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Development of a Modified Gentamicin Resistance Cassette for Genetic Manipulation of the Oral Spirochete *Treponema denticola*

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Herein, we report that a modified gentamicin cassette and a PCR-based method can be used for targeted mutagenesis of the oral spirochete *Treponema denticola*. This approach minimizes polar effects and spontaneous antibiotic resistance. Therefore, it can serve as a reliable tool for genetic manipulation of *T. denticola*.

The oral spirochete *Treponema denticola* is a member of the "red complex" bacteria. It is an important pathogen that is associated with human periodontal disease (7, 14), a chronic infection that occurs in 80% of the adult population worldwide (5, 22, 26). However, due to the paucity of genetic tools and its fastidious growth requirements, the biology and pathogenicity of *T. denticola* are poorly understood (7, 10, 15). Although significant progress has been achieved during the last 2 decades, tools for genetic manipulation of *T. denticola* are still very limited (2, 18, 19, 25, 31). The first tool applied to genetic studies in *T. denticola* is an erythromycin resistance cassette [*erm*(F)-*erm*(B)] developed by Li et al. (19). It has since become a standard method for genetic manipulations of *T. denticola*. However, the spirochete has a high spontaneous mutation rate to the presence of erythromycin, and the insertion of the *erm*(F)-*erm*(B) cassette in a targeted gene often has a polar effect on downstream genes (13, 20). These two drawbacks have substantially hampered the use of this cassette. Two modified *erm*(F)-*erm*(B) cassettes have been developed (13, 20), but the aforementioned problems still hold. Chloramphenicol and coumermycin antibiotic resistance cassettes have been used for *trans*-complementation of certain *T. denticola* mutants (4, 25). However, these two cassettes have not yet been successfully used for the targeted mutagenesis of *T. denticola*, as the resistance to chloramphenicol is not stable and the coumermycin resistance is not reliable due to the pleiotropic effects of gyrase mutation (24, 27).

Constructing a mutated gentamicin resistance cassette. A gentamicin cassette (*aacC1*) (6, 28–30) has been recently used as a selectable marker for transposon mutagenesis in the *T. denticola* ATCC 35405 (*Td*35405) strain (31). We reasoned that this cassette could also be used as a selectable marker for targeted mutagenesis in the *Td*35405 strain. Unexpectedly, all of the attempts to use it for targeted mutagenesis failed. Our recent work revealed that *Td*35405 carries genes encoding three type II DNA restriction endonucleases. One of these enzymes (TDE0911) recognizes and cleaves targeted DNAs containing the sequence GGNCC (2). Sequence analysis shows that the *aacC1* cassette contains a cleavage site recognized by TDE0911 (G_{97}GCCC). We hypothesized that the failure of the *aacC1* cassette in *Td*35405 could be due to the restriction cleavage mediated by TDE0911. To test this hypothesis, the cleavage site (G_{97}GCCC) within *aacC1* was mutated to A_{97}GCCC by site-directed mutagenesis. The wild-type and mutated (designated *aac*C_m) cassettes were treated with the crude cell extract of *Td*35405. As expected, the *aacC1* cassette was digested into two fragments; but the mutated cassette remained intact (Fig. 1), indicating that the mutation successfully protected *aac*C_m from cleavage.

Constructing a vector for deletion of the *prcA* gene. To test whether the *aac*C_m cassette is functional in *T. denticola*, we decided to choose the *prcA* gene for targeted mutagenesis. The *prcA* gene (*TDE0761*) encodes a lipoprotein that is a component of the outer membrane protease complex (dentilisin). The function of PrcA in the dentilisin complex has been extensively studied (11–13). In addition, *prcA* is located within the *prcB-prcA-prtP* operon. Previous studies report that the insertion of either *erm*(F)-*erm*(B) or *erm*(B) in *prcA* could decrease the expression level of the gene downstream, *prtP* (12, 13). Thus, targeting *prcA* could help us to determine whether the insertion of *aac*C_m cassette in a targeted gene has any polar effect on its downstream genes. A PCR-based method, which was recently used in the Lyme disease spirochete, *Borrelia burgdorferi* (21), was modified to construct a vector for the targeted mutagenesis of *prcA*. The scheme that is illustrated in Fig. 2 aims to replace the entire open reading frame of *prcA* (1,920 bp) in frame with the promoterless *aac*C_m cassette (the coding sequence from the start codon ATG to the TAA stop codon). The vector was constructed via three PCR amplification steps. In the first step, the upstream *prcB* gene (primers P_1/P_2), the downstream *prtP* gene (primers P_5/P_6), and the *aac*C_m cassette (primers P_3/P_4) were PCR amplified. In the second step, the *prcB* and *aac*C_m fragments were fused via PCR amplification with primers P_1/P_4. In the third step, the *prcB-aac*C_m and *prtP* fragments were linked via PCR amplification with primers P_1/P_6. The final fragment *prcB-aac*C_m-prtP[r] (2,132 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI). The final vector was named *PrcA*::*aacCm* and confirmed by DNA sequencing analysis (Roswell Park Cancer Institute DNA Sequencing Laboratory, Buffalo, NY). The primer sequences are listed in Table 1.

Isolation of gentamicin-resistant *prcA* mutants. The *PrcA*::*aacCm* plasmid was purified from an *Escherichia coli dam dcm* mutant strain (New England BioLabs, Ipswich, MA), as previously

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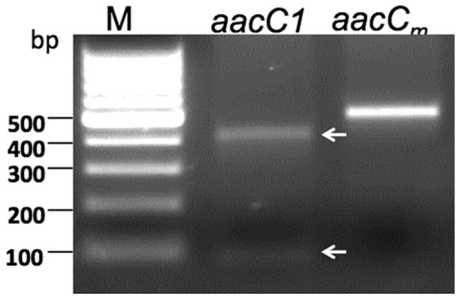

FIG 1 The mutated $aacC_m$ cassette is resistant to the cleavage of *T. denticola*. PCR amplified 534 bp of the wild-type *aacC1* cassette, and mutated $aacC_m$ fragments were treated with the crude cell extract of *Td*35405. The samples obtained were then analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. Lane M contains DNA markers. The two white arrows point toward the cleaved products.

TABLE 1 Oligonucleotide primers used in this study

Primer	Sequence[a]	Note	Direction[b]
P_1	CCGAATCTCGGCACCTGT	5′-Flanking region of *prcA*	F
P_2	GCGTAACATACTCGTCCTCCTATAAAT	5′-Flanking region of *prcA*	R
P_3	GGAGGACGAGTATGTTACGCAGCAGC AACG	$aacC_m$ cassette	F
P_4	GACCTCCTTAGGTGGCGGTACTTGGG	$aacC_m$ cassette	R
P_5	GCCACCTAAGGAGGTCTTTAAGATG AAG	3′-Flanking region of *prcA*	F
P_6	GATAGTGCCCGAACAGTG	3′-Flanking region of *prcA*	R
P_7	ATGTTACGCAGCAGCAACG	$aacC_m$	F
P_8	TTAGGTGGCGGTACTTGGG	$aacC_m$	R
P_9	GCTGCGGCGTTATGGTGT	Detected *prcA*	F
P_{10}	CCGTCGTCGAGGGGAAAG	Detected *prcA*	R
P_{11}	GCAGCGGGTACAAAGATAC	5′-Flanking region of *prcB*	F

[a] The underlined sequences are engineered overlapping sequences for PCR amplifications.
[b] F, forward; R, reverse.

described (2). The obtained unmethylated vector was linearized and transformed into *Td*35405 competent cells. The antibiotic-resistant clones were selected on TYGVS (1) semisolid agar plates containing gentamicin (20 μg/ml) (31). After a 14-day incubation, 18 antibiotic-resistant colonies appeared on the plates. Eight colonies were selected and analyzed by PCR for the presence of the $aacC_m$ cassette (primers P_7/P_8) and absence of the *prcA* gene (primers P_9/P_{10}). The results showed that all of the colonies examined contained the targeted mutation (data not shown). One clone (Δ*prcA* mutant clone) was further analyzed by PCR with different pairs of primers (Fig. 3a) and by Western blotting using a PrcA-specific antibody. The results showed that the entire *prcA* gene was replaced by $aacC_m$ as expected (Fig. 3b). Furthermore, the cognate gene product was completely abolished in the mutant (Fig. 3c), indicating that $aacC_m$ can be used for the targeted mutagenesis of *T. denticola*.

The insertion of $aacC_m$ in *prcA* has no polar effect on *prtP*. Insertion of an antibiotic resistance cassette in a targeted gene can interfere with expression of downstream genes. This effect has compromised the interpretation of results and needs to be ruled out via genetic complementation or by other studies. Given that there is no shuttle vector available for the *Td*35405 strain, an antibiotic resistance marker that avoids polar effects and a reliable method of targeting it to a desired locus would be very useful. In the Δ*prcA* mutant, the entire *prcA* gene was deleted and replaced in frame by the promoterless $aacC_m$ cassette. The $aacC_m$ cassette is transcribed from the promoter upstream of *prcA*. To test whether there are polar effects on the downstream gene, the level of PrtP in the Δ*prcA* mutant and two previously constructed *prcA* mutants (13, 16) was measured by Western blotting using a specific antibody against PrtP. A 72-kDa PrtP protein was detected in the wild type and the Δ*prcA* mutant (Fig. 4). However, PrtP was not detected in the PNE mutant [in which *prcA* was inactivated by an *erm*(F)-*erm*(B) cassette] (16) or the CF547 mutant [in which *prcA*

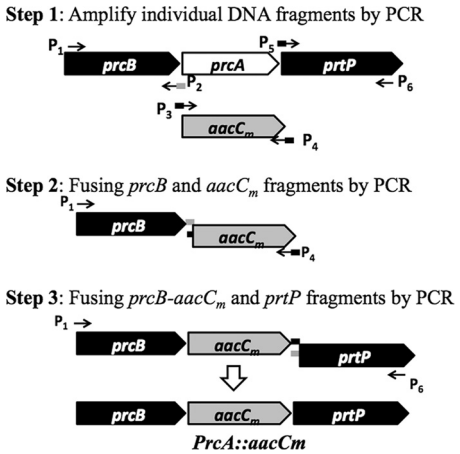

FIG 2 Schematic illustration of constructing the *PrcA::aacCm* vector for the deletion of *prcA* gene. The vector was constructed via three PCR amplification steps. Arrows indicate the relative positions of PCR primers for constructing the vector. The sequences of these primers are listed in Table 1.

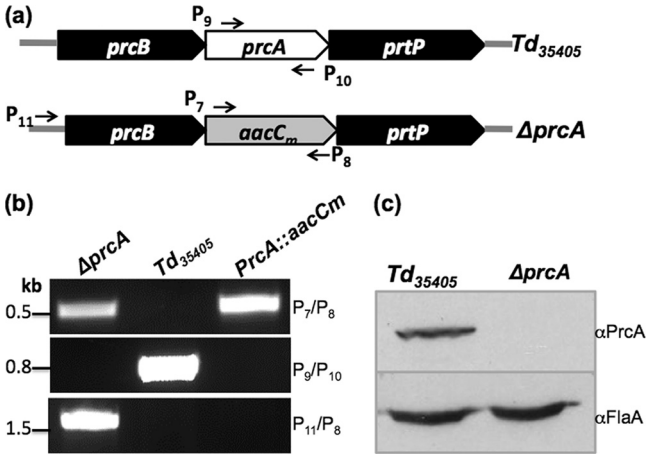

FIG 3 Isolation and characterization of Δ*prcA* mutant. (a) Illustration of the *prcA* locus in *Td*35405 (wild type) and the $aacC_m$ cassette in the Δ*prcA* mutant. (b) PCR analysis showing that the *prcA* gene was deleted and replaced by $aacC_m$ in the Δ*prcA* mutant. The primers used for PCR analysis are labeled in panel a. (c) Western blot analysis of the Δ*prcA* mutant. Similar amounts of whole-cell lysates from the wild type and the mutant were analyzed by SDS-PAGE and then probed with a specific PrcA antibody (αPrcA, anti-PrcA antibody). Immunoblots were developed using horseradish peroxidase secondary antibody with an enhanced chemiluminescence (ECL) luminol assay as previously described (2, 3).

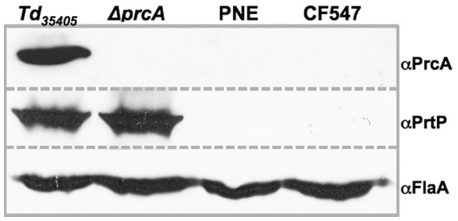

FIG 4 Western immunoblots of *Td*35405, Δ*prcA* mutant, and two previously constructed *prcA* mutants (PNE and CF547) (13, 16). The blots were probed with antibodies against *T. denticola* PrcA, PrtP, or FlaA. FlaA expression was used as a loading control (3, 13).

was disrupted by an *erm*(B) cassette] (13), two mutants that were constructed using different approaches. Taken together, these observations suggest that the approach reported here can avoid the potential polar effect in the targeted mutagenesis of *T. denticola* that has been observed previously.

***T. denticola* has a low spontaneous mutation rate to the presence of gentamicin.** Thus far, the *erm*(F)-*erm*(B) cassette and its *erm*(B) derivative have provided the only selectable marker for targeted mutagenesis of *T. denticola* (13, 19). However, *T. denticola* often develops spontaneous resistance to erythromycin. Thus, additional efforts are required to distinguish targeted mutant clones from spontaneous mutants. To determine whether a similar scenario exists with gentamicin, the spontaneous resistance to the antibiotic was measured. For this assay, *Td*35405 was continuously cultivated and passed in the liquid TYGVS medium for up to 28 generations. The cells (5×10^9) were collected every 4 generations, plated on semisolid TYGVS plates containing a MIC of erythromycin (40 μg/ml) (8) or gentamicin (20 μg/ml) (31), and incubated in an anaerobic chamber for 2 weeks. The frequencies of spontaneous resistance to the antibiotics were expressed as the number of colonies divided by the number of cells plated (17, 23). The development of gentamicin resistance in *T. denticola* is extremely rare (spontaneous mutation frequency of $<1.5 \times 10^{-10}$) and is substantially lower than that of erythromycin (approximate frequency of 5×10^{-8}) during the period of 28-generation cultivations, indicating that $aacC_m$ can serve as a reliable antibiotic selectable marker for constructing *T. denticola* mutants.

Summary. The main objective of this study was to develop an alternative and more reliable antibiotic marker for the genetic studies of *T. denticola*. Compared to the *erm*(F)-*erm*(B) cassette (2,145 bp) (9) and its derivative, *erm*(B) (741 bp) (13), the newly constructed $aacC_m$ cassette (534 bp) is much shorter, which will be more convenient for genetic manipulations. In addition, the spontaneously occurring gentamicin resistance in *T. denticola* is substantially less than that of erythromycin. Moreover, the application of $aacC_m$ in combination with the PCR-based method described herein can avoid potential polar effects in targeted mutagenesis. The $aacC_m$ cassette and the methodology developed in this report will facilitate genetic studies of *T. denticola* (e.g., providing an additional antibiotic marker for construction of double mutations, for complementation of a mutant, and for increasing the targeted mutagenesis efficiency in *T. denticola*).

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