recombination between the *lox71* and the *lox66* mutant creates the *loxP* double mutant site *lox72*, which contains mutations in both of the repeats (Fig. 1B) and therefore has a dramatically reduced affinity for Cre recombinase (4). To demonstrate the successful deletions of multiple genes by using the *loxP**-Ab^r cassettes, the *htrA* and *clpP* loci were selected for analysis; both of these genes were successfully inactivated in *S. mutans*, either individually or simultaneously. The recombination frequencies were found to be 1.7×10^{-3}, 0.4×10^{-3}, and 0.2×10^{-3} for *htrA*, *clpP*, and the simultaneous inactivation of both loci, respectively. In each case, single colonies were chosen and designated IBS508, IBS509, and IBS510, respectively (Table 2). To excise the *lox66*-Ab^r-*lox71* cassette from the mutant locus, the double-crossover mutants IBS508, IBS509, and IBS510 were transformed with pCrePA. The excision efficiencies of the Ab^r cassettes from the *htrA*, *clpP*, and simultaneously knocked-out loci were 31, 14, and 11% (500 colonies were tested), respectively. As described before, pCrePA was cured from these mutant strains after cultures were incubated at 37°C. Single-colony isolates, designated IBS511 (Δ*htrA*), IBS512 (Δ*clpP*), and IBS513 (Δ*htrA* Δ*clpP*), were selected. Deletion of the target loci was verified by PCR and by DNA sequencing, to confirm excision of the *loxP**-Ab^r cassettes (Fig. 2).

Successful generation of multiple gene deletions via Cre-*lox* recombination depends on the correct resolution events in the presence of the resident integrated *lox72* sites. Therefore, we attempted to delete *clpP* from a Δ*htrA* strain (IBS511), where

TABLE 2. *S. mutans* strains used in this study

Strain	Genotype	Source or reference
UA159	Wild type, serotype c	R. A. Burne, University of Florida
IBS501	UA159 *htrA*::*loxP*-Km-*loxP*	This study
IBS502	UA159 Δ*htrA*::*loxP*	This study
IBS508	UA159 *htrA*::*lox71*-Km-*lox66*	This study
IBS509	UA159 *clpP*::*lox71*-Sp-*lox66*	This study
IBS510	UA159 *htrA*::*lox71*-Km-*lox66*, *clpP*::*lox71*-Sp-*lox66*	This study
IBS511	UA159 Δ*htrA*::*lox72*	This study
IBS512	UA159 Δ*clpP*::*lox72*	This study
IBS513	UA159 Δ*htrA* Δ*clpP*::*lox72*	This study

the resident *lox72* site and the newly introduced *lox66*/*lox71* sites were well separated from each other; thus, the possibility of a recombination event between *lox72* and the *lox66*/*lox71* sites was remote. However, studies with *Lactobacillus plantarum* demonstrate that when *lox66* or *lox71* sites are in close proximity to a *lox72* site, Cre-mediated recombination between *lox66* and *lox71* sites occurs correctly in 99.4% of cases (24). Preferred Cre-mediated resolution between *lox66* and *lox71* sites occurs almost exclusively, whether the sites are distant or within close proximity to a *lox72* site, underscoring the selectivity of the Cre enzyme and the advantage of this system relative to methods that employ native *loxP* sites.

Stress-sensitive phenotype of the mutant strains. To verify the biological effects of the mutations using the Cre-*loxP** method, the phenotypes arising from the deletions of *htrA* and/or *clpP* were analyzed. The serine proteases HtrA and ClpP are involved in regulating the growth of *S. mutans* under various stress conditions (2, 11, 13, 28). The mutant strains were tested for their abilities to withstand superoxide stress generated by MV and thermal stress generated by puromycin (14, 48). We observed that IBS511 (Δ*htrA*) was highly sensitive to MV, while IBS512 (Δ*clpP*) was more sensitive to puromycin treatment (Fig. 3).

S. mutans rapidly adapts to an acidic environment by escalating a strong acid tolerance response (7, 17, 18). To determine the role of HtrA or ClpP in the acid tolerance response, mutant strains were grown in buffered THY broth at pHs 7.0 and 5.5. At pH 7.0, the Δ*clpP* and Δ*htrA* Δ*clpP* mutants grew more slowly than the wild type or the Δ*htrA* strain (Fig. 4A), but during incubation at pH 5.5, the growth rates of the Δ*htrA* and Δ*htrA* Δ*clpP* mutants were severely impaired (Fig. 4B). This suggests that HtrA and ClpP are essential for the survival of *S. mutans* under a variety of stress conditions, including high temperature, oxidative stress, and acid tolerance.

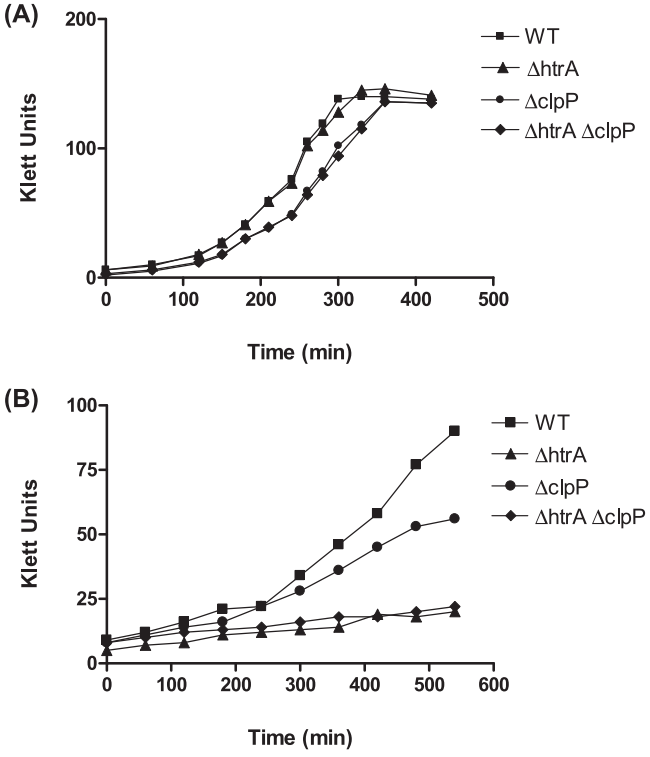


FIG. 4. Growth characteristics of the protease mutants. Growth of the wild-type and the *S. mutans* mutant strains, in buffered THY of pH 7.0 (A) and pH 5.5 (B), at 37°C were monitored with a Klett-Summerson colorimeter. The strains used for analysis include UA159 (■, WT), IBS511 (▲, Δ*htrA*), IBS512 (●, Δ*clpP*), and IBS513 (◆, Δ*htrA* Δ*clpP*). The experiments were repeated twice, and representative growth curves are shown.

DISCUSSION

Several markerless-gene-deletion mutagenesis methods have been developed for the construction of mutants of different bacterial species. In streptococci, markerless in-frame deletions have been constructed previously by using overlapping

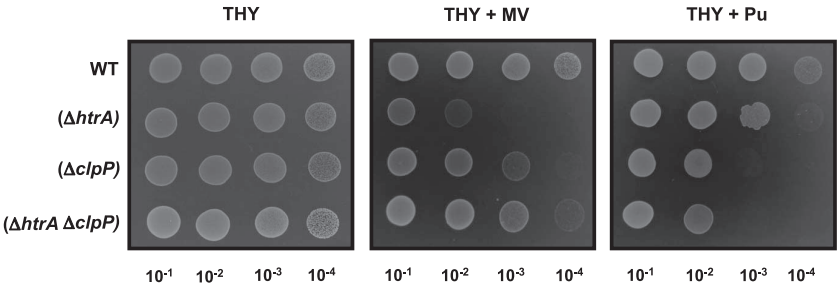


FIG. 3. Deletions of *htrA* and *clpP* affect the stress tolerance of *S. mutans.* Freshly grown overnight cultures of UA159 wild type (WT) and its derivatives were serially diluted with 0.85% NaCl. Portions of each dilution (7.5 μl) were spotted onto THY agar plates with no additions (control, THY), methyl viologen (10 mM, THY + MV), or puromycin (6 μg ml^{-1}, THY + Pu). The plates were incubated at 37°C under microaerophilic conditions. Experiments were repeated more than three times, and relevant areas of representative plates are shown.

extension PCR (21, 27) or by employing temperature-sensitive suicide vectors, such as pG+host (6, 10). In both approaches, mutants have been reported to occur at very low frequencies (1 to 2.5%). This is attributed to the low frequency of the recombination events that lead to excision. Moreover, there is an equal possibility for the formation of an in-frame deletion mutation or a reversion to the wild-type genotype (6, 10). Because of this, counter-selection strategies are integrated with these approaches to facilitate the screening process. One such method, which involves a galactokinase (*galK*)-based negative selection system, resulted in a markerless in-frame deletion of the *lacG* gene from *S. mutans* (31). In this case, 50% of the screened isolates contained the desired deletion; however, the plasmid excision occurred at a very low frequency (1/3,000). The main limitation of this method is the nature of the starting strain, which should be devoid of the galactose utilization operon; therefore, this method is very difficult to adapt to different *S. mutans* strains or to other bacteria. Two other methods, the cotransformation strategy with a thermosensitive plasmid (9) and the Janus cassette allele-exchange method (*rpsL*) (43), allow for markerless deletions and point mutations to be constructed. However, application of the *rpsL* method is limited to *S. pneumoniae*, which develops higher competency than *S. mutans*. In addition, a low rate of excision of the integrated plasmid would hinder the adaptation of this method for markerless gene deletion with *S. mutans* (6). With the cotransformation strategy, the efficiency of gene deletion was found to be approximately 10% (9), and therefore it is cumbersome to apply this method for multiple gene deletions.

To our knowledge, this is the first report of the Cre-*loxP* system being used for gene deletion analysis in a streptococcal species. The Cre-*loxP** system described here offers several advantages compared to the methods described above. The Cre-*loxP*-based method does not require any host cofactors or accessory proteins, exhibits high recombination efficiency, and is independent of the length of DNA located between the two *loxP* sites. The temperature-sensitive replicon in pCrePA is derived from the broad-host-range plasmid pWV01, which can replicate efficiently in streptococci and other gram-positive bacteria (30). It is a very powerful tool that can be used for functional genomics study. The efficiency of the process can be further improved if Cre is expressed from, or within, a cassette flanked by *loxP** sites, conferring antibiotic resistance. Recombination between the *loxP** sites in that case would lead to the removal of the antibiotic resistance marker and *cre*, such that the extra step of pCrePA removal could be avoided.

The efficiency of the Cre-*loxP** recombination system for multiple gene deletions was demonstrated by the deletion of *htrA* and *clpP*, which encode serine proteases essential to the virulence of *S. mutans*. Deletion of these genes resulted in the appearance of stress-dependent phenotypes when the mutant strains were exposed to stress induced by extremes of temperature and pH, as well as oxidative stress. In conclusion, Cre-*loxP** facilitates rapid genetic analysis of *S. mutans* in order to elucidate the molecular mechanisms of virulence in this pathogen and can be extended toward the study of other streptococci.

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