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Infectivity of the Highly Transformable BBE02⁻ lp56⁻ Mutant of *Borrelia burgdorferi*, the Lyme Disease Spirochete, via Ticks

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Infectious *Borrelia burgdorferi* strains that have increased transformability with the shuttle vector pBSV2 were recently constructed by inactivating the gene encoding BBE02, a putative restriction-modification gene product expressed by the linear plasmid lp25 (Kawabata et al., Infect. Immun. 72:7147–7154, 2004). The absence of the linear plasmid lp56, which carries another putative restriction-modification gene, further enhanced transformation rates. The infectivity of these mutants was assessed previously in mice that were inoculated with needle and syringe and was found to be equivalent to that of wild-type spirochetes. Here we examined the infectivity of spirochetes to ticks after capillary inoculation of *Ixodes scapularis* nymphs and the subsequent spirochetal infectivity to mice via ticks by using *B. burgdorferi* B31 clonal isolates lacking lp56 and/or BBE02. The absence of lp56 (but not BBE02) correlated with a lower number of spirochetes in ticks after feeding on mice; this plasmid thus may play a role, albeit not an essential one, in supporting spirochetal survival in the feeding tick. Importantly, however, the absence of lp56 and BBE02 did not detectably influence infectivity to mice via ticks.

Lyme borreliosis is a complex multisystem disorder with both early and late manifestations. It is caused by infection with the spirochete *Borrelia burgdorferi* sensu lato, which is transmitted to humans by the bite of infected ticks. In the United States, *B. burgdorferi* is most commonly transmitted by ticks of the genus *Ixodes*, chiefly *Ixodes scapularis*. Early signs and symptoms of Lyme disease include erythema migrans, acute meningitis, and Bell's palsy, whereas the late phase of the disease may manifest as arthritis and chronic neurologic abnormalities (8, 21).

Little is known about the pathogenesis of Lyme disease and the bacterial virulence factors involved. Bacterial lipoproteins have been implicated as generic virulence factors of *B. burgdorferi* as this spirochete genome bears more than 150 open reading frames putatively encoding such molecules (1, 4). Lipoproteins are potent immunopotentiators that are known to cause inflammation (2, 5, 12–14, 18, 19, 24, 25), which in turn is present in virtually every organ accessible to the spirochetes during infection. Aside from lipoproteins as generic inflammatory molecules, only a few *B. burgdorferi* proteins have thus far been shown definitively to play a role in spirochetal virulence. Outer surface protein C (OspC), itself a lipoprotein, has been reported to facilitate spirochetal invasion of tick salivary glands and to be essential in the initial phase of infection in mammals (7, 16). The lipoprotein OspA is essential for the survival of *B. burgdorferi* in ticks and binds to the tick receptor protein TROSPA (15, 26). Finally, the gene *bptA* (BBE16) is required for persistent infection of ticks and also increases the infectivity of *B. burgdorferi* in mice by needle inoculation (20) and BBE22, a gene that encodes a nicotinamidase, is essential for spirochetal survival in mice (6, 17).

More information on *B. burgdorferi* virulence factors is now likely to be forthcoming due to progress in the ability to efficiently transform this spirochete. Low transformation efficiencies had been repeatedly reported for infectious isolates of the B31 strain (3, 11, 22). The presence of the linear plasmids lp25 and lp56 of *B. burgdorferi* B31 was found to be strongly associated with decreased transformation efficiencies; these properties were tentatively attributed to the products of the putative restriction-modification genes BBE02 and BBQ67 (11). These genes, which encode motifs consistent with both DNA endonuclease and methlytransferase functions, are carried on lp25 and lp56, respectively. Recently, highly transformable and infectious *B. burgdorferi* B31 mutants were constructed by inactivating BBE02 by homologous recombination (10). The transformation efficiency with the shuttle vector pBSV2*C03*::*gnt*Δ*kan* increased from <1 to 10 colonies per microgram of DNA when BBE02 was disrupted in a wild-type (WT) B31 isolate (containing all plasmids) and from 14 to approximately 600 colonies per microgram of DNA when BBE02 disruption was performed in a B31 isolate lacking lp56 and lp28-4. BBE02 disruptants and pBSV2*C03*::*gnt*Δ*kan* transformants of these clones remained infectious in C3H/HeN mice as assessed by needle and syringe inoculation, and the 50% infective doses of the BBE02 disruptants were $<10^2$ organisms per mouse, values which were similar to that of the wild-type B31 strain (10).

The genes whose expression is essential for *B. burgdorferi* to be infectious to mice via needle and syringe are not necessarily the same as the genes required to mediate infectivity via ticks. For example, the supplementation of BBE22, a gene that en-

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TABLE 1. Effect of lp56 on survival and proliferation of *B. burgdorferi* clones 5A1 (lp56⁻) and 5A19 (lp56⁺) in capillary-inoculated ticks[a]

B. burgdorferi B31 isolate	Values for effects determined in expt 1						
	Before mouse feeding			After mouse feeding			
	No. of positive nymphs/total fed nymphs	Mean no. of spirochetes per field ± SD	*P*	Mouse	No. of positive nymphs/total fed nymphs	Mean no. of spirochetes per field ± SD	*P*
5A1 (lp56⁻)	5/5	0.64 ± 0.17		S327	14/14	41.84 ± 21.88	
				S328	4/5		
			0.13020	S329	6/6		0.000001
5A19 (WT)	5/5	0.48 ± 0.13		S330	12/12	92.83 ± 39.51	
				S331	4/4		
				S332	8/8		

[a] Culture positivity and mean number of spirochetes per field were determined 24 to 25 days after capillary inoculation (before mouse feeding group) or 7 days after mouse feeding (after mouse feeding group). Three and 10 fields were counted for each fed and unfed tick, respectively. Mean values of spirochetes per field were averaged over the total number of fed ticks in the three mice of each group. Student's *t* test was used to evaluate statistical significance. All mice were culture positive at all tissue sites examined after feeding.

codes a nicotinamidase and is located on lp25, renders lp25⁻ spirochetes infectious to mice when the spirochetes are inoculated via needle and syringe (17), although not via ticks (20, 23; but see reference 6). The *bptA* (BBE16) gene product and, potentially, the products of other genes located on lp25 are claimed to be necessary for infection to occur via the natural tick route (20).

Lest this also be the case with genes present on lp56 (and lp28-4), in this study, we examined whether BBE02 inactivation or the absence of these plasmids affects tick infection and the transmission of *B. burgdorferi* from ticks to mice. These experiments address the important question of whether the transformable mutant retained the ability to infect mice via the natural route, i.e., via ticks.

All spirochetal isolates had undergone two in vitro passages prior to being fed to ticks. Plasmid content was determined immediately prior to these two passages and was as stated. Spirochetes were cultured in complete BSK-H medium (Sigma Chemical Co., St. Louis, MO), to a concentration of 10^8 cells/ml. Transformants 5A18 NP1 and 5A4 NP1 (10) were cultured in the presence of 0.2 mg/ml kanamycin (Sigma) to assure the retention of lp25 carrying the BBE02::kan mutant allele. Nymphal *I. scapularis* ticks were capillary fed with spirochetes by a method described previously (9). Capillary-fed nymphs were kept at 22°C in a humidified environment for 24 to 25 days. Five ticks of each clonal group were crushed, and spirochetes were quantified after immunofluorescence labeling (see below) to evaluate comparative spirochetal survival in flat ticks. The spirochetes in 10 microscope fields were counted for each tick, and the average number of spirochetes per field was calculated. The mean value for the five ticks is reported. The remaining capillary-inoculated ticks were allowed to feed to repletion on 8-week-old female C3H/HeN mice (Charles River Labs, Wilmington, MA). Three mice were used for tick feeding with each spirochetal isolate. The fully engorged ticks were kept in a humidified environment at 22°C for 1 week, after which time, the spirochete burden of blood-fed ticks was assessed by quantification of spirochetes. Each blood-fed tick was cleaned by immersion in 3% H_2O_2-70% ethanol and then squashed in a volume of 25 μl of sterile phosphate-buffered saline. Half of the tick suspension volume was added to 5 ml of medium for culturing purposes, and the other half was spread on a microscope slide and fixed with acetone. The slides were then incubated with fluorescein-labeled goat anti-Borrelia antibody (Kirkegaard & Perry, Gaithersburg, MD) for 30 min at 37°C, washed, and examined by fluorescence microscopy. Several fields (magnification, 600×) on each slide were counted, so as to accumulate a count of at least 100 spirochetes, to arrive at a mean number of spirochetes per field for each tick. Tick cultures were examined for the presence of spirochetes. Infectivity to mice was assessed by culturing spirochetes from ear-punch biopsy tissue and from organ culture. Biopsies were obtained on days 7, 14, and 21 after the ticks had dropped off. Mice were sacrificed 4 weeks after this time. The ears, heart, bladder, and tibiotarsal joints were removed aseptically and cultured. All tick and mouse cultures were examined weekly for growth of *B. burgdorferi* by dark-field microscopy for up to 6 weeks. Differences in mean values of spirochetes per field in a tick population were evaluated for statistical significance by the Student *t* test when only two populations were compared (experiment 1) and by analysis of variance with Scheffe's post hoc analysis when more than two tick populations were compared (experiment 2). Differences were considered significant with P values of <0.01.

In experiment 1 (Table 1), we first compared survival in flat and blood-fed ticks of spirochetes of the clonal isolate 5A1, which lacks lp56, and the WT 5A19. Five capillary-inoculated flat nymphs were assessed per clonal isolate. The mean number of spirochetes per field (±standard deviations [SD]) was 0.64 ± 0.17 for 5A1, and 0.48 ± 0.13 for 5A19 (Table 1). This difference was not statistically significant ($P = 0.1302$).

In contrast, the mean number of 5A1 spirochetes per field postfeeding was 41.89 ± 21.88, less than half of the 92.83 ± 39.51 WT spirochetes per field (Table 1). This difference was statistically significant ($P = 0.000001$). The absence of lp56 thus resulted in a diminished spirochetal proliferation during and after tick feeding. The decreased number of spirochetes did not result in a significant difference in the number of fed ticks from which cultivable spirochetes could be recovered (Table 1). More importantly, ticks infected with either of the two B31 isolates were equally able to infect mice: all of the ear-punch biopsies collected at days 7, 14, and 21 postinfection

TABLE 2. Effects of BBE02 inactivation and absence of lp56 and lp28-4 on survival and proliferation of *B. burgdorferi* clones in capillary-inoculated ticks[a]

B. burgdorferi B31 isolate	Values for effects determined in expt 2						
	Before mouse feeding			After mouse feeding			
	No. of positive nymphs/total fed nymphs	Mean no. of spirochetes per field ± SD	*P*	Mouse	No. of positive nymphs/total fed nymphs	Mean no. of spirochetes per field ± SD	*P*
5A18 NP1 (lp56$^-$, lp28-4$^-$, and BBE02$^-$)	5/5	1.3 ± 0.2		S642	7/7	42.94 ± 17.72	
				S643	4/4		
			0.000063	S644	7/7		0.000412
5A4 NP1 (BBE02$^-$)	5/5	4.26 ± 0.84		S645	4/4	79.79 ± 38.42	
				S646	9/9		
			0.040845	S647	6/6		0.098377
5A4 (WT)	5/5	3.02 ± 0.79		S648	8/8	61.92 ± 19.55	
				S649	7/7		
				S650	9/9		

[a] Culture positivity and mean number of spirochetes per field were determined 24 to 25 days after capillary inoculation (before mouse feeding group) or 7 days after mouse feeding (after mouse feeding group). Five and 10 fields were counted for each fed and unfed tick, respectively. Mean values of spirochetes per field were averaged over the total number of fed ticks in the three mice of each group. Analysis of variance with Scheffe's post hoc analysis was used to evaluate statistical significance. All mice were culture positive at all tissue sites examined after feeding.

and all four organs of each of the three mice in each group yielded positive spirochetal cultures.

In experiment 2 (Table 2), we compared the survival of spirochetal isolates 5A18 NP1 (lp56$^-$, lp28-4$^-$, and BBE02::kan), 5A4 NP1 (BBE02::kan), and 5A4 (WT) in capillary-infected ticks before and after feeding on mice. The mean number of spirochetes per field in the group of unfed ticks that was inoculated with 5A18 NP1 spirochetes was 1.3 ± 0.2. This number was significantly lower than that in unfed ticks given 5A4 NP1 (4.26 ± 0.84; $P = 0.000063$) or 5A4 (3.02 ± 0.79; $P = 0.005868$). The difference in the mean number of spirochetes per field in flat ticks that were inoculated with 5A4 NP1 and 5A4 was marginal ($P = 0.040845$). Since the absence of lp56 alone did not seem to affect spirochetal persistence in the flat tick (Table 1), the difference observed in flat ticks infected with 5A18 NP1 and those given 5A4 NP1 or 5A4 may be due to the absence of lp28-4. Alternatively, it is possible that ticks that were capillary infected with 5A18 NP1 received a slightly less concentrated spirochetal inoculum than did the other two tick populations.

Differences in spirochete survival or replication rates in fed ticks in the second experiment (Table 2) were comparable to those observed in the first (Table 1). The mean number of spirochetes per tick in nymphs with 5A18 NP1 spirochetes was 42.94 ± 17.72, which was significantly lower ($P = 0.000412$) than that in nymphs with 5A4 NP1 spirochetes (79.79 ± 38.42) (Table 2). It was also lower than in WT spirochetes (42.94 versus 61.92) (Table 2) but not significantly ($P = 0.080034$). Thus, the absence of lp56 and/or lp28-4 correlated with a decrease in the rate of spirochetal survival or replication in fed ticks. As with experiment 1, however, this reduction did not translate itself into a detectable difference in the numbers of fed ticks bearing cultivable spirochetes or in spirochetal infectivity to mice, as all of the organ and biopsy cultures from each of the three mice in each group yielded cultivable organisms. The disruption of BBE02 per se also had no deleterious effect on the spirochetes in fed ticks (Table 2).

We have presented data to suggest that lp56 (and possibly lp28-4) plays a role, albeit not an essential one, in supporting spirochetal proliferation in the feeding tick. Importantly, however, the absence of lp56 and BBE02 did not detectably influence tick transmission of *B. burgdorferi* to mice. lp56 is a large plasmid that is thought to have derived from the integration of two progenitor plasmids, a much smaller (22,622 nt) linear plasmid and a cp32-like circular plasmid of 30,349 nt (1). The genome of *B. burgdorferi* contains seven cp32-type plasmids whose sequences are highly similar to each other and to the cp32-like portion of lp56 (1). This portion contains 36 possibly functional genes, i.e., open reading frames longer than 300 bp, two "genes" that are ≤300 bp, and four pseudogenes (1). In contrast, the rest of lp56 contains only 8 possibly functional genes, 6 genes that are ≤300 bp, and 22 pseudogenes (1). BBQ67, encoding a putative restriction endonuclease and an adenine-specific DNA methyltransferase, is contained within the unique segment of lp56 (1). The genetic composition of lp56 helps to explain the increased transformation efficiency associated with the loss of this plasmid, as BBQ67 is not otherwise represented in the *B. burgdorferi* genome. It may also explain the ability of lp56$^-$ spirochetes to survive in ticks and to be transmitted to mice during tick feeding, for it is likely that the gene functions involved in this process are also present in the cp32 plasmid family. The decreased number of lp56$^-$ *B. burgdorferi* isolates found in capillary-infected ticks before and after feeding on mice has no obvious explanation at this point, in that genes within the nonredundant regions of lp56 have no known roles in infectivity. In conclusion, the BBE02$^-$ lp56$^-$ mutant of *B. burgdorferi*, which is highly transformable with the pBSV2 shuttle vector, may be used in experiments involving infections with this spirochete via ticks, the natural route.

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