J. Mol. Microbiol. Biotechnol. (2000) 2(4): 433-442.

DNA Exchange and Insertional Inactivation in Spirochetes

Kit Tilly†, Abdallah F. Elias†, James L. Bono, Philip Stewart, and Patricia Rosa*

Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, 903 S. 4th Street, Hamilton, Montana USA

Abstract

Spirochetes have complex life cycles and are associated with a number of diseases in humans and animals. Despite their significance as pathogens, spirochete genetics are in their early stages. However, gene inactivation has been achieved in *Borrelia burgdorferi*, *Brachyspira hyodysenteriae*, and *Treponema denticola*. Here, we review methods that have been used in spirochetes for gene inactivation and DNA exchange, with a primary focus on *B. burgdorferi.* We also describe factors influencing electrotransformation in *B. burgdorferi*. In summary, optimal transformation frequencies are obtained with log phase bacteria, large amounts of DNA (up to 50 µg per transformation), and high field strength (12.5-37.5 kV/cm). Infectious *B. burgdorferi* isolates transform with frequencies 100-fold lower than those found for high passage, non-infectious strains. Surface characteristics of the bacteria, which often correlate with infectivity, are among the obstacles to effective transformation by electroporation.

Introduction

Spirochetes compose a diverse group of bacteria, first described in association with human disease by Obermeier in 1868 (Birkhaug, 1942). Pathogenic spirochete lifestyles are myriad. *Borrelia burgdorferi*, a Lyme disease agent, normally cycles between ticks and small rodents, as do most other Borrelia species. *Brachyspira* (formerly *Serpulina*) *hyodysenteriae* causes swine dysentery and is transmitted from pig to pig by the oral-fecal route (Harris *et al*., 1972). *Treponema denticola* lives in the complex human periodontal environment and is associated with periodontal disease, in combination with the other microbial inhabitants of the mouth. *Treponema pallidum* is the agent of syphilis and has yet to be established in culture. Spirochetes closely related to, if not within, the genus *Treponema* are acetogenic endosymbionts found in termite guts (Leadbetter *et al*., 1999). The genus *Leptospira* contains both pathogenic and saprophytic members, with the pathogenic species causing disease in both animals and humans (Vinetz, 1997).

Genetic tools for studying spirochetes are just being developed. Only recently has gene inactivation become possible and it is now becoming routine in a few spirochete systems. This mini-review covers published work describing state of the art methods of DNA exchange and gene inactivation for the spirochetes, focusing primarily on *B. burgdorferi*, with some coverage of *B. hyodysenteriae* and *T. denticola*. We also provide data from our laboratory addressing particular aspects of electroporation and other possible methods of gene transfer into *B. burgdorferi*. This information, in combination with the recently released genome sequences of *B. burgdorferi* (Casjens *et al*., 2000; Fraser *et al*., 1997) and *T. pallidum* (Fraser *et al*., 1998), should facilitate the development of genetic systems in other spirochetes.

Insertional Inactivation

Insertional inactivation has been used for mutagenesis of prokaryotic and eukaryotic genes for many years. Several methods achieve the desired result of inserting foreign sequences into a specific gene, thereby rendering it inactive. The first method is targeting by allelic exchange. The desired locus, with a selectable marker inserted into the gene of interest, is constructed *in vitro*. Transformation is used to introduce the mutant gene, which replaces the wild type copy by double recombination (Figure 1A). This technique requires a gene transfer mechanism and a selectable marker.

A gene of interest can also be insertionally inactivated by plasmid integration, using an internal fragment of the gene to target the insertion event (Figure 1B). Integration of the plasmid via a single crossover generates two copies of the targeted gene. Both are inactive, with one deleted at the 5' end and the other deleted at the 3' end of the gene. In theory, this single crossover should be more frequent than the double crossover required for allelic exchange. The resulting integrant contains a tandem duplication of part of the targeted gene, which could result in reversal of the integration by a single recombination event. Maintaining selection for the plasmid sequences should reduce this instability problem, but selection during growth of mutant bacteria in a natural host or environment may not always be possible. Plasmid integration can also occur without mutation at the insertion site (*e.g.*, Figure 2B), or can generate a wild type and a mutant copy of the targeted gene, depending on the nature of the sequences cloned into the plasmid to be integrated. Both allelic exchange and plasmid integration have been used in spirochetes (see below).

A third method, yet to be developed for spirochetes, is random insertional mutagenesis using a transposable element (Berg and Berg, 1996). Three ways of doing this are the following: 1. generation of a library of insertions into spirochete DNA cloned in *E. coli*, followed by transformation of the spirochete and selection for a marker carried on the transposon; 2. *in vitro* transposition into spirochete DNA, also followed by transformation and

† both authors contributed equally to this work

*For correspondence. Email prosa@nih.gov; Tel. (406) 363-9209; Fax. (406) 363-9394.

© 2000 Horizon Scientific Press

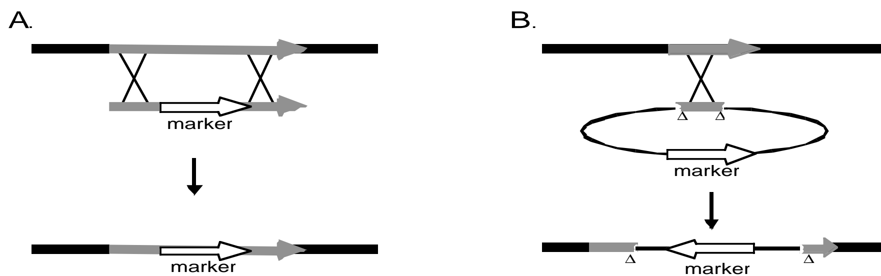

Figure 1. Insertional inactivation. A. Gene inactivation by allelic exchange with a selectable marker. B. Plasmid insertion yielding gene inactivation by recombination with a doubly-deleted gene. Δs refer to deletions. Gray arrows represent *B. burgdorferi* genes; black bars represent other genomic sequences; white arrows represent selectable markers, denoted "marker" ; and thin lines represent plasmid vector sequences.

selection; and 3. transformation with a minitransposon engineered to transpose within the target spirochete. In each case, designing a screen or selection for identifying mutations affecting the gene of interest is a crucial step. An advantage of this method is that it would generate a library containing insertions in most or all of the non-essential spirochete genes, which can be searched as screens or selections are developed.

The three methods of insertional inactivation described above require a usable selectable marker. For spirochetes without genetic systems, mutating an endogenous gene to confer drug resistance, as was done with the *B. burgdorferi gyrB* gene (Samuels *et al.*, 1994a), provides a good starting point. Since researchers studying other spirochetes (such as *T. denticola* and *B. hyodysenteriae*) have found drug-resistance markers that worked without alteration in their organisms, this approach is also worth trying (Li *et al.*, 1996; Rosey *et al.*, 1995).

Gene Inactivation in *B. hyodysenteriae*

Genes have been insertionally inactivated in *B. hyodysenteriae*, *T. denticola*, and *B. burgdorferi* (Table 1). The first gene to be inactivated in a spirochete was the *B. hyodysenteriae tly* gene, encoding a putative hemolysin (ter Huurne *et al.*, 1992). Subsequently, electroporation with the selectable markers *kan* and *cam*, conferring kanamycin- and chloramphenicol-resistance, respectively, was used to generate a series of single and double mutations affecting flagellar biosynthesis and NADH oxidase synthesis (Rosey *et al.*, 1995; Rosey *et al.*, 1996). Having these markers allowed the perspicacious researchers to realize that *B. hyodysenteriae* also undergoes another method of DNA exchange, generalized transduction (see below for further details), and to use this method to generate further combinations of mutants (Humphrey *et al.*, 1997). Recently, genes encoding several other flagellar components have been inactivated, allowing their roles in flagellar structure and motility to be determined (C. Li and N. W. Charon, personal communication).

B. hyodysenteriae mutants were generated in infectious strains, allowing the effects of the various mutations on pathogenicity to be determined. Flagellar structures (Kennedy *et al.*, 1997; Rosey *et al.*, 1996), *tly* gene product activity (Hyatt *et al.*, 1994; ter Huurne *et al.*, 1992), and NADH oxidase function (Stanton *et al.*, 1999) were all implicated in mouse and/or pig intestinal colonization and virulence.

Gene Inactivation in *T. denticola*

Genes have also been inactivated by directed insertion in *T. denticola*. Li and Kuramitsu (Li and Kuramitsu, 1996) first demonstrated that electroporation could be used to transform *T. denticola*, and then inactivated *flgE* by insertion of an erythromycin-resistance (*erm*) cassette (Li *et al.*, 1996). Subsequently, several other genes were inactivated using the same resistance cassette (Fenno *et al.*, 1998; Kataoka *et al.*, 1997; Limberger *et al.*, 1999) (Table 1) and the mutants were shown to have phenotypes affecting the predicted functions (flagellar structure, chemotaxis, and surface characteristics). Unfortunately, *T. denticola* has no experimental disease model, so mutant phenotypes have not been assessed in a natural infection system.

Gene Inactivation in *B. burgdorferi*

In *B. burgdorferi*, most mutations were generated by allelic exchange with selection for the *gyrB*^{r} gene (Table 1), a mutated version of the endogenous *gyrB* gene that confers coumermycin A_1-resistance (Cou^r) (Rosa *et al.*, 1996). Samuels *et al.* developed this marker by selecting for *B.*

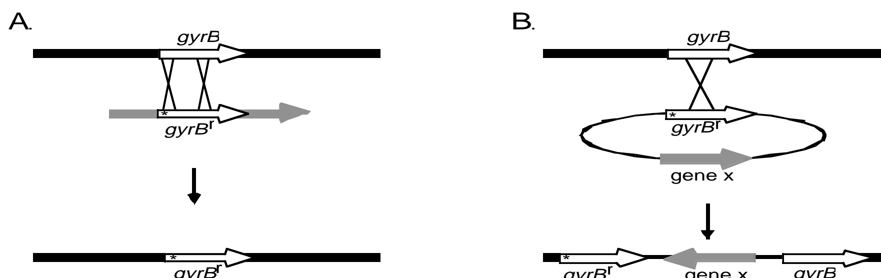

Figure 2. Recombination events at the *B. burgdorferi gyrB* locus. A. Recombination between transforming DNA and the chromosomal *gyrB* gene to generate a chromosomal *gyrB*^{r} gene. Gene conversion could yield the same product. B. Plasmid insertion via homologous recombination at the *gyrB* locus. Black bars represent *B. burgdorferi* genomic sequences; white arrows represent the *gyrB* or *gyrB*^{r} gene; and thin lines represent plasmid vector sequences. Asterisk (*) denotes the mutation in *gyrB*^{r} conferring coumermycin-resistance. It could occur in either copy of *gyrB* and is arbitrarily designated as shown.

Table 1. Spirochete Genes That Have Been Inactivated

Spirochete	Gene	Selected marker	Reference
B. hyodysenteriae	*tlyA*,	*kan*	ter Huurne *et al*., 1992
	flaA, *flaB*1	*kan*, *cat*	Kennedy *et al*., 1997; Rosey *et al*., 1995; Rosey *et al*., 1996
	*flaB*2, *flaB*3	*kan*, *cat*	Li and Charon, pers. comm.
	nox	*kan*, *cat*	Stanton *et al*., 1999
	nox-flaA	*kan*, *cat*	Humphrey *et al*., 1997
T. denticola	*flgE*	*erm*	Li *et al*., 1996
	dmcA	"	Kataoka *et al*., 1997
	msp	"	Fenno *et al*., 1998
	*tap*1	"	Limberger *et al*., 1999
B. burgdorferi	*ospC*	*gyrB*^{r}	Tilly *et al*., 1997
	*oppA*IV	"	Bono *et al*., 1998
	guaB	"	Tilly *et al*., 1998
	gac	"	Knight *et al*., 2000
	rpoS	"	Elias *et al*., 2000
	celB	"	Tilly, unpublished
	*oppA*V	*kan*	Bono *et al*., 2000
	*oppA*II	*kan*	Bono, unpublished
	fliF, *flaB*, *flaA*, *motA*	*kan*	Motaleb and Charon, pers. comm.

burgdorferi strains that were naturally Cou^r and then cloning the responsible gene (Samuels *et al*., 1994a; Samuels *et al*., 1994b). These studies marked a major breakthrough that allowed the first directed mutagenesis of *B. burgdorferi*, but the *gyrB*^{r} gene has a significant drawback as a selectable marker (Rosa *et al*., 1996). Since all *B. burgdorferi* carry a wild type copy of the *gyrB* gene in their chromosomes, the bacteria can also become Cou^r by recombination between the transforming DNA and the chromosomal copy (Figure 2A), without concomitant mutagenesis of the target gene. In fact, this is the vastly preferred event, found >99% of the time. Consequently, Cou^r transformants must be screened by PCR for those that actually have a mutation in the targeted gene (see, *e.g.*, Rosa *et al*., 1996).

Another breakthrough in selectable marker development for *B. burgdorferi* occurred when Stevenson *et al.* (1998) showed that recombinant plasmids could be integrated via homologous recombination, allowing assessment of gene activity without selection (Figure 2B, which is a variation on the event shown in Figure 1B). This method was used to show that the promoter of the Tn*903 kan* gene is expressed poorly in *B. burgdorferi* (Bono *et al*., 2000). However, linking a borrelial promoter to that gene increased expression levels, conferring kanamycin-resistance to high levels in *B. burgdorferi* (Bono *et al*., 2000). Since that innovation, the pace of gene inactivation has increased (Table 1; M.A. Motaleb and N.W. Charon, personal communication). Other methods that have been used in *B. burgdorferi* include inactivation via single crossover with a doubly-deleted gene, as diagrammed in Figure 1B (*oppA*V) (Bono *et al*., 2000), and point mutation via recombination linked to a selectable marker (*gac*) (Knight *et al*., 2000). Because we now have two selectable markers for *B. burgdorferi*, it has been possible to construct the first double mutant (J. Bono, unpublished). For reasons described below, these mutations have not been constructed in infectious isolates, so analysis of phenotypes with regard to infection of the tick or rodent host has not been possible.

Transformation of Infectious *B. burgdorferi*

Initial experiments demonstrated the feasibility of transforming *B. burgdorferi* by electroporation with non-clonal, culture-adapted spirochetes of the type strain B31 (Samuels, 1995; Samuels *et al*., 1994a). The objective of these experiments was to develop a method of transformation for *B. burgdorferi* and the choice of spirochetes was appropriate for this end. This strain grew well and formed colonies in solid medium at high efficiency, properties necessary for the selection of transformed bacteria. Subsequent experiments with this strain demonstrated the ability to inactivate genes by allelic exchange in *B. burgdorferi* (Rosa *et al*., 1996; Tilly *et al*., 1997). There was no reason to anticipate that genetic methods optimized with this strain would not be directly applicable to infectious clones, which in general grow more slowly and exhibit lower plating efficiencies.

Several years and many experiments later, we conclude that inactivating genes in infectious *B. burgdorferi* presents unanticipated challenges. The basic problem stems from their reduced capacity to be transformed by available methods. It is important to emphasize that transformation of infectious *B. burgdorferi* clones is possible, but at a significantly lower frequency than transformation of culture-attenuated clones. As discussed previously, the most common genetic event following electroporation with constructs containing the *gyrB*^{r} marker is allelic exchange at the chromosomal *gyrB* locus (Rosa *et al*., 1996). Allelic exchange at other targeted loci (using either the *gyrB*^{r} or *kan* gene as a selectable marker) occurs approximately 100-fold less frequently than at *gyrB* and is beyond the limit of detection in infectious *B. burgdorferi*. As a result, specific gene inactivation through allelic exchange has not been achieved in infectious *B. burgdorferi*.

There are two straightforward solutions to this problem: either the overall frequency of allelic exchange in *B. burgdorferi* (infectious and non-infectious alike) needs to be increased at least 100-fold, or the specific impediment to transformation of infectious spirochetes must be identified and overcome. We have pursued both routes in

attempts to introduce desired mutations into infectious *B. burgdorferi* clones. In subsequent sections, we discuss alternative methods of transformation or variations on electroporation as a means of improving overall transformation frequency.

Potential Reasons for Less Efficient Transformation of Infectious *B. burgdorferi*

A number of factors contribute to the difficulty in creating mutations in infectious *B. burgdorferi*. As discussed below, we have found that some infectious clones form dense aggregates in electroporation solution (EPS). Just as their surface properties prevent adequate resuspension in EPS, structural differences between infectious and non-infectious spirochetes could also affect the efficiency at which transforming DNA enters the bacteria during electroporation. This possibility could be addressed by a comparison of DNA delivery to both, using a transient assay with a reporter gene. Alternatively, a restriction/modification system present in infectious *B. burgdorferi*, but absent or inactive in culture-attenuated spirochetes, could result in rapid degradation of DNA even if it were efficiently introduced by electroporation.

Another possible reason for reduced transformation frequency could be that homologous recombination is more tightly constrained in infectious *B. burgdorferi*. In this case, transforming DNA would be efficiently delivered by electroporation and could persist transiently within infectious spirochetes, but recombination with the targeted locus would take place at a lower rate. Specific regulation of recombination has previously been demonstrated at the *vlsE* locus of infectious *B. burgdorferi*, in which recombination is undetectable in cultured spirochetes, but appears to be rapidly induced when spirochetes are introduced into the mammalian host (Zhang *et al.*, 1997; Zhang and Norris, 1998a; Zhang and Norris, 1998b). Identifying the reason for reduced transformation of infectious *B. burgdorferi* would suggest the best approach to overcoming this impediment.

Loss of Infectivity and Clonal Nature of *B. burgdorferi* with Laboratory Cultivation

The challenges associated with inactivating genes in infectious *B. burgdorferi* are not surprising when considered in the context of the organism. *Borrelia* are fastidious bacteria, with limited metabolic capacity (Barbour and Hayes, 1986; Fraser *et al.*, 1997; Fraser *et al.*, 1998; Fulton and Smith, 1960; Johnson, 1977). They exist in nature only within the arthropod vector or mammalian host (Felsenfeld, 1971). Although human diseases resulting from borrelial infections have been recognized for centuries, successful laboratory cultivation was only achieved by Kelly in 1971 (Kelly, 1971). His complex growth medium permitted the first continuous passage of the relapsing fever spirochete *B. hermsii*. Subsequent enrichments and modifications by Stoenner of the formulation facilitated growth from a smaller spirochetal inoculum and to higher cell densities (Stoenner *et al.*, 1982), and were instrumental in the isolation and identification of *B. burgdorferi* as the agent of Lyme disease (Barbour, 1984; Burgdorfer *et al.*, 1982).

The ability to establish and maintain laboratory strains of *B. burgdorferi* and *B. hermsii* led to the recognition that spirochetes become "culture-adapted," exhibiting growth rates and characteristics different from primary isolates (Barbour, 1984; Stoenner *et al.*, 1982). These and additional studies demonstrated that continuously passaged *Borrelia* lost infectivity for rodents (Hyde and Johnson, 1986; Johnson *et al.*, 1984; Schwan *et al.*, 1988; Stoenner *et al.*, 1982). Culture adaptation correlated with loss of some plasmids and occurred after limited *in vitro* passage (Barbour, 1988; Barbour *et al.*, 1982; Hyde and Johnson, 1986; Schwan *et al.*, 1988; Simpson *et al.*, 1990; Xu *et al.*, 1996). Presumably, laboratory propagation selects for variants that flourish in culture medium, but are compromised for growth in mammals. In contrast, Kelly's original medium formulation, while sub-optimal for growth to high cell density, permitted retention of spirochetal infectivity for mice after at least 8 months of continuous cultivation (Kelly, 1971).

The considerations mentioned above highlight the importance of establishing and maintaining an infectious, clonal population of *B. burgdorferi* for genetic studies. This is an essential component of experiments designed to assess the contribution of particular gene products to transmission, infection or disease. A mutant phenotype cannot be ascribed to an inactivated gene if other undefined differences exist between mutant and wild type organisms. These considerations also underline the importance of genetic complementation, which has yet to be performed in *B. burgdorferi*, in analyzing mutants.

Methods of Transformation

Gene inactivation in *T. denticola*, *B. hyodysenteriae*, and *B. burgdorferi* has been accomplished by electro-transformation using fairly similar conditions (Li and Kuramitsu, 1996; Li *et al.*, 1996; Limberger *et al.*, 1999; Rosey *et al.*, 1995). Differences are mainly due to specific physiological or growth requirements and the availability of selectable markers. Here, we review methodological aspects of gene transfer and gene inactivation for *B. burgdorferi*.

Strain and Growth Conditions

A clonal strain of high-passage, non-infectious B31 (B31-A), isolated from an individual colony, has been used for most of the transformation experiments that we describe (Bono *et al.*, 2000). B31-A grows relatively quickly, with a doubling time in liquid medium of approximately six hours. This strain forms dense colonies in solid medium that arise within five to eight days in the absence of selection. Colonies of transformed bacteria expressing an antibiotic resistance marker generally appear six to ten days after imposing selection. Plating efficiency, which is the ratio of the number of colonies formed to the number of viable spirochetes plated, is a useful measure of *B. burgdorferi* growth capacity in solid medium. The plating efficiency of B31-A ranges between 60% and 90%.

Preparation of Electrocompetent *B. burgdorferi*

Electrocompetent *B. burgdorferi* are prepared by an adaption of protocols previously used for other bacteria such as *E. coli* (Dower *et al.*, 1988; Miller, 1994). The original protocol devised for *B. burgdorferi* involves a series of washes in cold phosphate buffered saline (PBS) and cold EPS (0.27M sucrose, 15% (v/v) glycerol), during which the cell suspension is concentrated to a final concentration ranging from 10^9-10^{10} cells/ml in EPS, and subsequently

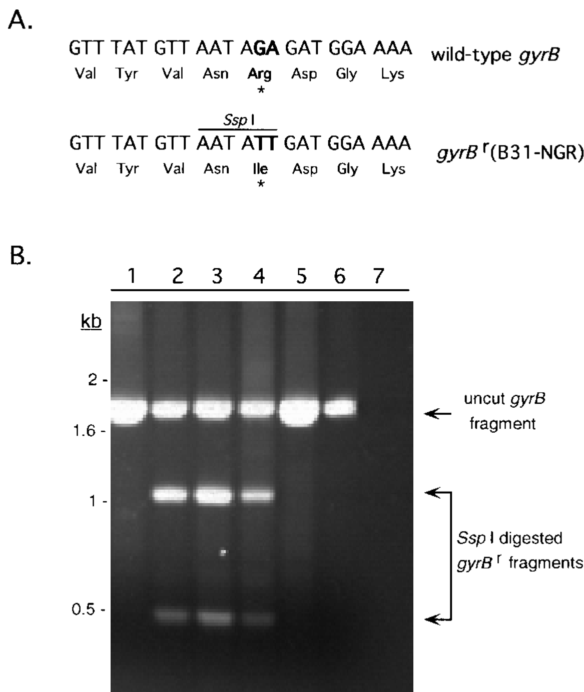

Figure 3. Discriminating between spontaneous mutants and transformed Cou^r colonies. A. Unique restriction site in *gyrB*^r of transformants. The *gyrB*^r gene from *B. burgdorferi* strain B31-NGR (Rosa *et al.*, 1996) contains an *Ssp*I site created by the combination of a silent mutation and the base pair change that results in the arginine 133 to isoleucine (R133I) mutation (Samuels *et al.*, 1994a). The dinucleotide change is highlighted in bold letters; asterisks indicate the R133I mutation. B. Ethidium bromide-stained gel of *gyrB* PCR products digested with *Ssp*I. *Ssp*I-digestion of *gyrB*^r amplified from transformants yields two fragments, whereas the *gyrB*^r product from spontaneous mutants is uncut. Some uncut B31-NGR *gyrB*^r-product remains because the digestions are incomplete. Sources of DNA from which *gyrB*^r was amplified: lane 1, spontaneous coumermycin-resistant *B. burgdorferi* mutant colony. Lane 2, *B. burgdorferi* transformant colony containing *gyrB*^r of NGR. Lane 3, B31-NGR genomic DNA. Lane 4, *E. coli* plasmid harboring *gyrB*^r from B31-NGR. Lane 5, coumermycin-sensitive *B. burgdorferi* colony. Lane 6, genomic DNA from B31 containing wild-type *gyrB*. Lane 7, no DNA.

stored at -70°C until use (Samuels, 1995). We have found that elimination of the PBS washes and reduction of the number of EPS washes to as few as two does not significantly change the electrocompetence of *B. burgdorferi*. By dark field microscopy, a suspension of electrocompetent spirochetes should be dense, but not aggregated. Since spirochetes are non-motile in EPS, motility cannot be used as an indication of viability of the competence preparation.

Standard Electroporation Conditions

Electroporation of a 50 µl suspension of electrocompetent *B. burgdorferi* is routinely performed in a 0.2 cm electroporation cuvette with a pulse generator set at 2.5 kV, 25µF, and 200Ω, resulting in an electrical field strength of 12.5kV/cm and a time constant between 4 and 5 ms (Samuels, 1995). Because survival of other bacteria in EPS after electroporation is very poor (Dower *et al.*, 1988), electroporated cells are resuspended in 5-10 ml liquid medium immediately after electroporation, followed by overnight recovery at 35°C.

Selection for Transformants

Selection for transformants after electroporation of B31-A with donor DNA containing *gyrB*^r or *kan* as a selectable marker is usually accomplished by growth in solid medium in the presence of antibiotic. If the transforming DNA contains both markers, selection with both antibiotics in liquid medium can also be used. Selection in liquid medium with only one antibiotic allows outgrowth of spontaneous resistance mutants. When *gyrB*^r is used for allelic exchange at a targeted locus, an additional problem of selection in liquid is the background of transformants with recombination at the endogenous *gyrB* locus, as discussed above.

Spontaneous resistance to coumermycin or kanamycin arises at a frequency of approximately 10^{-7} to 10^{-8}. Kan^r transformants can be distinguished from spontaneous mutants by PCR screening of resistant colonies, demonstrating the presence of the *kan* gene at the targeted site. Transformation with the NGR *gyrB*^r allele introduces an *Ssp*I site created by the combination of a silent mutation and the base pair change that causes the arginine 133 to isoleucine (R133I) mutation (Figure 3A)(Samuels *et al.*, 1994a). Therefore, the presence of NGR-*gyrB*^r can be assayed by PCR amplification of the gene from colonies, followed by *Ssp*I-digestion (Elias *et al.*, 2000). *gyrB*^r PCR products of *B. burgdorferi* transformants are cut by *Ssp*I, whereas amplified products from spontaneous mutants are not (Figure 3B).

Transformation with *gyrB*^r as a Selectable Marker

Preceding the availability of *kan* as a selectable marker, *gyrB*^r was used for allelic exchange to disrupt a number of *B. burgdorferi* genes (Table 1). When B31-A is transformed with *gyrB*^r, Cou^r colonies are obtained at a transformation frequency (number of transformants per total colony-forming units) of approximately 10^{-5}. However, since only 0.1-0.5% of the transformants are the result of allelic exchange at the targeted locus, targeted mutations in B31-A occur at a frequency of approximately 10^{-7}. Although we have seen some variation, similar frequencies were obtained in the majority of transformations involving targeted insertions on circular or linear plasmids, or the chromosome.

Transformation with *kan* as a Selectable Marker

The demonstration that the *kan* gene expressed from a borrelial promoter confers high-level kanamycin resistance in *B. burgdorferi* greatly facilitated targeted mutagenesis (Bono *et al.*, 2000). Gene inactivation in *B. burgdorferi* by allelic exchange with *kan* as a selectable marker occurs at a frequency of approximately 10^{-7} (Bono *et al.*, 2000). Although this is 100-fold lower than the overall transformation frequency with *gyrB*^r, allelic exchange at the targeted locus happens at the same frequency for both antibiotic resistance markers. The obvious advantage of *kan* over *gyrB*^r stems from the absence of an endogenous locus at which recombination could occur, resulting in 100-fold lower “background”.

Frequency of Integration

Since the demonstration by Stevenson *et al.* that heterologous DNA can be integrated in the borrelial genome via single cross-over mediated by homologous DNA, this method has been used on several occasions to

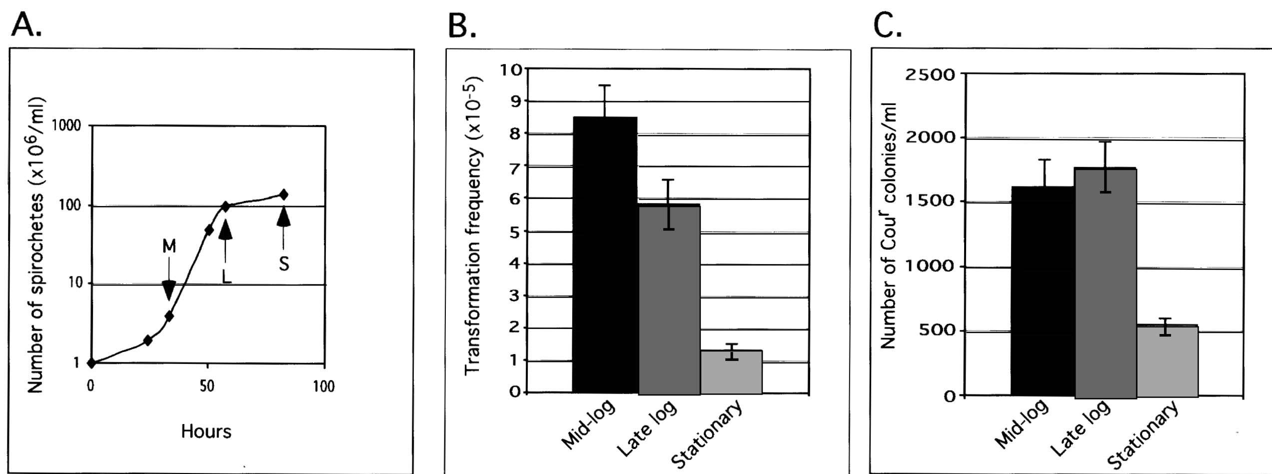

Figure 4. Effect of growth phase on transformation of *B. burgdorferi*. A. Growth curve of B31-A. Arrows indicate the time-points at which culture aliquots were harvested for preparation of electrocompetent cells. M, mid-log phase; L, late log phase; S, stationary phase. B. Transformation frequency of B31-A cells in mid-log, late log and stationary phase. Transformation was performed as described in the text, with 50 μg of a *gyrB*ʳ-containing plasmid (Stevenson *et al.*, 1998), followed by selection in solid medium in the presence of 0.5 μg/ml coumermycin A_1. The transformation frequency is the number of transformants (CFU on plates with coumermycin) relative to the total number of bacteria (CFU on plates without antibiotic). C. Number of Couʳ colonies/ml obtained in the same experiment. Values represent the mean and standard error obtained from three plates for each condition.

integrate circular DNA (Bono *et al.*, 2000; Stevenson *et al.*, 1998). Surprisingly, this event occurs at the same frequency as targeted allelic exchange, although the latter event requires a double cross-over. Similarly, when a genetic construct is introduced into *B. burgdorferi* as a circular DNA molecule containing *gyrB*ʳ, allelic exchange with the chromosomal *gyrB* gene (or gene conversion of that locus) is favored over integration of the entire plasmid at this locus (Elias *et al.*, 2000).

Parameters Affecting Electrotransformation

In many instances, it is difficult to investigate the effect of a specific parameter on transformation frequency in a quantitative fashion because of the slow and cumbersome nature of plating and otherwise manipulating *B. burgdorferi*. In the following experiments, we studied the effects of bacterial growth phase, amount and form of transforming DNA, and field strength on electrotransformation of *B. burgdorferi*.

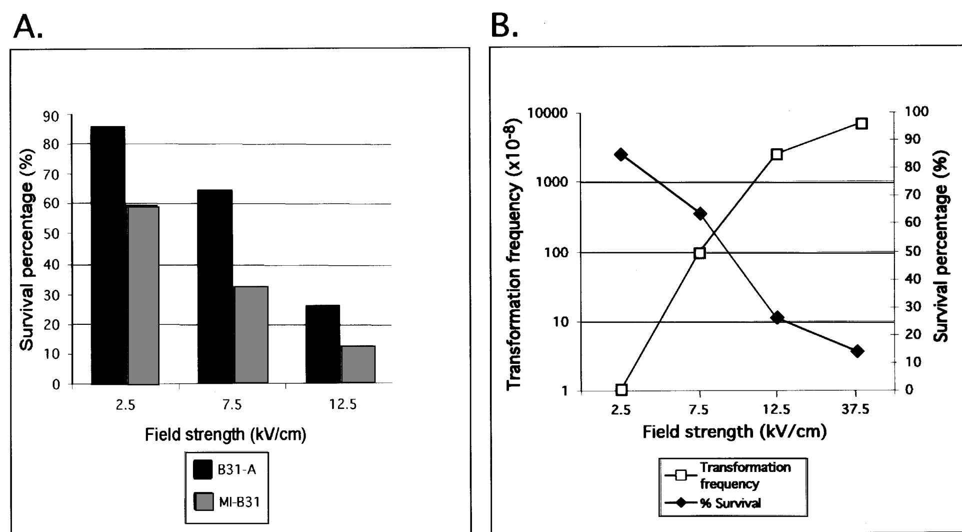

Figure 5. Effect of electroporation field strength on survival and transformation of *B. burgdorferi*. A. Survival of high-passage, non-infectious B31-A and low-passage, infectious MI-B31 at different field strengths. B. Effect of field strength on survival and transformation frequency of B31-A. The field-strength of 37.5 kV/cm was the sum of three pulses at electroporation conditions described in the text. The number of live spirochetes was determined before and after electroporation using the LIVE/DEAD® *Bac*Light™ bacterial viability kit. The survival percentage was calculated as the ratio of the number of live bacteria after electroporation relative to the number before electroporation, multiplied by 100. Transformation frequency was determined as described in the legend to Figure 4.

Growth Phase and Cell Density

Growth phase and cell density can influence transformation in different ways, depending on the bacterial species. For example, *E. coli* transforms best in early to mid-log phase (Miller, 1994), whereas late log and early stationary phase are optimal for some gram-positive organisms, including *Clostridium perfringens* (Allen and Blaschek, 1990; Phillips-Jones, 1990). Similarly, transformation efficiency (transformants per microgram of DNA) is a direct function of cell density for many bacteria including *E. coli* (Miller *et al.*, 1988). Some bacteria that secrete nucleases transform better at lower concentrations (Miller, 1994). Although *B. burgdorferi* cell density obviously increases with growth, there is not always an absolute correlation between the number of spirochetes and the growth phase. For example, a culture of B31-A at a density of 10^8cells/ml may be in late log or early stationary phase. Therefore, the point in the growth curve at which cells are harvested is the defining feature.

We compared the numbers of transformants obtained with B31-A cultures harvested in mid-log, late log and early stationary phase (Figure 4). Transformation frequency is highest with spirochetes harvested in mid-log phase and decreases in later growth phases (Figure 4B). This is also reflected in the total number of Cou^r colonies obtained (Figure 4C). These experiments show that *B. burgdorferi* transforms better in log than in stationary phase, but the overall yield of transformants is influenced by both the transformation frequency and the total number of spirochetes in the electroporation. Taking both these factors into account, the optimal timepoint to harvest a borrelial culture for transformation is late log phase.

Amount and Form of Transforming DNA

With regard to the amount of DNA used in the transformation, our general observation is that "more is better," at least in the range of 0.5 to 20 µg. We have seen no inhibition of transformation with up to 50 µg of DNA. This suggests that with the standard conditions used to transform *B. burgdorferi* by electroporation, both the amount of transforming DNA and the number of recipient bacteria can be limiting factors.

B. burgdorferi has been transformed with both linear and circular DNA from a variety of sources. Directly comparing these results is difficult because the molar equivalents of the relevant DNA (antibiotic resistance gene and/or DNA containing the targeted locus) differ greatly between experiments. For example, the molarity of a specific sequence contained within one microgram of a short PCR fragment or one microgram of total *B. burgdorferi* plasmid DNA differ by 1000-fold. Despite this caveat, transformations with circular or linearized plasmids result in similar transformation frequencies, suggesting that the physical shape of the DNA does not significantly affect transformation in *B. burgdorferi*. In contrast, *T. denticola* appears to be more efficiently transformed with linear than circular plasmid DNA (Limberger *et al.*, 1999).

Although spirochetes have been transformed with both methylated and unmethylated DNA from *E. coli*, it is unclear whether modification of the donor DNA has an effect on transformation. Allelic exchange at a targeted site in *B. burgdorferi* appears to be more efficient (if normalized to the molar equivalent of relevant DNA) when the transforming plasmid DNA is from *B. burgdorferi* rather than *E. coli*. There is evidence suggesting that *Borrelia* DNA can be methylated, although variable results were obtained with DNA from different *Borrelia* species (Hughes and Johnson, 1990). Homologs of DNA methyltransferases are present in the genomes of *B. burgdorferi* and *T. pallidum,* but no complete restriction-modification systems have been identified in either spirochete. (Casjens *et al.*, 2000; Fraser *et al.*, 1997; Fraser *et al.*, 1998; Subramanian *et al.*, 2000).

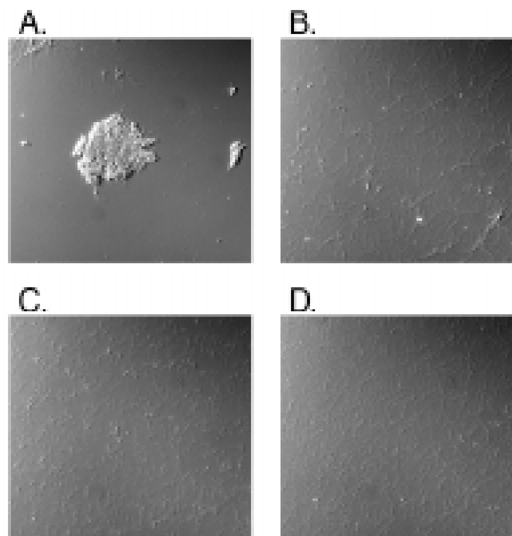

Figure 6. Resuspension of infectious *B. burgdorferi* in EPS. Comparable cultures of infectious *B. burgdorferi* clone B31-4A and strain MI-B31 were pelleted by centrifugation and resuspended in electroporation solution (EPS). Spirochetes in EPS were subsequently pelleted again and resuspended in culture medium. B31-4A in EPS (panel A) and culture medium (panel B); MI-B31 in EPS (panel C) and culture medium (panel D).

Effect of Field Strength on Survival and Transformation Frequency of *B. burgdorferi*

Electroporation conditions can have a significant effect on bacterial survival as well as transformation frequency and efficiency. Cell viability is crucial for recovery of transformants after electroporation, but an electrical field strong enough to kill an empirically determined portion of transformed cells is necessary to obtain an optimal transformation frequency (Dower *et al.*, 1988; Weaver, 1995). We, therefore, investigated the effect of the field strength on transformation frequency and survival of *B. burgdorferi*.

Cell viability was determined for high passage, non-infectious B31-A and low passage, infectious B31 (MI-B31) after electroporation with field strengths between 2.5kV/cm and 12.5kV/cm, using the LIVE/DEAD bacterial viability kit (Molecular Probes, Wilsonville, Oregon). Survival of both strains gradually decreased with increasing field strength, but MI-B31 was more sensitive to electroporation than B31-A (Figure 5A). Accurately determining cell viability after electroporation could be problematic because the permeability of the cell membrane is altered and membrane-based stains, such as the LIVE/DEAD kit used for this study, are not necessarily valid (Weaver, 1995). However, the results obtained with the vital stain were in good correlation with cell densities of cultures incubated overnight after electroporation, so we believe that this

method can be used to compare bacterial survival following different electroporation conditions.

In order to investigate the relationship between field strength, cell viability and transformation frequency, B31-A was transformed with field strengths ranging from 2.5 to 37.5 kV/cm (Figure 5B). Twenty-five µg of plasmid DNA containing *gyrB*^{r} was used and the number of Cou^r colonies was determined. A field strength of 2.5 kV/cm did not result in detectable transformants in a culture with approximately 10^8 viable spirochetes per ml. The maximum transformation frequency of 6.5×10^{-5} was obtained with 37.5kV/cm, which resulted in approximately 14% viable cells. The transformation frequency at 12.5kV/cm was slightly lower (2.5×10^{-5}), but approximately 25% of the cells remained viable after electroporation, so more transformants were obtained. A field strength between 12.5kV/cm and 37.5kV/cm for electroporation of B31-A, therefore, seems optimal.

Effect of Surface Properties on Electroporation

Initial attempts to recover allelic exchange events in a wild type background were done with infectious clone B31-4A. Transformation of B31-4A was impeded by the propensity of these spirochetes to aggregate in EPS. Attempts to prepare B31-4A for electroporation by centrifugation and resuspension in EPS were frustratingly unsuccessful because spirochetes formed dense aggregates (Figure 6A), adhered to the sides of tubes and pipettes and were inaccessible for electroporation. Aggregated spirochetes retained viability and were easily resuspended in culture medium (Figure 6B).

Differences in outer surface properties between infectious and non-infectious clones presumably dictate aggregation in EPS. These clones also differ in growth rates and cell densities achieved in liquid medium, as well as colony morphology in solid medium. Low passage, infectious isolates of *B. burgdorferi* frequently form macro-aggregates in culture medium, whereas culture-adapted strains grow as dispersed spirochetes. However, even B31-4A spirochetes growing freely in culture medium aggregate in the presence of EPS.

Many factors were varied in an attempt to understand aggregation of B31-4A in EPS. A 1:1 mixture of culture medium with EPS still yielded spirochetal aggregates, although a comparable mixture of medium with either sucrose or glycerol did not, but remained incompatible with electroporation. Adding cholesterol to EPS, although compatible with electroporation, had no effect on spirochete aggregation. Growth of B31-4A in sterilized "conditioned medium," in which high passage B31 was previously grown, also had no effect. Growing B31-4A at 23°C and then shifting to 35°C, which induces synthesis of a number of new outer surface proteins (Schwan *et al.*, 1995; Stevenson *et al.*, 1995), did not alter the EPS phenotype.

In contrast to these approaches, adding heparin (25µg/ml) did permit efficient resuspension of B31-4A in EPS. However, the elation was short-lived when it became apparent that the transformation frequency for B31-4A by electroporation was still approximately 100-fold less than non-infectious B31, even when both cultures were resuspended comparably in EPS with heparin. Clearly, properties other than clumping in EPS contribute to the reduced frequency of transformation with B31-4A. We have since confirmed this result with an independently maintained culture of infectious B31 (MI-B31) that does not aggregate in EPS (Figure 6C and D), yet is transformed at a comparably low frequency.

Other Methods of Gene Transfer

Transformation

A number of transformation methods have been developed for use in other bacteria and some have been tried in *B. burgdorferi*, with varying degrees of success. We found that $CaCl_2$-mediated transformation, which was the first method for naked DNA introduction into *E. coli* (Mandel and Higa, 1970), was successful for generating Cou^r transformants in high passage *B. burgdorferi* B31-A, but unsuccessful for low passage clone B31-4A (A. Elias, unpublished).

Treatment of *E. coli* with DMSO and PEG in the presence of Mg^{++} also promoted efficient uptake of plasmid DNA (Chung *et al.*, 1989). We applied this method to *B. burgdorferi*, using borrelial culture medium in place of the L broth component of the transformation and storage solution (K. Tilly and P. Rosa, unpublished). We found transformation frequencies (using a PCR fragment carrying the *gyrB*^{r} gene) similar to those found for electroporation, but infectious clone B31-4A still transformed approximately 100-fold less frequently than B31-A. This method worked best with relatively small DNA fragments (~2 kb) and worked progressively less well with larger fragments (~4 kb and ~14 kb). These results confirm that B31-4A has an obstacle to transformation other than differences in aggregation properties in EPS, consistent with effects at the level of DNA uptake, restriction, or recombination.

Encouraging results were recently obtained when looking for natural gene transfer among *B. burgdorferi* spirochetes (P. Rosa, unpublished). Phylogenetic evidence suggested that such events occur (Livey *et al.*, 1995), so Kan^r and Cou^r clones were co-inoculated at low density and allowed to grow together to stationary phase. The resultant mixed culture was plated on medium containing both drugs. Some colonies arose that carried both markers (*i.e.*, the Tn*903 kan* gene and the NGR *gyrB*^{r} allele), but we have not yet determined the direction of gene transfer. A number of other methods to promote gene transfer into *B. burgdorferi* and the other spirochetes are being tested or are theoretically possible. These include conjugation, which has been demonstrated to be useful for moving DNA from *E. coli* into other bacteria ranging from *Streptococcus* to *Streptomyces* (Amabile-Cuevas and Chicurel, 1992). Preliminary experiments have faced the problem of adequately killing the *E. coli* donor, which grows much faster than the *B. burgdorferi* recipient (K. Tilly and P. Rosa, unpublished). A second possibility is restriction-enzyme mediated integration (Brown *et al.*, 1996), which facilitated stable transformation of *Candida albicans* by electroporation with a DNA fragment with cohesive ends in combination with a small amount of the restriction enzyme that produces those cohesive ends. Transformants had the electroporated DNA integrated into one of the corresponding restriction sites within the recipient genome (Brown *et al.*, 1996). Liposome-mediated transformation works in Archaea of the genus *Methanosarcina* (Metcalf *et al.*, 1997), and may be worth testing in spirochetes. Direct transfer of genetic material from one spirochete to another by co-electroporation could also be investigated.

Treatments to stimulate recombination might also

increase transformation frequency. Possibilities include denaturing the transforming DNA (Oh and Chater, 1997) or treatment of spirochetes with mutagens like mitomycin C and ultraviolet light (Walker, 1996). Heating *T. denticola* for 30 min at 50°C prior to electroporation yielded increased numbers of transformants (J. P. Tsai and W. Shi, personal communication), and similar treatments may also facilitate transformation of other spirochetes.

Phage-Mediated Transduction

Currently, the only spirochete with a useful alternative gene transfer system is *B. hyodysenteriae*, in which a generalized transduction system is workable. The first observations leading to the discovery of the transducing phage were that *B. hyodysenteriae* has a unique size class of extrachromosomal DNA (Combs *et al*., 1992; Turner and Sellwood, 1997) and that it has a mitomycin-inducible prophage (Humphrey *et al*., 1995). Surprisingly, the DNA within the virion comprises total chromosomal DNA, in segments of 7.5 kb (Humphrey *et al*., 1997). These authors realized that this property of packaging host DNA should allow the phage to carry out generalized transduction and showed this to be true (Humphrey *et al*., 1997). Mixing mitomycin-induced and uninduced cultures carrying different selectable markers also allowed gene transfer, presumably via transduction. This phage appears to be defective and unable to form plaques, and a genome (encoding the virion proteins) has yet to be described.

B. burgdorferi and *Leptospira biflexa*, a saprophytic leptospire, also have phages (Eggers and Samuels, 1999; Saint Girons *et al*., 1990). The encouraging results with transduction in *B. hyodysenteriae* prompted these investigators to pursue various aspects of those phages as genetic tools. Saint Girons *et al.* (personal communication) have used part of one of the *L. biflexa* phages as the basis for a shuttle vector (see review by Saint Girons *et al*., this volume). Eggers *et al*. (personal communication) have demonstrated that the *B. burgdorferi* phage will move a drug-resistance marker from one *B. burgdorferi* strain to another, suggesting that it may be useful for genetic transduction (see review by Eggers *et al*., this volume).

Perspectives

This is an exciting time for researchers studying spirochetes. Recently developed genetic methods and selectable markers have facilitated directed mutagenesis of some of these bacteria. Comparing known techniques for genetic manipulation should help with developing genetic systems for spirochetes that currently lack them. Spirochetes, however, are diverse so specific methods or tools may not be generally applicable. Nevertheless, the burgeoning genomic information and sophisticated molecular biological techniques allow increasingly detailed questions to be asked and answered. This fortuitous combination of circumstances should further our understanding of the transmission and pathogenesis of spirochetal infections.

Acknowledgements

We thank Tom Schwan and Bob Karstens for assistance with testing low passage *B. burgdorferi* clones for infectivity in mice, Tom Schwan for photographs of *B. burgdorferi* in EPS and culture medium, and Brian Stevenson for an early version of Figure 1. Gary Hettrick and Anita Golden provided expert assistance with figure preparation. Paul Policastro and George Chaconas made useful comments on the manuscript. We thank Wenyuan Shi, Isabelle Saint Girons, Christian Eggers, and Nyles Charon for communicating results prior to publication.

References

Allen, S.P., and Blaschek, H.P. 1990. Factors involved in the electroporation-induced transformation of *Clostridium perfringens*. FEMS Microbiol. Lett. 58**:** 217-220.

Amabile-Cuevas, C.F., and Chicurel, M.E. 1992. Bacterial plasmids and gene flux. Cell 70**:** 189-199.

Barbour, A.G. 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. 57**:** 521-525.

Barbour, A.G. 1988. Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. J. Clin. Microbiol. 26**:** 475-478.

Barbour, A.G., and Hayes, S.F. 1986. Biology of *Borrelia* species. Microbiol. Rev. 50**:** 381-400.

Barbour, A.G., Tessier, S.L., and Stoenner, H.G. 1982. Variable major proteins of *Borrelia hermsii*. J. Exp. Med. 156**:** 1312-1324.

Berg, C.M., and Berg, D.E., 1996. Transposable element tools for microbial genetics, In: *Escherichia coli* and *Salmonella*. Neidhardt, F. C., Curtiss III, R. C., Ingraham, J. L., Lin, E. C. C., Low, K. B. *et al*., eds. ASM Press, Washington D.C. p. 2588-2612.

Birkhaug, K., 1942. Otto H.F. Obermeier: February 13, 1843 - August 20, 1873, In: Relapsing Fever in the Americas, Moulton, F. R., ed. Am. Assn. Adv. Sci., Dallas, Texas, p. 7-14.

Bono, J.L., Elias, A.F., Kupko III, J.J., Stevenson, B., Tilly, K., and Rosa, P. 2000. Efficient targeted mutagenesis in *Borrelia burgdorferi*. J. Bacteriol. 182**:** 2445-2452.

Bono, J.L., Tilly, K., Stevenson, B., Hogan, D., and Rosa, P. 1998. Oligopeptide permease in *Borrelia burgdorferi*: putative peptide-binding components encoded by both chromosomal and plasmid loci. Microbiol. 144**:** 1033-1044.

Brown, D.H., Jr., Slobodkin, I.V., and Kumamoto, C.A. 1996. Stable transformation and regulated expression of an inducible reporter construct in *Candida albicans* using restriction enzyme-mediated integration. Mol. Gen. Genet. 251**:** 75-80.

Burgdorfer, W., Barbour, A.G., Hayes, S.F., Benach, J.L., Grunwaldt, E., and Davis, J.P. 1982. Lyme disease - a tick-borne spirochetosis? Science 216**:** 1317-1319.

Casjens, S., Palmer, N., van Vugt, R., Huang, W.M., Stevenson, B., Rosa, P., Lathigra, R., Sutton, G., Peterson, J., Dodson, R.J., D., H., Hickey, E., Gwinn, M., White, O., and Fraser, C. 2000. A bacterial genome in flux: The twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi.* Mol. Microbiol. 35**:** 490-516.

Chung, C.T., Niemla, S.L., and Miller, R.H. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA 86**:** 2172-2175.

Combs, B.G., Hampson, D.J., and Harders, S.J. 1992. Typing of Australian isolates of *Treponema hyodysenteriae* by serology and by DNA restriction endonuclease analysis. Vet. Microbiol. 31**:** 273-285.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic. Acids Res. 16**:** 6127-6145.

Eggers, C.H., and Samuels, D.S. 1999. Molecular evidence for a new bacteriophage of *Borrelia burgdorferi*. J. Bacteriol. 181**:** 7308-7313.

Elias, A.F., Bono, J.L., Carroll, J.A., Stewart, P., Tilly, K., and Rosa, P. 2000. Altered stationary phase response in a *Borrelia burgdorferi rpoS* mutant. J. Bacteriol. 182**:** 2909-2918.

Felsenfeld, O., 1971. Borrelia. Strains, Vectors, Human and Animal Borreliosis. Warren H. Green, Inc., St. Louis, MO. p. 3-15.

Fenno, J.C., Wong, G.W., Hannam, P.M., and McBride, B.C. 1998. Mutagenesis of outer membrane virulence determinants of the oral spirochete *Treponema denticola*. FEMS Microbiol. Lett. 163**:** 209-215.

Fraser, C.M., Casjens, S., Huang, W.M., Sutton, G.G., Clayton, R., Lathigra, R., White, O., Ketchum, K.A., Dodson, R., Hickey, E.K., Gwinn, M., Dougherty, B., Tomb, J.-F., Fleischmann, R.D., Richardson, D., Peterson, J., Kerlavage, A.R., Quackenbush, J., Salzberg, S., Hanson, M., van Vugt, R., Palmer, N., Adams, M.D., Gocayne, J., Weidmann, J., Utterback, T., Watthey, L., McDonald, L., Artiach, P., Bowman, C., Garland, S., Fujii, C., Cotton, M.D., Horst, K., Roberts, K., Hatch, B., Smith, H.O., and Venter, J.C. 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi.* Nature 390**:** 580-586.

Fraser, C.M., Norris, S.J., Weinstock, G.M., White, O., Sutton, G.G., Dodson, R., Gwinn, M., Hickey, E.K., Clayton, R., Ketchum, K.A., Sodergren, E., Hardham, J.M., McLeod, M.P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J.K., Chidambaram, M., Utterback, T., McDonald,

L., Artiach, P., Bowman, C., Cotton, M.D., Venter, J.C., *et al.* 1998. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. Science 281**:** 375-388.

Fulton, J.D., and Smith, P.J.C. 1960. Carbohydrate metabolism in *Spirochaeta recurrentis*: 1. The metabolism of spirochaetes *in vivo* and *in vitro*. Biochem. J. 76**:** 491-499.

Harris, D.L., Glock, R.D., Christensen, C.R., and Kinyon, J.M. 1972. Swine dysentery. I. Inoculation of pigs with *Treponema hyodysenteriae* (new species) and reproduction of the disease. Vet. Med. Small Animal Clin. 67**:** 61-64.

Hughes, C.A.N., and Johnson, R.C. 1990. Methylated DNA in *Borrelia* species. J. Bacteriol. 172**:** 6602-6604.

Humphrey, S.B., Stanton, T.B., and Jensen, N.S. 1995. Mitomycin C induction of bacteriophages from *Serpulina hyodysenteriae* and *Serpulina innocens*. FEMS Microbiol. Lett. 134**:** 97-101.

Humphrey, S.B., Stanton, T.B., Jensen, N.S., and Zuerner, R.L. 1997. Purification and characterization of VSH-1, a generalized transducing bacteriophage of *Serpulina hyodysenteriae*. J. Bacteriol. 179**:** 323-329.

Hyatt, D.R., ter Huurne, A.A.H.M., van der Zeijst, B.A.M., and Joens, L.A. 1994. Reduced virulence of *Serpulina hyodysenteriae* hemolysin-negative mutants in pigs and their potential to protect pigs against a challenge with a virulent strain. Infect. Immun. 62**:** 2244-2248.

Hyde, F.W., and Johnson, R.C. 1986. Genetic analysis of *Borrelia*. Zbl. Bakt. Hyg. Ser. A 263**:** 119-122.

Johnson, R.C. 1977. The spirochetes. Ann. Rev. Microbiol. 31**:** 89-106.

Johnson, R.C., Marek, N., and Kodner, C. 1984. Infection of Syrian hamsters with Lyme disease spirochetes. J. Clin. Microbiol. 20**:** 1099-1101.

Kataoka, M., Li, H., Arakawa, S., and Kuramitsu, H. 1997. Characterization of a methyl-accepting chemotaxis protein gene, *dmcA*, from the oral spirochete *Treponema denticola*. Infect. Immun. 65**:** 4011-4016.

Kelly, R. 1971. Cultivation of *Borrelia hermsi.* Science 173**:** 443-444.

Kennedy, M.J., Rosey, E.L., and Yancey, R.L., Jr. 1997. Characterization of *flaA*$^-$ and *flaB*$^-$ mutants of *Serpulina hyodysenteriae* : both flagellin subunits, FlaA and FlaB, are necessary for full motility and intestinal colonization. FEMS Microbiol. Lett. 153**:** 119-128.

Knight, S.W., Kimmel, B.J., Eggers, C.H., and Samuels, D.S. 2000. Disruption of the *Borrelia burgdorferi gac* gene, encoding the naturally synthesized GyrA C-terminal domain. J. Bacteriol. 182**:** 2048-2051.

Leadbetter, J.R., Schmidt, T.M., Graber, J.R., and Breznak, J.A. 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283**:** 686-689.

Li, H., and Kuramitsu, H.K. 1996. Development of a gene transfer system in *Treponema denticola* by electroporation. Oral Microbiol. Immunol. 11**:** 161-165.

Li, H., Ruby, J., Charon, N., and Kuramitsu, H. 1996. Gene inactivation in the oral spirochete *Treponema denticola*: construction of an *flgE* mutant. J. Bacteriol. 178**:** 3664-3667.

Limberger, R.J., Slivienski, L.L., Izard, J., and Samsonoff, W.A. 1999. Insertional inactivation of *Treponema denticola tap1* results in a nonmotile mutant with elongated flagellar hooks. J. Bacteriol. 181**:** 3743-3750.

Livey, I., Gibbs, C.P., Schuster, R., and Dorner, F. 1995. Evidence for lateral transfer and recombination in OspC variation in Lyme disease *Borrelia*. Mol. Microbiol. 18**:** 257-269.

Mandel, M., and Higa, A. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53**:** 159-162.

Metcalf, W.W., Zhang, J.K., Apolinario, E., Sowers, K.R., and Wolfe, R.S. 1997. A genetic system for *Archaea* of the genus *Methanosarcina*: liposome-mediated transformation and construction of shuttle vectors. Proc. Natl. Acad. Sci. U S A 94**:** 2626-2631.

Miller, J.F. 1994. Bacterial transformation by electroporation. Meth. Enzymol. 235**:** 375-385.

Miller, J.F., Dower, W.J., and Tompkins, L.S. 1988. High-voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proc. Natl. Acad. Sci. U S A 85**:** 856-860.

Oh, S.H., and Chater, K.F. 1997. Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms. J. Bacteriol. 179**:** 122-127.

Phillips-Jones, M.K. 1990. Plasmid transformation of *Clostridium perfringens* by electroporation methods. FEMS Microbiol. Lett. 54**:** 221-226.

Rosa, P., Samuels, D.S., Hogan, D., Stevenson, B., Casjens, S., and Tilly, K. 1996. Directed insertion of a selectable marker into a circular plasmid of *Borrelia burgdorferi*. J. Bacteriol. 178**:** 5946-5953.

Rosey, E.L., Kennedy, M.J., Petrella, D.K., Ulrich, R.G., and Yancey, R.J., Jr. 1995. Inactivation of *Serpulina hyodysenteriae flaA1* and *flaB1* periplasmic flagellar genes by electroporation-mediated allelic exchange. J. Bacteriol. 177**:** 5959-5970.

Rosey, E.L., Kennedy, M.L., and Yancey, R.J., Jr. 1996. Dual *flaA1 flaB1* mutant of *Serpulina hyodysenteriae* expressing periplasmic flagella is severely attenuated in a murine model of swine dysentery. Infect. Immun. 64**:** 4154-4162.

Saint Girons, I., Margarita, D., Amouriaux, P., and Baranton, G. 1990. First isolation of bacteriophages for a spirochaete: potential genetic tools for *Leptospira*. Res. Microbiol. 141**:** 1131-1138.

Samuels, D.S., 1995. Electrotransformation of the spirochete *Borrelia burgdorferi*, In: Methods in Molecular Biology. Nickoloff, J. A., eds. Humana Press, Inc., Totowa, NJ. p. 253-259

Samuels, D.S., Mach, K.E., and Garon, C.F. 1994a. Genetic transformation of the Lyme disease agent *Borrelia burgdorferi* with coumarin-resistant *gyrB.* J. Bacteriol. 176**:** 6045-6049.

Samuels, D.S., Marconi, R.T., Huang, W.M., and Garon, C.F. 1994b. *gyrB* mutations in coumermycin A_1-resistant *Borrelia burgdorferi*. J. Bacteriol. 176**:** 3072-3075.

Schwan, T.G., Burgdorfer, W., and Garon, C.F. 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of *in vitro* cultivation. Infect. Immun. 56**:** 1831-1836.

Schwan, T.G., Piesman, J., Golde, W.T., Dolan, M.C., and Rosa, P.A. 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc. Natl. Acad. Sci. USA 92**:** 2909-2913.

Simpson, W.J., Garon, C.F., and Schwan, T.G. 1990. Analysis of supercoiled circular plasmids in infectious and non-infectious *Borrelia burgdorferi*. Microb. Pathogen. 8**:** 109-118.

Stanton, T.B., Rosey, E.L., Kennedy, M.J., Jensen, N.S., and Bosworth, B.T. 1999. Isolation, oxygen sensitivity, and virulence of NADH oxidase mutants of the anaerobic spirochete *Brachyspira* (*Serpulina*) *hyodysenteriae*, etiologic agent of swine dysentery. Appl. Environ. Microbiol. 65**:** 5028-5034.

Stevenson, B., Bono, J., L., Elias, A., Tilly, K., and Rosa, P. 1998. Transformation of the Lyme disease spirochete *Borrelia burgdorferi* with heterologous DNA. J. Bacteriol. 180**:** 4850-4855.

Stevenson, B., Schwan, T.G., and Rosa, P.A. 1995. Temperature-related differential expression of antigens in the Lyme disease spirochete, *Borrelia burgdorferi.* Infect. Immun. 63**:** 4535-4539.

Stoenner, H.G., Dodd, T., and Larsen, C. 1982. Antigenic variation in *B. hermsii.* J. Exp. Med. 156**:** 1297-1311.

Subramanian, G., Koonin, E.V., and Aravind, L. 2000. Comparative genome analysis of the pathogenic spirochetes *Borrelia burgdorferi* and *Treponema pallidum.* Infect. Immun. 68**:** 1633-1648.

ter Huurne, A.A.H.M., van Houten, M., Muir, S., Kusters, J.G., van der Zeijst, B.A.M., and Gaastra, W. 1992. Inactivation of a *Serpula (Treponema) hyodysenteriae* hemolysin gene by homologous recombination: importance of this hemolysin in pathogenesis of *S. hyodysenteriae* in mice. FEMS Microbiol. Lett. 92**:** 109-114.

Tilly, K., Casjens, S., Stevenson, B., Bono, J.L., Samuels, D.S., Hogan, D., and Rosa, P. 1997. The *Borrelia burgdorferi* circular plasmid cp26: conservation of plasmid structure and targeted inactivation of the *ospC* gene. Mol. Microbiol. 25**:** 361-373.

Tilly, K., Lubke, L., and Rosa, P. 1998. Characterization of circular plasmid dimers in *Borrelia burgdorferi.* J. Bacteriol. 180**:** 5676-5681.

Turner, A.K., and Sellwood, R. 1997. Extracellular DNA from *Serpulina hyodysenteriae* consists of 6.5 kbp random fragments of chromosomal DNA. FEMS Microbiol. Lett. 150**:** 75-80.

Vinetz, J.M. 1997. Leptospirosis. Curr. Opin. Infect. Dis. 10**:** 357-361.

Walker, G.C., 1996. The SOS response of *Escherichia coli*, In: *Escherichia coli* and *Salmonella*. Neidhardt, F. C., Curtiss III, R. C., Ingraham, J. L., Lin, E. C. C., Low, K. B. *et al.*, eds. ASM Press, Washington, D.C. p. 1400-1416.

Weaver, J.C., 1995. Electroporation theory. Concepts and mechanisms., In: Methods in Molecular Biology. Nickoloff, J. A., eds. Humana Press Inc., Totowa, New Jersey. p. 1-26.

Xu, Y., Kodner, C., Coleman, L., and Johnson, R.C. 1996. Correlation of plasmids with infectivity of *Borrelia burgdorferi* sensu stricto type strain B31. Infect. Immun. 64**:** 3870-3876.

Zhang, J.-R., Hardham, J.M., Barbour, A.G., and Norris, S.J. 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell 89**:** 1-20.

Zhang, J.R., and Norris, S.J. 1998a. Genetic variation of the *Borrelia burgdorferi* gene *vlsE* involves cassette-specific, segmental gene conversion. Infect. Immun. 66**:** 3698-3704.

Zhang, J.R., and Norris, S.J. 1998b. Kinetics and *in vivo* induction of genetic variation of *vlsE* in *Borrelia burgdorferi*. Infect. Immun. 66**:** 3689-3697.