

## *aadA* Confers Streptomycin Resistance in *Borrelia burgdorferi*

Kristi L. Frank,<sup>1†</sup> Sharyl F. Bundle,<sup>1‡</sup> Michele E. Kresge,<sup>1§</sup> Christian H. Eggers,<sup>2</sup> and D. Scott Samuels<sup>1\*</sup>

Division of Biological Sciences, The University of Montana, Missoula, Montana 59812,<sup>1</sup> and Center for Microbial Pathogenesis, University of Connecticut Health Center, Farmington, Connecticut 06030<sup>2</sup>

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**To enhance genetic manipulation of the Lyme disease spirochete *Borrelia burgdorferi*, we assayed the *aadA* gene for the ability to confer resistance to the antibiotics spectinomycin and streptomycin. Using the previously described pBSV2 as a backbone, a shuttle vector, termed pKFSS1, which carries the *aadA* open reading frame fused to the *B. burgdorferi* *flgB* promoter was constructed. The hybrid *flgB* promoter-*aadA* cassette confers resistance to spectinomycin and streptomycin in both *B. burgdorferi* and *Escherichia coli*. pKFSS1 has a replication origin derived from the 9-kb circular plasmid and can be comaintained in *B. burgdorferi* with extant shuttle vector pCE320, which has a replication origin derived from a 32-kb circular plasmid, or pBSV2, despite the fact that pKFSS1 and pBSV2 have the same replication origin. Our results demonstrate the availability of a new selectable marker and shuttle vector for genetically dissecting *B. burgdorferi* at the molecular level.**

*Borrelia burgdorferi* is the etiologic agent of Lyme disease, the most common arthropod-borne disease in the United States (22, 34). The progressive development of a system to genetically manipulate *B. burgdorferi* has provided many of the requisite tools to analyze molecular mechanisms in this pathogenic spirochete (4, 9, 10, 26, 29, 31, 33, 35).

Four antibiotic resistance markers have been used for molecular genetic analyses of *B. burgdorferi* (4, 12, 29, 31). The first marker developed, a mutant *gyrB* allele that confers coumermycin A<sub>1</sub> resistance (26, 29, 30), has been used for targeted gene disruption in *B. burgdorferi* (5, 11, 17, 38–40). However, caveats regarding the coumermycin A<sub>1</sub> resistance marker include the tendency of the *gyrB* gene to recombine at the chromosomal locus, which necessitates extensive screening of transformants (26), and pleiotropic effects that are likely caused by perturbed levels of DNA supercoiling (1, 2) (D. S. Samuels, C. R. Kuchel, and M. E. Kresge, unpublished data). In response to the need for an exogenous selectable marker, Bono et al. fused the open reading frame of *aphI*, a kanamycin resistance gene from Tn903, to either the *B. burgdorferi* *flaB* or *flgB* promoters, thereby developing kanamycin resistance cassettes that function in *B. burgdorferi* (4). These hybrid resistance markers have been used to disrupt genes (7, 12, 18, 20, 23), complement a gene (16, 25), demonstrate transduction by bacteriophage  $\phi$ BB1 (10), and construct *Escherichia coli*-*B. burgdorferi* shuttle vectors (9, 35, 36). A third selectable marker, the *ermC* gene conferring erythromycin resistance, was originally introduced into *B. burgdorferi* on a broad-host-range plasmid from gram-positive bacteria (31). It has been used to

disrupt genes (15, 16, 18) and to conduct complementation studies in *B. burgdorferi* (32). A gentamicin resistance marker was constructed by fusing the *aacC1* open reading frame to the *flgB* promoter and used to disrupt genes (12, 36). Other antibiotic resistance markers have been identified recently but not yet used to genetically manipulate *B. burgdorferi* (33).

With the new tools available to study *B. burgdorferi* at the molecular level, genetic manipulation is poised to become powerful and convenient. Currently, the genetic system needs to be augmented by developing additional selectable markers for complementation studies, multiple gene disruptions, and dual-plasmid experiments. Therefore, we investigated whether the *aadA* gene, which encodes an aminoglycoside-3'-adenylyl-transferase that confers resistance to spectinomycin and streptomycin (19, 24), can function in *B. burgdorferi*.

**Shuttle vector construction and transformation.** We constructed a hybrid antibiotic resistance marker by fusing the *aadA* open reading frame to the strong constitutive *flgB* promoter (13) on the backbone of the extant *E. coli*-*B. burgdorferi* shuttle vector pBSV2 (35). The *aadA* gene from R100, a broad-host-range plasmid originally isolated from *Shigella flexneri* (21, 37), was generously provided by Joachim Frey (Institute for Veterinary Bacteriology, University of Berne, Berne, Switzerland) on plasmid pHP45 $\Omega$  (24). Primers *aadA* F+NdeI (CAT ATGAGGGAAGCGGTGATC) and *aadA* R+AatII (GACG TCATTATTTGCCGACTACC), which introduced restriction sites for enzymes *Nde*I and *Aat*II, respectively, were used to amplify the *aadA* gene by PCR, essentially as described previously (17, 29). The 802-bp *aadA* PCR product was then cloned into plasmid pCR2.1-TOPO (Invitrogen) to create pTAadA1. pBSV2, generously provided by Philip Stewart (Rocky Mountain Laboratories, Hamilton, Mont.), and pTAadA1 were each digested with *Aat*II and *Nde*I. The digestion removes the kanamycin resistance open reading frame and part of the zeocin resistance gene from pBSV2. The appropriate restriction fragments were resolved by agarose gel electrophoresis and purified with a QIAEX II gel extraction kit (QIAGEN). pKFSS1 (Fig. 1) was constructed by ligating the open reading

\* Corresponding author. Mailing address: Division of Biological Sciences, 32 Campus Dr., #4824, The University of Montana, Missoula, MT 59812-4824. Phone: (406) 243-6145. Fax: (406) 243-4184. E-mail: samuels@mso.umt.edu.

† Present address: Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905.

‡ Present address: Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030.

§ Deceased.

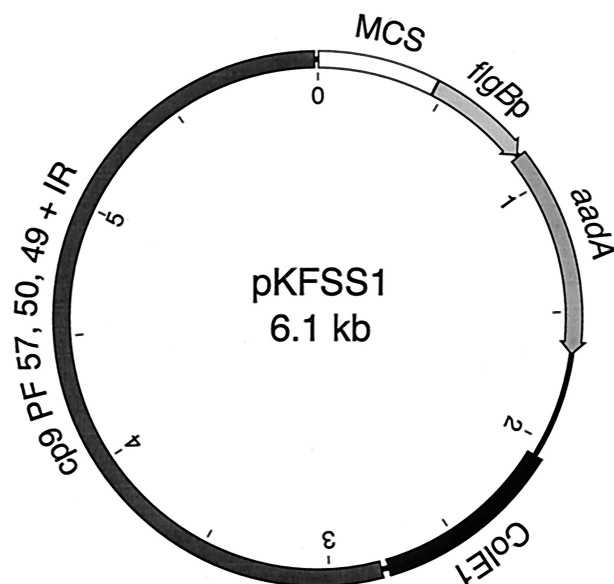


FIG. 1. Schematic of pKFSS1. pKFSS1 was derived from pBSV2 by replacing the kanamycin resistance open reading frame, under the control of the *flgB* promoter (*flgBp*), with a spectinomycin and streptomycin resistance open reading frame (*aadA*) from pHP45 $\Omega$ . pBSV2 and pKFSS1 contain the *lacZ* $\alpha$  fragment and multiple cloning site (MCS) and the replication origin (ColE1) of pCR-XL-TOPO. Replication in *B. burgdorferi* is mediated by a sequence containing genes in paralogous families (PF) 57, 50, and 49 (BBC01, BBC02, and BBC03; open reading frames 1, 2, and 3) and two inverted repeats (IR) from cp9, the 9-kb circular plasmid of *B. burgdorferi*.

frame of *aadA* (from pTAaadA1) to the *flgB* promoter (*flgBp*) carried on the 5.3-kb backbone of *Aat*II- and *Nde*I-digested pBSV2 (35).

*B. burgdorferi* sensu stricto strain B31-A, a high-passage avirulent clone of strain B31 (4), was routinely cultivated in modified Barbour-Stoenner-Kelly (BSK-H) complete medium (Sigma) at 34°C with a ~5% CO<sub>2</sub> atmosphere. *B. burgdorferi* was electroporated with 1 to 10  $\mu$ g of plasmid DNA and plated in semisolid medium as previously described (27), although 10 $\times$  medium 199 was fully or partially substituted for 10 $\times$  CMRL-1066 in some experiments. Transformants were selected in either 160 to 400  $\mu$ g of kanamycin ml<sup>-1</sup>, 1.5  $\mu$ g of spectinomycin ml<sup>-1</sup>, or 20 to 80  $\mu$ g of streptomycin ml<sup>-1</sup>. A mock electrotransformation (no DNA) served as a control.

**Selection of transformants.** *B. burgdorferi* was transformed with either pBSV2, which carries the *flgBp::aphI* kanamycin resistance cassette, or pKFSS1, which carries the *flgBp::aadA* spectinomycin and streptomycin resistance cassette. Cells were plated in medium containing kanamycin, spectinomycin, or streptomycin to assess whether these antibiotics can be used to select for *B. burgdorferi* transformants (Table 1). Transformation of *B. burgdorferi* with pKFSS1 yielded colonies on medium containing either streptomycin or spectinomycin, but not kanamycin. Electroporation with no DNA, pBSV2, or pKFSS1 yielded high numbers of colonies when plated on medium containing spectinomycin, but only cells transformed with pKFSS1 produced colonies on plates containing streptomycin.

*B. burgdorferi* transformants isolated by selection with spectinomycin or streptomycin were analyzed for the presence of

TABLE 1. Transformation efficiencies of *B. burgdorferi* with pBSV2 or pKFSS1 in semisolid medium containing different antibiotics<sup>a</sup>

Plasmid	No. of colonies/ $\mu$ g of DNA ( $\pm$ SEM)		
	Streptomycin	Spectinomycin	Kanamycin
pBSV2 ( <i>aphI</i> )	0 ( $\pm$ 0)	327 ( $\pm$ 41)	220 ( $\pm$ 134)
pKFSS1 ( <i>aadA</i> )	79 ( $\pm$ 27)	695 ( $\pm$ 136)	0 ( $\pm$ 0)

<sup>a</sup> Control transformations with no DNA yielded no colonies when plated on medium containing streptomycin or kanamycin, and the equivalent of 863 ( $\pm$ 234) colonies when plated on medium containing spectinomycin.

pKFSS1 by transforming total genomic DNA from these clones into *E. coli*. Genomic DNA was extracted from five randomly chosen spectinomycin-resistant and two streptomycin-resistant *B. burgdorferi* colonies as described previously (28). DNA was transformed into chemically competent *E. coli* JM109, and the cells were plated on semisolid Luria-Bertani medium containing 60 to 100  $\mu$ g of spectinomycin ml<sup>-1</sup>. *flgBp::aadA* confers resistance to both streptomycin and spectinomycin in *E. coli*, but many common cloning strains are resistant to streptomycin. Plasmid DNA was recovered from *E. coli* transformants by alkaline lysis (3). Only those *E. coli* cells transformed with DNA from streptomycin-resistant pKFSS1 transformants of *B. burgdorferi* acquired resistance to spectinomycin; pKFSS1 was recovered from these *E. coli* transformants. pKFSS1 was not recovered from any *B. burgdorferi* colonies isolated on plates containing spectinomycin, suggesting that they were background mutants.

**Plasmid stability.** A randomly chosen *B. burgdorferi* transformant carrying pKFSS1 was serially passaged in liquid BSK-H medium without streptomycin selection at 34°C. After about 50 generations, the culture was plated on semisolid medium without selection, and 10 colonies were isolated. Total genomic DNA was extracted from *B. burgdorferi* clones and used to transform *E. coli* DH5 $\alpha$  to screen for pKFSS1, as described above. Six of the 10 colonies isolated in the absence of streptomycin selection contained pKFSS1. Therefore, *B. burgdorferi* transformants maintained pKFSS1 for 50 generations. However, pKFSS1 is not as stable as the parental plasmid, pBSV2, which is maintained in all 20 colonies following ~90 passages in the absence of selection, as assayed by PCR (36). This difference in stability was unexpected, because the two plasmids have the same replication origin and are nearly identical except for the selectable marker; pKFSS1 also has a truncated zeocin resistance gene downstream of the *aadA* gene.

**Antibiotic resistance in *B. burgdorferi*.** The ability of pKFSS1 to confer resistance to selected aminoglycosides and spectinomycin was measured to assess whether the *flgBp::aadA* marker could be used in conjunction with the extant selectable markers that confer resistance to similar antibiotics. Resistance was assayed by inoculating media containing various concentrations of streptomycin, spectinomycin, kanamycin, and gentamicin antibiotics. Cultures of wild-type and transformed *B. burgdorferi* were inoculated into 4-ml cultures of BSK-H at approximately 10<sup>6</sup> cells ml<sup>-1</sup> and grown at 34°C for about 72 h in the presence of the antibiotics. Cultures were assayed for growth by spectrophotometry as previously described (28). Assays were replicated three to five times. The antibiotic concen-

TABLE 2. Antibiotic susceptibility of *B. burgdorferi* clones containing pBSV2 or pKFSS1

Plasmid	IC <sub>50</sub> (μg ml <sup>-1</sup> ) <sup>a</sup>			
	Streptomycin	Spectinomycin	Kanamycin	Gentamicin
None	7	0.1	7	2
pBSV2 ( <i>aphI</i> )	3	0.2	>1,400	6
pKFSS1 ( <i>aadA</i> )	650	1.4	10	3

<sup>a</sup> The IC<sub>50</sub> is the concentration of antibiotic that inhibits growth by 50%.

tration that inhibits 50% of bacterial growth was determined for wild-type *B. burgdorferi* and pBSV2 and pKFSS1 transformants (Table 2). The *flgBp::aadA* cassette in *B. burgdorferi* transformants confers 10-fold resistance to spectinomycin and approximately 100-fold resistance to streptomycin. No cross-resistance to kanamycin or gentamicin was observed with the *flgBp::aadA* cassette. Additionally, the *flgBp::aphI* cassette carried on pBSV2 does not confer resistance to either spectinomycin or streptomycin.

**Plasmid compatibility.** The ability to transform *B. burgdorferi* with two different shuttle vectors would expand experimental opportunities. Therefore, we transformed cells carrying pKFSS1 with the shuttle vector pCE320 (9), which uses a compatible replication origin from a 32-kb circular plasmid and the *flgBp::aphI* kanamycin resistance cassette. Plasmids carrying the same replication origin typically are not maintained together in the same cell (14), but plasmid incompatibility has not been extensively studied in *B. burgdorferi* (9, 35, 36). Therefore, pKFSS1 was also cotransformed with its parental plasmid pBSV2. Plasmids were electroporated into *B. burgdorferi*, and cells were plated in semisolid medium containing both kanamycin and streptomycin, as described above. As expected, transforming cells carrying pKFSS1 with pCE320 yielded colonies on plates with both kanamycin and streptomycin, indicating that the cells contained both resistance cassettes. Surprisingly, colonies resistant to both kanamycin and streptomycin were also obtained from cells carrying pKFSS1 transformed with pBSV2, although both plasmids carry the same replication origin derived from the 9-kb circular plasmid cp9 (35).

To test that these plasmids autonomously replicated in the cotransformant clones, DNA was isolated using a Wizard Plus Midipreps DNA purification system (Promega) and ~7 μg was loaded on a 1% agarose gel in 1× TAE (40 mM Tris–20 mM acetate–1 mM EDTA) buffer. Electrophoresis and Southern blotting to an Immobilon-Ny<sup>+</sup> membrane (Millipore) were performed essentially as described previously (1, 10), except the hybridization signal was detected using a FLA3000G radioisotope imaging system (Fuji film). The probes were amplified by two rounds of PCR using oligonucleotides *aadA* F+NdeI and *aadA* R+AatII or oligonucleotides *kanR* 88F (AATGTCGGGCAATCAGGTG) and *kanR* 488R (TCACTCGCATCAACCAAACC), and labeled using a Prime-It II random primer labeling kit (Stratagene). pKFSS1 and pCE320 differ in size, so they are readily resolved and detected in the cloned transformants by ethidium bromide staining (Fig. 2A). pKFSS1 and pBSV2 are similar in size, but they can be distinguished using either an *aadA* probe specific for pKFSS1 (Fig.

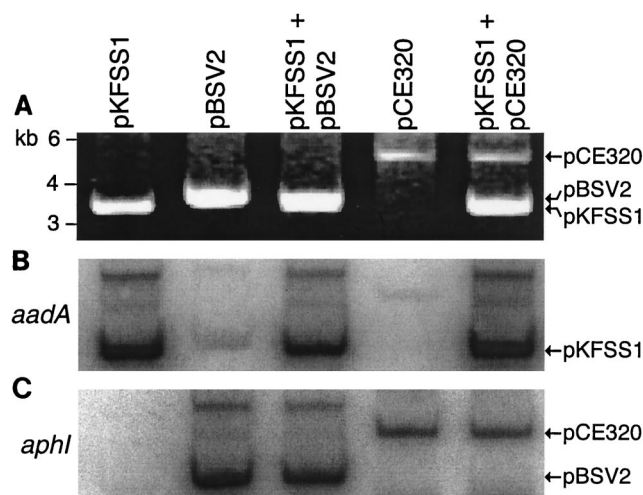


FIG. 2. Plasmid compatibility of pKFSS1 with pBSV2 and pCE320. A *B. burgdorferi* clone carrying pKFSS1 (6.1 kb; streptomycin resistance) was cotransformed with either pBSV2 (6.4 kb; kanamycin resistance) or pCE320 (8.1 kb; kanamycin resistance) and grown with both kanamycin and streptomycin in semisolid medium. Plasmid DNA, isolated from *B. burgdorferi* clones carrying pKFSS1 alone, pBSV2 alone, pKFSS1 and pBSV2, pCE320 alone, or pKFSS1 and pCE320, was resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining (A). The positions of molecular mass markers (in kilobase pairs [kb]) are indicated to the left of the gel. The gel was transferred to a nylon membrane and probed with either the spectinomycin and streptomycin resistance (*aadA*) open reading frame (B) or the kanamycin resistance (*aphI*) open reading frame (C). The *aadA* probe cross-reacts to a small extent with pBSV2 and pCE320.

2B) or an *aphI* probe specific for pBSV2 and pCE320 (Fig. 2C).

Because pKFSS1 and pBSV2 carry the same origin of replication, we assayed plasmid compatibility by growing the cotransformant in the presence or absence of both kanamycin and streptomycin for 40 generations, as described above. Cells were then plated in the absence of antibiotic or in the presence of kanamycin, streptomycin, or both kanamycin and streptomycin to assess the stability of pKFSS1 and pBSV2 (Table 3). We expected that the same number of colonies would be obtained on all four plates from cells grown in the presence of antibiotics, which would maintain selective pressure on those cells containing both plasmids. Interestingly, cells on plates with streptomycin (either alone or with kanamycin) formed

TABLE 3. Plating efficiencies in the presence of two antibiotics of a *B. burgdorferi* clone carrying both pBSV2 (kanamycin resistance) and pKFSS1 (streptomycin resistance) after 40 generations with or without selection

Treatment <sup>a</sup>	% of colonies compared to no antibiotic <sup>b</sup> (±SEM)		
	Kanamycin	Streptomycin	Kanamycin and streptomycin
With selection	95 (±1)	70 (±5)	79 (±8)
Without selection	93 (±12)	36 (±9)	27 (±11)

<sup>a</sup> Selection with kanamycin and streptomycin.

<sup>b</sup> Plates without antibiotics yielded a mean of 448 colonies (= 100%) from the cultures grown for 40 generations with selection (both kanamycin and streptomycin) and 379 colonies (= 100%) from the cultures grown for 40 generations without selection.



about 30% fewer colonies than cells on plates with kanamycin. We speculate that cells carrying pKFSS1 have a lower plating efficiency than cells carrying pBSV2, which may explain the observed lower transformation efficiency of pKFSS1 (Table 1). The mechanism for this lower plating efficiency is unknown and unanticipated, considering the similarity of pKFSS1 and pBSV2. Most cells grown in the absence of antibiotic maintained pBSV2. However, only about half the colonies were obtained on plates containing streptomycin (selecting for pKFSS1) from the cells grown without antibiotic compared to cells grown with an antibiotic. This is consistent with the above observation that pKFSS1 is not as stable as pBSV2. Several colonies from the *B. burgdorferi* cotransformant grown without antibiotic were isolated and assayed for plasmid DNA by transformation of *E. coli*, as described above. All colonies contained the expected plasmid(s) selected with the antibiotic(s) used for plating (data not shown).

**Conclusions.** Molecular genetic manipulation in *B. burgdorferi*, though relatively immature, has finally advanced to the stage where complex genetic studies can be conducted. Several methods to introduce foreign DNA into *B. burgdorferi* have been described, including electroporation (27, 29), transduction (10), and chemical transformation (12). Gene disruption and complementation experiments, made possible by the development of selectable markers (4, 12, 29, 31, 33) and shuttle vectors (9, 31, 35), are now available for probing gene function in *B. burgdorferi*.

We examined the ability of the *aadA* gene to confer spectinomycin and streptomycin resistance in *B. burgdorferi*. This resistance gene functions in several bacteria, including *Myxococcus xanthus*, another genetically challenging organism (19). We demonstrated that a hybrid *flgBp::aadA* cassette provides approximately 100-fold resistance to streptomycin in *B. burgdorferi* (Table 2) and allows for selection of streptomycin-resistant transformants in semisolid medium. The *aadA* gene with its native promoter confers low-level antibiotic resistance (B. J. Kimmel, M. E. Kresge, and D. S. Samuels, unpublished data). Spectinomycin-resistant *B. burgdorferi* arose at a high frequency, regardless of whether they had been electroporated with pKFSS1, pBSV2, or no DNA (Table 1). We were unable to recover plasmid DNA from these spectinomycin-resistant colonies, which suggests that they were naturally occurring background mutants.

The ability to use streptomycin resistance as a selectable marker adds significantly to the existing antibiotic-resistant genes. Streptomycin is not clinically used to treat *B. burgdorferi* infections in humans, and *aadA* does not have the potential for homologous recombination into the *B. burgdorferi* genome. Limitations imposed by the coumermycin A<sub>1</sub> marker have resulted in recent genetic studies relying on only the erythromycin resistance and kanamycin resistance markers (10, 12, 15, 16, 18, 20, 23, 25, 32). *flgBp::aadA* does not confer cross-resistance to either kanamycin or gentamicin (Table 2), allowing this resistance cassette to be used in conjunction with *flgBp::aphI* and *flgBp::aacC1*.

pKFSS1 is compatible with either pCE320 or pBSV2, even though pKFSS1 and pBSV2 have the same cp9 origin of replication. The comaintenance of these two plasmids is surprising because pBSV2 appears to displace the parental cp9 plasmid (35). In addition, other *B. burgdorferi* plasmids with the same

replication origin and compatibility locus demonstrate plasmid incompatibility (9, 35, 36). However, unlike the other larger *B. burgdorferi* plasmids, cp9 does not encode a gene for a paralogous family 32 (PF32) protein (6, 8, 35). PF32 proteins are homologous to the ParA proteins that play a critical role in the stable maintenance of several well-characterized plasmids, including P1 and F (14). A number of factors, including copy number and the absence of the ParA homolog, may contribute to the maintenance of pBSV2 and pKFSS1 in the same *B. burgdorferi* cell. Whatever the reason, compatibility of pKFSS1 with either pCE320 or pBSV2 enables experimental approaches that require introducing genes or regulatory sequences on two different vectors. *flgBp::aadA* and pKFSS1 add an essential new capability to the genetic study of *B. burgdorferi* by furthering techniques to complement mutants, simultaneously disrupt multiple genes, monitor several promoters, or assay gene interactions.

K. L. Frank and S. F. Bundle contributed equally to this work.

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