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A markerless protocol for genetic analysis of *Aggregatibacter actinomycetemcomitans*

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Abstract

Background/Purpose—The genomes of different *Aggregatibacter actinomycetemcomitans* strains contain many strain-specific genes and genomic islands (defined as DNA found in some but not all strains) of unknown functions. Genetic analysis for the functions of these islands will be constrained by the limited availability of genetic markers and vectors for *A. actinomycetemcomitans.* In this study we tested a novel genetic approach of gene deletion and restoration in a naturally competent *A. actinomycetemcomitans* strain D7S-1.

Methods—Specific genes' deletion mutants and mutants restored with the deleted genes were constructed by a markerless *loxP*/Cre system. In mutants with sequential deletion of multiple genes *loxP* with different spacer regions were used to avoid unwanted recombinations between *loxP* sites.

Results—Eight single-gene deletion mutants, four multiple-gene deletion mutants, and two mutants with restored genes were constructed. No unintended non-specific deletion mutants were generated by this protocol. The protocol did not negatively affect the growth and biofilm formation of *A. actinomycetemcomitans*.

Conclusion—The protocol described in this study is efficient and specific for genetic manipulation of *A. actinomycetemcomitans*, and will be amenable for functional analysis of multiple genes in *A. actinomycetemcomitans.*

Keywords

genomic islands; aggressive periodontitis; gene deletion; genetic analysis

Introduction

Gram-negative facultative *Aggregatibacter actinomycetemcomitans* is recognized as an etiology of periodontitis[1]. There are six serotypes of *A. actinomycetemcomitans* based on the

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structural distinction of O-antigen of lipopolysaccharide[2, 3]. Each serotype represents a distinct clonal lineage that shows little recombination with strains of other serotypes. Moreover, different serotypes or genotypes of *A. actinomycetemcomitans* may display distinct disease-association patterns[4–6]. However, little detailed information has been known of the underlying genomic variation among strains.

Recent studies from our laboratory have revealed remarkable genomic differences among *A. actinomycetemcomitans* strains[7, 8]. For example, 0.4–19.5% of the total protein-coding genes in each genome could differ between strains. Cumulatively among the 14 *A. actinomycetemcomitans* there are more than 1,200 accessory genes (ie, genes that are not shared by all strains), many of which reside in genomic islands and have no known functions. Approaches to assess the functions of these accessory genes need to be efficient, able to monitor multiple genes if necessary, and easily adaptable to assays in a variety of experimental conditions.

The genetic tools for *A. actinomycetemcomitans* are limited. The most common genetic markers used for *A. actinomycetemcomitans* are the resistance gene for spectinomycin, tetracycline, kanamycin or chloramphenicol[9–14]. In order to study the functions of multiple genes, more than one marker are required for deletion or complementation. This may pose some technical difficulties. This study was initiated to test a genetic protocol that is amenable for complex genetic analysis that involved multiple genes. Our future goal is to examine the functions of accessory genes (such as those carried on genomic islands) of *A. actinomycetemcomitans*. A markerless gene deletion protocol using *loxP* with different spacer regions was developed for single or sequential deletions of multiple DNA in *A. actinomycetemcomitans.* Both the accessory genes and core genes (ie, genes shared by all *A. actinomycetemcomitans* strains) were tested in deletion experiments. The results demonstrated that the protocol is highly efficient and specific in gene deletion and restoration. The protocol for genetic manipulation has not led to unintended deleterious effects to the growth and biofilm formation of *A. actinomycetemcomitans.*

Materials and Methods

Bacterial strains, plasmids and growth conditions

The bacteria strains and plasmids used in this study are listed in Table 1 and Table 2. Bacteria were grown either in a solid Trypticase Soy Broth agar (sTSB agar) containing 3% trypticase soy broth, 0.3% yeast extract, 5% horse serum and 1.5% agar, or Modified Trypticase Soy Broth (mTSB) composed of 3% trypticase soy broth and 0.6% yeast extract at 37°C in air supplemented with 5% CO_2. The pBluescript II KS plasmids (Stratagene, La Jolla, CA) were replicated in *Escherichia coli* host strain DH5α by standard methods [15]. For selection of transformants or mutants, spectinomycin (Spe, 50μg/ml), tetracycline (Tc, 4μg/ml) or ampicillin (Amp, 100μg/ml) were added to the media.

DNA manipulations

A. actinomycetemcomitans genomic DNA was prepared by phenol-chloroform method or GenElute Bacterial genomic Kit (Sigma, Saint Louis, MO). Plasmid DNA was isolated by

QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Transformation of *E coli* was carried out by electroporation using a MicroPulser® (BioRad, Hercules, CA). Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were purchased from New England Biolabs (Beverly, MA), and used as suggested by the manufacturer. The polymerase chain reactions (PCR) were performed as described previously[15] and the PCR products were purified with QIAquick PCR purification kit and GIAquick Gel Extraction kit (Qiagen, Valencia, CA). Table 3 lists the sequences of primers used for cloning, deletion, and mutation.

Construction of vectors containing the wildtype *loxP*-Spe-*loxP* cassette and its variants

Vectors cloned with a spectinomycin-resistance marker (Spe) flanked by two *loxP* sites or two of its variants were generated. As an example, for the construction of the Spe cassette with the wildtype *loxP* (*loxPW*) sites two partially complementary oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA):

5′

TCGACACCACGTGGATCCATAACTTCGTATA**ATGTATGC**TATACGAAGTTA TCTGCAGATAACTTCGTATA**ATGTATGC**TATACGAAGTTATGTCGACACGT GGTG3′, and

3′

GATCCACCACGTGTCGACATAACTTCGTATA**ATGTATGC**TATACGAAGTTA TCTGCAGATAACTTCGTATA**ATGTATGC**TATACGAAGTTATGGATCCACGT GGTG3′

(*loxP* sites were underlined, and bold letters indicated the spacer sequence of *loxP*. *Sal* I, *Dra* III, *Bam* HI, and *Pst* I were engineered in these two oligonucleotides). These two DNA fragments were annealed, and cloned into pBluescript II KS at the *Bam* HI and *Sal* I sites. A 1.1 kb Spe^r cassette released from *Pst* I-digested plox2-Spe plasmid[12] was inserted in the *Pst* I site between two *loxPW* sites to generate a plasmid bearing a *loxPW*-Spe-*loxPW* gene cassette. All recombinant plasmids were confirmed by sequencing the PCR products with T3 and T7 primers. With the same strategy plasmids with variants of the *loxP*-Spe-*loxP* cassette were generated (see Table 2 for variant *loxP* spacer sequences). The variant cassettes contained a pair of mutant *loxP* with one or two bases altered in the *loxP* spacer region based on their specificity in recombination[16]. This will allow successive deletions with the *loxP*/Cre system without interference from existing *loxP* sites of the genome.

Site-specific gene deletion with the *loxP* /Cre system

The strategy for gene deletion with the *loxP*/Cre system has been described previously[17]. Briefly, three steps were involved: (i) construction of the donor DNA with the *loxP*-Spe-*loxP* cassette flanked by homologous regions upstream and downstream of the gene to be deleted, (ii) allelic replacement of the target gene in *A. actinomycetemcomitans* by natural transformation, and (iii) removal of the *loxP*-Spe-*loxP* leaving a *loxP* scar at the deletion site (see Fig 1a for an example).

In this study, two approaches were used to construct the donor DNA. The first approach was by the *in vitro* ligation. Two pairs of primers were designed to amplify the upstream and

downstream homologous DNA fragments flanking the deletion target. A *Dra* III site was engineered in the proximal end of the two homologous DNA fragments (Table 3). Approximately 1600 ng of PCR amplicons of the upstream and downstream homologous DNA fragments isolated by QIAquick PCR purification kit (Qiagen, Valencia, CA), were mixed with approximately 800 ng of recombinant plasmid with a *loxP*-Spe-*loxP* cassette, which had *Dra* III sites in each end. The mixture was digested with *Dra* III at 37°C for an hour, then purified by the QIAquick PCR purification kit, and ligated at 16 °C overnight. After heat inactivation of the ligation reaction, the ligation mixture was used directly for transformation. The alternative method for donor DNA preparation involved cloning. Two pairs of primers were designed to generate the upstream and downstream homologous regions (>500 bp) flanking the deletion target by PCR, and the DNA fragments were cloned sequentially into the upstream and downstream of the *loxP*-Spe-*loxP* cassette in a recombinant plasmid constructed above. The DNA fragment containing the homologous regions and the *loxP*-Spe-*loxP* cassette was then released from the vector by restriction enzyme digestion, purified and served as donor DNA. Gene transfer by natural transformation was performed as described previously[18]. The transformants (with the target gene replaced by the *loxP*-Spe-*loxP* cassette) were verified by PCR and designated as "intermediate" gene deletion mutants. In the final step the spectinomycin-resistance marker was removed by introducing a vector containing *Cre* as described previously[17]. Mutants of *A. actinomycetemcomitans* strain D7S-1 with deletions of multiple genes were constructed step-wise with the *loxP*/Cre system as described above.

Restoration of the deleted genes

Essentially the reversal of the process of the deletion is applied for gene restoration (see Fig 1b for an example). Two fragments (one of which includes the deleted region) were constructed by PCR amplification. The two fragments were ligated to a *loxP*-Spe-*loxP* cassette *in vitro* and the DNA mixture was used directly for transformation. The transformants were identified from selective media and the resistance marker spliced out leaving a *loxP* scar in the genome. The gene-restored mutants were then confirmed by PCR analysis and sequencing as before.

Bacterial growth assays in broth

Bacterial suspensions were prepared by the indirect suspension method[19]. Briefly, bacteria were collected from agar plates with a sterilized plastic loop and spread as a thin layer slightly above the solution face on the test tube wall. The bacteria were dispersed into the mTSB broth by mixing with a Vortex, and the above steps were repeated several times. The optical density of each starter bacterial suspension was determined (OD of approximately 0.25–0.3) and then diluted 1:5 with fresh broth. Aliquots of 20 μl of the bacterial suspension were transferred to each well of a 100-well Bioscreen C plate preloaded with 180 μl of prewarmed fresh media. To prevent evaporation of the liquid culture, 15 μl of mineral oils were added in each well. The plate was incubated at 37°C for 40 hours with continuous shaking, and the absorbance at 420–580 nm visible light was recorded every 30 minutes. Wells containing media served as background controls. The data from duplicate wells for each strain were averaged to represent one data point. The growth of each strain was independently tested at least three times on different dates.

To calculate the doubling time, the exponential phase of the growth was first defined as the time period between two measurements of the optical density (OD): the minimum OD plus 0.1 and the maximum OD minus 0.1. After subtraction of the background OD (OD of the media only; without bacteria), the OD was transformed into $\log_2$ (optical density). A linear regression of the data points was performed and accepted if the R^2 was > 0.95, otherwise the experiments were repeated. The reciprocal of the slope gives the doubling time directly. The comparison of doubling time and maximum OD among strains was performed with SPSS 15.0 by using the Student *t* test or the analysis of variance (ANOVA).

Biofilm formation assay

Aliquots of 20 μl of bacterial suspension of *A. actinomycetemcomitans* were prepared as described above and transferred into wells preloaded with 180 μl of prewarmed media. The plate was incubated at 37°C for 48 hours in air supplemented with 5% CO_2. After incubation, the media was gently removed and the wells were washed twice with 200 μl PBS, then air dried. A solution of 0.1% crystal violet was used to stain the dried wells for 10 minutes. Subsequently, the staining solution was removed, and the wells were washed twice with 200 μl PBS, then air-dried again. The stained biofilms were destained with 100% (v/v) ethanol for 5 minutes. The absorbance of the dye was quantified at 540 nm visible light using a microplate reader. Strains were tested in triplicate for each condition tested.

Results

Construction of mutants

Eight single-deletion mutants were constructed (Table 1). Each set included an "intermediate" mutant with spectinomycin resistance marker and a final markerless mutant. The deletion targets were selected to include well-studied core genes that were unlikely to affect growth and biofilm formation (*ltxA* and *cdtB*), four small accessory genes of unknown functions and two genomic islands of unknown functions. Four sets of mutants with deletion of multiple genes were constructed (Table 1). They were made to test the protocol for sequential deletions by the Cre/*loxP* system. The step-wise process of a quadruple gene-deletion mutant strain D7SΔ*ltxA*Δ*cdtB*Δ16Δ285 is given here as an example. The construction began from the single gene deletion mutant D7SΔ*ltxA* with a *loxPW* scar. This mutant was transformed with the genomic DNA from the D7SΔ*cdtB*::Spe (with *loxP6* sites flanking the Spe^r marker) mutant. The transformants were identified and the resistance marker subsequently removed, leaving a *loxP6* scar in the genome. The same steps were repeated to generate a triple knockout mutant D7SΔ*ltxA*Δ*cdtB*Δ16 and the quadruple knockout mutant D7SΔ*ltxA*Δ*cdtB* 16Δ285 (leaving a *loxPW*, a *loxP6*, a *loxP5* and a *loxP1*scars in the genome).

Growth and biofilm formation

Table 4 provides a summary of the results of the growth assays. Most of the mutants did not show altered growth. However, it was noted that a mutant with a deletion of the 285-island appeared to grow faster than the wildtype. The restoration of the island in the deletion mutant reduced the growth rate to the level of the wildtype D7S-1. The genetic map of the 285-island and its deletion and restoration are illustrated in Figure 1a and 1b. The island is

6,660 bp, and has 6 predicted ORFs. The deleted region and the restored region are also marked. The difference in the wildtype and the mutant restored of the 285-island was the *loxP4* scar within the downstream hypothetical protein HP2. The insertion of a *loxP* within HP2 is not expected to change the phenotype.

Biofilms formation was evaluated for the wildtype D7S-1 and mutants. There were no significant differences in the biofilm formation among strains. Instead, the primary determinant for biofilm formation was the fimbriation status of the test strains (data not shown).

Discussion

The long-term goal of this study was to identify a suitable genetic approach to examine the functions of strain-specific genes of *A. actinomycetemcomitans.* Several genetic protocols have been developed for mutagenesis and gene complementation of *A. actinomycetemcomitans*, including allelic exchange and homologous integration[20–22], insertional mutagenesis[23, 24] and transposon-mediated mutagenesis[14, 25, 26]. These methods involve a cloning step, which could prolong the experiments or present an obstacle if the cloned products are toxic to the *E. coli* host. Our approach for genetic manipulation could prepare the recombinant DNA by PCR amplification and *in vitro* ligation, and use it directly as donor DNA for competence-mediated gene transfer. The transformation frequencies of were approximately 10^{-4} to 10^{-5} for naturally competent *A. actinomycetemcomitans*[12]. A typical experiment generated approximately 100 to 1000 transformants with 2×10^{7} competent bacteria. A gene deletion mutant could be generated in less than a week with minimal work.

In this study the Cre/*loxP* recombination system was employed to delete the antibiotic resistance marker. This approach was taken primarily for the ease of construction of mutants with deletion of multiple genes, and to avoid the possible effects of antibiotic resistance markers to the phenotypes of the bacteria. The Cre recombinase catalyzes cofactor independent recombination between two *loxP* sites, which consist of two 13 bp inverted repeats separated by an asymmetric 8 bp spacer region[27]. The Cre/*loxP* recombination system shows high recombination efficiency and has been widely used in eukaryotes. For prokaryotic organisms, Cre/*loxP* has been involved in genetic works for *Escherichia coli*[28, 29], *Lactobacillus plantarum*[30], and other species of Gram-negative bacteria[31–33], but limited in use for *A. actinomycetemcomitans*[17, 34].

A novel feature of our approach is the use of different *loxP* for construction of mutants with deletion of multiple genes. Consecutive deletion of genes using the same Cre/*loxP* may lead to unexpected deletions or inversions via recombination between the existing *loxP* of the genome and the new *loxP* introduced by the donor recombinant DNA, especially if the *loxP* sites were close to each other[33]. Therefore, we developed several *loxP* sites each with a variant spacer based on the previous study that showed incompatibility of certain *loxP* spacers in recombination[16]. We successfully generated deletion mutants of multiple genes, and did not notice any unexpected problem.

In this study we restored the deleted genes into the same locus as in the wildtype bacteria. By this process only a *loxP* scar was introduced into the genome in the gene-restored mutants. The *loxP* scar can be placed in a region that does not affect the phenotypes of the bacteria. Therefore, the restored mutants have exactly the same genetic background, and we avoid the problems with other complementation analysis that may affect gene expression due to variable copy numbers of the plasmid, different gene regulation mechanisms of the plasmid and the genome, or interference from the expression of an antibiotic resistance gene of the plasmid. Another advantage is the relative lack of size constraint for the restored genes. It is feasible and efficient to restore genes of 10–20 kb in one experiment.

The growth effect due to the mutation of 285-island was unexpected. The apparently functional ORFs in the 285-island are *secA*, *acrA*, a *marR*-family transcriptional regulator and a small hypothetical protein. In *E. coli*, SecA protein is a major component of the cellular mechanism that mediates the translocation of proteins across the plasma membrane[35], and AcrA and AcrB are related to the multidrug efflux pump, known as two major components of the tripartite efflux system[36, 37]. The MarR (multiple antibiotic resistance regulators) family of prokaryotic transcriptional regulators includes proteins critical for control of virulence factor production, bacterial response to antibiotic and oxidative stresses, and catabolism of environmental aromatic compounds[38, 39]. Therefore, the function of the 285-island could be for regulation of antibiotic resistance. However, strain D7S-1 has another efflux pump homologue. The deletion of the 285-island may or may not affect its resistance to antibiotics. The E test was performed to examine the antibiotics resistance profiles of the 285-island deletion and complementation mutants, but no corresponding results were shown. More studies are needed to verify the possible modulation of growth by the 285-island and its mechanism of *A. actinomycetemcomitans*.

In conclusion, this study has demonstrated the feasibility of employing Cre/*loxP* recombination system in genetic manipulation of *A. actinomycetemcomitans*. The protocols are relatively easy and efficient in the construction of mutants with deletion of single or multiple genes. The approach could be used as a tool to identify functional strain-specific genes or genomic islands for further hypothesis testing.

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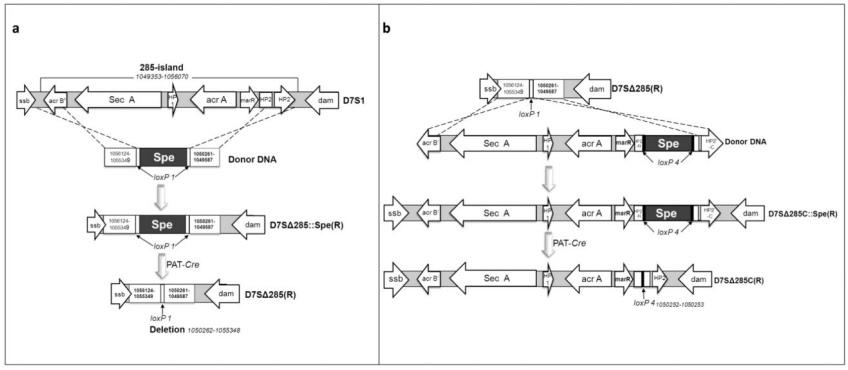

Figure 1. Deletion (a) and gene restoration (b) of the 285-island of *A. actinomycetemcomitans* strain D7S-1

(a) The upstream (1056124-1055349) and downstream (1050261-1049587) regions of the 285-island (1049353-1056070) were amplified and ligated to the *loxP1*-Spe-*loxP1* cassette *in vitro*. The 2.7 kb fragment was then used as donor DNA by natural transformation to generate an intermediate deletion mutant Δ285::Spe(R). Finally, the plasmid pAT/Cre was introduced and the recombination between two *loxP1* sites removed the Spe^r marker, resulting in the markerless deletion mutant Δ285(R). (b) The upstream (1055865-1050253) and downstream (1050252-1049645) regions of the 285-island were amplified and ligated to the *loxP4*-Spe-*loxP4* cassette *in vitro*. The 7.4 kb fragment was then used as donor DNA by natural transformation to generate an intermediate 285-island restored mutant Δ 285C::Spe(R). Recombination between two *loxP4* sites removed the Spe^r marker, leaving one copy of *loxP4* in the final markerless 285-island restored mutant Δ285C(R).

Table 1

Bacterial strains

Strains	Feature descriptions	Annotation of affected gene(s); deletion size in bp
D7S-1	Wildtype, fimbriated	N/A
D7SΔ*ltxA*::Spe	Deletion of *ltxA* replaced with a *loxPW*-Spe-*loxPW*	LtxA; 934
D7SΔ*ltxA*	Derivative of D7SΔ*ltxA*::Spe; Spe removed leaving a *loxPW*	
D7SΔ*cdtB*::Spe	Deletion of *cdtB* replaced with a *loxP6*-Spe-*loxP6*	CdtB; 289
D7SΔ*cdtB*	Derivative of D7SΔ*cdtB*::Spe; Spe removed leaving a *loxP6*	
D7SΔ*acrB*::Spe	Deletion of *acrB* replaced with a *loxP1*-Spe-*loxP1*	acriflavine resistance protein; 566
D7SΔ*acrB*	Derivative of D7SΔ*AcrB*::Spe; Spe removed leaving a *loxP*1	
D7SΔ*pulG*::Spe	Deletion of *pulG* replaced with a *loxP1*-Spe-*loxP1*	pseudopilin; 617
D7SΔ*pulG*	Derivative of D7SΔ*pulG*::Spe; Spe removed leaving a *loxP*1	
D7SΔ*p2579*::Spe	Deletion of gene "*p2579*" replaced with a *loxP3*-Spe-*loxP3*	PTS system; 989
D7SΔ*p2579*	Derivative of D7SΔ*p2579*::Spe; Spe removed leaving a *loxP*3	
D7SΔ*p2639*::Spe	Deletion of gene "*p2639*" replaced with a *loxP1*-Spe-*loxP1*	oligopeptide transport system permease protein (*p2639*); 662
D7SΔ*p2639*	Derivative of D7SΔ*p2639*::Spe; Spe removed leaving a *loxP*1	
D7SΔ16::Spe	Deletion of the 16-island replaced with a *loxP5*-Spe-*loxP5*	helicase, hypothetical protein, type III DNA modification methylase, protein of unknown function and hypothetical protein; 7,845
D7SΔ16	Derivative of D7SΔ16::Spe; Spe cassette removed leaving a *loxP*5	
D7SΔ285::Spe(R)	Deletion of the 285-island replaced with a *loxP1*-Spe-*loxP1*	integral membrane protein, MarR-family transcriptional regulator, AcrA protein, hypothetical protein, SecA-related protein and acriflavine resistance protein; 5,087
D7SΔ285(R)	Derivative of D7SΔ285::Spe(R); Spe cassette removed leaving a *loxP1*	
D7SΔ*ltxA*Δ*cdtB*::Spe	Derivative of D7SΔ*ltxA* with a deletion in *cdtB* replaced with a *loxP6*-Spe-*loxP6*	See above; 1,223
D7SΔ*ltxA*Δ*cdtB*	Derivative of D7SΔ*ltxA*Δ*cdtB*::Spe; Spe removed leaving a *loxP6* at *cdtB* and a prior *loxPW* at *ltxA*	
D7SΔ*ltxA*Δ*cdtB*Δ16::Spe	Derivative of D7SΔ*ltxA*Δ*cdtB* with a deletion in the 16-island replaced with a *loxP5*-Spe-*loxP5*	See above; 9,068
D7SΔ*ltxA*Δ*cdtB*Δ16	Derivative of D7SΔ*ltxA*Δ*cdtB*Δ16::Spe; Spe removed leaving a *loxP5*	
D7SΔ*ltxA*Δ*cdtB*Δ285::Spe	Derivative of D7SΔ*ltxA*Δ*cdtB* with a deletion in the 285-island replaced with *loxP1*-Spe-*loxP1*	See above; 6,310
D7SΔ*ltxA*Δ*cdtB*Δ285	Derivative of D7SΔ*ltxA*Δ*cdtB*Δ285::Spe; Spe removed leaving a *loxP1*	
D7SΔ*ltxA*Δ*cdtB*Δ16Δ285::Spe	Derivative of D7SΔ*ltxA*Δ*cdtB*Δ16 with a deletion within the 285-island replaced with *loxP1*-Spe-*loxP1*	See above; 14,155
D7SΔ*ltxA*Δ*cdtB*Δ16Δ285	Derivative of D7SΔ*ltxA*Δ*cdtB*Δ16Δ285::Spe; Spe cassette removed leaving a *loxP1*	
D7SΔ285C::Spe(R)	Derivative of D7SΔ285(R) restored with a *loxP4*-Spe-*loxP4* and the 285-island	N/A
D7SΔ285C(R)	Derivative of D7SΔ285C::Spe(R); Spe removed leaving a *loxP4*	
D7SΔ*ltxA*Δ*cdtB*Δ285C::Spe	Derivative of D7SΔ*ltxA*Δ*cdtB*Δ285 restored with a *loxP4*-Spe-*loxP4* and the 285-island	See above; 1,223
D7SΔ*ltxA*Δ*cdtB*Δ285C	Derivative of D7SΔ*ltxA*Δ*cdtB*Δ285C::Spe; Spe removed leaving a *loxP4*	

Table 2

Plasmids used in this work

Plasmid	Feature descriptions	Reference
pBluescript II KS	Amp^r; Cloning vector	Stratagene
pAT/Cre	Tc^r; shuttle plasmid of *E. coli* and *A. actinomycetemcomitans*; pPK1 derivative containing the *cre* gene	[17]
ploxw-Spe	Amp^r,Spe^r; pBluescript II KS derivative containing two wildtype *loxP* sites (the *loxPW* spacer sequence: ATGTATGC) flanking a *Spe*^{r} gene	This work
plox1-Spe	Amp^r,Spe^r; pBluescript II KS derivative containing two mutant *loxP1* sites (the *loxP1* spacer sequence: ATGcATGC*) flanking a *Spe*^{r} gene	This work
plox2-Spe	Amp^r,Spe^r; pBluescript II KS derivative containing two mutant *loxP2* sites (the *loxP2* spacer sequence: ATGgATGC*) flanking a *Spe*^{r} gene	This work
plox3-Spe	Amp^r,Spe^r; pBluescript II KS derivative containing two mutant *loxP3* sites (the *loxP3* spacer sequence: ATGTATaC*) flanking a *Spe*^{r} gene	This work
plox4-Spe	Amp^r,Spe^r; pBluescript II KS derivative containing two mutant *loxP4* sites (the *loxP4* spacer sequence: AaGTATcC*) flanking a *Spe*^{r} gene	This work
plox5-Spe	Amp^r,Spe^r; pBluescript II KS derivative containing two mutant *loxP5* sites (the *loxP5* spacer sequence: ATGTgTaC*) flanking a *Spe*^{r} gene	This work
plox6-Spe	Amp^r,Spe^r; pBluescript II KS derivative containing two mutant *loxP6* sites (the *loxP6* spacer sequence: AgaTcTGC*) flanking a *Spe*^{r} gene	[17]

*the lower case letter(s) indaicated the altered base(s) located in the variant *loxP* spacer sequences

Table 3

List of Primers

Primers	Sequences(5′→3′)[a]	Location and usage[b]
ltxA-UpF-Apa	ATAGGGCCCGGCATTAACTAATG	Upstream of *ltxA* (600424-600411); for deletion of *ltxA*
ltxA-UpR-Xho	TATCTCGAGTATTCCTCAAGCATTC	Upstream of *ltxA* (599669-599684); for deletion of *ltxA*
ltxA-DwF-Spe	TATACTAGTAAAGGTCGCACCGGT	Downstream of *ltxA* (598753-598738); for deletion of *ltxA*
ltxA-DwR-Sac	TACGAGCTCTTGAGGTGAAATTGT	Downstream of *ltxA* (597736-597750); for deletion of *ltxA*
ltxA-IntF	TGAACATATCGCGAATCAGC	Inside of *ltxA* (599431-599412); for confirmation of *ltxA* deletion
ltxA-IntR	ACGTGTAACGGCATGTTGAA	Inside of *ltxA* (598876-598895); for confirmation of *ltxA* deletion
cdtB-UpF-Apa	ATAGGGCCCATTGATACGCCAACGAA	Upstream of *cdtB* (2171088-2171072); for deletion of *cdtB*
cdtB-UpR-Xho	CACCTCGAGAGCAAGCACGTGAA	Upstream of *cdtB* (2170488-2170501); for deletion of *cdtB*
cdtB-DwF-Spe	TACACTAGTACCAATGCGGATACCTA	Downstream of *cdtB* (2170192-2170176); for deletion of *cdtB*
cdtB-DwR-Sac	AATGAGCTCTCTTCATCCAAGAATGG	Downstream of *cdtB* (2169234-2169250); for deletion of *cdtB*
cdtB-IntF	CGTGGTAAATGTGCGTCATG	Inside of *cdtB* (2170482-2170463); for confirmation of *cdtB* deletion
cdtB-IntR	TTACAGTGCATGCTTTGGCC	Inside of *cdtB* (2170206-2170225); for confirmation of *cdtB* deletion
16-UpF	GGCGTCTGCTCGGTGATAA	Upstream of the 16-island (1997280-1997262); for deletion of the 16-island
16-UpR-Dra	TGCACGTGGTGTTGTATGACGTAGA	Upstream of the 16-island (1996774-1996787); for deletion of the 16-island
16-DwF-Dra	ATCACGTGGTGTTCCAACACGGC	Downstream of the 16-island (1988918-1988907); for deletion of the 16-island
16-DwR	CCAATTCGGTGCGGACATTCC	Downstream of the 16-island (1988335-1988355); for deletion of the 16-island
16-IntF	CGGCGGTCAGAAGATTGGG	Inside of the 16-island (1991452-1991434); for confirmation of the 16-island deletion
16-IntR	AGTCCTGCTCACCGCCACG	Inside of the 16-island (1990908-1990926); for confirmation of the 16-island deletion
285-UpF	TAAACCTACCGCCGAAGCG	Upstream of the 285-island (1056124-1056106); for deletion of the 285-island;
285-UpR-Dra	CTCACGTGGTGATGGTGTTGTTA	Upstream of the 285-island (1055356-1055367); for deletion of the 285-island
285-DwF-Dra	TACACGTGGTGCTGATTATTTGGGT	Downstream of the 285-island (1050256-1050243); for deletion of the 285-island
285-DwR	CTGCCTTATTCCACTTCCACCC	Downstream of the 285-island (1049587-1049608); for deletion of the 285-island
285-IntF	GCGCTGCAACATCATAAAGC	Inside of the 285-island (1052447-1052428); for confirmation of the 285-island deletion
285-IntR	CCAGCCACAGCCATAATCAA	Inside of the 285-island (1051915-1051934); for confirmation of the 285-island deletion
285s-UpF	TGGAATTCTATCCTCCGGTGTTACTGA	Upstream of the 285-island (1055865-1055839); for complementation of the 285-island
285s-UpR II	TTCACGTGGTGTCAGCACCATTAAGACG	Upstream of the 285-island (1050253-1050269); for complementation of the 285-island

Primers	Sequences(5′→3′)[a]	Location and usage[b]
285s-DwF II	TACACGTGGTGTTATTTGGGTGTGGGGT	Downstream of the 285-island (1050252-1050236); for complementation of the 285-island
285s-DwR II	CCAATCTTGGTGCGTCATCAAGGCTAAT	Downstream of the 285-island (1049645-1049672); for complementation of the 285-island
acrB-UpF	TCTCGCAGAACGGGTTTATCAAACAGTAT	Upstream of *acrB* (1054510-1054538); for deletion of *acrB*
acrB-UpR	ATCACGTGGTGTTCGCCTAAGGATATTCA	Upstream of *acrB* (1055313-1055296); for deletion of *acrB*
acrB-DwF	CGCACGTGGTGTAATCTTTTTGAAAAAATACC	Downstream of *acrB* (1055878-1055898); for deletion of *acrB*
acrB-DwR	GATAAGTTTGTCGCGTGGAATGGTTAAGT	Downstream of *acrB* (1056805-1056777); for deletion of *acrB*
acrB-IntF	GCGTGGTGATGGTGTTGTTA	Inside of *acrB* (1055348-1055367); for confirmation of *acrB* deletion
acrB-IntR	ATCCTCCGGTGTTACTGACG	Inside of *acrB* (1055856-1055837); for confirmation of *acrB* deletion
p2579-UpF	GGCGTTTTTACAACCTGCAGAAGCCTTGAAA	Upstream of gene encoding protein with p-*02579* (429323-429353) ; for deletion of p-cluster *02579*
p2579-UpR	TTCACGTGGTGTAGACGGCGTTTCTTTCTAC	Upstream of gene encoding protein with p-*02579* (429918-429937); for deletion of p-cluster *02579*
p2579-DwF	ATCACGTGGTGAAATTCTCTCCTCTTCACGA	Downstream of gene encoding protein with p-*02579* (430925-430944); for deletion of p-cluster *02579*
p2579-DwR	CGTTAAGCGGATAAATTCCGGCCATGATGTT	Downstream of gene encoding protein with p-*02579* (431650-431620); for deletion of p-cluster *02579*
p2579-IntF	GGCAATTTGGCACTTTTGTT	Inside of gene encoding protein with p-*02579* (430732-430713); for confirmation of p-cluster *02579* deletion
p2579-IntR	GAAACGCGTACCTTCGGTAA	Inside of gene encoding protein with p-*02579* (430142-430161); for confirmation of p-cluster *02579* deletion
p2639-UpF	ATCGTGCAGGAGATTTGGACATCACCAG	Upstream of gene encoding protein with p-*02639* (651694-651721); for deletion of p-cluster *02639*
p2639-UpR	ATCACGTGGTGGAAATTGTCCCCAACGTA	Upstream of gene encoding protein with p-*02639* (652650-652633); for deletion of p-cluster *02639*
p2639-DwF	AACACGTGGTGTAAAGCACGCGCTGCGTC	Downstream of gene encoding protein with p-*02639* (653311-653328); for deletion of p-cluster *02639*
p2639-DwR	CATCACCATGTCGATTTTGCCGCCGAAAT	Downstream of gene encoding protein with p-*02639* (654021-653993); for deletion of p-cluster *02639*
p2639-IntF	GAAGTGATGGCGAACATTGA	Inside of gene encoding protein with p-*02639* (652780-652799); for confirmation of p-cluster *02639* deletion
P2639-IntR	CCTTGGCGGTACGAATAAAA	Inside of gene encoding protein with p-*02639* (653280-653261); for confirmation of p-cluster *02639* deletion
pulG-UpF	GCGCTGAGCAACAATAACAAACTCATCGT	Upstream of *pulG* (611176-611204); for deletion of *pulG*
pulG-UpR	ATCACGTGGTGTAGAGCGTTTTTCCACGT	Upstream of *pulG* (611960-611943); for deletion of *pulG*
pulG-DwF	ATCACGTGGTGTTGATGGGTATTTTTGGG	Downstream of *pulG* (612576-612593); for deletion of *pulG*
pulG-DwR	GGTGGCTATTCCGTTCAAGATTTACAACA	Downstream of *pulG* (613537-613509); for deletion of *pulG*
pulG-IntF	TGCCGTTCGTTTATTTTTCC	Inside of *pulG* (612543-612524); for confirmation of *pulG* deletion
pulG-IntR	TGACGCAAGCACTCAAAGAT	Inside of *pulG* (611972-611991); for confirmation of *pulG* deletion
Spe-UpF	GCAGGTCGATTTTCGTTC	Inside of Spe^r gene; for identification of Spe^r intermediate deletion mutants
Spe-DwR	GCCACTGCATTTCCCGCATA	Inside of Spe^r gene; for identification of Spe^r intermediate deletion mutants

[a] Underlined sequences are the restriction sites

[b] The nucleotide coordinates of the primers are based on strain D7S-1

Table 4

Doubling times of *A. actinomycetemcomitans* wildtype and mutants

Fimbriated wildtype and derived mutants		
Strain	**Doubling Time**	**P value[a]**
D7S-1	4.86 ± 0.09	-
D7SΔ*ltxA*	4.79 ± 1.71	1.000
D7SΔ*cdtB*	5.53 ± 0.55	0.840
D7SΔ*acrB*	5.38 ± 0.21	0.357
D7SΔ*pulG*	4.94 ± 0.27	1.000
D7SΔ*p2579*	5.82 ± 0.30	0.233
D7SΔ*p2639*	5.42 ± 0.31	0.576
D7SΔ16	5.47 ± 0.62	0.230
D7SΔ285(R)	2.86 ± 0.24	0.004
D7SΔ*ltxA*Δ*cdtB*	5.65 ± 0.15	0.054
D7SΔ*ltxA*Δ*cdtB* Δ16	3.74 ± 0.28	0.135
D7SΔ*ltxA*Δ*cdtB* Δ285	2.17 ± 0.15	0.001
D7SΔ*ltxA*Δ*cdtB* Δ16Δ285	2.09 ± 0.24	0.011
Gene-restored mutants		
Strain	Doubling Time	P value[b]
D7SΔ285C(R)	4.86 ± 0.53	0.678*
D7SΔ*ltxA*Δ*cdtB* Δ285C	6.58 ± 0.57	0.239**

The data are the averages of three independent experiments and standard deviations are shown. Significant levels are determined by [a] One-way ANOVA or [b]the Student *t* test.

* compared to D7S-1,

** compared to D7SΔ*ltxA*Δ*cdtB*.