Characterization of antibiotic resistance mutations in Borrelia burgdorferi

Daniel C. Criswell

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CHARACTERIZATION OF ANTIBIOTIC RESISTANCE MUTATIONS
IN BORRELIA BURGDORFERI

by

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B.S. Weber State College, Ogden, Utah 1982

Presented in partial fulfillment of the requirements for the degree of

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Characterization of Antibiotic Resistance Mutations in *Borrelia burgdorferi*

Director: D. Scott Samuels

This study has identified and characterized five *Borrelia burgdorferi* naturally occurring mutations conferring resistance to spectinomycin, kanamycin, gentamicin or streptomycin. These antibiotics target the small subunit of the ribosome, which catalyzes protein synthesis. A naturally occurring coumermycin A1-resistant mutant has also been characterized. Four other *B. burgdorferi* mutants have been created using site-directed mutagenesis conferring resistance to coumermycin A1. Coumermycin A1 targets the B subunit of DNA gyrase, which catalyzes DNA supercoiling.

16S rRNA mutations, A1185G and C1186U, homologous to *Escherichia coli* nucleotides 1191 and 1192, confer 1500-fold and 2500-fold resistance, respectively, to spectinomycin. These mutations appear in a population of *B. burgdorferi* wild-type parental strain B31-A at a high frequency of \(6 \times 10^{-6}\). After 100 generations in competition with wild-type B31-A, 18% of the cells in a B31-A+ C1186U culture was mutants and 8% of the cells in a B31-A+ A1185G culture were mutants. A 16S rRNA A1402G mutation confers over 100-fold resistance to kanamycin and gentamicin. These mutants appear at a frequency of \(1 \times 10^{-8}\) in a population of B31 and are unable to compete with B31-A after 100 generations. Two streptomycin-resistant mutations were identified in the S12 ribosomal protein. A K88R mutation conferred seven-fold resistance and a K88E mutation conferred 10-fold resistance to streptomycin. Both exhibited only the streptomycin-resistant, SmR, phenotype. The frequency of the streptomycin-resistant mutants was \(3 \times 10^{-9}\) and the K88E mutant was unable to compete with B31-A at 100 generations.

Coumermycin A1-resistant mutants were constructed with amino acid substitutions in gyrase B of T162F, T162L, T162M and T162S. These mutants were from 5 to 6000-fold resistant to coumermycin A1. The naturally occurring T162I mutant was 420-fold resistant to coumermycin A1.
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Chapter 1
Introduction

1.1 *Borrelia burgdorferi*

*Borrelia burgdorferi* is a tick-borne pathogen that causes Lyme disease in humans (1). Although the entire genome of *B. burgdorferi* has been sequenced (2) a limited number of molecular tools are available to use in manipulating this genome (3). An important factor in developing these tools is choosing selectable markers that allow molecular biologists to identify clones of interest. A high mutation rate conferring resistance to an antibiotic used for selecting clones, or a high frequency in the population of organisms resistant to the antibiotic of choice, interferes with selection. In addition to the utility for molecular manipulation, many microorganisms have become resistant to a host of antibiotics that are, or have been, medically significant (4, 5, 6). Studies on the mechanisms of how resistance is acquired and maintained may further the understanding of how to avoid or eradicate resistant organisms that were once susceptible to a class of antibiotics.

The genome of *B. burgdorferi* is unusual for bacteria. It has a number of linear plasmids with covalently-closed hairpin loops at the telomeres and several circular plasmids in addition to a single linear 910 kb chromosome (2). Within the linear chromosome are two copies of the 23S and 5S rRNA genes and a single copy of the 16S rRNA gene (7, 8). The secondary structure and sequence of the *B. burgdorferi* 16S rRNA is nearly identical to that of many bacteria including
Escherichia coli and Thermus thermophilus and to eukaryotic chloroplast 16S rRNA found in Chlamydomonas and Nicotiana (9). These sequence and structural homologs are particularly helpful in providing clues of where possible mutations that confer resistance to a particular antibiotic would be located in the B. burgdorferi genome.

Many antibiotics, such as spectinomycin and the aminoglycosides, target bacterial ribosomes preventing translocation of tRNAs during translation (spectinomycin) or causing errors in accurately selecting cognate tRNAs (aminoglycosides) (10, 11, 12, 13). Other antibiotics, such as the coumarins, prevent DNA gyrase from introducing negative supercoiling, which is necessary for normal replication, recombination, and transcription. Some mutations can prevent binding by these antibiotics or compensate for antibiotic binding, while maintaining or restoring near normal levels of ribosomal or DNA gyrase function. A short review of ribosomal function and a literature review of mutations that confer resistance to these antibiotics will assist in understanding the results of this study, which identified the mechanisms of antibiotic-resistance in Borrelia burgdorferi.

1.2 Ribosomes

In E. coli, a typical prokaryote and model organism for ribosomal function, the 16S rRNA combines with 21 proteins to form the 30S ribosomal subunit. The 23S and 5S rRNAs combine with 31 proteins to form the 50S ribosomal subunit (10). These subunits assemble to form a 70S ribosome during
protein synthesis. Initiation of protein synthesis occurs when translation initiation sequences on mRNA bind by complementary sequences along the 16S rRNA of the 30S subunit. An initiation complex forms when initiation factors, f-Met-tRNA_{fMet} (the initiator tRNA), the 30S subunit, mRNA and the 50S subunit assemble (10). The peptidyl sites (P-site) located on both the 30S and 50S subunits are occupied by f-Met-tRNA_{fMet} upon assembly of the initiation complex (10). Elongation of the polypeptide chain begins as a cognate tRNA recognizes the correct codon on mRNA, occupies the A-site (which is also comprised of portions of both ribosomal subunits), and forms a peptide bond with the polypeptide chain attached to tRNA in the P-site (peptidyl tRNA) of the 70S ribosome (10). The now uncharged (no amino acid) peptidyl tRNA is released as the A-site tRNA (aminoacyl tRNA) possesses the polypeptide chain becoming the peptidyl tRNA. Translocation occurs when the aminoacyl tRNA with the polypeptide chain in the A-site moves to the P-site with the assistance of elongation factor G and the expenditure of energy provided by hydrolyzed GTP (10). These procedures are repeated until the polypeptide is formed and translation terminates when the ribosome reaches a stop codon on mRNA. A review of this process is illustrated in Figure 1.
Figure 1. Basic ribosome structure and the translation cycle. (A) 16S rRNA is part of the 30S subunit. (B) An initiation complex forms when initiation factors (IF2), f-Met-tRNA\textsubscript{Met} (the initiator tRNA), the 30S subunit, mRNA and the 50S subunit assemble. (C) The E (exit) site, P (peptidyl) site, and A (aminoacyl) site on the 70S ribosome with f-Met-tRNA\textsubscript{Met}. (D) Selection of cognate tRNA. A charged aminoacyl tRNA binds at the A-site. (E) Translocation of the peptidyl tRNA to the E-site and the aminoacyl tRNA to the P-site after a peptide bond was formed between the peptidyl tRNA amino acid and the aminoacyl tRNA amino acid.
1.3 Antibiotic Resistance

There are only a few reports of antibiotic resistance in *B. burgdorferi* and only one example of resistance to an antibiotic that targets the ribosome (14, 15). *B. burgdorferi* strain N40 was shown to be >3000 times more resistant to the macrolide erythromycin than strain B31-A (14). *In vitro* experiments have shown *B. burgdorferi* to be susceptible to this antibiotic (16, 17), which contrasts with clinical trials that were ineffective in treating Lyme disease in humans and animals (18, 19). Differences in the physiology of *B. burgdorferi* when grown in culture instead of a host have been proposed as a possible mechanism for this discrepancy (19). Although the mechanism of resistance has not been identified in strain N40, erythromycin-resistant clones selected on solid medium showed changes in colony morphology and lack of growth in liquid medium indicating resistance may be due to changes in metabolism (14). Metabolic changes associated with antibiotic resistance and affecting normal functions such as colony morphology and size, cell aggregation, and cell adhesion have been identified previously in *Pseudomonas aeruginosa* (20). Sequencing the 23S rRNA gene revealed that the mechanism of erythromycin resistance conferring resistance in *B. burgdorferi* strain N40 is not a mutation in the 23S rRNA, a common site in other bacteria such as *Streptococcus pneumoniae* (21). An A2059G mutation in the 23S rRNA of *S. pneumoniae* conferred resistance to erythromycin, and as the number of alleles with this mutation increased, the level of resistance also increased.
1.4 Spectinomycin Resistance

Spectinomycin is an aminocyclitol antibiotic produced by several species of *Streptomyces*, including *S. flavopersicus* and *S. glebosus* (22), which targets the 30S ribosomal subunit of many gram-negative bacteria. In *E. coli* and *T. thermophilus*, spectinomycin blocks elongation of the polypeptide during protein synthesis by preventing the translocation of the peptidyl tRNA from the A-site to the P-site (11, 12, 13). The antibiotic inhibits the activity of elongation factor G, which binds GTP and promotes translocation of the ribosome along mRNA (11, 13). Associated with the translocation process is helix 34 (H34, Figure 2) of 16S rRNA that comprises a portion of the A-site. Rotation of H34 and other elements of the 16S rRNA head enable the movement of mRNA along the ribosome and subsequently the translocation of tRNA from the A-site to the P-site. This movement is inhibited by the rigid spectinomycin molecule when it forms hydrogen bonds with several bases including G1064 and C1192 homologous to *B. burgdorferi* 1058 and 1186, respectively, in the minor groove of H34 (11, 12, 23). Translocation of tRNA not only involves movement of H34 but possibly H35 and H38 as well (11). Spectinomycin binding stabilizes H34 and could sterically hinder its movement, or prevent conformational changes necessary for translocation (11).

*E. coli* has been shown to be resistant to spectinomycin when nucleotide 1064 or the base-paired partner at 1192 in H34 of the 16S rRNA are mutated (12). *T. thermophilus* is also spectinomycin resistant when the base-pair G1064 and C1192 are mutated (11). (*E. coli* numbering will be used throughout the text)
unless otherwise noted.) Currently, most mutations at these sites are hypothesized to reduce the overall stability of H34 restoring the movement necessary for the translocation of tRNA even while spectinomycin is bound (11). Thermodynamic studies of all mutations at these sites have been shown to reduce the stability of H34 with the exception of the C1192G mutant (12, 24, 25). A C1192G mutation combined with the compensatory mutation, G1064C, actually increases the stability of H34 (25) and are accompanied by a five-fold decrease in growth rates when compared to wild-type (12). This double mutant interferes with the assembly of the 70S ribosome, reducing ribosomal activity and causing decreased growth rates in E. coli (11, 12). This might be particularly important for B. burgdorferi since it has only one copy of the 16S rRNA gene (rrs) compared to the seven copies in E. coli. The C1192G and G1064C mutations may prevent the assembly of functional ribosomes, and consequently inhibit growth of B. burgdorferi since it lacks another 16S rRNA. Analysis of translational efficiency, using chloramphenicol acetyl transferase (CAT) as a reporter, with the mutations G1064A, C, or U and C1192U showed little effect on ribosomal activity in E. coli in the presence of spectinomycin indicating these mutations have little effect on growth rates (12). By comparison, CAT activity for the C1192G-G1064C double mutant was 20% of normal, which explains the observed decrease in growth rates (12). In addition to the changes observed in ribosomal activity and growth rates, differences in levels of spectinomycin resistance by different base substitutions have also been observed. A C1192A mutation gives low levels of resistance while
substitutions at this site to U or G give high levels of resistance to spectinomycin (24).

Mutations at sites 1191 and 1193 in the chloroplast 16S rRNA of *C. reinhardtii* and *N. tabacum* confer resistance to spectinomycin for these eukaryotic organisms as well (26, 27). As with *E. coli* mutants, different levels of resistance correspond to mutations at different sites. A G1193A mutation confers low-level resistance to spectinomycin while A1191G and A1191C mutants result in high-levels of resistance in *C. reinhardtii* (26).

Mutations that result in amino acid substitutions in the S5 protein of the 30S ribosomal subunit confer resistance to spectinomycin in *E. coli* and *T. thermophilus*. Two different strains of *E. coli* have been shown to be resistant to spectinomycin when serine of the VSK tripeptide (residues 20-22) is mutated to proline creating a S21P substitution (27, 28, 29). Although S5 does not directly interact with the antibiotic (11, 28), resistance is probably due to destabilization of the tertiary structure formed by interactions of the 16S rRNA with the protein (11, 30). Structural changes in the S5 protein may cause a shift in the structure of the upper stem of helix 34 making it inaccessible to spectinomycin (11).

Spectinomycin is not used as an antibiotic to treat Lyme disease but is currently used to treat infections by strains of *Neisseria gonorrhoeae*. Many antibiotic-resistant strains of *N. gonorrhoeae* have emerged in the past few years, but spectinomycin continues to be effective in treating gonococcal infections world-wide (31, 32, 33). However, clinical isolates of *N. gonorrhoeae* that are
spectinomycin-resistant have been identified. As expected, these resistant strains had G1064C and C1192U mutations in the 16S rRNA (34).

1.5 Kanamycin and Gentamicin Resistance

Kanamycin and gentamicin are aminoglycosides produced by *Micromonospora* and *Streptomyces* species. They are both 2-deoxystreptamine antibiotics belonging to the 4, 6-disubstituted class of aminoglycosides (35). The two antibiotics are structurally similar with the main difference of two additional amine groups on gentamicin (35). Kanamycin is of particular interest since many organisms have become resistant to this antibiotic by mutations in their 16S rRNA genes or by possessing genes whose products catalyze acetylation, phosphorylation or methylation of aminoglycosides, or by modifications to the 16S rRNA gene (36, 37, 38). Both antibiotics cause miscoding of mRNA upon binding to the 16S rRNA (35, 36). These antibiotics bind to the major groove of helix 44 (Figure 2) in a pocket formed by the non-canonical base-pairing of A1408 and A1493 and the bulged nucleotide A1492 (39). This region encompasses a portion of the A-site and includes the universally conserved A1492 and A1493 sites that are essential for *E. coli* ribosomal function and viability (39, 40). Gentamicin and kanamycin form several hydrogen bonds along the major groove of H44 including G1494, U1495, G1405 and U1406 (39, 41). Hydrogen bonds are also formed with A1408 (G1408 in eukaryotic organelles) and stacks against G1491 causing the phosphate backbone of A1492 and A1493 to flip out and lock into this position (11, 36). In this locked position, the affinity
for cognate and noncognate tRNAs at the A-site is increased causing errors in reading mRNA transcripts (11). In the absence of antibiotic, this region would be free to rotate during the decoding step, reducing or eliminating translational errors (11).

A mutation at site U1406 or A1408 confers resistance to gentamicin and kanamycin in *E. coli* (36). A U1406A mutation in *E. coli* confers low level resistance to kanamycin and gentamicin while increasing the culture doubling time from 60 to 90 minutes (36). The slower growth rate, which is typical of hyperaccuracy, would indicate there is some interference of ribosomal function. Hyperaccuracy occurs when the ribosome becomes too selective for tRNAs, slowing down elongation of polypeptides and subsequently growth. An A1408G mutation in *E. coli* causes no change in the doubling time and apparently does not interfere with ribosomal function, but does confer high levels of resistance to these antibiotics (36). Footprinting studies have demonstrated that resistance in these two mutants results from decreased binding affinity of kanamycin and gentamicin to H44 (36, 42). This inability to bind is caused by the distortion created in the binding pocket as a result of the base substitutions at either 1406 or 1408 (42).

Many microorganisms can become kanamycin and gentamicin-resistant. For example, an A1408G mutation and several C1409 mutations confer high levels of resistance (>500 fold) to kanamycin in several species of *Mycobacterium* (43, 44). An A1408G mutation in *C. reinhardtii* resulted in high levels of
resistance to kanamycin, while a C1409U mutation confers lower levels of resistance (26).

1.6 Streptomycin Resistance

The aminoglycoside streptomycin, like spectinomycin, is an antibiotic produced by Streptomyces species. This antibiotic disrupts translational accuracy by increasing the affinity for noncognate aminoacyl tRNA molecules and interfering with the ability to accurately proofread during translation (45, 46). This error-prone activity is induced when streptomycin forms hydrogen bonds at several sites along the 16S rRNA phosphate backbone and the ribosomal S12 protein (11, 47). In E. coli and T. thermophilus, studies involving mutagenesis, cross-linking, and crystallography have identified several bases in the 16S rRNA and a residue in S12 as binding sites for streptomycin (11, 48, 49, 50). These sites include 16S rRNA C526 and G527 in the 530 loop located within H18, A913 and A914 of the 900 stem loop in H27, C1490 and G1491 of H44, and U14 in the 16S 5' end (Figure 2) (11). The single contact in S12 is at residue K45. Single mutations in the 16S rRNA or the S12 gene, rpsL, in E. coli have been shown to cause resistance to streptomycin (47, 48, 49, 51). These include, but are not limited to, the 16S rRNA sites 523, 885-890, and 910-912, and the S12 residue K45. Mutations at homologous sites in the 16S rRNA and the S12 protein also confer resistance to streptomycin in many other organisms (52, 53, 54, 55). A K88N mutation in the S12 protein of Mycobacterium tuberculosis (52) and a C912U mutation in the chloroplast 16S rRNA of the eukaryote Euglena gracilis.
have been shown to confer streptomycin resistance in these organisms. Wild-type 18S rRNA in yeast has a U at site 912 that confers natural resistance to streptomycin. When the reverse mutation is made, U912C in yeast 18S rRNA, these organisms also become susceptible to the antibiotic (56).

Mutations in the 16S rRNA and S12 protein can produce several different *E. coli* phenotypes with respect to streptomycin resistance. These phenotypes result from mutations of sites in the helix 27 "accuracy switch" (49). Helix 27 has been proposed to have two possible base-pairing patterns during translation. Bases 910-912 are paired with bases 885-887 in what is termed the ribosomal ambiguity or *ram* state, which has a high affinity for tRNA (49, 57, 58). Transient switching of the base pairing of 910-912 to bases 888-890 moves the 16S rRNA into the restrictive or proofreading state that lowers its affinity for tRNA (49). In the restrictive state, the S-turn motif of H27 that was present in the *ram* state is disrupted, changing the packing between H27 and H44. This change in conformation by H27, which contacts several other helices, probably causes conformational adjustments by these adjacent helices as well (11). Chemical protection experiments show that residues of these helices are more chemically reactive in the restrictive state, which is consistent with the proposed conformational changes (11). The contacts that S12 makes in this region probably facilitate the switch mechanism in H27 (49). Streptomycin binding is hypothesized to stabilize the area and prevent switching to the restrictive state, increasing the affinity for noncognate tRNA and, consequently, errors in translation (11). With the addition of streptomycin, the 16S rRNA is locked into
the error-prone *ram* state and unable to switch to the hyperaccurate restrictive state to proofread and eject non-cognate tRNAs (11). The presence of the S12 protein is also believed to stabilize this region. An S12 mutation may destabilize the region compensating for the increased stability conferred by streptomycin (11). This possibility would allow the ribosome to switch to the restrictive state, edit the translational errors and function fairly normally even while bound to streptomycin (11). This streptomycin-resistant *E. coli* phenotype is designated SmR (59). A mutation at one of the previously mentioned sites in 16S rRNA, destabilizing the region, also produces a SmR phenotype (11). Mutations that produce the SmR phenotype do not provide cross-resistance to other aminoglycosides such as kanamycin and gentamicin (60).

Another streptomycin phenotype results from mutations in the small subunit proteins or multiple mutations to 16S rRNA. This phenotype is called streptomycin-dependent and is designated SmD (61). Mutations in the S4, S5 and S12 proteins or multiple mutations in 16S rRNA force the ribosome to remain predominately in a restrictive state. Streptomycin binding stabilizes the *ram* state restoring ribosomal function by permitting the ribosome to switch between the error-prone *ram* state and the hyperaccurate restrictive state (11). The SmD phenotype is cross-resistant to kanamycin and gentamicin, which provides another method for verifying the SmD phenotype (60).

Specific mutations to the S12 protein have been shown to produce either the SmR or SmD phenotypes in several organisms, including *E. coli*, *T. thermophilus* and *Salmonella typhimurium* (62, 63, 64). In *T. thermophilus*,
single mutations in the \textit{rpsL} gene resulting in amino acid substitutions P41S, K42R, K88E or K88R in the S12 protein resulted in a SmR phenotype (62). Each of these mutations gave high levels of resistance to streptomycin with the P41S mutant being about half as resistant as the others. Culture doubling times for each of the mutants were increased over the wild-type. K88E had the longest increase in doubling time at 90 minutes, while K88R had the smallest increase at 56 minutes compared to the doubling time for wild-type \textit{T. thermophilus} of 50 minutes (62). The frequency of these mutants was reported to be $10^{-7}$ (62), which is about three orders of magnitude more frequent than the homologous mutations in \textit{E. coli} ($2 \times 10^{-10}$) (63, 65).
Figure 2. *Borrelia burgdorferi* 16S rRNA secondary structure map. Each box is labeled with the appropriate helix. Modified from www.ma.icmb.utexas.edu (9)
1.7 Coumermycin A₁ Resistance

Coumermycin A₁ is an antibiotic in the coumarin family produced by *Streptomyces* species (66). This antibiotic targets DNA gyrase (67), a type II topoisomerase that introduces negative supercoiling in DNA, which relieves torsional stress during replication or transcription (68). Gyrase is an A₂B₂ tetramer encoded by two genes, *gyrA* and *gyrB*. Gyrase A catalyzes the breakage and reunion of double-stranded DNA and gyrase B contains the ATP binding site involved in ATP hydrolysis (68). Coumermycin A₁ binds to gyrase B inhibiting ATP hydrolysis and growth (68).

The coumarin antibiotic novobiocin interacts with the universally conserved residue T165 (T162 in *B. burgdorferi*) of GyrB in *E. coli* (69). A mutation at *E. coli* T165, and homologous residues in other bacteria, has been postulated to confer resistance to coumarin antibiotics including coumermycin A₁ (69, 70, 71). Mutations at other residues have also been shown to confer resistance to coumermycin A₁ in several organisms. Several mutations in *Staphylococcus aureus* have been shown to confer high resistance to coumermycin A₁, including a T165N mutation (70). Mutations in *Bartonella bacilliformis* at residues G124, R184 and T214 (homologous to G77, R136, and T165 in *E. coli*) are up to five-fold resistant to coumermycin A₁ (71). These residues correspond to residues G74, R133, and T162 in *B. burgdorferi*. In *E. coli*, R136 substitutions, homologous to *B. burgdorferi* R133, conferred a 10-fold increase in resistance to coumermycin A₁ (72). Three different amino acid substitutions, R136C, R136H, and R136S, conferred high levels of resistance to
coumermycin A₁; however, gyrase activity was impaired (72). Mutations in \( B. burgdorferi \) gyrB, which resulted in substitutions at residue R133 to G or I also provide resistance to coumermycin A₁ (73). Furthermore, our laboratory has found that any amino acid substitution at R133 will confer resistance to coumermycin A₁ (B. Eggers, C. Eggers and D.S. Samuels, unpublished data.) The naturally occurring mutations G74S or T162I also have been identified as conferring resistance to coumermycin A₁ in \( B. burgdorferi \) (D.S. Samuels, unpublished data).

1.8 Objectives

The goal of this project was to identify and characterize \( B. burgdorferi \) mutants that are resistant to the antibiotics coumermycin A₁, spectinomycin, kanamycin, streptomycin, and gentamicin. Mutants resistant to spectinomycin, streptomycin, kanamycin and gentamicin have been found resulting from mutations in the genes encoding the 30S ribosomal subunit, including the 16S ribosomal RNA and the S12 protein. Mutants resistant to coumermycin A₁ have been constructed by site-directed mutagenesis in the gene encoding the B subunit of DNA gyrase. This study focused on genetically identifying the site of resistance to these antibiotics and the fitness of each antibiotic-resistant mutant.

1.9 Specific Aims

1. Spectinomycin resistant mutants were isolated and characterized. We have observed naturally occurring mutants of \( B. burgdorferi \) that are resistant to the
antibiotic spectinomycin. The frequency of mutation was determined by plating $10^8$ and $10^7$ cells of *B. burgdorferi* in different concentrations of spectinomycin and counting the number of resistant colonies that appeared on the plate. The location of the mutation conferring resistance was determined by sequencing the single gene for the 16S rRNA. Susceptibility assays, measuring the concentration that inhibits growth of 50% of the cells (IC$_{50}$), were used to gauge the level of resistance to spectinomycin by the resistant mutants. A mutant 16S rRNA gene that was spectinomycin-resistant was cloned into the vector pBSV2RII and transformed into wild-type B31-F to determine if resistance in the resulting merodiploid was recessive or dominant. The pBSV2RII plasmid alone was also transformed into wild-type B31-A as a control.

2. **Aminoglycoside-resistant mutants were isolated and characterized.**

We have found mutants resistant to the aminoglycosides streptomycin, kanamycin and gentamicin. As with the spectinomycin-resistant mutants, plating $10^8$ and $10^7$ cells in 50 $\mu$g/ml of the respective antibiotic and counting the number of colonies that appeared on the plates was done to determine the frequency of these mutations. The location of these mutations was determined by sequencing the gene for the 16S rRNA. Streptomycin-resistant mutations were also found in the *rpsL* gene that codes for the S12 protein of the 30S ribosomal subunit. Susceptibility assays (IC$_{50}$) were performed to determine the levels of resistance to each of the antibiotics. Each antibiotic-resistant mutant was tested for cross-resistance to the other aminoglycosides and spectinomycin to assist in identifying the cause of resistance.
3. **The genetic fitness of the spectinomycin- and aminoglycoside-resistant mutants was assayed.** A competition assay was performed to test each of the mutants against wild-type *B. burgdorferi* to see if there is a growth advantage or disadvantage in possessing a particular mutation to one of the antibiotics. We hypothesized that the high frequency of the spectinomycin-resistant mutants is a result of no cost or an attenuated cost to the organism that does not significantly affect growth.

4. **Coumermycin A₁-resistant mutants were constructed and characterized.**

Random site-directed mutagenesis was used to introduce mutations into the gene for the B subunit of DNA gyrase. These mutations change amino acid residue 162 from threonine to any of the other nineteen amino acids. Mutants *gyrB* genes were sequenced to identify the amino acid conferring resistance to coumermycin A₁. Each of the coumermycin A₁-resistant clones underwent a susceptibility assay to determine its level of resistance. A growth assay was done to determine if there is any significant difference in doubling times for the mutants when compared to the wild-type.
Chapter 2
Materials and Methods

2.1 Isolation of Antibiotic-Resistant Mutants

Spectinomycin Resistance. *Borrelia burgdorferi* strain B31-A was grown in 10 ml of liquid Barbour-Stoenner-Kelly (BSK-H) medium (Sigma) at 34°C to a concentration of $10^8$ cells/ml. The cell density was determined by a spectrophotometer at an optical density (OD) of 600nm. One ml of liquid culture was centrifuged at 10,000 rpm for 10 minutes, resuspended in one ml of dPBS++ (8.0g NaCl, 0.2g KCl, 1.15g Na$_2$HPO$_4$, 0.2g KH$_2$PO$_4$, 0.1g CaCl$_2$, and 0.213g MgCl$_2$/L) and the optical density at 600nm (OD$_{600}$) was determined. The OD$_{600}$ was multiplied by $1.4 \times 10^9$ to calculate the number of cells/ml (74). To determine the frequency of spectinomycin resistance cells in the *B. burgdorferi* population and to estimate the resistance levels of these strains, one ml (10$^8$ cells) of this culture was plated in semi-solid BSK medium (75) with spectinomycin (Sigma) at concentrations of 1.5 μg/ml, 6.0 μg/ml, and 30 μg/ml. 100 μl (10$^7$ cells) were plated at the same spectinomycin concentrations. The plates were incubated at 34°C for 11 days before colonies appeared. This procedure was repeated independently seven times. Seven spectinomycin-resistant colonies were isolated from the plates and grown in 10 ml of BSK medium containing 1.5 μg/ml of spectinomycin, until $10^8$ cells were present in the growth medium. DNA was extracted from each of the clones grown in liquid BSK as previously described (74). Spirochetes from the 10 ml cultures were centrifuged at 10,000 rpm for 10
minutes. The pellet was washed with one ml dPBS++ and then resuspended in 200 μl of 50 mM TES (Tris-HCl pH 8, 50 mM EDTA, 15% sucrose). The cells were lysed by adding 23 μl of 10% sodium dodecylsulfate (SDS) and incubated at 37° C for 30 minutes following the addition of 3 μl of proteinase K. The lysate was extracted using an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated by adding 8 μl of 5M NaCl and 400 μl of 100% ethanol and incubated at -20° C. The precipitated DNA was recovered by centrifugation for 25 minutes at 13,000 rpm, washed with 70% ethanol, and suspended in 50 μl of 10 mM Tris-HCl (pH 8.0).

The 3' end of the 16S rRNA gene was amplified using 16S 40F and 16S 659R (Table 1) in a polymerase chain reaction (PCR) since this is where known spectinomycin-resistant mutations are located in other organisms. The reaction produced a 620 base pair DNA segment. DNA for the sequencing template was purified using a Qiaquick® PCR purification kit and 15 μl at a concentration of 10 ng/μl were sent with the two primers to the Murdock Molecular Biology Facility for sequencing. The DNA sequencing template was also amplified using the same technique to sequence the rpsE gene, which codes for the S5 protein. The primers used in this PCR reaction were rpsG U322F and rpsE 441R (Table 1).

**Aminoglycoside Resistance.** The same techniques were used to isolate and identify streptomycin-, kanamycin-, and gentamicin-resistant mutants, as described above for the spectinomycin-resistant mutants. BSK plates were made with 40 μg/ml kanamycin, 35 μg/ml streptomycin, or 30 μg/ml gentamicin. Two sets of plates, one with 10^8 cells and one with 10^7 cells, were made using the
antibiotic concentrations above. This procedure was repeated five times. When antibiotic-resistant colonies were not consistently isolated on streptomycin and gentamicin plates at concentrations of $10^8$ cells, $10^9$ cells were plated for all three antibiotics.

Three kanamycin-resistant strains, designated KAN1, KAN3 and KAN4, were picked and grown as described for spectinomycin-resistant colonies, except with 40 μg/ml of kanamycin added to each culture tube. DNA was extracted using standard protocols and the 16S rRNA gene was sequenced for mutations associated with resistance to kanamycin. The primers 16S 40F and 16S 659R (Table 1) were used for amplifying the 16S rRNA gene by PCR. The same procedures used for the spectinomycin-resistant mutants were followed for purifying the PCR product and sequencing DNA.

One gentamicin-resistant strain was selected and grown in liquid medium with 25 μg/ml gentamicin. From this 10 ml culture, DNA was extracted and purified for sequencing the 16S rRNA along with the spectinomycin- and kanamycin-resistant isolates using the same primers. The gentamicin-resistant strain was designated GEN2.

Four strains from the 35 μg/ml streptomycin plates were isolated and grown in BSK medium with streptomycin following the previously described procedures. DNA was extracted following standard protocols from the cultures designated SmR1, SmR2, SmR3 and SmR4. Due to the possibility that a mutation in several sites of the 16S rRNA gene might confer resistance to
streptomycin, the entire gene was sequenced using the primers indicated in Table 1. The location of these primers is shown in Figure 3.

Primers for sequencing the \textit{rpsL} gene that encodes the S12 protein were a 25 nucleotide reverse primer, \textit{rpsG} 58R and a 25 nucleotide forward primer, \textit{rpsL} U106F (Table 1).
Table 1. Oligonucleotides used for sequencing the 16S rRNA gene and the ribosome proteins S5 and S12.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Gene</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpsE U322F</td>
<td>rpsE (S5)</td>
<td>AATAAGGACAAAAATAGGG</td>
</tr>
<tr>
<td>rpsE 441R</td>
<td>rpsE (S5)</td>
<td>CAAAACCTAAATCAAATGCCC</td>
</tr>
<tr>
<td>16S U169F</td>
<td>16S rRNA</td>
<td>AATGACCTAAAAATTAAGTCTAA</td>
</tr>
<tr>
<td>16S 40F</td>
<td>16S rRNA</td>
<td>TTGTTACGACTTCACCCCCCCT</td>
</tr>
<tr>
<td>16S 553F</td>
<td>16S rRNA</td>
<td>TCAAGCCCTGGTAAAGGTTC</td>
</tr>
<tr>
<td>16S 659R</td>
<td>16S rRNA</td>
<td>GAGTATGCTGCAAGAGTGT</td>
</tr>
<tr>
<td>16S 993R</td>
<td>16S rRNA</td>
<td>CGTTGTTCGGGATTATTG</td>
</tr>
<tr>
<td>16S 1060F</td>
<td>16S rRNA</td>
<td>TCATCACTTTGTCTTC</td>
</tr>
<tr>
<td>16S 1538R</td>
<td>16S rRNA</td>
<td>AAATAACGAAGAGTTTGATCC</td>
</tr>
<tr>
<td>BB425 3F</td>
<td>16S rRNA</td>
<td>GGAAGATGAGAGAGGGAGAG</td>
</tr>
<tr>
<td>BB426 288F</td>
<td>16S rRNA</td>
<td>AGGATTTCGCCTTTGCAAG</td>
</tr>
<tr>
<td>rpsG 58R</td>
<td>rpsL (S12)</td>
<td>AATTATATCTGGTATCAAACAAAAAC</td>
</tr>
<tr>
<td>rpsL U106F</td>
<td>rpsL (S12)</td>
<td>AAAATTAAGTTAGTGAAATATCG</td>
</tr>
</tbody>
</table>

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Figure 3. A map of the annealing sites for each primer. These primers were used in PCR to amplify 500-600 bp DNA fragments for sequencing the 16S rRNA gene in *B. burgdorferi*. 

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**Coumermycin A₁ Resistance.** Random site-directed mutagenesis was performed by Betsy Eggers on the T162 codon of the gyrB gene by overlap extension as previously described (76). Two overlapping PCR fragments were produced from two sets of primers and then amplified using the flanking primers of the first reactions. In the first PCR, a 357 nucleotide product was amplified using a 24 nucleotide forward primer 141F (5'-GGCTTTAGCTGGGTTTTGTGATAG-3') and a 27 nucleotide reverse primer 498R/T162X (5'-AAATGAAACCTTNNNCCCCGTAACAGA-3'). A second 497 nucleotide PCR product was amplified using a 27 nucleotide forward primer 472F/T162X (5'-TCTGTACGNGGNNAAGGTACTTTT-3') and a 24 nucleotide reverse primer 969R (5'-GACAGAAATAACAGCTGTAAGCCC-3'). Sites with N (the triplet for T162) had any of the four DNA bases to yield all possible codons. The purified fragments were then amplified in a second PCR with the two flanking primers, 141F and 969R, from the initial reactions. The resulting PCR product, a population of DNA molecules presumably encoding all codons for residue 162, was purified and 5 μg of the DNA was electroporated into *B. burgdorferi* strain B31-A. The transformed culture was plated in semi-solid BSK medium containing 0.1 μg/ml of coumermycin A₁. (Coumermycin A₁ was first dissolved in DMSO.) Colonies that appeared on the plates in 14 days were isolated and grown in liquid BSK medium containing 0.1 μg/ml coumermycin A₁. DNA from the cultures was extracted using standard protocols described above. A PCR product from the putative mutant gyrB gene was obtained using the primers.
141F and 969R. The PCR product was purified by ammonium acetate purification and sent to the Murdock Molecular Biology Facility for sequencing.

2.2 Susceptibility

Spectinomycin Susceptibility. A susceptibility assay was done for each identified mutant class represented by SPR3 (C1186U) and SPR6 (A1185G) as previously described (74, 77). Cultures were grown in 10 ml of BSK until 10^8 cells/ml was present. From these cultures, 10^6 cells, determined by an OD<sub>600</sub>, were inoculated into 4 ml of BSK with the following concentrations in µg/ml of spectinomycin: 0, 60, 120, 180, 240, 300, 360, and 480. After incubating at 34°C for 72 hours, the cultures were pelleted, resuspended in one ml of dPBS++ and the OD<sub>600</sub> was determined. The percent growth was the OD<sub>600</sub> of each sample compared to the OD<sub>600</sub> of a control with no antibiotic, which was defined as 100% growth. This procedure was repeated four times for SPR3 and six times for SPR6. A susceptibility assay was also done for wild-type B. burgdorferi at spectinomycin concentrations in µg/ml of 0, 0.015, 0.03, 0.06, 0.15, 0.30, 0.60 and 1.5. An IC<sub>50</sub>, the inhibitory concentration that prevents 50% growth at 72 hours, was used to compare the level of resistance for the mutants. The IC<sub>50</sub> was determined by graphing the results in SigmaPlot®. Susceptibility assays were performed on both spectinomycin-resistant mutants, SPR3 and SPR6, to test for cross-resistance to the antibiotics streptomycin, kanamycin and gentamicin. 10^6 cells of each mutant were added to 4 ml of BSK in 10 ml tubes. Antibiotics were added at the following concentrations in µg/ml and the cultures incubated for 72
hours at 34°C: streptomycin, 0, 0.7, 1.8, 3.5, 7, 18, 35, 70; kanamycin, 0, 0.8, 2, 4, 8, 20, 40, 80; and gentamicin, 0, 0.6, 1.5, 3, 6, 15, 30, 60. An OD<sub>600</sub> was taken for each culture tube to determine the percent growth and plotted in SigmaPlot<sup>®</sup> to determine the IC<sub>50</sub> for each mutant in each antibiotic.

To determine if the spectinomycin-resistant mutation was dominant or recessive, a plasmid expressing the mutant SPR3 (C1186U) 16S rRNA gene was constructed. The plasmid vector, pBSV2RII (Figure 4), was derived from pBSV2 (78) by removing the EcoRI restriction site in open reading frame 2 (ORF2) (S. Bundle and D.S. Samuels, unpublished data). The 16S rRNA gene was amplified from SPR3 using the proofreading polymerase KOD. The primers used in the PCR were 16S U169F and 16S 1538R (Table 1). The approximately 1.7 kb PCR product includes the entire 16S rRNA gene with its promoter. A 3' adenine overhang was added to the KOD polymerase PCR product with Taq polymerase. The overhang reaction was incubated for 10 minutes at 72° C. The adenine overhang is required for cloning into the pCR2.1-TOPO plasmid. The fragment was cloned into pCR2.1 using a TOPO TA cloning kit (Invitrogen<sup>®</sup>). The pCR2.1 plasmid was transformed into E. coli TOP10 F' cells and extracted from a 10 ml culture using a WizardPlus<sup>®</sup> mini prep kit. The 1.7 kb 16S rRNA gene insert was removed from pCR2.1 with EcoRI and ligated into the single EcoRI site of pBSV2RII, yielding plasmid pVSPR3. The plasmid was transformed into E. coli DH5α cells, which were grown on 40 µg/ml kanamycin plates to select for the plasmid. Colonies from the plates were PCR-screened for the presence of the plasmid and insert using the primers to construct the insert (16S U169F and 16S
Plasmid DNA was purified with a Wizard Plus® midi prep kit and 5 μg of each plasmid was electroporated into competent *B. burgdorferi* strains B31-F and B31-A as described (75). These cells were plated in semi-solid BSK medium with 40 μg/ml of kanamycin. When colonies appeared, they were picked and grown in liquid BSK with 160 μg/ml of kanamycin to continue selective pressure. To verify the presence of the pBSV2RII and pVSPR3 plasmids in B31-A and B31-F, DNA was extracted from the cultures (as previously described) and 2 μg was transformed back into *E. coli* DH5α. DH5α was plated on Luria-Bertani plates with 40 μg/ml kanamycin to screen for colonies with the respective plasmid that was present in the B31-F DNA extraction.

Susceptibility assays were done as described to determine the level of resistance conferred by each of the constructed plasmids in wild-type and SPR3 cells. The SPR3 16S rRNA gene was sequenced to check for mutations other than the C1186U previously identified as spectinomycin resistant. The 16S U169F primer was used to sequence the 5' end, BB426 288F was used to sequence the 3' end, and 553F was used to sequence the center of the SPR3 16S rRNA gene (Table 1).

To confirm if the SPR3 16S rRNA gene was expressed from the pBSV2RII plasmid in B31-F, sequencing of cDNA was done using the following protocols from strain B31-F/pVSPR3 to evaluate the amount of mutant RNA present in the cell. A culture containing $10^{10}$ cells of strain B31-F/pVSPR3 was centrifuged at 8000 rpm for 15 minutes to pellet the cells. The pellet was resuspended in 4 ml of TRIzol™ and incubated for 5 minutes at ambient
temperature. 1 ml of chloroform was added and incubated for 2.5 minutes. The mixture was centrifuged at 10,000 rpm for 15 minutes and the top layer containing RNA was transferred to a separate culture tube. RNA was precipitated by adding 1 ml of isopropanol, mixing by inversion, and incubating at ambient temperature for 10 minutes. RNA was pelleted by centrifuging at 12,000 rpm for 10 minutes. The pellet was washed with 75% ethanol, centrifuged again and air dried for 10 minutes. The final RNA pellet was resuspended in 25 ml of RNase-free water. An OD$_{260}$ was taken to determine the concentration of RNA.

The following procedures were used to obtain cDNA from the RNA. To insure no DNA was present, the RNA was treated with DNase RQ1 (Gibco). 5 µg of the treated RNA, 2 µl of random decamers, and 2 µl of water from a RETROscript™ RNA kit (Ambion) were incubated at 85°C for 3 minutes. 10 µl of this reaction was added to 2 µl of 10X RT-Buffer, 4 µl dNTP (25nm), 1 µl RNase inhibitor and 2 µl of Reverse Transcriptase (RETROscript™) to transcribe cDNA. This reaction was incubated at 44°C for one hour. The reaction was stopped by incubating at 92°C for 10 minutes. A PCR was performed using the primers 659R and 40F as described above and the product was sent to the Murdock Molecular Biology Laboratory for sequencing.
Figure 4. The pBSV2RII plasmid used as a cloning vector for the SPR3 16S rRNA gene. The 16S rRNA gene from SPR3 with the C1186U mutation was cloned into the EcoRI site in the multiple cloning site. The plasmid was transformed into B. burgdorferi strain B31-F to test for spectinomycin-resistance of the resulting merodiploid.
Aminoglycoside Susceptibility. A susceptibility assay was done to determine the level of resistance of the KanR mutant. Culture tubes were “set up” the same way as the spectinomycin experiment with the following concentrations in µg/ml of kanamycin: 0, 160, 320, 400, 640, 800, 960 and 1120. After 72 hours the cells were centrifuged, resuspended in dPBS++, and an OD₆₀₀ was taken to determine the IC₅₀. Susceptibility assays were also performed to test for cross-resistance to spectinomycin, streptomycin, and gentamicin. Again, 10⁶ cells of the mutant were added to 4 ml of BSK, grown for 72 hours at 34°C with the following concentrations in µg/ml added to individual tubes: spectinomycin, 0, 0.015, 0.03, 0.06, 0.15, 0.3, 0.6, 1.5; streptomycin, 0, 0.7, 1.8, 3.5, 7, 18, 35, 70; and gentamicin, 0, 0.6, 1.5, 3, 6, 15, 30, 60. The amount of gentamicin used in this assay was increased to 0, 120, 180, 240, 300, 360, 480, and 600 as a result of the resistance at lower levels. To determine the increase in resistance to both kanamycin and gentamicin, a wild type B31-A culture was assayed at the following concentrations in µg/ml: kanamycin; 0, 0.8, 2, 4, 8, 20, 40, 80; gentamicin; 0, 0.6, 1.5, 3, 6, 15, 30, 60; and streptomycin; 0, 0.7, 1.8, 3.5, 7, 18, 35, 70.

Susceptibility assays of the two streptomycin-resistant mutants SmR3 (K88R) and SmR4 (K88E) were performed as described with the following concentrations in µg/ml of streptomycin: 0, 70, 140, 210, 280, 350, 420, and 490. Susceptibility assays were also done to test for cross-resistance to the other three antibiotics.
Coumermycin A1 Susceptibility. A susceptibility assay, as described for the other antibiotic-resistant mutants, was performed on each of the coumermycin A1-resistant T162 mutants to determine the level of resistance (IC$_{50}$). These assays included a naturally occurring T162I mutant that was previously isolated (D.S. Samuels, unpublished data). *B. burgdorferi* strain B31-A was also assayed to determine the wild-type level of resistance to coumermycin A$_1$. The concentrations in µg/ml of coumermycin A$_1$ added to the B31-A cultures were: 0, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5. Because coumermycin A$_1$ is dissolved in DMSO, the protocol was modified to add equal amounts of DMSO to each culture tube in the assay, but not to exceed 1% (40 µl) of the total volume. At high levels of coumermycin A$_1$, the culture tubes had to be vortexed and warmed to 34°C to prevent coumermycin A$_1$ from precipitating out of solution. Each culture in the assay was grown to $10^7$ to $10^8$ cells/ml before an OD$_{600}$ was taken, which took 4-6 days. The identified mutants were assayed for coumermycin A$_1$ resistance at levels found in Table 2.

2.3 Metabolism of Antibiotic-Resistant Mutants

**Competition Assays.** An in vivo competition assay between wild-type B31-A and the mutant antibiotic resistant strains similar to the experiment done by Moine et al. was performed (79). Cultures of *B. burgdorferi* strains B31-A, SPR3, SPR6, SmR4 and KanR/GenR were grown, without selection, in 10 ml of BSK to a cell density of $10^8$ cells/ml. The concentration of cells was determined by a spectrophotometry reading at OD$_{600}$. From these cultures, approximately $10^5$
cells of each strain were added to 10 ml of BSK to make the following nine
different cultures: B31-A, SPR3, SPR6, SmR4, KanR/GenR, B31-A+SPR3, B31-
A+SPR6, B31-A+SmR4, and B31-A+KanR/GenR. After mixing, approximately
10^3 cells from each culture were plated using techniques already described to
evaluate the number of cells in each culture at the beginning of the assay. The
cultures were incubated for 96 hours until a cell density of about 10^8 cells was
reached. Each time growth reached 10^8 cells/ml (96 hours) another 10^5 cells were
passed into 10 ml of BSK. Cultures were passed 10 times, or ~ 100 generations.
Three of the experiments included plating cultures at 5 passages in addition to the
five sets of plates that were made after 10 passages to determine if the mutants
were still present with wild-type strain B31-A after 50 generations. At the end of
the assay, 10^3 cells from each of the nine different cultures were plated into semi-
solid BSK medium with no antibiotic and in BSK plates with the appropriate
antibiotic to screen for the ratio of antibiotic-resistant colonies to the wild-type.
The first two sets of competition assay experiments were plated using the same
method described above for plating B31-A on plates with spectinomycin,
streptomycin and kanamycin to search for strains resistant to these antibiotics
(74). The last two competition assays were plated using the following technique
(80): Two parts BSK were added to one part 2% agarose after equilibrating both
at 55°C. 15 ml of the BSK-agarose medium was pipetted onto 100 mm plates.
After this layer solidified, 20 ml of molten media at 42°C was mixed with the
appropriate B. burgdorferi strain and pipetted on top of the solidified 15 ml. An
example of the procedure for adding cells to both methods of plating was as
follows: \(10^3\) cells of SPR3, SPR6, B31-A + SPR3 and B31-A + SPR6 cultures were plated with no antibiotic or with 30 \(\mu\)g/ml spectinomycin to determine the number of spectinomycin mutants relative to the wild-type. This was done with each strain on plates without selection and on plates with their respective antibiotic. The plates were incubated for two weeks at 34°C at which time the colonies on each plate were counted. This experiment was repeated five times.

At the end of the last growth assay, 20 colonies were picked from the B31-A+SPR3 plate with no selection and 20 colonies were picked from the B31-A+SPR6 plate with no selection. Each of these colonies was added to one of the wells in a 24-well culture dish containing 2 ml BSK with 30 \(\mu\)g/ml of spectinomycin. SPR3 mutants were added to three of the wells in the B31-A+SPR3 dish for a positive control and no cells were added to one of the wells to serve as a negative control. SPR6 mutants were added to three of the wells in the B31-A+SPR6 dish and one well had no cells added. A color change from red to yellow caused by an increase in acidity from the metabolic activity of the \(B. burgdorferi\) strains was used to determine if the well was positive or negative for spectinomycin-resistant mutants.

**Growth Assay.** A simple growth assay was performed to determine the doubling time of the respective mutants compared to wild-type B31-A (81). As with the competition assay, each of the strains (B31-A, SPR3, SPR6, SmR4, and KanR/GenR) was grown in 10 ml of BSK until a concentration of \(10^8\) cells/ml was reached. From these cultures, \(10^6\) cells of each strain were added to separate tubes containing 50 ml of BSK. Every 24 hours for three days, 10 ml of each
culture were removed and an OD\textsubscript{600} was taken to determine the number of cells/ml. The doubling time, when cells were in exponential growth phase, was calculated using the following equation for the growth between day 1 and 2:

\[
T_2 - T_1 = \log_2 \frac{C_2}{C_1}
\]

Where, \(T_2 - T_1\) represents the time difference between day 2 and day 1, which was 24 hours. \(\frac{C_2}{C_1}\) represents the concentration of cells on day 2 and day 1. This procedure was repeated three times. The growth rates were plotted using SigmaPlot®, and a student’s t-test using SigmaPlot® was performed to evaluate the significance of the differences in doubling times of each strain.

A growth assay was done to determine the doubling time of the coumermycin A\textsubscript{1}-resistant mutants following the same procedure described for the spectinomycin and aminoglycoside-resistant mutants.
Table 2. Concentrations of coumermycin A₁ used in susceptibility assays for each T162 mutant.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Coumermycin A₁μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>T162I</td>
<td>0, 1, 2, 5, 10, 25, 50, 100</td>
</tr>
<tr>
<td>T162M</td>
<td>0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0</td>
</tr>
<tr>
<td>T162F</td>
<td>0, 50, 100, 150, 200, 300, 400, 500</td>
</tr>
<tr>
<td>T162S</td>
<td>0, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25</td>
</tr>
<tr>
<td>T162L</td>
<td>0, 10, 25, 50, 100, 150, 200, 250</td>
</tr>
</tbody>
</table>
Chapter 3

Spectinomycin-Resistant and Aminoglycoside-Resistant Mutants

3.1 Identification and Characterization of Spectinomycin-Resistance Mutants

In an effort to develop a new selectable marker for manipulating the *B. burgdorferi* genome, Frank et al. discovered a high frequency of background mutants on 1.5 μg/ml spectinomycin plates, which precluded using the antibiotic for selection (77). A number of other bacteria have been identified with mutations conferring spectinomycin resistance so we hypothesized that *B. burgdorferi* strain B31-A may harbor a large subpopulation with the same type of mutation. With this knowledge we set out to identify and characterize the source of the large number of spectinomycin-resistant mutants.

**Spectinomycin Mutation Frequency.** The frequency of the spectinomycin-resistant mutants was determined by plating *B. burgdorferi* in three different concentrations of spectinomycin: 1.5 μg/ml, 6.0 μg/ml and 30 μg/ml which are 7, 29 and 143-fold higher than the concentration that inhibits growth of the wild-type by 50%. $10^8$ and $10^7$ strain B31-A cells were plated at each of these concentrations and colonies typically appeared within 11 days. When $10^8$ B31-A cells were plated, an average of 572, 694, and 514 colonies grew in spectinomycin concentrations of 1.5, 6.0 and 30 μg/ml, respectively. Similar results were achieved when $10^7$ cells were plated, producing an average of 69, 62 and 62 colonies in 1.5, 6.0 and 30 μg/ml spectinomycin, respectively. These procedures were repeated at least four times with the results shown in...
Table 3. The frequency of spectinomycin mutants in the *B. burgdorferi* population is ~ $6 \times 10^6$.

To test the hypothesis that there was a high rate of mutation that confers resistance to spectinomycin, three spectinomycin plates were inoculated with $10^8$ cells of a recently isolated clone, the kanamycin and gentamicin-resistant mutant (KanR/GenR), with a known 16S rRNA sequence that did not include a mutation specific for spectinomycin-resistance (Figure 5). No colonies grew on any of the three plates inoculated with KanR/GenR mutants suggesting that the high frequency of spectinomycin-resistant mutants was due to a large subpopulation and not a high mutation rate. Therefore, we hypothesized the mutant had a low fitness cost so that it would be maintained in the population.

**Identification of the Spectinomycin-Resistant Mutations.**

Spectinomycin-resistant mutations have been mapped to the 16S rRNA gene in other bacteria (11, 13). Using this information we sequenced the 16S rRNA gene in seven of the spectinomycin-resistant mutants to determine the mechanism of resistance. Results of the sequencing revealed that three mutants, SPR5, SPR6 and SPR7 had an 16S rRNA gene T354C mutation resulting in a 16S rRNA A185G mutation. The other four mutants, SPR1, SPR2, SPR3 and SPR4, had a G353A 16S rRNA gene mutation resulting in a C186U 16S rRNA mutation (Figure 5). The C1192U spectinomycin-resistant mutation is homologous to the site identified in spectinomycin-resistant *E. coli* and *T. thermophilus* (11, 13), while the A1185G mutation is homologous to that found in *C. reinhardtii* (26) and has not been previously described in bacteria. The 1185-1186 16S rRNA sites are
located in helix 34 that can be seen in Figure 6. The sequence reveals no compensatory mutations at the based-paired partners G1058 (1186) and U1059 (1185) (data not shown), which correspond to E. coli sites G1064 and U1065. The rpsE gene encoding the S5 protein was also sequenced and no mutations were found at sites 60-66 where spectinomycin-resistant mutations are typically found (Figure 7) (11, 24).

**Antibiotic Resistance.** Susceptibility assays with wild-type B. burgdorferi strain B31-A, SPR3 (C1186U), and SPR6 (A1185G) revealed that both mutations confer a high level of resistance to spectinomycin. The IC\(_{50}\) for wild-type B. burgdorferi is 0.2 μg/ml spectinomycin. The C1186U mutant, SPR3, has an IC\(_{50}\) of ~300 μg/ml for a 1500-fold increase in resistance to spectinomycin. The A1185G mutant, SPR6, has an IC\(_{50}\) greater than 500 μg/ml, making it >2500 times more resistant than the wild-type (Figure 8 and Table 4).

SPR3 and SPR6 show no high levels of cross-resistance to related antibiotics, streptomycin, kanamycin and gentamicin (Figures 9, 10, 11 and Table 4). The IC\(_{50}\) for wild-type B. burgdorferi strain B31-A is 11 μg/ml, 9 μg/ml, and 2.5 μg/ml for streptomycin, kanamycin, and gentamicin, respectively. Aminoglycoside-resistance levels for B31-A used in these assays to compare cross-resistance were nearly identical to those reported previously (77). Both streptomycin and kanamycin had an IC\(_{50}\) of 7 μg/ml determined previously (77), which is slightly lower than the 11 μg/ml and 9 μg/ml, respectively, determined in this study (Table 4). The IC\(_{50}\) for SPR3 was 6 μg/ml, 17 μg/ml, and 2.5 μg/ml for streptomycin, kanamycin, and gentamicin, respectively. The IC\(_{50}\) for SPR6 was

40

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9, 13 and 2.5 μg/ml, respectively, for the same set of antibiotics. The results from all of the antibiotic-resistance assays for SPR3 and SPR6 are summarized in Table 4.

A merodiploid was constructed to test the dominance of the spectinomycin-resistant mutant using the pVSPR3 plasmid, constructed from pBSV2RII and the C1186U mutant 16S rRNA. Sequencing this plasmid identified no additional mutations. Because of difficulties in cloning pVSPR3 into strain B31-A, which is the strain used throughout these experiments, a closely related but more transformable strain, B31-F, was used. Susceptibility assays revealed no increase in spectinomycin resistance when pVSPR3 was present in B31-F. Four different colonies were used in the susceptibility assays of B31-F with pVSPR3 and the results from all the susceptibility assays with these plasmids are listed in Table 5. Sequencing the cellular 16S rRNA in strain B31-F/pVSPR3 showed only wild-type 16S rRNA present in the cell, suggesting that mutant 16S rRNA was not being transcribed from the plasmid, which explains the lack of a resistant phenotype.
Table 3. The frequency of spectinomycin-resistant mutations in *B. burgdorferi* strain B31-A (wild-type).

<table>
<thead>
<tr>
<th>Spectinomycin Concentration (µg/ml)</th>
<th>Number of Cells Plated</th>
<th>Avg. No. of Colonies (±S.E.M.)</th>
<th>Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>$10^8$</td>
<td>572 (± 83)</td>
<td>$5.7 \times 10^{-6}$</td>
</tr>
<tr>
<td>1.5</td>
<td>$10^7$</td>
<td>69 (± 22)</td>
<td>$6.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>6.0</td>
<td>$10^8$</td>
<td>694 (± 126)</td>
<td>$6.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>6.0</td>
<td>$10^7$</td>
<td>62 (± 21)</td>
<td>$6.2 \times 10^{-6}$</td>
</tr>
<tr>
<td>30</td>
<td>$10^8$</td>
<td>514 (± 118)</td>
<td>$5.1 \times 10^{-6}$</td>
</tr>
<tr>
<td>30</td>
<td>$10^7$</td>
<td>62 (± 5)</td>
<td>$6.2 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

The experiment was repeated four times for all spectinomycin concentrations with the exception of the 30 µg/ml with $10^8$ cells, which was repeated seven times.
Figure 5. 16S rRNA gene sequences for seven mutant colonies picked from spectinomycin plates and four other aminoglycoside-resistant mutants. Wild-type *B. burgdorferi* strain B31-A sequence is on the top row. The DNA mutations are a G353A in SPR 1-4 corresponding to a 16S rRNA C1186U and a T354C mutation in SPR 5-7 corresponding to a 16S rRNA A1185G. Kanamycin (KAN1, 3 and 4) and gentamicin (GEN2) resistant mutants have no mutations in this region.
Figure 6. 16S rRNA secondary structure map of the helix 34 region (boxed). Both spectinomycin-resistant mutations are in helix 34. Modified from: www.rna.icmb.utexas.edu/ (9)
**Figure 7.** SPR1, SPR2, SPR3 and SPR6 *rpsE* gene sequences that encode the ribosomal S5 protein. These spectinomycin-resistant mutants have no mutations in the 60-66 nucleotides that are known to confer spectinomycin-resistance in other bacteria.
Figure 8. Spectinomycin susceptibility assay of the two mutants SPR3 (C1186U) and SPR6 (A1185G). Percent growth was determined from a spectrophotometer reading at OD_{600}. Error bars represent the SEM. B31=B31-A. n = 5 for B31 and 4 for SPR3 and SPR6.
Figure 9. Streptomycin susceptibility assays for SPR3, SPR6, and KanR/GenR. This plot, from the susceptibility assays to determine cross-resistance to streptomycin, shows that the spectinomycin-resistant mutants SPR3 and SPR6 and the kanamycin/gentamicin-resistant mutant KanR/GenR, are not resistant to streptomycin. The percent growth was determined from a spectrophotometer reading at OD$_{600}$ and the error bars represent the SEM. B31=B31-A. n = 3 for B31 and KanR/GenR and, 6 for SPR3 and SPR6.
Figure 10. Kanamycin susceptibility assays for SPR3 and SPR6. This graph, from the susceptibility assays for cross-resistance to kanamycin of the C1186U (SPR3) and A1185G (SPR6) shows only a slight increase in resistance to kanamycin. The percent growth was determined from a spectrophotometer reading at OD$_{600}$ and the error bars represent the SEM. B31=B31-A. n = 3 for B31, SPR3 and SPR6.
Figure 11. Gentamicin susceptibility assays for SPR3 and SPR6. This graph shows no increase in resistance to gentamicin in the SPR3 C1186U and SPR6 A1185G mutants. The percent growth is from a spectrophotometer reading at OD_{600} and the error bars represent the SEM. B31=B31-A. n = 3 for B31, SPR3 and SPR6.
**Table 4.** The IC₅₀ (µg/ml) for each of the antibiotic-resistant strains to four antibiotics.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Sp¹</th>
<th>Sm¹</th>
<th>Km¹</th>
<th>Gm¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31-A</td>
<td>None</td>
<td>0.22</td>
<td>11</td>
<td>9</td>
<td>2.5</td>
</tr>
<tr>
<td>SPR3</td>
<td>16S rRNA C1186U</td>
<td>300</td>
<td>6</td>
<td>17</td>
<td>2.5</td>
</tr>
<tr>
<td>SPR6</td>
<td>16S rRNA A1185G</td>
<td>&gt;500</td>
<td>9</td>
<td>13</td>
<td>2.5</td>
</tr>
<tr>
<td>KanR/GenR</td>
<td>16S rRNA A1402G</td>
<td>0.12</td>
<td>6</td>
<td>&gt;800</td>
<td>&gt;600</td>
</tr>
<tr>
<td>SmR3</td>
<td>S12 K88R</td>
<td>0.10</td>
<td>80</td>
<td>13</td>
<td>1.5</td>
</tr>
<tr>
<td>SmR4</td>
<td>S12 K88E</td>
<td>0.08</td>
<td>110</td>
<td>16</td>
<td>1.5</td>
</tr>
</tbody>
</table>

¹Antibiotics: spectinomycin (Sp), streptomycin (Sm), kanamycin (Km), and gentamicin (Gm).

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Table 5. The susceptibility assays and resulting levels of resistance of the 16S rRNA merodiploid.

<table>
<thead>
<tr>
<th>Strain</th>
<th>IC$_{50}$ Spectinomycin (µg/ml)</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31-A</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>B31-F</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>B31-A + pBSV2RII$^a$</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>B31-F + pVSPR3$^b$</td>
<td>0.2</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$The pBSV2RII plasmid does not have a gene for 16S rRNA.

$^b$The pVSPR3 plasmid contains a 16S rRNA gene with the G353A mutation (C1186U 16S rRNA mutation).
3.2 Identification and Characterization of Aminoglycoside Resistant Mutants

The high frequency of spectinomycin-resistant mutants identified in our studies motivated us to examine the frequency and mechanism of resistance to closely related aminoglycoside antibiotics. The maintenance of spectinomycin-resistant mutants at high frequency indicates that there is a low fitness cost for these mutants. The structurally similar aminoglycosides were tested to discover if there is a corresponding fitness cost associated with the frequency of these mutations. Identification of the frequency and levels of resistance for these mutants may also aid in choosing reliable selectable markers when working with *B. burgdorferi*. Already, several markers have been constructed with aminoglycoside resistance cassettes for identifying clones of interest (77, 78, 82). Identifying and characterizing the frequency of aminoglycoside resistant strains should aid in choosing the appropriate plasmids for manipulating the *Borrelia* genome.

Identification of Kanamycin and Gentamicin Resistant Mutations. The frequency of kanamycin resistance was determined by plating $10^7$, $10^8$ and $10^9$ cells of *B. burgdorferi* strain B31-A in semi-solid BSK medium containing 40 μg/ml kanamycin. Based on these results, the frequency of this mutation is between $1.3 \times 10^{-8}$ and $1.4 \times 10^{-7}$. Kanamycin plates that had $10^8$ cells added produced 3-28 colonies. $10^9$ cells plated five times produced an average of 13 colonies per plate (ranging from 3 to 29 colonies per plate). Colonies took 14 days to appear on the kanamycin plates, which is three days longer than the appearance of spectinomycin-resistant colonies to appear on spectinomycin plates. Plating
B31-A on gentamicin plates gave similar results. The frequency of gentamicin-resistant colonies was $1 \times 10^{-8}$ when $10^9$ cells were plated in 30 μg/ml of gentamicin. Results from these experiments are compiled in Table 6.

To determine the location of the mutation that conferred resistance to kanamycin and gentamicin, three colonies were isolated from kanamycin plates and grown in liquid BSK medium with 40 μg/ml of kanamycin and one colony from the gentamicin plate was grown in liquid BSK medium with 25 μg/ml gentamicin. Sequencing of the 16S rRNA gene revealed an A1402G mutation in each of the clones designated KAN1, KAN3, and KAN4 (Figure 12), which corresponds to the A1408G mutation in *E. coli* conferring resistance to kanamycin and gentamicin (36). Sequencing the 16S rRNA gene from a colony grown on plates containing 30 μg/ml of gentamicin identified the same mutation, A1402G, conferring resistance to gentamicin (Figure 12 and Table 4). The gentamicin-resistant strain GEN2 has an identical 16S rRNA sequence as the kanamycin-resistant strain making a single designation, KanR/GenR, convenient for the kanamycin- and gentamicin-resistant mutant carrying the A1402G mutation. Because the kanamycin-resistant mutants and the gentamicin-resistant mutant had identical sequences they are represented by KanR/GenR in this paper. The location of the A1402G mutation is in helix 44 as shown in Figure 13.

**Antibiotic Resistance.** The KAN3 mutant, now designated KanR/GenR, was assayed for susceptibility to kanamycin and the other antibiotics. The results of this assay, which are graphed in Figure 14, reveal an IC$_{50}$ > 800 μg/ml, which is more than 90 times the wild-type level of resistance to kanamycin (Table 4). As
expected a susceptibility assay revealed that the kanamycin-resistant mutant was also cross-resistant to gentamicin at high levels. The \( IC_{50} \) for the KanR/GenR A1402G mutant is over 600 \( \mu g/ml \) of gentamicin, which is 240-fold more resistant than wild-type B31-A (Figure 15). The \( IC_{50} \) of the KanR/GenR mutant in gentamicin steadily decreases to 75% of wild-type at 360 \( \mu g/ml \) then increases to nearly 90% of the wild-type at 600 \( \mu g/ml \) (Figure 15). The cause of this increase in \( IC_{50} \) is unknown.

In addition to the susceptibility assays done to determine the level of resistance to kanamycin, assays were done to identify cross-resistance to the antibiotics streptomycin, and spectinomycin. The assays reveal that the A1402G mutation provides no resistance to spectinomycin or streptomycin. The \( IC_{50} \) for the KanR/GenR A1402G mutant is 0.12 \( \mu g/ml \) spectinomycin (Figure 16) and 6 \( \mu g/ml \) streptomycin (Figure 9), which is similar to wild-type B31-A (Table 4).
Table 6. The frequency of aminoglycoside–resistant mutants.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (µg/ml)</th>
<th>Cells Plated</th>
<th>Avg. Number Colonies (± SEM)</th>
<th>Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectinomycin</td>
<td>30.0</td>
<td>$10^8$</td>
<td>514 ($± 118$)</td>
<td>5.1 x 10$^{-6}$</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>30.0</td>
<td>$10^7$</td>
<td>62.0 ($± 5$)</td>
<td>6.2 x 10$^{-6}$</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>40.0</td>
<td>$10^9$</td>
<td>13.2 ($± 4.3$)</td>
<td>1.3 x 10$^{-8}$</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>40.0</td>
<td>$10^9$</td>
<td>14.0 ($± 6$)</td>
<td>1.4 x 10$^{-7}$</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>35.5</td>
<td>$10^9$</td>
<td>3.4 ($± 1.7$)</td>
<td>3.4 x 10$^{-9}$</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>35.5</td>
<td>$10^8$</td>
<td>2.0 ($± 1$)</td>
<td>2.0 x 10$^{-8}$</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>30.0</td>
<td>$10^9$</td>
<td>10.3 ($± 1.2$)</td>
<td>1.0 x 10$^{-8}$</td>
</tr>
</tbody>
</table>

$a_n = 7$

$b_n = 4$

$c_n = 5$

$d_n = 3$
Figure 12. 16S rRNA gene sequences from three kanamycin-resistant mutants, seven spectinomycin-resistant mutants and one gentamicin-resistant mutant. Wild-type *B. burgdorferi* sequence is on the top row. The DNA mutation is a T137C in KAN 1, 3 and 4, and in GEN2 corresponding to a 16S rRNA A1402G mutation. Sequences for the four kanamycin- and gentamicin-resistant mutants are identical for the rest of the 16S rRNA gene.
Figure 13. 16S rRNA secondary structural map of the helix 44 region. The A1402G mutation conferring resistance to kanamycin and gentamicin is in helix 44. Modified from: www.rna.icmb.utexas.edu/ (9)
Figure 14. Kanamycin susceptibility assay for KanR/GenR. The percent growth was determined by a spectrophotometer reading at OD$_{600}$. Error bars represent the SEM. B31=B31-A. n = 3 for B31 and KanR/GenR.
Figure 15. Gentamicin susceptibility assay for KanR/GenR. The percent growth was determined from a spectrophotometer reading at OD$_{600}$. Error bars represent the SEM. B31=B31-A. n = 3 for B31 and KanR/GenR.
Figure 16. Spectinomycin susceptibility assay for aminoglycoside-resistant mutants. The percent growth was determined from a spectrophotometer reading at OD$_{600}$. Error bars represent the SEM. B31=B31-A. n = 5 for B31 and 3 for KanR/GenR, SmR3 and SmR4.
Identification of Streptomycin-Resistant Mutants. The frequency of streptomycin resistance is $2.0 \times 10^8$ to $3.4 \times 10^9$, based on the number of colonies that grew on BSK plates containing 35 µg/ml of streptomycin (Table 6). BSK plates with $10^8$ cells typically had one-two colonies, while plates with $10^9$ cells ranged from 3-9 colonies. Colonies appeared within 11 days after plating.

Sequence analysis of the four streptomycin-resistant mutants, designated SmR1, SmR2, SmR3, and SmR4, revealed two different mutations in the *rpsL* gene encoding the S12 protein (Figure 17). An A263G *rpsL* mutation in SmR1, SmR2, and SmR3 resulted in a K88R substitution in the S12 protein in each of these strains (Figure 18 and Table 4). An A262G mutation in the *rpsL* gene resulted in a K88E substitution in the S12 protein of SmR4 (Figure 18 and Table 4).

Antibiotic Resistance. Susceptibility assays revealed a slight difference in the resistance conferred by these two mutations. The IC$_{50}$ was 80 µg/ml for the SmR3 mutant (K88R), and 110 µg/ml for the SmR4 mutant (K88E) (Figure 19 and Table 4). Complete sequencing of the 16S rRNA gene revealed no mutations in these streptomycin-resistant strains (data not shown). Ten different primers were used to sequence the entire gene on both strands to identify any possible additional mutations (Figure 4).

Susceptibility assays for spectinomycin, kanamycin, and gentamicin showed small changes in cross-resistance for SmR3 or SmR4. These two streptomycin-resistant mutants are slightly more susceptible to spectinomycin and gentamicin than wild-type B31-A. The IC$_{50}$ in µg/ml of SmR3 for spectinomycin,
kanamycin and gentamicin was 0.10, 13, and 1.5, respectively. The IC$_{50}$ of SmR4 for the same three antibiotics was 0.08, 16 and 1.5 µg/ml, respectively. By comparison, the IC$_{50}$ for B31-A is 0.22 µg/ml for spectinomycin, 9 µg/ml for kanamycin and 2.5 µg/ml for gentamicin. The results from these assays are graphed in Figures 16, 20, and 21, and listed in Table 4.
Figure 17. Nucleotide sequence of the rpsL gene encoding the S12 protein in streptomycin-resistant mutants SmR1, SmR2, SmR3 and SmR4. Wild-type B31-A is the top line. SmR4 has an A262G mutation and SmR1-3 have an A263G mutation corresponding to a K88E and K88R amino acid substitution in the ribosome S12 protein, respectively.
Figure 18. Amino acid sequences for the S12 protein in SmR3 and SmR4. Wild-type B31-A has lysine (K) at residue 88 while the SmR3 mutant has an arginine (R) substitution and SmR4 has a glutamic acid (E) substitution.
Figure 19. Streptomycin susceptibility assay for SmR3 and SmR4. The percent growth was determined by a spectrophotometer reading at OD_{600} and the error bars represent the SEM. B31=B31-A. n = 3 for B31, SmR3 and SmR4.
Figure 20. Kanamycin susceptibility assay for SmR3 and SmR4. The percent growth was determined from a spectrophotometer reading at OD$_{600}$ and the error bars represent the SEM. B31=B31-A. n = 3 for B31, SmR3 and SmR4.
Figure 21. Gentamicin susceptibility assays for SmR3 and SmR4. The percent growth was determined from a spectrophotometer reading at $OD_{600}$ and the error bars represent the SEM. $B31 = B31-A$. $n = 3$ for B31 and SmR4. $n = 5$ for SmR3.
3.3 Competition and Growth Assays

The frequency of spectinomycin-resistant mutants in the B31-A population was approximately 100-fold higher than the frequency of the aminoglycoside-resistant mutants. We hypothesized that this difference was a result of a lower fitness cost of the SPR3 (C1192U) and SPR6 (A1185G) mutations conferring spectinomycin resistance. This lower fitness cost enables spectinomycin-resistant mutants to compete well enough with wild-type B31-A to be maintained at frequencies higher than the typical mutation frequency observed for most bacteria. To test this hypothesis, growth and competition assays were done with B31-A and four of the mutants.

Growth Assays. Three independent growth assays resulted in no significant differences in doubling time for the mutants and wild-type B31-A. The doubling time was calculated during log phase growth that took place between day one and day two (Figure 22). From these experiments, the doubling time for B31-A was calculated as 7.9 hours while the doubling time was 8.8 hours for KanR/GenR, 8.1 hours for SPR3 and SPR6, and 8.0 hours for SmR4 (Table 7). A paired t-test for each mutant versus B31-A indicates that the differences in doubling times are not statistically significant (p = 0.53 for SPR3, 0.77 for SPR6, 0.96 for SmR4, and 0.30 for KanR/GenR, α = 0.05, Table 7).
Figure 22. Growth curves of four antibiotic-resistant mutants. Growth was determined from a spectrophotometer reading at OD<sub>600</sub> at the start (day 0) and at days 1, 2 and 3. n = 5
Table 7. Doubling time of four antibiotic-resistant mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling Time (±SEM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B31-A = 7.9 hours</td>
<td></td>
</tr>
<tr>
<td>SPR3</td>
<td>8.1 (± 0.3)</td>
<td>0.53</td>
</tr>
<tr>
<td>SPR6</td>
<td>8.1 (± 0.1)</td>
<td>0.77</td>
</tr>
<tr>
<td>SmR4</td>
<td>8.0 (± 0.2)</td>
<td>0.96</td>
</tr>
<tr>
<td>KanR/GenR</td>
<td>8.8 (± 0.6)</td>
<td>0.30</td>
</tr>
</tbody>
</table>
Competition Assays. At the start of each competition assay, approximately $10^3$ cells from each culture were plated to estimate what percent of the competition cultures were antibiotic-resistant mutant cells compared to the B31-A wild-type cells. The number of colonies on plates without antibiotic selection versus plates with selection indicates that approximately equal numbers of mutant and wild-type cells were plated (Table 8). Comparing the percent of colonies that grew on selection at the beginning of the assay and the percent of colonies that grew on selection at the end of the assay indicates that SmR4 and KanR/GenR were completely outgrown between 50 and 100 generations while SPR3 and SPR6 were still present at 100 generations (Table 9).

The spectinomycin-resistant mutants cultured with B31-A fared much better than the aminoglycoside-resistant mutants, surviving in the population for 100 generations. Comparing the number of cells from the B31-A+SPR3 cultures plated on selection versus no selection indicates that 18% of the cells remaining after 100 generations were SPR3. Since there is no selection for wild-type B31-A or against SPR3 cells on the plates without selection, a test was designed to quantify what percent of the colonies on the plates without antibiotic were SPR3. Twenty colonies from a B31-A+SPR3 plate with no selection were picked and grown in 2 ml BSK with 30 μg/ml spectinomycin to assess what percentage of the colonies were SPR3 on the plate without selection. The results of this experiment were similar to the one comparing the number of colonies on selection versus no selection with 4 of the 20 colonies (20%) being spectinomycin-resistant. Similar numbers were also obtained for the B31-A+SPR6 culture. Comparing the number
of colonies on spectinomycin plates versus the number of colonies on plates without spectinomycin indicated that only 8% of the cells in the culture were resistant to spectinomycin (SPR6) after 100 generations. Picking 20 B31-A+SPR6 colonies from plates with no selection and growing them in 2 ml BSK with 30 μg/ml spectinomycin produced two spectinomycin-resistant cultures, which yield 10% of the colonies in the culture that are still spectinomycin-resistant (SPR6) after 100 generations. The experiment at 50 generations had more spectinomycin-resistant mutants present than at 100 generations, as was expected. Plating on selection versus no selection showed that 41% of the cells were SPR3 in the B31-A+SPR3 culture and 41% were SPR6 in the B31-A+SPR6 culture. These results are listed in Table 9.

No colonies from the B31-A+KanR/GenR culture grew on plates with kanamycin, nor did any colonies from the B31-A+SmR4 culture appear on plates with streptomycin after 100 generations. However, colonies from the same cultures grew on plates with no selection indicating that wild-type B31-A was the only cell type in the culture. Plating the B31-A+SmR4 culture at 50 generations did produce colonies on the streptomycin plates. Two separate experiments produced similar results indicating that 6% of the cells in the B31-A+SmR4 culture were SmR4 at 50 generations of the competition assay. Less than one percent of the B31-A+KanR/GenR culture were KanR/GenR mutants at 50 generations. Two independent experiments produced only one colony each on kanamycin plates while the two plates with no selection produced 104 and 152 colonies from the B31-A+KanR/GenR culture.
Single cultures used as controls for the competition assays revealed that each of the mutant strains, SPR3, SPR6, SmR4, and KanR/GenR, grew on plates with and without antibiotic selection after 100 generations indicating that these cells were still viable and antibiotic-resistant (Table 10). Approximately $10^3$ cells were plated.
Table 8. The number of colonies that grew on plates from each culture at the beginning of the competition assay.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No Selection (±SEM)</th>
<th>Selection (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31-A</td>
<td>411 (±24.2)</td>
<td>0 (±0)</td>
</tr>
<tr>
<td>SPR3</td>
<td>1173 (±25.7)</td>
<td>837 (±35.0)</td>
</tr>
<tr>
<td>SPR6</td>
<td>1316 (±208)</td>
<td>1285 (±40.0)</td>
</tr>
<tr>
<td>B31-A + SPR3</td>
<td>1136 (±73.9)</td>
<td>775 (±19.2)</td>
</tr>
<tr>
<td>B31-A + SPR6</td>
<td>1385 (±130)</td>
<td>1172 (±109)</td>
</tr>
<tr>
<td>SmR4</td>
<td>1061 (±65.8)</td>
<td>836 (±89.9)</td>
</tr>
<tr>
<td>B31-A + SmR4</td>
<td>1178 (±86.0)</td>
<td>862 (±70.0)</td>
</tr>
<tr>
<td>KanR/GenR</td>
<td>762 (±114)</td>
<td>918 (±214)</td>
</tr>
<tr>
<td>B31-A + KanR/GenR</td>
<td>1072 (±4.00)</td>
<td>1286 (±138)</td>
</tr>
</tbody>
</table>

Spectinomycin (30 µg/ml) was the antibiotic used for selection with all SPR3 and SPR6 cultures, streptomycin (35 µg/ml) was the antibiotic used for all SmR4 cultures and kanamycin (40 µg/ml) was used for all KanR/GenR cultures. The chart shows that antibiotic-resistant strains are present in large numbers in the culture tubes at the beginning of the competition assays. n = 5.
**Table 9.** The number of antibiotic-resistant cells compared to wild-type.

<table>
<thead>
<tr>
<th>Strain</th>
<th>50 Generations (±SEM)</th>
<th>100 Generations (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31-A + SPR3</td>
<td>SPR3(^a) 41% (±4.4)(^a)</td>
<td>SPR3 18% (±4.4)(^b)</td>
</tr>
<tr>
<td>B31-A + SPR6</td>
<td>SPR6 41% (±2.1)(^a)</td>
<td>SPR6 8% (±3.1)(^b)</td>
</tr>
<tr>
<td>B31-A + SmR4</td>
<td>SmR4 6.0% (±2.0)(^c)</td>
<td>SmR4 0% (^d)</td>
</tr>
<tr>
<td>B31-A + KanR/GenR</td>
<td>KanR/GenR 0.75% (±0.25)(^c)</td>
<td>KanR/GenR 0% (^d)</td>
</tr>
</tbody>
</table>

\(^a\) n = 3  
\(^b\) n = 4  
\(^c\) n = 2  
\(^d\) n = 5
Table 10. The number of mutant viable colonies on plates with antibiotic and no antibiotic selection after 100 generations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Avg. Colonies with No Selection (±SEM)</th>
<th>Avg. Colonies on Antibiotic Selection (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31-A</td>
<td>234 (±119)</td>
<td>0 (±0)</td>
</tr>
<tr>
<td>SPR3</td>
<td>821 (±308)</td>
<td>940 (±95)</td>
</tr>
<tr>
<td>SPR6</td>
<td>866 (±189)</td>
<td>1079 (±338)</td>
</tr>
<tr>
<td>SmR4</td>
<td>927 (±170)</td>
<td>840 (±85)</td>
</tr>
<tr>
<td>KanR/GenR</td>
<td>542 (±132)</td>
<td>361 (±206)</td>
</tr>
</tbody>
</table>

SPR3 and SPR6 were plated in 30 μg/ml spectinomycin, SmR4 was plated in 35 μg/ml, and KanR/GenR was plated in 40 μg/ml kanamycin for selection. B31-A was also plated in each one of these antibiotic concentrations and no colonies were found on the plates. n = 5

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Chapter 4

Coumermycin A₁-Resistant GyrB T162 Mutants

Coumermycin A₁ has been used as a model for antibiotic-resistance in *Borrelia burgdorferi*. This antibiotic targets DNA gyrase (67), a type II topoisomerase that introduces negative supercoiling in DNA, which relieves torsional stress during replication or transcription (68). Mutations in *B. burgdorferi* gyrB, a subunit of DNA gyrase, that result in amino acid substitutions at residue R133 confer resistance to coumermycin A₁ (73). Lewis et al. predicted that amino acid substitutions in *E. coli* GyrB T165 (homologous to T162 in *B. burgdorferi* GyrB) would result in reduced gyrase activity and resistance to coumarin drugs (69). We performed site-directed mutagenesis to identify mutations in *B. burgdorferi* gyrB resulting in amino acid substitutions at residue T162 of GyrB that would confer coumermycin A₁ resistance but maintain GyrB function. Characterization of coumermycin A₁-resistant mutants at residue T162 of GyrB may further elucidate *B. burgdorferi* gyrase structure and function.

A naturally occurring coumermycin A₁-resistant mutant designated CR12E, but referred to here as T162I, with a T162I substitution in GyrB was previously isolated (D.S. Samuels, unpublished data). Sequencing revealed a C to T substitution in *gyrB* changing the triplet for threonine at position 162 from ACT to ATT, which codes for isoleucine (D.S. Samuels, unpublished data). Susceptibility assays showed this mutant to have an IC₅₀ for coumermycin A₁ of 21 μg/ml, which is 420-fold more resistant than the wild-type (Figure 23 and...
Four other mutants were created using random site-directed mutagenesis targeting the codon for T162 in GyrB (B. Eggers and D.S. Samuels, unpublished data). Several different base substitutions in the *gyrB* gene, TTT, CTT, TCA, and ATG resulted in four different amino acid substitutions from threonine to phenylalanine, leucine, serine, and methionine, respectively at position T162. These mutants were designated T162F, T162L, T162S and T162M. The IC$_{50}$ for each of these mutants was considerably higher than the wild-type B31, which was determined to be 0.02 μg/ml and is identical to previously reported levels of resistance for B31 (73, 83). The IC$_{50}$ for the mutants ranged from 0.5 μg/ml for T162S to 300 μg/ml for T162F. T162L had an IC$_{50}$ of 200 μg/ml, T162I had an IC$_{50}$ of 21 μg/ml and T162M had an IC$_{50}$ of 4.0 μg/ml (Table 11). A plot of the T162F, T162I and T162L coumermycin A$_1$-resistance assays is in Figure 23. The assays for T162S and T162M are graphed in Figure 24.

Doubling times for each of the mutants were calculated based on the log phase growth that took place between days one and two after passage. These data show that the mutants with the highest resistance to coumermycin A$_1$ have the slowest doubling times. All mutants had slower doubling times than wild-type B31-A (7.9 hours). The T162F and T162L mutants, which were the most resistant to coumermycin A$_1$, had the slowest doubling times of 9.2 and 8.7 hours, respectively. The slower growth of the T162F mutant was statistically significant with a p-value from a t-test at 0.05. The mutants T162I, T162M, and T162S had doubling times of 8.6, 8.6, and 8.2 hours, respectively (Table 11). Figure 25
shows the growth of each of the T162 mutants for three days with log phase occurring between days one and two. Two of the doubling times for T162F were determined from day two to three when the culture was log phase growth. Log phase growth in these two experiments did not occur until day two.
Figure 23. Coumermycin A₁ susceptibility assay of three T162 mutants. The percent growth was determined from a spectrophotometer reading at OD₆₀₀ and the error bars represent the SEM. n = 5.
Figure 24. Coumermycin A₁ susceptibility assay for two T162 mutants. The percent growth was determined from a spectrophotometer reading at OD₆₀₀ and the error bars represent the SEM. n = 5.
Table 11. Coumermycin A₁ resistance by five GyrB T162 mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA Sequence</th>
<th>GyrB Residue 162</th>
<th>IC₅₀ (µg/ml)</th>
<th>Fold Resistance</th>
<th>Doubling Time Hours (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31 (Wild-type)</td>
<td>ACT</td>
<td>Threonine</td>
<td>0.02</td>
<td>-</td>
<td>7.9 (± 0.5)</td>
</tr>
<tr>
<td>T162I</td>
<td>ATT</td>
<td>Isoleucine</td>
<td>21</td>
<td>420</td>
<td>8.6 (± 0.3)</td>
</tr>
<tr>
<td>T162F</td>
<td>TTT</td>
<td>Phenylalanine</td>
<td>300</td>
<td>6000</td>
<td>9.2 (± 0.1)</td>
</tr>
<tr>
<td>T162L</td>
<td>CTT</td>
<td>Leucine</td>
<td>200</td>
<td>4000</td>
<td>8.7 (± 0.6)</td>
</tr>
<tr>
<td>T162S</td>
<td>TCA</td>
<td>Serine</td>
<td>0.5</td>
<td>25</td>
<td>8.2 (± 0.3)</td>
</tr>
<tr>
<td>T162M</td>
<td>ATG</td>
<td>Methionine</td>
<td>4.0</td>
<td>80</td>
<td>8.6 (± 0.6)</td>
</tr>
</tbody>
</table>
Figure 25. Growth curve of the GyrB T162 mutants. Growth was determined from a spectrophotometer reading at OD$_{600}$. Error bars represent the SEM. $n = 5$. 

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Chapter 5

Discussion

The high frequency of spectinomycin-resistant colonies appearing on 30 µg/ml spectinomycin plates in our laboratory (76) prompted an investigation to identify the mechanism of resistance. We hypothesized that mutations to the 16S rRNA or small ribosomal subunit proteins were the cause of spectinomycin resistance based on previous studies in other bacteria (13, 24, 28, 34). We hypothesized that the frequency of these mutants in the *B. burgdorferi* B31-A population was due to a low fitness cost. We also theorized that there were other mutants present in the population resistant to the related aminoglycoside antibiotics. We have identified five different mutations conferring high levels of resistance to spectinomycin, streptomycin, kanamycin or gentamicin. We have also characterized five different coumermycin A₁-resistant mutants. One of these mutants is naturally occurring and the other four were constructed using random site-directed mutagenesis.

5.1 Spectinomycin Resistance

Spectinomycin is an antibiotic that prevents elongation of the polypeptide by blocking translocation of the peptidyl tRNA from the A-site to the P-site during translation (11, 12, 13). Mutations at several sites including 1191-1193 in the 16S rRNA of *Escherichia coli, Thermus thermophilus*, and *Chlamydomonas reinhardtii* have been shown to confer resistance to spectinomycin (12, 24, 26).
These mutations provide enough instability to allow helix 34 the movement necessary for translocation even while spectinomycin is bound (11).

We have identified A1185G and C1186U mutations (homologous to *E. coli* A1191 and C1192) in *Borrelia burgdorferi* 16S rRNA that confer a high level of resistance to spectinomycin (Figure 5). Susceptibility assays show a difference in resistance to spectinomycin by the two mutants. The C1186U mutant has an IC$_{50}$ of 300 µg/ml compared with an IC$_{50}$ well over 500 µg/ml for the A1185G mutant (Table 4). Sequencing the 16S rRNA gene revealed no other mutations, including the complementary portion of helix 34 at sites 1057-1058 (*E. coli* 1063-1064). In addition, no mutations were found in the *rpsE* gene encoding the S5 protein, a site of spectinomycin-resistant mutations in other bacteria (Figure 7). The absence of a second mutation in these genes suggests that the difference in susceptibility of the two mutants to spectinomycin is due to the different nucleotide sites that were mutated. This also suggests that the mutants are not spectinomycin-dependent. Secondary mutations in *Bacillus subtilis* and *E. coli* have been shown to confer spectinomycin dependence when more than one mutation occurs in the 16S rRNA or when there is a mutation in the S5 protein in addition to a mutation in the 16S rRNA (84, 85). This lack of dependence was confirmed by the ability of SPR3 and SPR6 to grow in the absence of spectinomycin (Table 8).

The 1500-fold increase in resistance to spectinomycin by the C1186U mutation is similar to the resistance levels reported for *Salmonella enterica*, which is more than 500-fold resistant to spectinomycin when a homologous mutation is
present (86). Resistance levels conferred by plasmid-borne 16S rRNA C1192U mutation in *E. coli* have been measured at a more modest 16-fold increase (87). This difference is probably due to the seven wild-type 16S rRNA genes still present in *E. coli*.

Of more interest is the A1185G mutation that confers over 2500-fold resistance to spectinomycin. A homologous mutation has been identified in the chloroplast of the eukaryote *C. reinhardtii* (A1123G, *E. coli* 1191) and confers a high level of resistance to spectinomycin (26). Why the A1185G mutation confers more resistance than the adjacent C1186U mutation is perplexing. There are no other bacterial mutations at this site (*E. coli* 1191) to compare resistance levels with an 1192 mutation. Mutations deeper in helix 34 have been hypothesized to confer more resistance to spectinomycin than those that are closer to the surface (88). This may be due to conformational changes that disrupt the right side of helix 34 and provide the flexibility needed in helix 34 for normal ribosomal function when spectinomycin is bound (11, 88). This model arises from experiments that show a *Nicotiana* G1193A mutation that caused a 30% reduction in protein synthesis in 80 µg/ml spectinomycin while a G1064A mutation, which is deeper in the helix, had only 5% reduced activity (88). This seems plausible if A1185 in *B. burgdorferi* is deeper in the helix than C1186 (11). The homologous site in *T. thermophilus* appears to be deeper in the 30S subunit based on 16S rRNA models from high resolution crystallography (11). Another factor determining the level of resistance is the specific base substitution as well as the site of the substitution. For example, a C1192A mutation confers low levels of
resistance to spectinomycin in *E. coli* whereas a C1192 to G or U resulted in high levels of resistance (24). The data in this study demonstrate that a G substitution provides more resistance at site 1185 than a U substitution at 1186 in *B. burgdorferi*. The wild-type base at the homologous site in *Euglena gracilis*, and the blue-green algae *Anacystis* is a C at position 1141 and 1132, respectively (89, 90). The wild-type C instead of a U in 16S rRNA in both of these organisms provides natural resistance to spectinomycin. However, *Euglena* is 20-fold more resistant to spectinomycin than *Anacystis* (88). Fromm et al. hypothesized that species-specific interactions with spectinomycin and the translocation machinery are likely sources of the differences observed in levels of resistance (88). All of these factors, the position of the base substitution on helix 34, the particular base at the mutated site, and the species-specific characteristics of the translational machinery are probably involved with the observed differences in levels of resistance between the two *B. burgdorferi* mutants, C1186U and A1185G. The A1185G mutation matches the resistance levels of the KanR/GenR mutants. The homologous kanamycin resistant *E. coli* A1408G mutant reduces the binding affinity of kanamycin and gentamicin for helix 44. The A1185G mutation may also reduce the binding affinity of spectinomycin to helix 34 reducing the resulting in resistance levels similar to those of kanamycin resistant mutants.

Susceptibility assays with aminoglycosides revealed that both mutants, SPR6 (A1185G) and SPR3 (C1186U), had the same susceptibility to streptomycin and gentamicin as wild-type B31-A. However, both were nearly twice as resistant to kanamycin (Table 4). The small increase in resistance may be a result of slight
global changes along the 16S rRNA that are caused by a decrease in the stability of helix 34 (12, 24). Many of the helices involved in antibiotic resistance have been shown to interact with each other as well as several 30S proteins during translation (11, 91, 92). The decrease in stability of helix 34, which allows movement in the head region of 16S rRNA even while the rigid spectinomycin molecule is bound, has been postulated to create changes in the position of helix 28, 35, 36, and the S5 protein (11). These structural changes could, in turn, cause minor shifts in the position of other helices such as helix 27 and helix 44, which binds kanamycin, resulting in the small increase in resistance to kanamycin. All three helices, H27, H34 and H44 are structurally very close to each other and all three interact with the S5 protein either directly or indirectly (11, 91, 92). Helix 27 stacks on helices 1 and 28 and the conformational switch that is made by H27 has been postulated to change the packing interactions between these three helices (11, 49). Because of the helix-helix interaction between H34 and H28, any change in the conformation of H34 due to base substitutions may affect the packing of the helices of the H27 switch mechanism involving H1, H27 and H28. One model proposes a series of switches involving H34 and H44 as a result of the activities of the H27 switch (11). Therefore, modifications to any of these helices may cause slight alterations in the functioning of the other helices that are contacted. Slight changes in susceptibility to antibiotics due to a mutation in a helix that is not associated with resistance to that antibiotic may be mediated through helix-helix interactions or the S5 protein, which interacts with all three helices involved in spectinomycin and aminoglycoside resistance.
The frequency of the spectinomycin-resistant phenotype is $6 \times 10^{-6}$ in our population of *B. burgdorferi* strain B31-A. This frequency includes both the C1186U and A1185G mutations. The frequency of individual mutations was not determined since both of the mutant types appear on plates used to derive mutation rates. Additional point mutations in the 16S rRNA gene, which have been identified in other organisms conferring spectinomycin-resistance (12, 26, 27, 34), may contribute to the phenotypic mutation rate as well, but were not represented in the seven mutants sequenced. The same number of recently cloned *B. burgdorferi* with a known 16S rRNA gene sequence (KanR/GenR, Figures 5 and 12) were plated on spectinomycin plates and no colonies appeared. This suggests that either the mutation frequency experiments with B31-A simply selected for spectinomycin-resistant phenotypes already present in the population, or that spectinomycin resistance and kanamycin/gentamicin resistance are not compatible in *B. burgdorferi*. The concentration of spectinomycin that B31-A was exposed to and the number of cells in the culture media did not affect the frequency of mutants. Plating $10^7$ or $10^8$ cells at concentrations of 1.5, 6.0, and 30 μg/ml of spectinomycin resulted in similar numbers of colonies appearing on plates (Table 3). This indicates that neither of these factors affects the spectinomycin-resistance phenotype. Similar observations were made with *B. burgdorferi* N40 resistant to erythromycin suggesting that the frequency of resistant cells was probably due to the maintenance of a small population of erythromycin-resistant N40 *B. burgdorferi* in the population (14).
The frequency of spectinomycin-resistant mutants is considerably higher than the frequencies of the aminoglycoside-resistant mutants also reported in this study. The frequency of kanamycin-resistant mutants is approximately $1.3 \times 10^8$, the frequency of streptomycin mutants is $3.4 \times 10^9$ and gentamicin $1.0 \times 10^8$.

Although the methods used in our experiments are different from those of other researchers who used methods pioneered by Luria and Delbrück (93), Newman (94) or Lederberg and Lederberg (95), comparisons of the mutation rates for other organisms that have mutations conferring spectinomycin-resistance are informative. The $B. burgdorferi$ spectinomycin-resistant phenotypic frequency recorded in this study is considerably higher than the spectinomycin-resistant mutation frequency reported for $E. coli$ and $Salmonella enteritidis$ (96). Spectinomycin-resistant mutants appear at a frequency of $2 \times 10^9$ in $E. coli$ and $3 \times 10^8$ in $S. enteritidis$ (96). The frequency of spectinomycin-resistance in 60 μg/ml spectinomycin is approximately $10^8$ for $Mycoplasma mycoides$ (97).

These mutation rates, along with rifampicin-resistant mutation rates of $1.2 \times 10^8$ for $E. coli$ and $2 \times 10^8$ for $S. enteritidis$, have been identified as typical random mutation rates for bacteria (45, 96, 98). Increases in mutation rates have been identified in organisms that have defective methyl-directed DNA mismatch repair systems, which are catalyzed by proteins encoded by the $mut$, $dam$ and $uvrD$ genes (96, 98, 99). Mutations in these genes, referred to as mutator genes, can increase the incidence of mutation 10-100 times (96). The increased mutation frequency in mutator strains matches the mutation frequency for spectinomycin-resistance mutants observed in our experiments with $B. burgdorferi$. Mutator
genes have not been sequenced to determine if this is the cause of the increase in mutation rate in *B. burgdorferi*. However, if any of these mutants were a mutator strain we would predict that there would be an increase in resistance to all of the antibiotics tested.

The frequency of spectinomycin resistance in *B. burgdorferi* is similar to the frequency observed for spectinomycin-dependent mutants in *E. coli*, which is $10^6$ (85). It is lower than the $4 \times 10^5$ frequency for the secondary mutation in *B. subtilis* that confers resistance for spectinomycin-dependent *B. subtilis* (84). Although the high mutation frequencies hint at spectinomycin dependence in the *B. burgdorferi* mutants, plating the SPR3 and SPR6 mutants on BSK plates with no antibiotic produced as many colonies as plating with spectinomycin (Tables 8 and 10). There also were no secondary mutations found in the *rpsE* gene encoding S5 or additional mutations in the 16S rRNA gene that would be characteristic of this phenotype. This suggests that a secondary mutation, conferring spectinomycin-dependence, is not present in SPR3 or SPR6 to account for the apparently high mutation rates.

The doubling times (growth rates) for SPR3 and SPR6 were both 8.1 hours during log phase, which is not significantly different from the 7.9 hours for B31-A. The aminoglycoside-resistant mutant doubling times, 8.0 hours for SmR4 and 8.8 hours for KanR/GenR, were also not significantly different than those of B31-A, suggesting that there was too much variability in the data to detect the differences. Statistically significant changes in doubling times have been found in B31 that possess *gyrB* mutations although the doubling time reported by Samuels
et al. of 10 hours for B31 was calculated over several days and not just at log phase, which accounts for the discrepancy with the current study (73). The difference in the doubling times between SPR3 or SPR6 and B31-A was not statistically significant, which reflects the results found for other spectinomycin-resistant organisms with mutations in 16S rRNA. Spectinomycin-resistant ribosomes in \textit{E. coli} had no effect on growth rates (79) and C1192U mutant ribosomes were as efficient as the wild-type in \textit{E. coli} (12). There is little or no difference between growth rates of wild-type \textit{E. coli} and 16S rRNA C1192U mutants (\textit{B. burgdorferi} C1186U) (87). In this experiment, a plasmid was constructed carrying the C1192U mutation in a 16S rRNA gene and inserted into \textit{E. coli}. The doubling time, 42 minutes, for this spectinomycin-resistant clone containing 80\% mutant ribosomes was the same as wild-type \textit{E. coli} (87). A similar experiment was performed with \textit{Salmonella enterica} where the C1192U mutant 16S rRNA gene was cloned into a plasmid and tested for resistance and doubling time (86). The doubling time of the mutant clone was 38 minutes compared to 33 minutes for the wild-type (86).

The small and statistically insignificant differences in doubling time among the spectinomycin-resistant mutants may not account for the reduced numbers of these mutants compared to B31-A in competition assays spanning 100 generations. However, SPR3 and SPR6 were better able to compete with B31-A than either of the aminoglycoside-resistant mutants, SmR4 and KanR/GenR. After 100 generations of competition between B31-A and SPR3 or SPR6, 18\% and 8\%, respectively, of the cells were the mutant spectinomycin-resistant type.
The increase in B31-A cells in the cultures of B31-A+SPR3 and B31-A+SPR6 is evident from the plating data at the beginning of the assay, which show more mutants present (Table 8), the plating data at 50 generations, which show 41% of the cells to be SPR3 or SPR6 (Table 9), and the plating data at 100 generations showing a further decrease in mutant cells (Table 9). In a competition assay with wild-type E. coli and three strains that had mutations in the 16S rRNA binding site with S8, wild-type ribosomes were able to out-compete mutants when grown in M9 medium with glucose as the only carbon source for metabolism (79). The doubling time of the variants was reported to be the same as wild-type, but after 30 doublings in M9 medium, 100% of the cells in the culture were wild-type E. coli (79). However, culturing the wild-type and mutants in Luria broth (LB) resulted in one of the variants being as numerous as the wild-type after 30 doublings (79). These experiments suggest that frequency of a mutant in a population depends on the environment and the resources that are available for the mutant. Based on these results, and the results of the competition assays with spectinomycin- and aminoglycoside-resistant mutants in B. burgdorferi, mutations in 16S rRNA may affect the ability of the mutants to utilize the resources in the culture medium as efficiently as the wild-type, resulting in reduced ability to compete with wild-type. The high frequency of the spectinomycin-resistant mutants (6 x 10^{-6}) compared to the aminoglycoside-resistant mutants in the B31-A population is likely the result of the lower fitness cost and the increased ability to compete.
Cloning the SPR3 16S rRNA gene into the pBSV2RII plasmid and transforming it into \textit{B. burgdorferi} strain B31-F did not produce spectinomycin-resistance. Sequencing cellular 16S rRNA from strain B31-F/pVSPR3 suggests there is only wild-type 16S rRNA in the cell and the mutant 16S rRNA is not being transcribed from the plasmid. This explains why the plasmid did not confer any spectinomycin resistance. There could be several reasons for the gene not being expressed. The gene may be missing part of the promoter element, it may not be transcribed efficiently on the plasmid, the plasmid may be unstable, or the wild-type 16S rRNA gene may have recombined with the plasmid.

\textit{E. coli} merodiploids carrying spectinomycin-resistant and sensitive genes have been made and characterized by other researchers (100, 101). There was no apparent increase in resistance of heterozygous individuals carrying both resistant and sensitive ribosomes. Unfortunately, the source of mutation conferring the resistance was not reported (100, 101). \textit{E. coli} heterozygous merodiploids carrying streptomycin-resistant \textit{rrn} genes have also been shown to be recessive (100, 101, 102). A plasmid carrying a 16S rRNA gene with the Cl 192U mutation confers high levels spectinomycin resistance in \textit{E. coli} and \textit{Salmonella} as previously mentioned (86, 87). Merodiploids with plasmid Cl 192U 16S rRNA in \textit{E. coli} and \textit{Salmonella} did confer spectinomycin resistance indicating the mutant 16S rRNA provides at least a cumulative effect if not a dominant one (86). In addition to these experiments, \textit{Salmonella} 16S rRNA with the Cl 192U mutation was transformed into two strains of \textit{E. coli} (86). In the first experiment, \textit{E. coli} that had all seven \textit{rrn} (16S rRNA) genes knocked-out was transformed with a
plasmid carrying the *Salmonella* spectinomycin-resistant 16S rRNA gene. The resulting transformants were spectinomycin-resistant. However, using an *E. coli* strain with its own wild-type *rrn* genes and the plasmid carrying the *Salmonella* spectinomycin-resistant C1192U 16S rRNA did not provide resistance to spectinomycin. Although *Salmonella* and *E. coli* 16S rRNA are 97% identical in sequence (103), *E. coli* 16S rRNA was hypothesized to out-compete the *Salmonella* mutant 16S rRNA for ribosomal proteins, transcription factors and ribosomal assembly (86). The *B. burgdorferi* spectinomycin-resistant plasmid-borne 16S rRNA gene is identical in sequence (with the exception of the C1186U mutation) to the wild-type, making competition for ribosomal proteins and ribosomal assembly unlikely. The copy number of the plasmid in the cell may factor into the amount of C1186U 16S rRNA present and it may also be out-competed for transcription factors by chromosomal *rrn* genes. The pBSV2 plasmid, from which the pBSV2RII was derived, appears to have a higher copy number than chromosomal 16S rRNA, but the copy number for pBSV2RII is unknown (76, 77). Furthermore, the conflicting results from *E. coli* studies determining spectinomycin resistance of the heterozygous merodiploid may be a function of the different plasmids that were used as vectors in the individual experiments.

There are additional factors that should be considered as well. The difficulty in transforming B31-A with pVSPR3 may indicate that the plasmid is not very stable. Although several plasmids with antibiotic-resistant markers have been successfully transformed in *B. burgdorferi* (77), including pBSV2, there
have been a few problems cloning and transforming plasmids in *B. burgdorferi* (78). For example, the *B. burgdorferi* plasmid cp9 from strain N40 was successfully cloned into pCR-XL-TOPO and reported to be quite stable. However, when cp9 was cloned into pBluescript, the resulting plasmid was unstable in spite of having the same origin of replication, and similar size and copy number as pCR-XL-TOPO (78).

5.2 Kanamycin and Gentamicin Resistance

The aminoglycosides, kanamycin and gentamicin, form several hydrogen bonds along the major groove of helix 44 in 16S rRNA (39, 41) causing miscoding of mRNA during translation of the nascent polypeptide (36, 39). Resistance to these antibiotics in *E. coli* is conferred as a result of U1406A or A1408G mutations in 16S rRNA that reduce the binding affinity of the antibiotic for helix 44 (36, 42).

We have identified an A1402G mutation in *B. burgdorferi* strain B31-A conferring a high level of resistance to kanamycin and gentamicin (Figure 12). This mutation appears at a frequency of $1.3 \times 10^{-8}$ on kanamycin plates and $1.0 \times 10^{-8}$ on gentamicin plates, which is in the range of frequencies considered typical for random mutations in bacteria (47, 95, 97). With an IC$_{50}$ > 800 µg/ml for kanamycin and > 600 µg/ml for gentamicin, this mutation is about 100-fold or more resistant to both antibiotics (Table 4). This high level of resistance to kanamycin and gentamicin is also found in *E. coli* with the homologous A1408G
mutation (36, 42) and *Mycobacterium tuberculosis* with the homologous A1400G mutation (44).

Susceptibility to the antibiotics spectinomycin and streptomycin increases with the A1402G mutation. The IC₅₀ of spectinomycin and streptomycin for the KanR/GenR mutant is about half the IC₅₀ for wild-type B31-A (Table 4). The increased susceptibility of the KanR/GenR mutant to streptomycin eliminates the possibility that this mutant carries an additional mutation that would produce a streptomycin-dependent phenotype (59). One possibility for the observed changes in susceptibility to these antibiotics caused by the A1402G mutation has already been discussed regarding spectinomycin resistance. A change in susceptibility to other antibiotics is likely due to structural changes along the 16S rRNA that alter helix-helix interactions or increase the affinity that antibiotics have for their binding sites. Helix 44, the site of kanamycin and gentamicin binding, interacts directly with helices 27 and 34 (11, 91). These two helices harbor binding sites for streptomycin (H27 and H44) and spectinomycin (H34) (11, 91). Additional interactions that may be affected involve the S5 protein that interacts with all three helices and the S12 protein that interacts with helices 27 and 44 (91, 92).

Although not statistically significant, the KanR/GenR growth rate of 8.8 hours is slower than B31-A (7.9 hours) and the other antibiotic-resistant mutants SPR3, SPR6 and SmR4 (Table 7). The growth rate for the homologous *E. coli* A1408G mutant is identical to the wild-type even with the addition of 160 µg/ml kanamycin (36). The *E. coli* A1408G mutant also does not affect ribosomal
activity suggesting this mutant may be able to compete with wild-type *E. coli* (36, 42). *B. burgdorferi* carrying the A1402G mutation may have deleterious effects on ribosomal function responsible for the slower growth rates. In addition to slower growth rates, the KanR/GenR mutants were unable to compete with wild-type B31-A in competition assays. After 50 generations, less than 1% of the cells in the B31-A+KanR/GenR culture tubes were KanR/GenR. At 100 generations, no KanR/GenR cells were detected indicating that all of the remaining cells were B31-A. The slower growth rate alone may account for the inability of KanR/GenR mutants to compete with B31-A. However, the B31-A+SmR4 competition assay produced the same results despite SmR4 having a growth rate virtually identical to B31-A, indicating there may be other metabolic factors involved in the competition with wild-type. The frequency of the KanR/GenR mutant in the B31-A population may reflect how well this mutant is maintained as well as how often the mutation arises. This scenario matches the data for mutation frequencies and competition assays, which show SPR3 and SPR6 are more able to compete with wild-type and, therefore, more frequent than SMR4, which is unable to compete with wild-type and less frequent than the spectinomycin-resistant mutants. It also reconciles the results of the statistical analysis that indicate the differences in growth rates are not significant although the less frequent mutants KanR/GenR and SmR4 do not compete as well as the spectinomycin-resistant mutants with B31-A.
5.3 Streptomycin Resistance

Streptomycin disrupts translational accuracy by increasing the affinity for noncognate aminoacyl tRNA molecules at the A-site and interfering with the ability to accurately proofread during translation (45, 46). This error-prone activity is induced when streptomycin binds at several sites along the 16S rRNA phosphate backbone and to the ribosomal S12 protein (11, 47, 48, 49, 50). Mutations at several sites in the 16S rRNA gene and the rpsL gene encoding the S12 protein have been identified as conferring resistance to streptomycin in many organisms (47, 48, 51, 52, 53, 54, 55).

We have identified two mutations in the B. burgdorferi rpsL gene conferring resistance to streptomycin. These mutations encode K88R and K88E amino acid substitutions in the ribosomal S12 protein. The frequency of the streptomycin-resistant mutations, which is between $2 \times 10^{-8}$ and $3.4 \times 10^{-9}$ for B. burgdorferi, is in agreement with published reports (47, 96, 98) for bacterial mutation rates and similar to those for B. burgdorferi KanR/GenR. This frequency includes both the K88R (SmR3) and K88E (SmR4) mutations and falls within the range given for other streptomycin-resistant bacteria. The mutation frequency for the rpsL gene in M. smegmatis is $10^{-8}$ to $10^{-10}$ (47), and $2 \times 10^{-10}$ for E. coli (65). rpsL mutation frequencies in T. thermophilus are considerably higher at $10^{-7}$ (62). The frequency of K88R and K88E mutants is similar to that of the kanamycin-resistant mutation and 100-fold less frequent than the spectinomycin-resistant mutants.

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Neither mutant exhibited any of the characteristics associated with streptomycin dependence. Complete sequencing of the \textit{rpsL} and 16S rRNA genes revealed no secondary mutations associated with streptomycin dependence.

Another characteristic of this phenotype is an increased resistance to kanamycin and gentamicin. Although the level of resistance to kanamycin did increase two-fold, the SmR4 mutant was able to grow in BSK plates without streptomycin. The ability to grow without streptomycin in the medium is not characteristic of the streptomycin-dependent phenotype. Plates with 35 $\mu$g/ml streptomycin and plates without streptomycin grew nearly identical numbers of SmR4 colonies indicating that SmR4 was not dependent on streptomycin for growth in a solid medium.

The level of streptomycin-resistance was approximately 7-fold for SmR3 and 10-fold for SmR4 compared to the wild-type (Table 4). These resistance levels are much lower than those for SPR3, SPR6 and KanR/GenR, which are all >100-fold more resistant than wild-type to their respective antibiotics. Apparently in \textit{B. burgdorferi}, mutations in 16S rRNA gene encoding the region of spectinomycin and kanamycin binding are able to confer much higher levels of resistance compared to mutations located in the genes for 30S proteins (S12) that interact with streptomycin. The levels of resistance in \textit{B. burgdorferi} are typical of other streptomycin-resistant mutants. Similar susceptibilities have been identified in \textit{M. smegmatis} that have the homologous mutation in \textit{rpsL} encoding the S12 protein as well as in 16S rRNA (47). The S12 K88R, K88E and the 16S rRNA 530 stem loop substitutions identified in \textit{M. smegmatis} were all 5 to 10-fold more
resistant to streptomycin than the wild-type (47). An *E. coli* C912U substitution conferred four-fold resistance (50).

The slight differences in streptomycin-resistance between SmR3 and SmR4 are likely caused by a change in the electrochemical charge at residue 88 in the S12 protein, although this difference was not observed in either *M. smegmatis* or *T. thermophilus* (47, 62). The arginine substitution in SmR3 retains the positive charge of the wild-type lysine residue. In spite of the charge remaining the same, conformational changes in the S12 protein must occur upon substituting arginine and its two additional side chain amino groups. The glutamic acid substitution creates a residue at position 88 with a negatively charged side chain altering the S12 structure from the wild-type. These changes perturb the structure of S12 and alter the global structure of the ribosome reducing the stability of the region around helix 27 in *T. thermophilus* (11, 104). The decrease in stability from the K88R and K88E mutations may compensate for the increased stability created by streptomycin binding and allow the ribosome to switch between the error prone *ram* state and the hyper accurate restrictive state, restoring nearly normal ribosome function while providing streptomycin resistance (11).

Susceptibility assays revealed changes in susceptibility to spectinomycin, gentamicin, and kanamycin in both K88E and K88R strains. The two mutants were half as resistant to spectinomycin and gentamicin and two-fold resistant to kanamycin (Table 4). As discussed previously, these changes in susceptibility must be due to global changes along the 16S rRNA. Several lysine and arginine residues of *T. thermophilus* S12 including the homologous K88 contact helix 27...
with additional residues contacting helices 18 and 44 (11, 90, 104). The K88R and K88E mutations could disrupt the S12 interactions with any of these three helices and alter the binding affinity of spectinomycin, kanamycin or gentamicin. S12 has been proposed to facilitate the helix 27 accuracy switch (48) that enables the ribosome to change from the error prone ram state to the hyper accurate proofreading state (11, 48). Mutations causing modifications to the S12-helix 27 interaction may disrupt other helix-helix interactions as well. The helix 27 switch may activate a series of switches along the 16S rRNA including helix 34, which is the site of spectinomycin binding, and helix 44, which is the site of kanamycin and gentamicin binding (11). S12 also has direct contact with the upper portion of helix 44 (91, 104). The K88E and K88R substitutions may cause conformational changes along helix 44 that alter binding affinities for kanamycin and gentamicin, thus changing the susceptibilities to these antibiotics.

The doubling time during log phase was 8.0 hours for SmR4, which is roughly the same as wild-type B31-A (7.9 hours). In competition assays, SmR4 was unable to compete with B31-A indicating a significant fitness cost for this mutation. SmR4 comprised only 6% of the cell population at 50 generations and was undetectable at 100 generations. This follows the pattern of the slower growing KanR/GenR mutant that also had no detectable cells after 100 generations in co-culture with B31-A. The homologous A1408G 16S rRNA and S12 mutations have been shown to affect the selection of cognate tRNAs and the rates of polypeptide chain elongation in *E. coli* and *T. thermophilus* (36, 105, 106). Mutations in the *E. coli rpsL* gene slow the rates of polypeptide chain elongation.
elongation by nearly 20% (105). A slower rate of translation could explain the
inability of SmR4 and KanR/GenR to compete with wild-type B31-A, but should
also be accompanied by slower growth rates that are found only in the
KanR/GenR mutants. It may also help to explain why the SPR3 and SPR6
mutants are able to compete much better in competition with B31-A.
Spectinomycin-resistant mutations have been shown to have little if any affect on
ribosome function even in the absence of antibiotic (24, 25). Normal ribosome
function in SPR3 and SPR6 certainly could provide a fitness advantage that
accounts for the differences in ability to compete with B31-A among the four
mutants SPR3, SPR6, SmR4 and KanR/GenR. The decreased fitness, based on
ribosome function, for the SmR4 and KanR/GenR mutants likely explain why
they are less common in a population of B31-A than SPR3 and SPR6.

Slower translation rates should result in significantly slower growth rates.
Since SmR4 grows at the same rate as wild-type B31-A, the inability of SmR4 to
compete may be from deleterious effects on another S12 cellular role. If S12 is a
bifunctional protein this would provide an alternative explanation for the near-
normal growth, coupled with the inability of SmR4 to compete with B31-A for
resources in the medium. Another ribosomal protein, S3, is bifunctional in
Drosophila with roles in both translation and DNA repair (107), and several other
ribosomal proteins have been shown to be bifunctional as well (108, 109).
5.4 Coumermycin A<sub>1</sub> Resistance

Coumermycin A<sub>1</sub> is a competitive inhibitor of the enzymatic action of GyrB (69). Coumermycin A<sub>1</sub> binds to GyrB at residue T165 in *E. coli* (homologous to T162 in *B. burgdorferi*) preventing ATP hydrolysis necessary for the catalytic activity of DNA gyrase (68). This reaction relieves torsional stress caused by supercoiling resulting from transcription and replication (68).

Mutations in *gyrB* encoding substitutions at T165 and other residues have been identified conferring resistance to coumermycin A<sub>1</sub> and other coumarin drugs such as novobiocin (69, 70, 71).

We have identified one natural coumermycin A<sub>1</sub>-resistant mutation and four mutants generated by site-directed mutagenesis. The naturally occurring mutant has a T162I amino acid substitution and the four site-directed mutants are T162F, T162L, T162M and T162S. Resistance levels vary considerably for each of the mutations and are probably a result of disrupting the water-mediated hydrogen bond between the side chain hydroxyl group of threonine 162 and coumermycin A<sub>1</sub> (69). The T162I mutant is 420-fold more resistant than wild type. This level of resistance is considerably more than reported for a *Bartonella bacilliformis* T162I mutant, which was only 5-fold more resistant (71). However, it is similar to the *B. burgdorferi* R133I GyrB mutation that is 300-fold more resistant (73). As noted previously with spectinomycin resistance in *E. gracilis* and *Anacystis*, species-specific interactions with the antibiotic could be responsible for the observed difference in resistance (88). The least resistant of the mutants is T162S, which is 5-fold resistant to coumermycin A<sub>1</sub>. The side
chain of serine is structurally similar to that of threonine. This similarity probably allows some hydrogen bonding, or does not change the conformation of GyrB sufficiently to interrupt the other coumermycin A₁ contacts at residues G74 and R133. In *E. coli*, the homologous T165S mutant does not confer resistance to novobiocin, a structurally similar, but less potent inhibitor of gyrase activity (67, 72, 110). This may explain why mutations in *E. coli* R136 are the main sources of resistance to coumermycin A₁ (72). Alternatively, the reported difference in resistance between *E. coli* and *B. burgdorferi* at this residue may be the result of species differences in the interaction of GyrB and coumermycin A₁, or biochemical differences between novobiocin and coumermycin A₁, which is essentially a dimer of novobiocin.

The T162L mutant that is 4000-fold more resistant to coumermycin A₁ than the wild-type or about ten-fold more resistant than the T162I mutant is particularly puzzling. These two amino acids have side chain isomers that would seem to provide a similar chemical milieu for coumermycin A₁. To further confuse this situation, R133I and R133L mutants have these levels of resistance reversed with R133I conferring 10-fold more resistance than R133L (B. Eggers and D.S. Samuels, unpublished data). The T162M mutation is 80-fold resistant and nearly matches the 100-fold resistance of an R133M mutation (B. Eggers and D. S. Samuels, unpublished data). The most resistant of the T162 mutants is T162F. This is likely due to the bulky aromatic ring of the phenylalanine side chain, and along with the nonpolar leucine (T162L) and isoleucine (T162I), lacks the ability to form hydrogen bonds. T162F conferred resistance up to 6000-fold
more than B31-A. A nonpolar side chain mutant T166A (B. burgdorferi T162) in the spirochete Brachyspira hyodysenteriae conferred coumermycin A₁ resistance 400- to 500-fold over the wild-type (111), which is within the range of resistance levels found in this study. Other nonpolar side chain mutants, the homologous E. coli T165A and T165V, have been reported to increase the novobiocin IC₅₀ for ATPase to 18 and 13-fold (110). The T173N (B. burgdorferi T162) mutation in Staphylococcus aureus conferred 120-fold resistance to coumermycin A₁, but produced just two-fold resistance to novobiocin indicating that nonpolar mutations may provide more resistance to coumermycin A₁ than novobiocin (70). These levels are considerably less than the resistance in B. burgdorferi.

The doubling time of T162F is significantly slower than B31-A, and is probably a result of the substitution from the polar side chain of threonine to the nonpolar aromatic ring of phenylalanine. The conformational changes in gyrase resulting from this substitution would provide a logical explanation for a decrease in gyrase function that is manifested in the slower growth rate. Substituting residues in gyrase from charged or polar residues to nonpolar alanine residues in E. coli has been shown to decrease activity (110). This could explain the decreases in growth rates for the T162 mutants that have nonpolar side chains. The slowest growing mutants, T162L (8.7 hours), T162I (8.6 hours), T162M (8.6 hours) and T162F (13.9 hours), all have nonpolar side chains that may reduce the ATPase activity of GyrB. This in turn could interfere with replication and transcription associated with growth. The doubling time of B. burgdorferi coumermycin A₁ resistant R133I mutants was about 10% slower than the wild-
type (73), which is similar to the growth rate of the T162I mutant. The growth rate of T162S is slightly slower than the wild-type at 8.2 hours but faster than the nonpolar substitutions, which may be a reflection of near normal gyrase activity resulting from the retention of a polar amino acid at residue 162.

5.6 Conclusion

Several mutations in the 16S rRNA gene and rpsL encoding 30S ribosomal protein S12 have been identified conferring resistance to spectinomycin, streptomycin, kanamycin or gentamicin in B. burgdorferi, including an A1191G (B. burgdorferi A1185G) mutation that has not been previously reported in bacteria. Random site-directed mutagenesis generated additional B. burgdorferi mutants resistant to coumermycin A1 in addition to a naturally occurring mutant.

The frequency of spectinomycin-resistant mutants was approximately 6 x 10^6. Growth and competition assays suggest this frequency results from the ability of these mutants to compete with wild-type B31-A for resources. Typical bacterial mutation rates are 100-fold lower than those for the SPR3 and SPR6 spectinomycin-resistant mutants. Mutation rates for the three aminoglycoside-resistant mutants are similar to those typically reported for bacteria. Neither of these mutants, SmR4 or KanR/GenR, competed with wild-type B31-A indicating a higher fitness cost than the SPR3 and SPR6 mutants to maintain these mutations in the population. Although the growth rate for SmR4 was essentially the same as the wild type, this mutant was unable to compete with B31-A. The slower growth
rate of the KanR/GenR mutants is not statistically significant although it could be a reason for the inability of this mutant to compete with B31-A. Reduced ribosomal function, which has not been observed in homologous *E. coli* mutants, may also account for the inability of this mutant to compete with wild-type B31-A. The higher fitness cost measured by the inability of the KanR/GenR mutants to compete with wild-type B31-A is likely the reason for the lower frequencies of these mutants in the population compared to the spectinomycin-resistant mutants.

Levels of antibiotic resistance were very high for SPR3 (1500-fold), SPR6 (>2500-fold) and KanR/GenR (>100-fold) all of which had mutations in the 16S rRNA gene. Streptomycin resistance conferred by the two S12 mutations K88R and K88E were considerably lower at 7- and 10-fold, respectively, but were similar to those reported for other streptomycin-resistant microorganisms. Cross-resistance in SPR3, SPR6, SmR3, SmR4 and KanR/GenR suggest each of the mutations cause conformational changes along the 16S rRNA that affect interactions not associated with the respective antibiotics.

None of the mutants exhibited an antibiotic-dependent phenotype. All had comparable plating efficiencies and growth rates with or without antibiotic selection.

Levels of coumermycin *A*1 resistance and growth rates appear to be directly related to the side chain substitutions at residue 162 of GyrB.

Substitutions of the polar side chain of threonine to nonpolar side chains (T162F, T162L, T162I) increase resistance to coumermycin *A*1 over 420-fold with the most dramatic increase from the aromatic side chain of phenylalanine (6000-fold).
Consistent with this observation is the substitution of serine with a polar side chain conferring only a five-fold increase in resistance. Growth rates also followed this pattern, with T162F being the slowest growing mutant and T162S growing faster than the nonpolar substitutions. T162M, although possessing a nonpolar side chain, had a growth rate equal to the wild-type B31-A. This may be due to the unique side chain with sulfur providing resistance to coumermycin A1, but not inhibiting normal gyrase function.

5.7 Future Research

The experiment to determine if the spectinomycin-resistant 16S rRNA was dominant or recessive was inconclusive. A new vector, such as pBSV2, might be used to provide adequate expression of the mutant gene. Ribosomal activity using a CAT reporter/assay could be done to determine mutant ribosome activity versus the wild-type. Reduced ribosomal activity may be responsible for the slower growth rates and one of the fitness costs of the mutation. Footprinting of the mutant 16S rRNA with each of the antibiotics may confirm whether antibiotic affinity is affected by the mutations. Spectinomycin binding affinity should be assessed in the A1185G 16S rRNA mutant. A more accurate evaluation of the competition assays could be achieved by performing an RNA primer extension from the competition cultures to measure the relative amounts of mutant 16S rRNA versus wild-type. Additional searches should be made with infectious strains of *B. burgdorferi* to see if the mutants are associated only with non-infectious strains or if they are present in all populations of *B. burgdorferi*. A
dominant antibiotic-resistant mutation in an infectious strain may prove to be clinically relevant.
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