Published in final edited form as:

J Microbiol Methods. 2010 October ; 83(1): 66–68. doi:10.1016/j.mimet.2010.07.020.

A simplified erythromycin resistance cassette for *Treponema denticola* mutagenesis

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Abstract

The primary selectable marker for genetic studies of *Treponema denticola* is a hybrid gene cassette containing both *ermF* and *ermAM* (*ermB*) genes. ErmB functions in *Escherichia coli*, while ErmF has been assumed to confer resistance in *T. denticola*. We demonstrate here that ErmB is sufficient for erythromycin selection in *T. denticola* and that the native *ermB* promoter drives ErmB expression.

Keywords

spirochete; selectable marker; mutagenesis

Progress in molecular analysis of the anaerobic oral spirochete *Treponema denticola* has been limited by factors including fastidious nutrient requirements, slow growth rate and poor plating efficiency. In addition, genetic analysis of *T. denticola* is limited by its extremely low transformation efficiency and strain-limited shuttle plasmid as well as by the small number of validated selectable markers. Erythromycin has been the most reliable and widely used selectable marker; chloramphenicol, gentamicin and coumermycin have also been reported. However, some *Treponema* strains are relatively resistant to chloramphenicol (and J.C. Fenno, data not shown), and coumermycin is generally regarded as unsuitable due to pleiotropic phenotypes of *gyrB* mutants.

The *ermF* gene isolated from the *Bacteroides* plasmid pBF4 is used widely as a selectable marker for mutagenesis studies in organisms in the *Cytophaga-Flavobacterium-Bacteroides* group. The *ermAM* gene (hereinafter designated *ermB* in agreement with current suggested taxonomy), was first isolated from enterococcal conjugative plasmid pAMβ1 and subsequently developed as part of a shuttle vector for other streptococci. The transmethylase encoded by *ermB* confers erythromycin resistance in a wide range of Gram-positive organisms and also confers high-level resistance in *E. coli*.

Fletcher *et al.* designed a hybrid *ermF-ermB* gene cassette for use as a selectable marker for *Porphyromonas gingivalis* allelic replacement mutagenesis. The cassette structure permits selection in both *E. coli* (*ermB*) and *Bacteroides*-family organisms (*ermF*), thus facilitating intermediate cloning steps. In addition to its use in numerous studies in *P. gingivalis*, it has

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become a standard selectable marker in *Treponema denticola*, *Fusobacterium nucleatum* and *Tannerella forsythia*. While spirochetes (including *T. denticola*) are classified as Gram-negative due to the presence of an outer membrane and thin peptidoglycan layer, they carry large numbers of genes having closest homologies with Gram-positive organisms, particularly genes encoding various membrane proteins, transcriptional regulators and membrane trafficking functions. We thus hypothesized that *ermB*, primarily characterized as active in Gram-positive organisms, might be sufficient for erythromycin resistance in *T. denticola*. This had the potential to substantially simplify construction of defined isogenic mutants in *T. denticola*.

To characterize activity of *ermB* in *T. denticola*, we constructed two isogenic mutant strains in the dentilisin protease locus: one each in *prcA* and *prcB*, the first two genes in the *prcB-prcA-prtP* operon (Figure 1). A 1142 bp BstZ17I-PmeI fragment of pVA2198 including the *ermB* coding region and putative promoter originally derived from pAM β 1 was cloned in plasmids carrying the *T. denticola* target gene sequences such that each end of the *ermB* cassette was flanked by at least 300 bp of target gene DNA. The resulting plasmids, based on plasmid vector pSTBlue-1 (Novagen, Inc., Madison, WI, USA), conferred both kanamycin resistance ($30 \mu\text{g ml}^{-1}$) and erythromycin ($300 \mu\text{g ml}^{-1}$) in the *E. coli* JM109 host. Plasmids carrying the mutant constructs were linearized to facilitate allelic replacement by homologous recombination. *T. denticola* ATCC 35405 (the Type strain, which has been passaged extensively in various laboratories) was electroporated with the resulting linear DNA s and plated in NOS-GN soft agar medium containing erythromycin ($40 \mu\text{g ml}^{-1}$, Sigma Chemical Co., St. Louis, MO) as described previously. Resulting erythromycin-resistant colonies growing under the agar surface were picked using Pasteur pipets, grown in NOS broth medium containing erythromycin ($40 \mu\text{g ml}^{-1}$) and screened for the expected mutations by PCR and DNA sequencing. The mutation constructed in *prcB* is identical to that in *prcB* mutant strain *T. denticola* P0760, except that P0760 carries the *ermF-ermB* cassette. The mutation constructed in *prcA* was constructed by replacing a 287 bp StuI fragment with *ermB* yielding a mutant essentially identical to *prcA* mutant strain *T. denticola* PNE, which carries the *ermF-ermB* cassette. *T. denticola* parent and mutant strains were tested for expression of PrcA and PrtP proteins, using FlaA expression as a positive control. Immunoblots of *T. denticola* cell lysates were probed with rabbit polyclonal antibodies raised against PrtP, PrcA or FlaA and immunoreactive bands were detected using horseradish peroxidase-conjugated goat anti-rabbit antibodies and a chemiluminescent detection as described previously. As shown in Figure 2, neither mutant produced PrcA or PrtP, while all strains expressed wildtype levels of FlaA. The *ermB* cassette is in opposite transcriptional orientation relative to the target gene in both mutant strains (Figure 1). These results indicate that *ermB* transcription is driven by a promoter sequence present on the *ermB* cassette, presumably the native promoter carried on pAM β 1.

We previously demonstrated that insertion of the *ermF-ermB* cassette results in interruption of transcription downstream of the insertion site in the target gene or operon. Furthermore, the *ermF-ermB* cassette contains a promoter that is active in *T. denticola*, since its function is not dependent on its orientation relative to the target gene. While we have not addressed transcription or activity of *ermF* in *T. denticola*, we demonstrate that the *ermB* locus from pAM β 1 contains a promoter that is functional in *T. denticola*. We recently reported that nonpolar deletion of *prcB* does not block expression of PrcA protein. The polar effects on expression downstream of *ermB* insertion in *prcB* or *prcA* (Figure 2) indicate that the *ermB* cassette contains a transcription termination signal.

To date, well over 40 *T. denticola* mutants have been constructed using the *ermF-ermB* cassette, most of which have been reported in the literature. It is our intention here to provide improved characterization of this extremely useful antibiotic resistance cassette so that its utility can be expanded. A single gene cassette known to contain its own functional promoter is generally

preferable to a cassette with two genes encoding the same activity driven by uncharacterized promoter(s). Furthermore, reducing the size of the cassette by fifty percent can be expected to facilitate the required preliminary cloning steps and may also increase transformation efficiency in this recalcitrant organism.

Acknowledgments

This work was supported by United States Public Health Service grants DE018221 (National Institute of Dental and Craniofacial Research Bethesda, MD) and AI079325 (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

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Figure 1.

Construction of *T. denticola* mutants using *ermF-ermB* and *ermB* cassettes. The protease operon (*prcB-prcA-prtP*) and adjacent gene TDE0759 in wildtype parent strain ATCC 35405 and isogenic strains mutated in *prcB* (P0760 and CF548) and *prcA* (PNE and CF547) showing locations of *ermF-ermB* and *ermB* insertions. The symbol *x-* indicates gene disruption in *prcA* or *prcB*.

Figure 2.

Western immunoblots of *T. denticola* parent and mutant strains, probed with antibodies raised against *T. denticola* PrcA, PrtP or FlaA. Strains are as in Figure 1. Lanes: 1, 35405 (parent); 2, P0760; 3, CF547; 4, CF548.