

Development of Shuttle Vectors for Spirochetes

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Abstract

Constructions of *Escherichia coli*-spirochete shuttle vectors are based on naturally occurring plasmids, broad host range plasmids or bacteriophages. This review primarily focuses on genetic tools for *Treponema denticola* which is associated with periodontal diseases. The *T. pallidum* FlaA protein, *E. coli* β -galactosidase, and the green fluorescent protein were successfully expressed in *T. denticola* from a shuttle vector system.

Introduction

Spirochetes have unique morphology and motility due to their periplasmic flagella, located between the outer membrane and the cytoplasmic membrane. It is an ancient bacterial phylum consistent with modern molecular typing based on 16S rRNA sequence comparisons (Woese, 1987). This phylum includes the main genera *Treponema*, *Borrelia*, *Brachyspira* (*Serpulina*) and *Leptospira* with saprophytic and pathogenic species. Spirochetes cause diseases such as syphilis, Lyme disease, swine dysentery, Leptospirosis, some of them known for centuries. No extensive genetic analysis has been reported for any spirochete (for a review see (Rosa *et al.*, 1999)). We shall present the strategies for constructing shuttle vectors with a focus on *Treponema denticola* (Chi *et al.*, 1999; Li and Kuramitsu, 1996).

T. denticola and Other Treponemes

T. denticola, a small cultivable spirochete, is a potential periodontal pathogen (Fenno and Mc Bride, 1998). However, its role in periodontal disease remains to be demonstrated. It is, moreover, a potential model for the study of other spirochetes which are still uncultivable, including oral spirochetes associated with periodontitis and *T. pallidum*, the causative agent of syphilis. Therefore, development of gene transfer systems in *T. denticola* should significantly enhance our knowledge of the pathogenesis of Treponemes.

Spirochetal Genomes With Several Replication Origins

Genomes of spirochetes vary in size, structure and base composition (30 to 50 % GC). While the genome of *Borrelia*

burgdorferi consists of a linear chromosome with several linear and circular plasmids, *Leptospira interrogans* contains two circular chromosomes, and *T. denticola*, a circular chromosome and endogenous plasmids (Caudry *et al.*, 1995; Fraser *et al.*, 1997; Ivic *et al.*, 1991; Mac Dougall and Saint Girons, 1995; Zuerner *et al.*, 1993). The complete nucleotide sequences of the genomes of two spirochetes (*B. burgdorferi* and *T. pallidum*) have been reported (Fraser *et al.*, 1997; Fraser *et al.*, 1998). The *T. denticola* genome is currently being sequenced by TIGR (<http://www.ncbi.nlm.nih.gov/htbinpost/Taxonomy/wgetorg?id=158&lvl=3>). Analysis of the sequence of pTD1, a small cryptic circular *T. denticola* plasmid revealed homology with plasmids from Gram-positive bacteria (Mac Dougall *et al.*, 1992). Such homology was also apparent for another *T. denticola* plasmid pTS1 (S. Chauhan and H. K. Kuramitsu, unpublished). The presence of bacteriophages has been documented for several spirochetes (for a review see Rosa *et al.*, 1999), but none for *T. denticola*.

Shuttle Vectors for *T. denticola*

An important goal for spirochete research was thus to construct shuttle vectors which will replicate within spirochetes. Strategies to find such vectors rely on broad host range plasmids, endogenous cryptic plasmids, or bacteriophages. Broad host range plasmids of the IncQ compatibility group, such as derivatives of RSF1010, have been used as shuttle vectors for Gram-negative as well as Gram-positive bacteria and for the spirochete *T. denticola* (Li and Kuramitsu, 1996). Their replication is independent of host replication functions, rendering them appropriate for trial in a phylogenetically remote host. Although the plasmid pKT210, derived from RSF1010, was initially introduced successfully into *T. denticola*, this strategy was later abandoned since this plasmid vector proved to be unstable in *T. denticola* (Li and Kuramitsu, 1996). However, these experiments provided the groundwork for further development of genetic tools. Another approach consisted of engineering the pTS1 cryptic plasmid as a shuttle vector (Chi *et al.*, 1999). The sequence of pTS1 (S. Chauhan and H. K. Kuramitsu, unpublished) revealed an open reading frame homologous to the *rep* gene of a cryptic plasmid from the Gram positive bacterium *Selenomonas ruminantium* subsp. *lactilytica* (Attwood and Kuramitsu, 1992). The 2.8 kb-long fragment from pTS1 which encodes the potential Rep protein was ligated to pKMOZ19, an *E. coli* plasmid vector. An erythromycin resistance gene was chosen as the selective marker for the shuttle vector. Transformation of *T. denticola* cells by the *T. denticola*-*E. coli* shuttle vector pKMR4PE was confirmed by extraction of the corresponding plasmid. The presence within the recipient strain of the endogenous pTD1 plasmid did not interfere with the introduction of pKMR4PE, thus demonstrating the compatibility of pTD1 with pTS1. To facilitate cloning, the superlinker MCS of plasmid pSL301 (Invitrogen, Carlsbad, CA) was ligated into the *Xba*I site of pKMR4PE to generate the more user friendly shuttle vector pKMMCS.

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Restriction and Modification for Spirochetes

The second potential difficulty for constructing suitable shuttle vectors was to find an efficient way of introducing DNA into spirochetes. The work of S. Samuels was seminal in using electroporation for *B. burgdorferi* (Samuels, 1995). Basically, the electroporation conditions were not very different from those used with other bacteria. It should be noted that usually the efficiency of transformation was quite low, less than 1000 transformants per microgram of DNA. A likely explanation for such a result could be the presence of restriction enzymes within some spirochetes such as *Leptospira* sp. (Boursaux-Eude, D.E.A.) and *T. denticola* (Li and Kuramitsu, 1996). The fact that the transformation efficiency of *T. denticola* with the shuttle vector isolated from *T. denticola* was more than 100-fold higher than with the same plasmid isolated from *E. coli* further confirmed the presence of restriction enzymes in *T. denticola* (Chi *et al.*, 1999). However, for transformation of *B. burgdorferi*, the modification state of the DNA does not appear to be critical (Rosa *et al.*, 1999).

Expression of Heterologous Genes in *T. denticola*

In order to determine the utility of the *T. denticola* shuttle vectors, expression of heterologous genes was studied. The *T. pallidum* endoflagellum protein FlaA was successfully expressed in *T. denticola* using the shuttle vector pKMR4PE (Chi *et al.*, 1999), suggesting the potential usefulness of *T. denticola* in studying the virulence factors of other uncultivable spirochetes.

To monitor promoter activities in *T. denticola*, reporter genes were examined for expression in *T. denticola*. The extremely low endogenous β -galactosidase activity in *T. denticola* makes *lacZ* a suitable reporter gene. β -galactosidase was expressed successfully in *T. denticola* and expression made it possible to select blue-white *T. denticola* colonies on X-Gal agarose plates (Chi and Kuramitsu, 1999). Green fluorescent protein (GFP), which has the extra advantage of labeling bacteria, was also evaluated in *T. denticola*. The same strategy utilized for expressing *T. pallidum* FlaA in pKMR4PE was used.

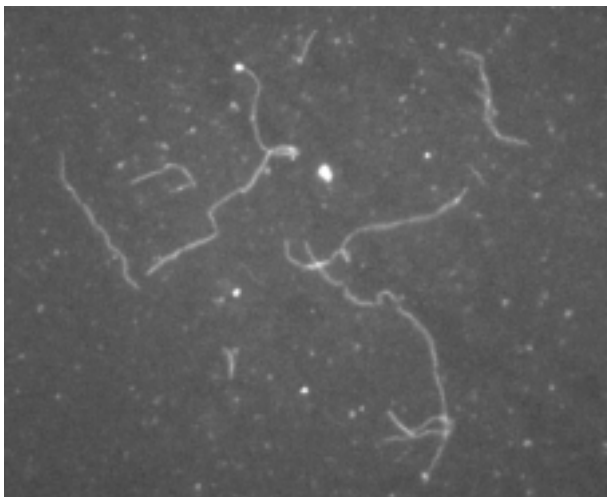


Figure 1. *T. denticola* GFP transformants under epifluorescence microscope.

GFPmut2 (Cormack *et al.*, 1996) was cloned into the *Xba*I site of the pKMR4PE vector downstream of the erythromycin resistance gene cassette. GFP was expressed from the upstream *T. denticola* protease gene promoter, *prtB* (Chi *et al.*, 1999). *T. denticola* transformants showed green fluorescence under epifluorescence microscopy (Figure 1). However, the fluorescence of *T. denticola* transformants was weaker than *E. coli* transformed with the same shuttle vector. Attempts to express GFP from stronger *T. denticola* promoters are in progress in order to increase the fluorescence of *T. denticola* GFP transformants.

Shuttle Vectors for Other Spirochetes

Finally, we mention several potential promising genetic tools for other spirochetes including *B. burgdorferi* and *L. biflexa*. An extrachromosomal cloning vector was very recently developed for expressing enhanced green fluorescent protein in *B. burgdorferi* under the control of the *flaB* promoter (Sartakova *et al.*, 2000). This *B. burgdorferi*-*E. coli* shuttle vector is based on pGK12, a broad-host-range plasmid active in Gram positive bacteria. It confers erythromycin resistance (and not chloramphenicol resistance) to *B. burgdorferi*. The knowledge of the whole genome sequence of *B. burgdorferi* (Fraser *et al.*, 1997) helped in defining potential origins of replication for its numerous circular and linear plasmids. Indeed, two *E. coli*-*B. burgdorferi* shuttle vectors were constructed, one based on the linear plasmid lp28-2 (J. García-Lara, M.J. Osborn and S. Padula unpublished), the other on the cp9 circular plasmid (P. Stewart, J.L. Bono and P. Rosa, unpublished). Three bacteriophages (LE1, LE3 and LE4) specific for *L. biflexa* were found in French sewage water (Saint Girons *et al.*, 1990). A small part of the DNA of the LE1 temperate phage was engineered as an *E. coli*-*L. biflexa* shuttle vector (I. Saint Girons, P. Bourhy, C. Ottone, M. Picardeau, D. Yelton, R. Hendrix, P. Glaser and N. Charon, unpublished). Therefore, it is likely that shuttle vectors could be utilized in several different spirochetes to enhance our knowledge of the genetics of these organisms.

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