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ORIGINAL ARTICLE

A markerless protocol for genetic analysis of *Aggregatibacter actinomycetemcomitans*

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Background/Purpose: The genomes of different *Aggregatibacter actinomycetemcomitans* (*A 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*.

Conflicts of interest: The authors have no conflicts of interest relevant to this article.

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Introduction

Gram-negative facultative *Aggregatibacter actinomycetemcomitans* (*A actinomycetemcomitans*) is recognized as an etiology of periodontitis.[1] There are six serotypes of *A actinomycetemcomitans* based on the 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 >1200 accessory genes (i.e., 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 is required for deletion or complementation. This may pose some technical difficulties. This study was initiated to test a genetic protocol, which is amenable for complex genetic analysis, which 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 (i.e., 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 Tables 1 and 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, USA) were replicated in *Escherichia coli* host strain DH5α by standard methods.[15] For selection of transformants or mutants, spectinomycin (Spe, 50 μg/mL), tetracycline (Tc, 4 μg/mL) or ampicillin (Amp, 100 μg/mL) were added to the media.

DNA manipulations

A actinomycetemcomitans genomic DNA was prepared by the phenol-chloroform method or GenElute Bacterial genomic Kit (Sigma, Saint Louis, MO, USA). Plasmid DNA was isolated by QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA). Transformation of *E coli* was carried out by electroporation using a MicroPulser (BioRad, Hercules, CA, USA). Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were purchased from New England BioLabs (New England BioLabs Inc., Ipswich, MA, USA), 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 a QIAquick PCR purification kit and a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Table 3 lists the sequences of primers used for cloning, deletion, and mutation.

Construction of vectors containing the wildtype loxP-Spe^r-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^r cassette with the wildtype *loxP* (*loxPW*) sites, two partially complementary oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA): 5′-TCGACACCACGTGGATCCATAACTTCGTATAATGTATGCTATACGAAGTTATCTGCAGATAACTTCGTATAATGTATGCTATACGAAGTTATGTCGACACGTGGTG-3′, and 3′-GATCCACCACGTGTCGACATAACTTCGTATAATGTATGCTATACGAAGTTATCTGCAGATAACTTCGTATAATGTATGCTATACGAAGTTATGGATCCACGTGGTG-3′ (*loxP* sites are underlined, and bold letters indicate 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^r-*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^r-*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: (1) construction of the donor DNA with the