

Antibiotic Selective Markers and Spirochete Genetics

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Abstract

Until very recently, the pathogenic spirochetes have been refractory to genetic manipulation. This has been due, in part, to difficulties with *in vitro* growth and the genetic distance that spirochetes are from typical Gram-negative and Gram-positive organisms. Insertional mutagenesis and other genetic techniques are now possible in some of the pathogenic spirochetes such as *Borrelia burgdorferi*, *Brachyspira (Serpulina) hyodysenteriae*, *Leptospira sp.*, and *Treponema denticola*. However, organisms such as *Treponema pallidum*, which cannot be grown *in vitro*, are still not amenable to genetic manipulation. These recent advances have paved the way for more detailed genetic studies of transcriptional regulation, protein function, protein localization, metabolic capabilities, motility, and pathogenic nature of this group of spirochetes. This review article will discuss the current repertoire of antibiotic markers that are useful for spirochetal genetic manipulation. Further advances in selectable markers and shuttle vectors will allow researchers to complete Koch's molecular hypothesis for various virulence genes of the pathogenic spirochetes and increase the overall understanding of these challenging bacteria.

Introduction

The pathogenic spirochetes cause a wide variety of animal and human diseases, ranging from sexually transmitted diseases (*Treponema pallidum* subsp. *pallidum*) to intestinal diseases (*Brachyspira hyodysenteriae* and *B. pilosicoli*). The types of disease symptoms can also vary depending on the specific disease, ranging from localized (*Treponema pallidum* subsp. *pertenue*) to fully systemic (*Treponema pallidum* subsp. *pallidum*, *Borrelia burgdorferi*, etc.). In general, spirochetes tend to cause chronic diseases, potentially due to their unique abilities to evade the host immune response and find protected niches within the host.

The pathogenic spirochetes are notoriously difficult to work with. They tend to be difficult or unable to grow *in vitro*. The spirochete growth or maintenance media are generally very complex, time consuming to prepare, and costly. The pathogenic spirochetes also have a propensity to be very fragile and are therefore easily disrupted by standard manipulations. This facet of the spirochete

membrane structure makes protein localization an arduous task. For these and other reasons, little information is available on the virulence properties of this family of bacteria. Lack of defined genetic systems has made the task of characterizing potential virulence factors quite difficult. In order to define a given gene product as a virulence factor, scientists must fulfill the molecular version of Koch's postulates. These postulates define criteria that must be met in order for a gene to be termed a "virulence factor". First, the gene should be found in virulent strains and may or may not be found in avirulent strains. Second, disrupting a gene in a virulent organism should reduce its virulence. The corollary to this postulate is that expressing the wild-type gene from an autonomously replicating plasmid should restore virulence to the before mentioned avirulent mutant. Third, the virulence gene's protein product should be expressed during the infectious process. Finally, antibodies directed against the gene product should elicit protective immunity. It is critical to this process to be able to routinely insertional inactivate open reading frames. Until recently, this capability has not been available for spirochete researchers. However, this began to change in the early 90's when ter Huurne and coworkers (ter Huurne *et al.*, 1992a) inactivated a *B. hyodysenteriae* gene whose product imparts hemolytic activity and opened the door to spirochetal genetic manipulation. Following this publication, there has been a significant effort to increase the genetic tools available for spirochetal research. There has been much progress over the last decade, but there is still a long way to go.

In this article, selective markers that have been developed for *B. burgdorferi*, *B. hyodysenteriae*, *Leptospira sp.*, and *Treponema denticola* will be reviewed. Potential applications and possible routes for future work will also be discussed. For more detailed reviews of spirochete shuttle vectors and insertional mutagenesis, the readers are referred to other articles found within this issue by Charon and Saint Girons and Tilly *et al.*, respectively.

Brachyspira hyodysenteriae

B. hyodysenteriae, an anaerobic β -hemolytic spirochete, is the major etiologic agent of the highly contagious mucohemorrhagic diarrheal disease swine dysentery. It is fitting that the first report of targeted gene disruption in the pathogenic spirochetes was the successful inactivation of a gene (*tlyA*) from *B. hyodysenteriae* C5 which imparted hemolytic activity when expressed in *E. coli* (ter Huurne *et al.*, 1992a and Table 1). Although the exact role of hemolysin in the pathogenesis of swine dysentery is not known, there is a correlation between hemolytic activity and disease. *B. hyodysenteriae* is differentiated from the non-pathogenic, weakly β -hemolytic *Brachyspira innocens* (Kinyon and Harris, 1979; Joens *et al.*, 1980) which lacks the *tlyA* gene (Muir *et al.*, 1992; ter Huurne *et al.*, 1992b). The hemolysin gene was inactivated by insertion of a kanamycin resistance cassette from *Tn903 in vitro* followed by electroporation mediated allelic exchange in *B.*

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hyodysenteriae C5 and also later inactivated using the same method in the more virulent *B. hyodysenteriae* B204 strain (Hyatt *et al.*, 1994). Since these initial reports, *B. hyodysenteriae* has proven itself to be perhaps the most amenable spirochete for targeted gene disruption.

The work of Rosey *et al.* (Rosey *et al.*, 1995) has led to the application of the *kan* marker for targeted disruption

of a number of flagellar genes in *B. hyodysenteriae* B204 (Table 1). The first report of specific inactivation of motility-associated genes in spirochetes included the use of the *kan* marker from *Tn903* (pUC4K; Pharmacia) to disrupt the *flaA* flagellar sheath protein gene and the *flaB1* flagellar core protein gene (Rosey *et al.*, 1995). One limitation to the use of the *kan* selectable marker in *B. hyodysenteriae*

Table 1. Selective Markers Utilized in Spirochetal Research

Host	Target	Selective marker	Selective marker source	Reference
<i>Borrelia burgdorferi</i>				
hp ^a <i>B. burgdorferi</i> B31	WT ^b <i>gyrB</i>	<i>gyrB</i> ^c	<i>B. burgdorferi</i>	(Samuels <i>et al.</i> , 1994b)
hp <i>B. burgdorferi</i> B31	WT <i>gyrB</i> ^c	<i>gyrB</i> ^c	<i>B. burgdorferi</i>	(Stevenson <i>et al.</i> , 1998)
hp <i>B. burgdorferi</i> B31	<i>oppAV-guaB</i> intergenic region	<i>gyrB</i> ^c	<i>B. burgdorferi</i>	(Rosa <i>et al.</i> , 1996)
hp <i>B. burgdorferi</i> B31	<i>gyrB</i> oligonucleotides	<i>gyrB</i> ^c	Synthetic	(Samuels and Garon, 1997)
hp <i>B. burgdorferi</i> B31	<i>oppAV</i>	<i>gyrB</i> ^c	<i>B. burgdorferi</i>	(Bono <i>et al.</i> , 1998)
hp <i>B. burgdorferi</i> B31	<i>gac</i>	<i>gyrB</i> ^c	<i>B. burgdorferi</i>	(Knight <i>et al.</i> , 2000)
hp <i>B. burgdorferi</i> B31	<i>ospC</i>	<i>gyrB</i> ^c	<i>B. burgdorferi</i>	(Tilly <i>et al.</i> , 1997)
hp <i>B. burgdorferi</i> B31	<i>guaB</i>	<i>gyrB</i> ^c	<i>B. burgdorferi</i>	(Tilly <i>et al.</i> , 1998)
hp <i>B. burgdorferi</i> B31	N/A ^d	<i>cat</i>	<i>S. agalactiae</i> (identical to the <i>cat</i> gene of pC194 from <i>S. aureus</i>)	(Sohaskey <i>et al.</i> , 1997; Sohaskey and Barbour, 1999)
hp <i>B. burgdorferi</i> B31	<i>oppAV</i> ^e	<i>kan</i>	pOK12/Tn903	(Oka <i>et al.</i> , 1981; Bono <i>et al.</i> , 2000)
hp <i>B. burgdorferi</i> B31	<i>flaB</i> , <i>fliF</i> , <i>motA</i>	<i>kan</i>	pOK12/Tn903	(Bono <i>et al.</i> , 2000; Motaleb and Charon, 2000)
hp <i>B. burgdorferi</i> B31	N/A ^f	<i>erm</i>	<i>B. burgdorferi</i>	(Gherardini, 2000)
<i>Borrelia hermsii</i>				
<i>B. hermsii</i>	<i>gyrB</i>	<i>gyrB</i> ^c	<i>B. burgdorferi</i> / <i>B. hermsii</i>	(Samuels, 2000)
<i>Brachyspira (Serpulina) hyodysenteriae</i>				
<i>B. hyodysenteriae</i> C5 and B204	<i>tlyA</i>	<i>kan</i>	<i>Tn903</i> (GenBlock, Pharmacia)	(Oka <i>et al.</i> , 1981; ter Huurne <i>et al.</i> , 1992a)
<i>B. hyodysenteriae</i> B204	<i>flaA</i> <i>flaB1</i> <i>flaB2</i> <i>flaB3</i> <i>flaA + flaB1</i> <i>flaA + flaB2</i> <i>flaA + flaB3</i> <i>flaB1 + flaB2</i> <i>fliG</i>	<i>cat</i> or <i>kan</i> <i>kan</i> <i>cat</i> <i>cat</i> <i>cat + kan</i> <i>kan + cat</i> <i>kan + cat</i> <i>kan + cat</i> <i>cat</i>	<i>cat</i> : <i>S. aureus</i> pC194 <i>kan</i> : pUC4K	(Rosey <i>et al.</i> , 1995; Rosey <i>et al.</i> , 1996; Li and Charon, 2000)
<i>B. hyodysenteriae</i> B204	<i>nox</i>	<i>cat</i> <i>kan</i>	<i>cat</i> : <i>S. aureus</i> pC194 <i>kan</i> : pUC4K	(Stanton <i>et al.</i> , 1999)
<i>B. hyodysenteriae</i> B204	None, defective phage VSH-1	<i>gyrB</i> ^c , tylosin/lincomycin	<i>B. hyodysenteriae</i> B204	(Stanton, 2000)
<i>Treponema denticola</i>				
<i>T. denticola</i> 35405	<i>fliG</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Li <i>et al.</i> , 1996)
<i>T. denticola</i> 33520	<i>tap1</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Limberger <i>et al.</i> , 1999)
<i>T. denticola</i> 35405	<i>msp</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Fenno <i>et al.</i> , 1998)
<i>T. denticola</i> 35405	<i>prtP</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Fenno <i>et al.</i> , 1998)
<i>T. denticola</i> 35405	<i>dmcB</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Li <i>et al.</i> , 1999)
<i>T. denticola</i> 35405	<i>dmcA</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Kataoka <i>et al.</i> , 1997)
<i>T. denticola</i> 33520	<i>fliG</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Limberger, 2000)
<i>T. denticola</i> 33520	<i>cfpA</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Limberger, 2000)
<i>T. denticola</i> 35405	<i>oppA</i> , <i>oppF</i>	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Fenno <i>et al.</i> , 2000)
<i>T. denticola</i> 33520	None, shuttle vector pKMR4PE	<i>ermF/AM</i>	<i>Bacteroides fragilis</i>	(Chi <i>et al.</i> , 1999)
<i>T. denticola</i> 33520	None, shuttle vector pKT210 ^g	<i>cat</i>	pKT210	(Bagdasarian <i>et al.</i> , 1981; Kuramitsu, 2000)
<i>Leptospira sp.</i>				
<i>Leptospira biflexa</i>	None, shuttle vector	<i>kan</i>	<i>Streptococcus faecalis</i>	(Saint Girons, 2000)

^aHigh passage.

^bWild type.

^cSingle cross-over insertion of circular plasmid. Used for heterologous gene expression.

^dNot used for insertional mutagenesis or selection in *Borrelia*. The *cat* marker was used on plasmid pGOΔ1 to analyze promoter activity.

^eSingle cross-over of circular plasmid. Plasmid integration inactivated *oppAV*.

^fNot used for insertional mutagenesis. The marker is present on a conjugal element.

^gDiscontinued use of *cat* marker due to high spontaneous resistance.

is a result of the organism's basal resistance to several antibiotics (Table 3). The sensitivity of *B. hyodysenteriae* B204 to ampicillin, chloramphenicol, erythromycin, kanamycin, rifampin, streptomycin and tetracycline was examined and the organism was found to be resistant (MIC > 128 µg/ml) to all compounds except chloramphenicol (MIC, 8 µg/ml) and tetracycline (MIC, 64 µg/ml) (Rosey *et al.*, 1995). This basal resistance can be managed by increased selective levels for kanamycin in the range of 150-400 µg/ml as was done in the previous inactivation studies. However, this limitation and the desire for more than a single selective marker for genetic manipulation, led to the selection and application of the chloramphenicol (*cat*) gene from the *S. aureus* plasmid pC194 (Horinouchi and Weisblum, 1982). The *cat* gene was also modified into an antibiotic cassette to facilitate gene mutagenesis studies (Rosey *et al.*, 1996) and unlike the case in *T. denticola* and *B. burgdorferi*, results in extremely low background residual growth of *B. hyodysenteriae* at levels >10 µg/ml chloramphenicol.

Subsequently, the *kan* and *cat* markers have been routinely used to create *flaA::kan*, *flaA::cat*, *flaB1::kan*, *flaB2::cat*, *flaB3::cat*, *fliG::cat*, and NADH oxidase (*nox*) *nox::kan* and *nox::cat* single gene disruptions in *B. hyodysenteriae* (Rosey *et al.*, 1995; Li and Charon, 2000; and Table 1). The additional *cat* marker allowed the creation of a dual *flaA::cat*, *flaB1::kan* mutant (Rosey *et al.*, 1996). More recently Li and Charon (Li and Charon, 2000) have greatly expanded the capability to analyze the structure and function of the complex flagella and to begin to determine interactions between the multiple flagellar proteins of *B. hyodysenteriae*. They have combined these markers to create *flaA::kan*, *flaB2::cat*; *flaA::kan*, *flaB3::cat*; *flaB1::kan*, *flaB2::cat*; and *flaB1::kan*, *flaB3::cat* double mutants within the flagellar genes of this spirochete. These *B. hyodysenteriae* strains represent the only spirochete double mutants reported to date.

The creation of double mutant strains was not an easy process. Initial strains were created by first inactivating a single gene target then preparing the resulting strain for a second round of electroporation-mediated allelic exchange with the remaining available selectable marker. Although it is conceivably possible to carry out this dual inactivation simultaneously, the electroporation and/or homologous recombination frequencies observed for this organism would not be expected to allow a positive outcome with such an approach. Many of the latter dual mutants were constructed by a less laborious method utilizing the phage VSH-1 to combine single disrupted genes to create multiply-tagged mutant strains (see below). Interestingly, in *B. hyodysenteriae*, there has been no evidence for targeted disruption resulting from simple Campbell-type plasmid integration at the target or at spontaneous sites (Rosey *et al.*, 1995; Rosey *et al.*, 1996; Li and Charon, 2000). All confirmed knockout strains have resulted from a double crossover event resulting in allelic exchange of the target DNA and replacement by the inserted *kan* or *cat* selectable marker.

Although targeted mutagenesis in *B. hyodysenteriae* has been reasonably successful, additional selectable markers as well as methods for natural gene transfer would be valuable tools for the study of structure/function and pathogenesis. There are currently no well characterized plasmids or shuttle plasmids available for use in *B.*

hyodysenteriae. However, Humphrey and coworkers (Humphrey *et al.*, 1995; Humphrey *et al.*, 1997) have identified and characterized a mitomycin C-inducible gene transfer agent, VSH-1, from numerous strains of *B. hyodysenteriae* and *B. innocens*. VSH-1 is capable of packaging and transducing chromosomal, antibiotic-marked genes (eg. *flaB1::kan*, *flaA::cat*) between *B. hyodysenteriae* B204 strains (Humphrey *et al.*, 1997). Recent evidence suggests VSH-1 related agents may be present in *B. pilosicoli* (Stanton, 2000), although it is not clear whether such agents are capable of generalized transduction in this species.

Additional selectable markers that may be applied for *B. hyodysenteriae* genetic manipulation include naturally occurring tylosin/lincomycin resistance. Recent exploitation of the DNA gyrase (*gyrB*) gene from *B. hyodysenteriae* has led to the generation of several UV-induced point mutations within *gyrB* which impart coumermycin A₁ resistance on *B. hyodysenteriae* B204 (Stanton, 2000). In addition to mobilization of *kan* and *cat*, the VSH-1 phage has been used to successfully move tylosin/lincomycin and coumermycin resistance within *Brachyspira sp.* The current status and future potential for gene transfer in *B. hyodysenteriae* and closely related spirochetes is exemplified by the recent construction of a strain containing four different antibiotic resistant phenotypes (Stanton, 2000). This strain will be a useful substrate for gene transfer studies. Further development of VSH-1 or other gene transfer agents including new phage, plasmids, or antibiotic markers will allow even greater flexibility in genetic manipulation of this organism. Additionally, *B. hyodysenteriae* may be considered a potential surrogate model system when appropriate for other spirochetes with less robust selectable markers.

Borrelia burgdorferi

B. burgdorferi is the causative agent of Lyme disease, a chronic tick-borne disease with both local and systemic manifestations. *B. burgdorferi* and the closely related *B. hermsii* (the causative agent of relapsing fever) are capable of *in vitro* cultivation in BSK medium (Barbour, 1988; Pollack *et al.*, 1993). Culture adapted, high passage, non-infectious *B. burgdorferi* have a faster doubling time, a higher plating efficiency, and are more genetically amenable than low-passage, infectious *B. burgdorferi*. The specific reasons for these differences are not known, but have limited genetic research to mostly the high passage, non-infectious strains that apparently no longer express many of the important *Borrelia* virulence genes.

Samuels *et al.* (Samuels *et al.*, 1994a) identified mutations in the *B. burgdorferi gyrB* gene that conferred resistance to coumermycin A₁. A *gyrB* gene containing three point mutations, *gyrB_{ngr}*, was found to confer a high level of resistance and has been extensively utilized for insertional mutagenesis in *B. burgdorferi* (Table 1). The main problem with the use of the *gyrB_{ngr}* marker in electroporation mediated allelic exchange experiments is that approximately 99.5% of the coumermycin A₁ resistant transformants are the result of an insertion of the *gyrB_{ngr}* gene into the wild-type *gyrB* locus instead of the gene of interest. Given the difficulties of growing, transforming, and plating *B. burgdorferi*, this single problem has hindered insertional mutagenesis with this marker. In light of this,

there are no reported insertionally inactivated genes in low-passage, high-infectivity *B. burgdorferi* isolates.

Table 1 shows the genes that have been successfully inactivated in *B. burgdorferi*. Five of the nine reported gene inactivation experiments have utilized the *gyrB'* marker. The first insertion of the *gyrB'* marker placed the marker on lp54 in the *oppAV* and *guaB* intergenic region. This was done to assess whether or not insertion was possible without the potential deleterious effects caused by gene inactivation. Following this success, *oppAV*, *gac*, *ospC*, and *guaB* have been inactivated using the *gyrB'* marker (Table 1).

A large amount of work has been done in an attempt to identify other non-*Borrelia* markers that could be utilized in *B. burgdorferi*. Numerous markers have been assessed using the electroporation protocol of Samuels (Samuels *et al.*, 1994b). The drawback of this approach is that if a given marker fails to yield resistant colonies following transformation and plating, the investigators are unable to determine if the marker in question failed due to an inability to function in the spirochetal background or if the gene was inefficiently transcribed and/or translated.

Sohaskey and coworkers (Sohaskey *et al.*, 1997) developed a transient expression system that utilized the *cat* gene from *Streptococcus agalactiae* as a reporter. This system was utilized to determine the efficiency with which various *Borrelia* promoters could transcribe the *cat* gene from an exogenous plasmid in electroporated *Borrelia* cells. While CAT expression was detected in *Borrelia* cells electroporated with the *cat* gene transcribed off various promoters, no chloramphenicol-resistant mutants were isolated. One reason for this apparent paradox was later resolved (Sohaskey and Barbour, 1999). These investigators showed that esterases present in serum-containing medium can convert diacetyl chloramphenicol (inactivated form) back into chloramphenicol (active form). This medium activity can convolute any attempts to use the *cat* gene as a selective marker for *Borrelia* sp. grown in serum containing medium. A second potential explanation for the lack of chloramphenicol resistance comes from an analysis of the *B. burgdorferi* genome (Fraser *et al.*, 1997). Based on DNA and protein sequence homologies of the various enzymes, there is no fatty acid oxidation pathway or pyruvate dehydrogenase complex. This would result in a lack of the substrate for chloramphenicol acetyl transferase, namely Acetyl CoA, in the *Borrelia* cytoplasm (Samuels, 2000). Therefore, even with CAT expression, the enzyme would be unable to function at full capacity to confer resistance. These results further underscore the drawbacks of testing potential selective markers by electroporation and direct selection.

Stevenson and coworkers (Stevenson *et al.*, 1998) developed a procedure in which a plasmid containing the *gyrB'* marker and a second potential resistance marker is recombined into the genome via a single crossover event at the *gyrB* locus with selection on coumermycin A₁. Once a stable transformant is identified, the strain can be tested for resistance to the second antibiotic. This method was done using a *kan* marker from pOK12/Tn903 (Oka *et al.*, 1981). In this publication, the *kan* marker failed to provide resistance to kanamycin.

Bono and coworkers (Bono *et al.*, 2000) have since determined that the failure of the above mentioned *kan* marker was due to a lack of transcription of the *kan* gene.

These investigators replaced the *kan* promoter with either the *B. burgdorferi* *flaB* or *flgB* promoters and successfully isolated a coumermycin A₁^r, kanamycin-resistant *Borrelia* mutant. These investigators subsequently utilized the P_{*flaB*}-*kan* marker to insertionally inactivate the *oppAV* gene on lp54. Utilizing the P_{*flaB*}-*kan* marker, 90% of the Kan^r colonies contained the *kan* marker inserted in the target gene. This percentage is immensely better than the 0.5% observed with the *gyrB'* selectable marker. The P_{*flgB*}-*kan* marker has also recently been used to create gene replacement disruptions in the *B. burgdorferi* *flaB*, *flfI*, and *motA* genes (Motaleb and Charon, 2000).

Recently, Hudson and coworkers (Hudson *et al.*, 2000) have described a conjugal element in *B. burgdorferi* that contains an erythromycin-resistance determinant. Mating experiments demonstrated that erythromycin resistance could be transferred from *B. burgdorferi* to either *Bacillus subtilis* or *Enterococcus faecalis*. Erythromycin resistant cells were resistant not only to erythromycin, but also to other macrolide antibiotics such as lincomycin, clindamycin, and streptogramin. Additionally, isolated ribosomes from resistant cells were incapable of binding erythromycin. Therefore, the erythromycin-resistance was conferred by an erythromycin-resistance methylase (*erm*) that alters the antibiotic binding site on the rRNA. Since some isolates of *B. burgdorferi* are sensitive to erythromycin while others (potentially containing this *erm* determinant) are resistant, utilization of this *erm* determinant as a selective marker in sensitive strains would likely yield positive results.

The *ermC* gene has been utilized in the first reported *B. burgdorferi* extrachromosomal cloning vector, pGK12 (Sartakova *et al.*, 2000). This vector contains origins of replication from *Lactococcus lactis* pWV01 and two antibiotic resistance genes (*ermC* and *cat* from *Staphylococcus aureus* pE194 and pC194). While the *ermC* and *cat* genes are able to provide erythromycin and chloramphenicol resistance in *E. coli*, *Staphylococcus*, *Streptococcus*, *Bacillus*, and *Lactococcus*, only the *ermC* gene appears to provide resistance in *Borrelia* (Sartakova *et al.*, 2000). This observation lends more support to the hypothesis suggesting that CAT is nonfunctional in *Borrelia* due to a lack of available substrate (Acetyl CoA) for chloramphenicol acetyl transferase (Samuels, 2000).

Multiple antibiotic resistance markers would clearly be a benefit to *Borrelia* research. Currently only two markers have successfully been utilized for insertional mutagenesis, *gyrB'* and *kan*. The erythromycin resistance determinants described by Hudson *et al.*, (2000) and Sartakova (Sartakova *et al.*, 2000) may prove to be useful for this application as well. Other antibiotic resistance markers are currently being tested and evaluated (Samuels, 2000).

Treponema denticola

Treponema denticola is an anaerobic oral pathogen that is capable of being continuously cultivated *in vitro*. The ability to culture this organism and the ease of genetic manipulation has made *T. denticola* an attractive spirochete for research. The *ermF/AM* cassette (Fletcher *et al.*, 1995) has been utilized to insertionally inactivate a number of *T. denticola* genes (Table 1). This 2.1-kb cassette, originally from the *Bacteroides fragilis* transposon Tn4351, contains the *ermF* and *ermAM* genes in tandem. The *ermF* gene is believed to be expressed from an endogenous promoter

(Kuramitsu, 2000). For this reason, Chi and coworkers (Chi *et al.*, 1999) replaced the *ermF* promoter with the promoter region of the *T. denticola prtB* gene to ensure appropriate transcription of the *ermF/AM* cassette in their shuttle plasmid pKMR4PE.

A second antibiotic marker that has been utilized in *T. denticola* is the *cat* gene from pKT210 2000^o (Kuramitsu, 2000). This marker was used to select for transformants that carried the shuttle vector pKT210. However, due to high background levels of chloramphenicol-resistant colonies during selection, use of the *cat* marker was discontinued.

At present, there is only one antibiotic resistance marker commonly used in this genetically amenable organism. Clearly, there is a need for additional markers. For example, if two markers were present, the *T. denticola msp* gene could be inactivated with one marker while a second marker is utilized for selection of a shuttle vector expressing modified Msp or even the *T. pallidum* Tpr proteins. This would be a useful surrogate system for non-cultivable spirochetes such as *T. pallidum*. Additionally, *T. denticola* has proven to be a good model system for spirochete motility. Several of the motility-associated genes have been inactivated using the *ermF/AM* cassette (Li *et al.*, 1996; Kataoka *et al.*, 1997; Limberger *et al.*, 1999; Li *et al.*, 1999; Limberger, 2000). An autonomously replicating shuttle vector with a second selectable marker would permit researchers to inactivate a given gene and complement with specifically mutated variants. This process would allow for a detailed analysis of protein function.

The *gyrB'* marker would seem to be a good candidate for a second selectable marker. The benefits of having a second resistance marker, in this case, outweigh the drawbacks of having a high recombination rate at the wild-type *gyrB* locus. Since the *T. denticola* GyrB protein and the *B. burgdorferi* GyrB protein are ~60% identical, the chances of success are high. The potential for the *B. burgdorferi gyrB'* gene to confer coumermycin A₁ resistance would make this approach attractive. The ability of the *B. burgdorferi* GyrB protein to interact with the *T. denticola* machinery, however, may limit or inhibit the utility of this marker. The *T. denticola gyrB* gene, however, may be utilized in a similar fashion to that of its borrelial counterpart. Other markers in addition to these are currently under consideration as well (Limberger, 2000).

Leptospira sp.

To date, there have been no reports of insertional mutagenesis being performed in any *Leptospira sp.* Given the advancing nature of selectable markers in spirochetes, insertional mutagenesis should be possible in this genus once appropriate markers are identified. The observation that antibiotic resistance patterns vary between serovars may confound the application of a single antibiotic resistance marker to multiple serovars (Faine *et al.*, 1999). For example, *L. interrogans* sv. *grippotyphosa* is sensitive to 3.5 µg/ml streptomycin, while some isolates of *L. interrogans* sv. *canicola* can grow in up to 2000 µg/ml streptomycin. Similar observations have also been made for other antibiotics such as neomycin, vancomycin, penicillin, tobramycin, kanamycin, and polymixin (Faine *et al.*, 1999). This only serves to underscore the requirement to double check not only serovar resistance to a given

antibiotic, but also spontaneous resistance under selection prior to initiating an insertional mutagenesis experiment in a *Leptospira sp.*

While there has been little progress in the utilization of antibiotic markers for insertional mutagenesis in *Leptospira*, an *E. coli* – *Leptospira biflexa* shuttle vector has been developed (Saint Girons, 2000). This shuttle vector is based on the LE1 bacteriophage and utilizes the *kan* gene from *Streptococcus faecalis*. Whether or not this shuttle vector and antibiotic marker are useful in other serovars remains to be determined. Additionally, the utility of the *kan* marker for insertional mutagenesis should be investigated. This, however, marks the first successful attempt to introduce foreign DNA into any *Leptospira sp.* The LE1-type shuttle vector and other spirochetal shuttle vectors are the topic of a separate review article found within this issue and therefore will not be discussed in further detail.

Discussion

Over the years, research involving the pathogenic spirochetes has been severely limited by several factors including the inability to cultivate some of the spirochetes *in vitro*, limited or no genetic systems, and difficulties associated with the genetic distance separating spirochetes from the most commonly studied Gram-positive and Gram-negative bacteria. Slowly, these obstacles are being overcome through a consolidated and persistent effort on behalf of a large number of investigators in the spirochete research community. Aside from *in vitro* growth, the key element in the advancement of spirochetal genetics is the development of multiple antibiotic selection markers for use in insertional mutagenesis, shuttle vectors, and development of other genetic tools.

There are currently four antibiotic selection markers that have been utilized in various spirochetes. Table 1 shows a summary of the various markers, their usage, and the spirochetes involved. Unfortunately, only *B. burgdorferi* (very recently in 2000) and *B. hyodysenteriae* have at least two markers that have been used effectively. The remainder of the spirochetes either have only one reliable marker or none. This effectively limits the genetic studies that can be carried out in these spirochetes. There is a clear need to develop several new antibiotic selection markers for the various pathogenic spirochetes.

The first step in developing new antibiotic markers is to create a system in which potential markers can be screened effectively. Simply placing a potential marker in a gene and electroporating spirochetal cells is not enough. A negative result could be the result of a lack of transcription of the inserted antibiotic gene cassette. Stevenson and coworkers (Stevenson *et al.*, 1998) created a system for *B. burgdorferi* that is useful for screening potential antibiotic selection markers. This system allows for positive selection of single cross-over insertion of a plasmid containing the *gyrB'* marker as well as a second gene of interest. Once coumermycin A₁ resistant clones are identified, they can be screened for resistance to the second marker. At the same time, transcription of the second marker can be assessed by RT-PCR with specific primers. Therefore, both the function (antibiotic resistance) and the transcription of each potential antibiotic resistance gene can be checked. This system could be adapted for use in other spirochetes

Table 2. Antibiotics Recommended for Treatment of Various Spirochetal Diseases

Infection	Causative agent	Recommended antibiotics ^a	Reference
Lyme disease	<i>Borrelia burgdorferi</i>	TT, DX, MN, AM, CF, PN, CT	(Gorbach <i>et al.</i> , 1998)
Relapsing fever	<i>Borrelia hermsii</i>	TT, ER, PN	(Gorbach <i>et al.</i> , 1998)
Syphilis	<i>Treponema pallidum</i> subspecies <i>pallidum</i>	PN, ER, CT, TT	(Gorbach <i>et al.</i> , 1998)
Yaws	<i>Treponema pallidum</i> subspecies <i>pertenueae</i>	PN, ER, CT, TT	(Gorbach <i>et al.</i> , 1998)
Periodontal disease	<i>Treponema denticola</i>	MT, DX	(Gorbach <i>et al.</i> , 1998)
Leptospirosis	<i>Leptospira sp.</i>	PN, DX, ER	(Gorbach <i>et al.</i> , 1998)
Swine dysentery	<i>Brachyspira hyodysenteriae</i>	BT, CB, LN, NT, TM, VM	(Aiello and Mays, 1998)
Intestinal spirochetosis ^b	<i>Brachyspira pilosicoli</i>	MT, NM	(Hampson and Stanton, 1997)

^aAbbreviations: AM, Amoxicillin; BT, Bacitracin; CB, Carbadox; CF, Cefuroxime; CT, Ceftriaxone; DX, Doxycycline; ER, Erythromycin; LN, Lincomycin; MT, Metronidazole; MN, Minocycline; NM, Neomycin; NT, Nitroimidazole; PN, Penicillin; TT, Tetracycline; TM, Tiamulin; VM, Virginiamycin

^bMT and NM have shown efficacy in humans but are not formally recommended for treatment. Veterinary treatment for intestinal spirochetosis generally employs those antibiotics utilized for swine dysentery.

as well. Additionally, this system could be utilized to assess promoter function and/or regulation.

In identifying new antibiotic selection markers, there are two main criteria that need to be met. First, the antibiotic in question cannot be clinically useful for treatment of the associated disease. Second, the spirochete cannot already be resistant to the antibiotic. Tables 2 and 3 summarize the available data on these two topics. Due to the antibiotic resistance variations observed between the different *Leptospira* serovars, the authors refer the reader to the excellent resistance summary of *Leptospira* antibiotic resistance patterns by Faine and coworkers (Faine *et al.*, 1999). An obvious first step would be to consider antibiotic markers that have already proven themselves in one spirochete for use in another. For example, erythromycin resistance has proven to be an excellent insertional marker for *T. denticola*. It's utility for other spirochetes should be assessed. The currently used *ermF/AM* cassette, the *erm* determinant described by Hudson and coworkers (Hudson *et al.*, 2000), and the *ermC* gene from pGK12 (Sartakova *et al.*, 2000) could be tested in the system of Stevenson and coworkers

(Stevenson *et al.*, 1998). Additionally, many of the markers currently in use for *B. burgdorferi*, *B. hyodysenteriae*, *T. denticola*, and *Leptospira sp.* could be utilized in *B. hermsii* and *B. pilosicoli* in an attempt to create genetic systems in these spirochetal pathogens.

A group of antibiotic resistance markers that have not been extensively assessed to date include the antibiotic export pumps. For example, the *Escherichia coli acrD* (aminoglycoside resistance), *E. coli emrE* (lipophilic cation resistance), *Staphylococcus aureus cadA* (cadmium resistance), *S. aureus qacA/abr/smr* (quaternary ammonium compound resistance), or *S. aureus/Bacillus subtilis/Streptococcus pneumoniae norA/bmr/pmrA* (fluoroquinolone, norfloxacin, ethidium bromide, and puromycin resistance) genes could be tested. These efflux pumps utilize either an antiport mechanism or membrane potential to actively export compounds out of the cytoplasm. Therefore, these pumps should not be subjected to limitations such as limiting amounts of enzyme cofactors (*i.e.* CAT and acetyl-CoA levels). The potential drawback of efflux pumps would be the slow doubling time of

Table 3. Natural Resistances Observed for Various Spirochetes

Spirochete	Antibiotic and resistance level or MIC ($\mu\text{g/ml}$) ^{a,b}	Reference
<i>Borrelia burgdorferi</i>	TM (S), SM (R), CP (23), CD (45), CC (91), CF (0.13), CI (0.8), CT (0.02-0.03), PN (0.06), ER (0.007-0.06), CL (0.003-0.03), HC (0.007-0.03), AZ (0.003-0.03), VN (0.5-2.0), EV (0.06-0.5), MR (0.125)	(Agger <i>et al.</i> , 1992; Dever <i>et al.</i> , 1992; Dever <i>et al.</i> , 1993a; Dever <i>et al.</i> , 1993b; Kazragis <i>et al.</i> , 1996; Reisinger <i>et al.</i> , 1997; Dever <i>et al.</i> , 1999)
<i>Borrelia hermsii</i>	VN (0.5-2.0)	(Kazragis <i>et al.</i> , 1996)
<i>Borrelia turicatae</i>	VN (0.5)	(Kazragis <i>et al.</i> , 1996)
<i>Treponema pallidum</i> subspecies <i>pallidum</i>	ER (R) ^c	(Stamm <i>et al.</i> , 1988; Stamm and Bergen, 2000)
<i>Treponema denticola</i>	TT (R), ER (R)	(Roberts <i>et al.</i> , 1996)
<i>Leptospira sp.</i>	Refer to (Faine <i>et al.</i> , 1999)	(Faine <i>et al.</i> , 1999)
<i>Brachyspira hyodysenteriae</i>	TY (100), LN (100), NV (50-200), GN (3-25), AP (100), ER (100), KN (50), NL (500), NM (200), PB (500), RF (200), SC (500), SP (200), ST (50), VN (500)	(Weber and Earley, 1991; Trott <i>et al.</i> , 1996)
<i>Brachyspira pilosicoli</i>	ER (100), KN (20), NL (200), NM (100), PB (500), RF(20), SC (500), ST (50), VN (500)	(Trott <i>et al.</i> , 1996)

^aAbbreviations: AM, Amoxicillin; AP, Ampicillin; AZ, Azithromycin; CC, Cefaclor; CD, Cefadroxil; CF, Cefuroxime; CI, Cefixime; CL, Clarithromycin; CT, Ceftriaxone; CP, Cephalexin; DX, Doxycycline; ER, Erythromycin; EV, Everninomicin; GN, Gentamycin; HC, 14-hydroxycyclarithromycin; KN, Kanamycin; LN, Lincomycin; MT, Metronidazole; MN, Minocycline; MR, Meropenem; NL, Naladixic Acid; NM, Neomycin; NV, Novobiocin; PN, Penicillin; PB, Polymyxin-B; R, resistant; RF, Rifampicin; S, Sensitive; SC, Spectinomycin; SP, Spiramycin; ST, Streptomycin; SM, Sulfamethoxazole; TM, Trimethoprim; TT, Tetracycline; TY, Tylosin; VN, Vancomycin.

^bR or S indicate that the MIC was not reported.

^c*T. Pallidum* subspecies *pallidum* Nichols strain is sensitive while Street Strain 14 is resistant.

spirochetes would force the pump to maintain low intracellular levels of compound over a longer time period.

The work of Sohaskey and coworkers (Sohaskey *et al.*, 1997; Sohaskey and Barbour, 1999) with *B. burgdorferi* chloramphenicol resistance brings an interesting point to bear. The complex nature of medium required for spirochetal growth may interfere with the utilization of certain antibiotics. It is possible that using the serum free *Borrelia* medium described by Posey and Gherardini (personal communication) may mitigate the issues discussed herein for chloramphenicol acetyl transferase. The absence of a serum esterase activity in this serum free medium may allow for selection of resistant *Borrelia* cells with chloramphenicol despite the low intracellular acetyl-CoA levels. Moreover, this defined medium may be useful in screening other antibiotic markers as well. However, high levels of magnesium, calcium, or other cations may still effect enzyme activities.

While not a selective marker, the green fluorescent protein (Cormack *et al.*, 1996) may be a useful tool to spirochete research. Indeed, it has already been utilized to demonstrate the utility of the extrachromosomal cloning vector, pGK12, developed by Sartakova and coworkers (Sartakova *et al.*, 2000). Another potential use of the green fluorescent protein would be as an enrichment agent. Spirochete cells expressing Gfp may be enriched by a fluorescent activated cell sorter (FACS). For spirochetes that can be plated, FACS-sorted cells could be plated and fluorescing colonies identified. While this method may be more time consuming, it may have utility by providing an additional marker that may not be present in a given spirochete.

With two spirochetes (*B. burgdorferi* and *B. hyodysenteriae*) now having 2 or more selective markers, three spirochetes now having shuttle vectors (*B. burgdorferi*, *T. denticola* and *Leptospira biflexa*), and the potential for gene manipulation using phage, the goal to fulfill Koch's molecular postulates is realistic. It will now be possible for a potential virulence gene to be inactivated using one selective marker and complemented by a wild-type gene present either at a separate insertion site or on a shuttle vector using a second resistance marker. The creation of multiple selectable markers for each of the pathogenic spirochetes will allow for detailed studies of pathogenic factors, metabolism, physiology, motility, and membrane architecture.

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