AMERICAN SOCIETY FOR MICROBIOLOGY | Clinical and Vaccine Immunology

Live Attenuated *Borrelia burgdorferi* Targeted Mutants in an Infectious Strain Background Protect Mice from Challenge Infection

Beth L. Hahn,[a] Lavinia J. Padmore,[a]* Laura C. Ristow,[b]* Michael W. Curtis,[b] Jenifer Coburn[a,b]

Department of Medicine, Division of Infectious Diseases,[a] and Department of Microbiology and Molecular Genetics,[b] Medical College of Wisconsin, Milwaukee, Wisconsin, USA

***Borrelia burgdorferi*, *B. garinii*, and *B. afzelii* are all agents of Lyme disease in different geographic locations. If left untreated, Lyme disease can cause significant and long-term morbidity, which may continue after appropriate antibiotic therapy has been administered and live bacteria are no longer detectable. The increasing incidence and geographic spread of Lyme disease are renewing interest in the vaccination of at-risk populations. We took the approach of vaccinating mice with two targeted mutant strains of *B. burgdorferi* that, unlike the parental strain, are avirulent in mice. Mice vaccinated with both strains were protected against a challenge with the parental strain and a heterologous *B. burgdorferi* strain by either needle inoculation or tick bite. In ticks, the homologous strain was eliminated but the heterologous strain was not, suggesting that the vaccines generated a response to antigens that are produced by the bacteria both early in mammalian infection and in the tick. Partial protection against *B. garinii* infection was also conferred. Protection was antibody mediated, and reactivity to a variety of proteins was observed. These experiments suggest that live attenuated *B. burgdorferi* strains may be informative regarding the identification of protective antigens produced by the bacteria and recognized by the mouse immune system *in vivo*. Further work may illuminate new candidates that are effective and safe for the development of Lyme disease vaccines.**

Lyme disease (LD) is the most common vector-borne disease in North America. In addition to *Borrelia burgdorferi*, the major LD agent in North America, *B. garinii* and *B. afzelii* are agents of LD in Europe and Asia. While antibiotic treatment is available and effective in the majority of cases diagnosed early in infection, significant morbidity is associated with LD, in some cases, with symptoms continuing beyond the standard antibiotic therapy regimens. In addition, the Centers for Disease Control and Prevention (CDC) recently investigated possible *B. burgdorferi* infection as a trigger of sudden cardiac death in relatively young, active people (1, 2). Recent estimates from the CDC also suggest that the number of cases may be as much as 10-fold higher than the number actually reported (3), with current estimates of ~300,000 cases/year in the United States.

No vaccine against LD is currently available for use in humans. A single dose of lyophilized whole-cell *Borrelia* produced limited protection as a vaccine in hamsters (4, 5), and the authors suggested that further studies were warranted. In fact, whole-cell vaccines are currently available for veterinary use, but a less reactogenic multivalent subunit vaccine has more recently been developed (6–8). Live attenuated flagellumless *Borrelia* cells in a high-passage-number noninfectious background also elicited protective immunity in mice for a limited duration (9). A vaccine targeting outer surface protein A (OspA) (10, 11), an abundant protein on the surface of the bacteria grown in the laboratory, was available for human use from 1998 to 2002. Interestingly, the anti-OspA antibodies killed *B. burgdorferi* while the bacteria were still in the feeding tick, the primary site of OspA production in the life cycle of the bacterium (12–14). This recombinant, lipidated OspA vaccine was approximately 80% effective in large-scale human trials in the United States and postmarket monitoring. The genomics era revealed that different LD species and strains carry different *ospA* alleles, so a multivalent vaccine would likely be necessary for global applicability. While no evidence was ever obtained that raised concerns regarding safety, anti-OspA immune responses (cellular and humoral) are seen in patients with Lyme arthritis, particularly treatment-resistant Lyme arthritis (15–19). This led to concerns in some that the vaccine could potentially induce arthritis, although the incidence of arthritis in prelicensure trials and postlicensure monitoring was not different from that in the general population (10, 11).

With the continued increase in cases, interest in a new vaccine against LD has been renewed and recent reports include a variety of approaches. A human trial with a multivalent anti-OspA vaccine was effective against multiple *Borrelia* strains (20). A chimeric recombinant OspA vaccine candidate showed broad protection in a mouse trial (21). Other vaccination approaches have targeted reservoir animals (22–27).

An ideal vaccine candidate would be a protein highly conserved among LD *Borrelia* species in order to be broadly applicable to protection against LD caused by different strains and across a wide geographic area. To address this need, we have taken a different approach, the use of live attenuated vaccines generated in an infectious strain background, which allows us to evaluate antigens identified on the basis of recognition by a protective immune response. This includes antigenic elements encoded on genome

Received 14 June 2016 **Accepted** 17 June 2016

Accepted manuscript posted online 22 June 2016

Citation Hahn BL, Padmore LJ, Ristow LC, Curtis MW, Coburn J. 2016. Live attenuated *Borrelia burgdorferi* targeted mutants in an infectious strain background protect mice from challenge infection. Clin Vaccine Immunol 23:725–731. doi:10.1128/CVI.00302-16.

Editor: D. L. Burns, Food and Drug Administration

Address correspondence to Jenifer Coburn, jcoburn@mcw.edu.

* Present address: Lavinia J. Padmore, Research Institute, ProHealth Care, Inc., Waukesha, Wisconsin, USA; Laura C. Ristow, Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin, USA.

TABLE 1 *Borrelia* strains used in this study

Strain	Description	Antibiotic resistance	Reference
B. burgdorferi B31-A3	Wild-type, infectious clone of North American tick isolate B31, missing cp9	None	49
B. burgdorferi B31-A3 Δ*p66* mutant	Deletion of *p66* in B31-A3; noninfectious	Kanamycin	31
B. burgdorferi B31-A3 *p66*^{cp}	Complement of Δ*p66* on shuttle vector pBSV2G; overproduces P66, noninfectious	Kanamycin, gentamicin	31
B. burgdorferi N40 D10/E9	Wild-type, infectious clone of North American tick isolate N40	None	50
B. garinii PBr	Wild-type, infectious human CSF isolate from Germany	None	37

segments (plasmids) that are frequently lost during *in vitro* propagation. In this work, we focused on mutations affecting P66, a *Borrelia* outer membrane protein with integrin binding function (28, 29). Native P66 purified from *B. burgdorferi* had previously been shown to raise a protective immune response in animals (30), but a recombinant version of the protein did not. A *B. burgdorferi* mutant lacking P66 (Δ*p66* deletion mutant) was cleared from the inoculation site within 48 h and had a 50% infective dose (ID_{50}) of $>10^9$ bacteria (31). While complementation by restoration of *p66* to the chromosome restored infectivity, complementation by introducing a shuttle plasmid containing *p66* did not. The plasmid-complemented (*p66*^{cp}) strain overproduced P66 and was avirulent in mice, with an ID_{50} of $>10^9$ bacteria (31).

In this study, we tested both the Δ*p66* and *p66*^{cp} strains as live attenuated vaccine candidates in a mouse model. Our results indicated that vaccinated mice were protected from a challenge with the parental strain or a different *B. burgdorferi* strain by either needle inoculation or tick bite. Partial immunity to *B. garinii* was generated. Vaccination of mice resulted in elimination of the homologous but not the heterologous strain from the ticks after feeding. Protection was also conferred by passive transfer of sera from immunized mice to naive mice. Thus, the Δ*p66* and *p66*^{cp} strains appear to generate broadly protective, antibody-mediated immunity in mice, warranting further investigation in the pursuit of an LD vaccine.

MATERIALS AND METHODS

Mice. Three-week-old female C3H/HeN mice were obtained from Charles River Laboratories, Wilmington, MA, and housed in the Medical College of Wisconsin biosafety level 2 animal facility. The mice were fed and watered *ad libitum*. All of the procedures used were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Bacteria. The bacterial strains used in this study are described in Table 1. Bacteria from frozen stocks were cultured at 33°C in Barbour-Stoenner-Kelly (BSK) II medium (32) with selective antibiotics as indicated. Antibiotics were used at concentrations of 40 μg/ml for gentamicin and 200 μg/ml for kanamycin. When a cell density of 1×10^7 to 5×10^7 cells/ml was reached, analysis to confirm genomic plasmid presence was performed for B31-A3 and mutants in this background by using multiplex PCR essentially as previously described (33). Plasmid profiling protocols have not been established for strain N40 D10/E9 or PBr, so these analyses were not performed.

Immunizations of mice. Cultured Δ*p66* or *p66*^{cp} organisms with the full complement of plasmids present in the parental strain were washed in phosphate-buffered saline (PBS) plus 0.2% normal mouse serum (NMS), counted by dark-field microscopy, and diluted to 1×10^6 cells/ml. C3H/HeN mice were inoculated subcutaneously between the scapulae with 1×10^5 bacteria delivered in a 0.1-ml volume. Immunization groups consisted of mice inoculated with Δ*p66* organisms, *p66*^{cp} organisms, and PBS-NMS (control). Immunizations were given at 2-week intervals, with a total of two, three, or four immunizations delivered.

Challenge infections of mice. One week to 6 months after the final immunization, mice were challenged subcutaneously with 1×10^5 infectious *B. burgdorferi* cells (for B31-A3, confirmed to contain the full complement of genomic plasmids) delivered in a 0.1-ml volume. Mice were euthanized 2 to 4 weeks after the challenge infection. Tissue samples of mouse bladder, heart, tibiotarsal joint, skin at the site of inoculation, and ear were placed in BSK medium supplemented with 50 μg/ml rifampin, 20 μg/ml phosphomycin, and 2.5 μg/ml amphotericin B for culture analysis of infection. Cultures were incubated at 33°C and checked weekly for 8 weeks for the presence of live borreliae *via* dark-field microscopy. A mouse was considered infected when at least one culture was positive.

Ticks. *Ixodes scapularis* ticks were obtained as egg masses from the Oklahoma State University Tick Rearing Facility, Stillwater, OK. Ticks were housed over saturated KNO_3 at ambient temperature. Cohorts of larval ticks were allowed to feed on mice that had been infected via subcutaneous inoculation with *B. burgdorferi* strain B31-A3 or N40 D10/E9 (Table 1). After repletion, the ticks were collected, housed as described above, and allowed to molt. Emerged nymphs were sampled for evidence of *B. burgdorferi* infection by culture as previously described (34). The infectivity rate of the B31-A3-infected ticks was 20%; that of the N40-infected cohort was 30%.

Tick challenge infections of mice. Four weeks after the final immunization, infected nymphal ticks were placed on immunized mice by using techniques adapted to our work as previously described (31). Briefly, mice were anesthetized and shaved between the scapulae and a plastic tick chamber was affixed to each mouse with rosin-beeswax. After the placement of five ticks into each chamber, the chambers were sealed with Parafilm, which was punctured with a 27-gauge needle for air exchange, and the ticks were allowed to feed to repletion. Replete ticks were collected for infection analysis via culture as described above. Cultures were incubated at 33°C and checked weekly for 8 weeks via dark-field microscopy for the presence of live borreliae. The mice were euthanized 2 weeks after the last tick dropped off, and tissue samples were collected, placed in culture medium, and assessed for the presence of *B. burgdorferi* as described above. Mice were considered infected when at least one culture was positive.

Passive immunization of mice. Naive mice were intraperitoneally inoculated with 0.1 ml of pooled serum from mice immunized four times with Δ*p66* organisms, *p66*^{cp} organisms, or PBS-NMS (control) and harvested 4 weeks after the last immunization. One day later, mice were challenged subcutaneously with 1×10^5 infectious *B. burgdorferi* B31-A3 cells. One day after the challenge, 0.1 ml of the pooled immune serum was given to the mice. Mice were euthanized 14 days after the challenge, and organs were harvested. Tissues were cultured as previously described to assess the presence of live borreliae.

Immunoglobulin blot assay. A line blot IgG assay (Gold Standard Diagnostics, Davis, CA) was used to assay *B. burgdorferi* B31 IgG antigens recognized by sera from mice immunized four times but not challenged (prechallenge). While this assay is used commercially for human serum analysis, we modified it as a screening technique to identify antigens recognized by immunized mice by using the secondary antibody conjugate directed against mouse IgG. Sera from mice immunized and then infected via B31-A3-infected ticks were also tested (postchallenge). The assay was performed under standard conditions essentially in accordance with the

TABLE 2 Number of immunizations required to protect mice against syringe inoculation with *B. burgdorferi*[a]

Immunogen	No. of immunizations	Challenge strain	No. of tissue samples culture positive/total	No. of mice protected/ total
PBS-NMS	2	B31-A3	22/25	0/5
Δ*p66* bacteria	2	B31-A3	8/25[b]	2/5[c]
p66^{cp} bacteria	2	B31-A3	9/25[b]	1/5[c]
PBS-NMS	3	B31-A3	24/25	0/5
Δ*p66* bacteria	3	B31-A3	0/25[b]	5/5[d]
p66^{cp} bacteria	3	B31-A3	1/25[b]	4/5[e]
PBS-NMS	4	B31-A3	24/25	0/5
Δ*p66* bacteria	4	B31-A3	0/25[b]	5/5[d]
p66^{cp} bacteria	4	B31-A3	0/25[b]	5/5[d]

[a] All mice were challenged 4 weeks after the last boost with 1×10^5 *B. burgdorferi* B31-A3 bacteria via subcutaneous injection. Mice were euthanized 2 weeks after the bacterial challenge. In each group, Δ*p66*- or *p66*^{cp}-immunized mice were compared to the PBS-NMS-treated controls with Fisher's exact test in GraphPad Prism. No statistical information is provided for the PBS-NMS group compared to itself.
[b] $P < 0.0001$.
[c] No statistically significant difference.
[d] $P < 0.01$.
[e] $P < 0.05$.

TABLE 3 Time after final immunization required to confer protection on mice challenged by syringe inoculation[a]

Immunogen	Time (wk) between last immunization and challenge	No. of tissue samples positive/total	No. of mice protected/total
PBS-NMS	1	19/25	1/5[b]
Δ*p66* bacteria	1	0/25[c]	5/5[d]
p66^{cp} bacteria	1	2/25[c]	4/5[e]
PBS-NMS	26	18/25	0/5
Δ*p66* bacteria	26	3/25[c]	2/5[f]
p66^{cp} bacteria	26	0/25[c]	5/5[d]

[a] All mice received four immunizations and were challenged with 1×10^5 *B. burgdorferi* B31-A3 bacteria via subcutaneous injection. Mice were euthanized 2 weeks after the bacterial challenge. In the Δ*p66*-immunized group challenged 26 weeks later, three ear tissue samples were culture positive. No other tissue samples from the immunized mice were culture positive. In each group, Δ*p66*- or *p66*^{cp}-immunized mice were compared to the PBS-NMS-treated controls with Fisher's exact test in GraphPad Prism.
[b] One mouse in the group was not infected.
[c] $P < 0.0001$.
[d] $P < 0.01$.
[e] $P < 0.05$.
[f] No statistically significant difference.

manufacturer's protocol. Nitrocellulose strips were supplied with 10 antigenic proteins affixed (molecular masses of 18, 23 [OspC], 30, 31 [OspA], 39 [BmpA], 41 [Fla], 45, 58 [OppA-2], 66, and 93 kDa); strips were placed in individual reservoir channels. Serum from each mouse was diluted 1:100, added to a strip, and incubated for 30 min, after which the strips were washed three times. A conjugate was added that consisted of anti-human antibody as supplied in the kit for the control strips and anti-mouse IgG-alkaline phosphatase for the mouse sera. After incubation and washing, substrate was added to visualize proteins that reacted with the test sera. Bands equal to or greater in intensity than the cutoff control were considered positive.

Statistics. Statistical analyses were performed with Fisher's exact test in GraphPad Prism. In each group, mice immunized with Δ*p66* or *p66*^{cp} bacteria were compared to the PBS-NMS-treated controls. Because the number of tissue samples per group is five times the number of mice per group in some analyses, the *P* values in the tissue analyses differ from those in the whole-mouse analyses.

RESULTS

Initial experiments were performed to determine the minimal number of immunizations with the Δ*p66* or *p66*^{cp} avirulent *B. burgdorferi* strains required to fully protect mice from a challenge 4 weeks later with wild-type, infectious, *B. burgdorferi* strain B31-A3. The data presented in Table 2 demonstrate that two immunizations with either Δ*p66* or *p66*^{cp} bacteria were insufficient to protect mice from a *B. burgdorferi* challenge. When three immunizations with Δ*p66* bacteria were administered, complete immunity was observed (5/5 mice protected), while partial protection was noted when *p66*^{cp} bacteria were used (4/5 mice protected). Four immunizations with either Δ*p66* or *p66*^{cp} bacteria conferred complete immunity to a *B. burgdorferi* challenge (5/5 mice in both groups were protected).

We then tested the amount of time required to generate fully protective immunity, and the duration of that immunity, by challenging immunized mice at only 1 week or at 26 weeks after the completion of four immunizations. As shown in Table 3, when mice were challenged 1 week after their final immunization, complete protection was seen with Δ*p66* bacteria (5/5 mice protected), while *p66*^{cp} bacteria provided partial protection (4/5 mice protected), although our results described above showed complete protection when both groups were challenged at 4 weeks postimmunization. However, at 26 weeks, full protection was conferred by immunization with *p66*^{cp} bacteria (5/5 mice protected) but not by immunization with Δ*p66* bacteria (only 2/5 mice protected).

One critical way to determine the efficacy of any vaccine against organisms as diverse as the LD agents is to evaluate cross-protection conferred against other strains and even species of LD borreliae. B31-A3, a clone derived from northeastern U.S. tick isolate B31, has been classified as OspC type A and ribosomal spacer type (RST) 1 (35). Since OspC is produced by the bacteria as they are making the transition from tick to mammal (13), it is a major antigen seen by the host immune system early in infection. We therefore tested the effectiveness of immunization with our live attenuated strains against infection by *B. burgdorferi* strain N40 clone D10/E9, which differs from widely used clone "cN40" (36). N40 D10/E9 is OspC type M and RST 3B (35). We also tested a challenge with a *B. garinii* strain, PBr, a human cerebrospinal fluid (CSF) isolate from Germany (37). As shown by the results in Table 4, immunization with Δ*p66* or *p66*^{cp} bacteria conferred complete protection against a challenge with strain N40 D10/E9 (even though the interval between the last boost and the challenge was only 1 week), and immunization with Δ*p66* bacteria protected against dissemination from the inoculation site after a challenge with *B. garinii* strain PBr. Since *B. garinii* is maintained primarily in avian reservoirs in nature and is not associated with the development of arthritis in humans, it is not necessarily surprising that not all mouse tissues were culture positive in the control mice mock immunized with PBS-NMS. Although the protection against PBr infection afforded by immunization with Δ*p66* bacteria was not complete, it should be noted that the only site that was culture positive in the animals that were infected was the inoculation site. For the group immunized with *p66*^{cp} bacteria, only the inoculation sites and ears were culture positive, so dissemination was limited. These results suggest that further experiments with

TABLE 4 Protection against different strains of LD borreliae inoculated via syringe[a]

Immunogen	Challenge strain	No. of tissue cultures positive/total					No. of mice protected/total
		Bladder	Heart	Tibio	Skin	Ear	
PBS-NMS	*B. burgdorferi* N40 D10/E9	5/5	5/5	5/5	5/5	5/5	0/5
Δ*p66* bacteria	*B. burgdorferi* N40 D10/E9	0/5[b]	0/5	0/5	0/5	0/5	5/5[c]
p66^{cp} bacteria	*B. burgdorferi* N40 D10/E9	0/5[b]	0/5	0/5	0/5	0/5	5/5[c]
PBS-NMS	*B. garinii* PBr	1/5	2/5	0/5	5/5	3/5	0/5
Δ*p66* bacteria	*B. garinii* PBr	0/5	0/5	0/5	2/5	0/5	3/5[d]
p66^{cp} bacteria	*B. garinii* PBr	0/5	0/5	0/5	5/5	5/5	0/5[d]

[a] All mice were immunized four times at 2-week intervals. Mice were challenged with 1×10^5 N40 D10/E9 bacteria only 1 week after the last immunization and euthanized 4 weeks after the challenge. Those challenged with PBr (1×10^5 bacteria) were challenged at 4 weeks after the last immunization and euthanized 2 weeks after the challenge. Tibio refers to tibiotarsal joint, skin refers to the area of skin between the shoulder blades at which the mice were injected with bacteria, and ear refers to a sample of the margin of the ear distal to the head. In each group, Δ*p66*- or *p66*^{cp}-immunized mice were compared to the PBS-NMS-treated controls with Fisher's exact test in GraphPad Prism.
[b] $P < 0.0001$.
[c] $P < 0.01$.
[d] No statistically significant difference.

additional *B. burgdorferi* strains, as well as fully infectious *B. garinii* and *B. afzelii*, are warranted. In addition, our results suggest that immunization with live attenuated *B. burgdorferi* cells may generate responses directed against antigens that are highly, but not completely, conserved between the two species.

A second critical parameter by which to evaluate the efficacy of any vaccine against LD is protection against infection via tick bite, as this is the biologically relevant route of infection. We therefore challenged immunized mice with *I. scapularis* nymphs infected with *B. burgdorferi* strains B31-A3 and N40 D10/E9. The infectivity rates of tick cohorts placed on immunized mice were 20% for B31-A3-infected ticks and 30% for N40-infected ticks. The results shown in Table 5 indicate that mice immunized with either Δ*p66* or *p66*^{cp} bacteria were protected (5/5 and 5/5, respectively) when challenged with ticks infected with either *B. burgdorferi* strain. It should be noted that one PBS-immunized mouse was not infected in the B31-A3-infected tick challenge group. Replete ticks recovered from this mouse did not show infection upon culture, and thus, we could not conclude that the mouse had been exposed to *B. burgdorferi*. This mouse was therefore excluded from the data analysis.

The fed ticks were then evaluated for the presence of *B. burgdorferi* infection after repletion. The percentage of ticks infected with *B. burgdorferi* after feeding on sham-immunized mice increased compared to that in the unfed cohort, possibly because of tick-to-mouse skin-to-tick transmission within the confined feeding area (Table 6). The data in Table 6 show that B31-A3 infection was cleared from 87.5% of the fed ticks that were immunized with Δ*p66* bacteria, while 100% of the fed ticks immunized with *p66*^{cp} bacteria were cleared of infection. In contrast, N40 infection was not cleared from the ticks that fed on mice immunized with either Δ*p66* bacteria (8% cleared of infection) or *p66*^{cp} bacteria (0% cleared). Since the homologous but not the heterologous strain was eliminated from ticks, immunity to multiple antigens may play a role in the prevention of mammalian infection via tick bite.

To investigate the role of humoral immunity in protection against a challenge in the mice immunized with Δ*p66* and *p66*^{cp} bacteria, we passively immunized naive mice with pooled sera from mice that had been immunized four times but not chal-

TABLE 5 Protection against a *B. burgdorferi* challenge by infected tick bite[a]

Immunogen	Challenge strain	No. of tissue samples culture positive/total	No. of mice protected/total
PBS-NMS	*B. burgdorferi* B31-A3	15/25	0/4[b]
Δ*p66* bacteria	*B. burgdorferi* B31-A3	0/25[c]	5/5[d]
p66^{cp} bacteria	*B. burgdorferi* B31-A3	0/25[c]	5/5[d]
PBS-NMS	*B. burgdorferi* N40 D10/E9	18/25	1/5
Δ*p66* bacteria	*B. burgdorferi* N40 D10/E9	0/25[c]	5/5[e]
p66^{cp} bacteria	*B. burgdorferi* N40 D10/E9	0/25[c]	5/5[e]

[a] All mice were immunized four times and challenged with *Borrelia*-infected nymphal ticks 4 weeks after the last immunization. Five ticks were placed on each mouse for the challenge. Mice were euthanized 2 weeks after the tick challenge. In each group, Δ*p66*- or *p66*^{cp}-immunized mice were compared to the PBS-NMS-treated controls with Fisher's exact test in GraphPad Prism.
[b] One mouse was not infected in this group. This was probably due to the low infectivity rate of the unfed ticks. Replete ticks recovered from one mouse in this group did not show infection upon culture. Thus, we could not conclude that this mouse was actually exposed to infectious organisms able to be transmitted via tick bite. We therefore excluded this mouse from the denominator.
[c] $P < 0.0001$.
[d] $P < 0.01$.
[e] $P < 0.05$.

TABLE 6 Clearance of *B. burgdorferi* from infected ticks fed on immunized mice[a]

Immunogen	Challenge strain	No. of ticks positive/total (%)
PBS-NMS	*B. burgdorferi* B31-A3	6/9 (67)
Δ*p66* bacteria	*B. burgdorferi* B31-A3	1/8 (12.5)[b]
p66^{cp} bacteria	*B. burgdorferi* B31-A3	0/8 (0)[c]
PBS-NMS	*B. burgdorferi* N40 D10/E9	8/8 (100)
Δ*p66* bacteria	*B. burgdorferi* N40 D10/E9	12/13 (92)[d]
p66^{cp} bacteria	*B. burgdorferi* N40 D10/E9	4/4 (100)[d]

[a] All mice were immunized four times and challenged with infected ticks 4 weeks after the last immunization. Five ticks were placed on each mouse for challenge. Replete ticks were collected and cultured to assess for the presence of *Borrelia*. In each group, Δ*p66*- or *p66*^{cp}-immunized mice were compared to the PBS-NMS-treated controls with Fisher's exact test in GraphPad Prism.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] No statistically significant difference.

TABLE 7 Protection of mice from infection by syringe-inoculated *B. burgdorferi* B31-A3 by passive immunization[a]

Serum from mice immunized with:	No. of mice protected/total
PBS	1/5[b]
Δ*p66* bacteria	5/5[c]
p66^{cp} bacteria	5/5[c]

[a] Naive mice received 0.1 ml of serum from immunized mice intraperitoneally on day −1, were subcutaneously challenged with 1×10^5 *B. burgdorferi* B31-A3 bacteria on day 0, and were reimmunized on day 2. Mice were harvested on day 14, and organs were cultured to check infectivity. Mice passively immunized with Δ*p66* or *p66*^{cp} bacteria were compared to the PBS-immunized controls with Fisher's exact test in GraphPad Prism.
[b] One mouse was not infected in this group.
[c] $P < 0.01$.

TABLE 8 *B. burgdorferi* antigens recognized by immunized mouse serum[a]

Band size (kDa) or parameter	No. of samples positive/total					
	Prechallenge			Postchallenge with B31-A3-infected tick		
	PBS	Δ*p66*	*p66*^{cp}	PBS	Δ*p66*	*p66*^{cp}
18	0/4	0/5	0/5	2/5	0/5	0/5
23	0/4	1/5	0/5	3/5	1/5	3/5
30	0/4	5/5	5/5	3/5	5/5	5/5
31	0/4	2/5	5/5	0/5	4/5	3/5
39	0/4	2/5	0/5	2/5	3/5	2/5
41	0/4	4/5	5/5	2/5	5/5	5/5
45	0/4	1/5	3/5	0/5	4/5	3/5
58	0/4	0/5	0/5	1/5	0/5	0/5
66	0/4	1/5	3/5	0/5	4/5	3/5
93	0/4	0/5	0/5	0/5	0/5	0/5
All bands	0/40	16/50[b]	21/50[b]	13/50	26/50[c]	24/50[c]
No. of mice seropositive/ total[d]	0/4	1/5[e]	3/5[e]	2/5	4/5[e]	3/5[e]

[a] Prechallenge mice were immunized four times at 2-week intervals. Two weeks after the last immunization, serum samples were collected. Postchallenge mice were immunized four times and then challenged 4 weeks after the last immunization with B31-A3-infected ticks. Serum samples were collected 2 weeks after the challenge. Serum samples were used in a Gold Standard Diagnostics IgG line blot assay to detect *B. burgdorferi* proteins. Band intensity equal or greater than the cutoff control intensity was interpreted as positive.
[b] $P < 0.0001$.
[c] $P < 0.05$.
[d] A sample was considered *B. burgdorferi* IgG seropositive if five or more bands were present. The molecular masses of the test proteins used were 18, 23 (OspC), 30, 31 (OspA), 39 (BmpA), 41 (Fla), 45, 58 (OppA-2), 66, and 93 kDa. The number of bands to which each serum sample reacted and the seropositivity (defined as five or more bands reactive) of Δ*p66*- or *p66*^{cp}-immunized mice were compared to those of PBS controls with Fisher's exact test in GraphPad Prism.
[e] No statistically significant difference.

lenged. Results shown in Table 7 indicate that the immune response generated was antibody mediated for mice immunized with Δ*p66* (5/5) or *p66*^{cp} bacteria (5/5). While these results do not rule out a role for cellular immunity, they do demonstrate the importance of humoral immunity in protection.

To further analyze the humoral response, we probed commercially available strips arrayed with 10 *B. burgdorferi* antigens with sera from immunized mice. As shown in Table 8, sera from mice immunized with Δ*p66* bacteria recognized bands at 23 kDa (1/5), 30 kDa (5/5), 31 kDa (2/5), 39 kDa (2/5), 41 kDa (4/5), 45 kDa (1/5), and 66 kDa (1/5). The reactivity to P66 may be due to the production of a fragment of the amino-terminal end of P66 in the Δ*p66* mutant strain (29, 31). Sera from mice immunized with *p66*^{cp} bacteria produced bands at 30 kDa (5/5), 31 kDa (5/5), 41 kDa (5/5), 45 kDa (3/5), and 66 kDa (3/5). PBS-immunized mouse sera produced no visible bands in any of the four mice tested. After a challenge with live borreliae, sera from mice treated with PBS recognized antigens at 18 kDa (2/5), 23 kDa (3/5), 30 kDa (3/5), 39 kDa (2/5), 41 kDa (2/5), and 58 kDa (1/5). Sera from mice immunized with Δ*p66* bacteria and challenged with B31-A3-infected ticks reacted with bands at 23 kDa (1/5), 30 kDa (5/5), 31 kDa (4/5), 39 kDa (3/5), 41 kDa (5/5), 45 kDa (4/5), and 66 kDa (4/5). Sera from mice immunized with *p66*^{cp} bacteria and challenged with B31-A3-infected ticks recognized bands at 23 kDa (3/5), 30 kDa (5/5), 31 kDa (3/5), 39 kDa (2/5), 41 kDa (5/5), 45 kDa (3/5), and 66 kDa (3/5). While antigenic reactivity varied greatly, all of the mice immunized with either bacterial strain showed reactivity with a 30-kDa protein. After a challenge, a range of reactivity was also seen. With the exception of the one mouse that was not infected (as assessed by culture), tick-challenged, PBS-treated mice showed reactivity to at least one *B. burgdorferi* protein on this commercial test strip. We did not assess reactivity to *B. burgdorferi* lysates generated after *in vitro* culture, as the organism is known to produce different proteins in the mammalian versus tick versus laboratory environments.

DISCUSSION

The public health concern of increasing geographic distribution and case rates of LD has been expanding since the disease was identified. Vaccination could help reduce the morbidity and economic burden of LD, but no vaccines are currently available for human use. The OspA vaccine was demonstrated to be safe, but it was withdrawn from the market only a few years after introduction, as acceptance by the public was low because of a number of factors (38–41). Several candidate vaccines investigated more recently have targeted proteins produced in abundant amounts by *B. burgdorferi* grown in the laboratory and some that are exposed on the bacterial surface, although only some of these proteins have also been assigned activities or functions *in vitro* and/or *in vivo* (6, 8, 42–48). An immune response that blocks the function of a protein may prove more efficacious in preventing disease than a response that does not interfere with function. Unfortunately, few have generated long-lasting immunity or a response that is protective against the bacteria introduced by a tick bite, although others remain promising candidates. Several investigators have built on the success of the OspA vaccine and designed new-generation candidates that are multivalent or reservoir targeted (20, 22, 23, 25, 26).

We reasoned that noninfectious *B. burgdorferi* mutants in an infectious strain background might serve as live attenuated vaccines for several reasons. First, the strains, aside from the targeted mutation in the *p66* gene (*bb0603*), encode antigens that might be recognized by the immune system in a biologically relevant context, i.e., in the native conformation. This is often difficult to achieve for proteins in recombinant form. Second, the two strains used in this study can be cultured from the site of inoculation for 24 h, and occasionally for as long as 48 h, postinoculation (31). This time window may provide the opportunity for the bacteria to adjust gene expression to that of the mammalian environment as opposed to laboratory culture, and repeated immunization likely boosts the protective response to at least some of these *in vivo*-

produced antigens. Third, it is possible that this approach will identify several antigens that, in combination, confer protective immunity, while each individual antigen does not.

Both of the live attenuated vaccine strains tested here did confer protective immunity in mice. Although four immunizations were required to generate a fully protective immune response, immunity was protective against not only the parental strain, B31-A3 (OspC type A and RST 1), but also against *B. burgdorferi* strain N40 clone D10/E9 (OspC type M and RST 3B) (35). This was true for both subcutaneous injection of laboratory-grown bacteria and inoculation by tick bite. Interestingly, the Δ*p66* mutant strain appeared to generate a somewhat more protective response than did the *p66*^{cp} strain early after immunization was completed, but the protective response to the *p66*^{cp} strain appeared superior in terms of duration of protection between immunization and a challenge.

Although several live attenuated vaccines are in current use in humans, subunit vaccines are generally considered less risky, particularly for immunocompromised individuals. It will therefore be of great interest in future work to determine whether there is a difference between the Δ*p66*- and *p66*^{cp}-vaccinated groups of animals in terms of antigens recognized, whether T and/or B cell responses are involved, and whether the nature of the responses (e.g., IgG subtypes) differs between the groups. While much work remains to be done to address these issues, the strategy used here, i.e., allowing the bacteria and the mice to collaborate in informing us of the nature of the protective antigens and responses, should facilitate the future development of a safe and effective vaccine against infection by LD spirochetes.

ACKNOWLEDGMENTS

This work was supported by National Institute of Allergy and Infectious Diseases National Institutes of Health grant R01AI084873. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. In addition, support from the Medical College of Wisconsin Department of Medicine, Advancing a Healthier Wisconsin, and the Center for Infectious Disease Research at the Medical College of Wisconsin is gratefully acknowledged.

FUNDING INFORMATION

This work, including the efforts of Beth L. Hahn, Lavinia J. Padmore, Laura C. Ristow, Michael W. Curtis, and Jenifer Coburn, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (R01AI084873).

REFERENCES

1. **Centers for Disease Control and Prevention (CDC).** 2013. Three sudden cardiac deaths associated with Lyme carditis—United States, November 2012–July 2013. MMWR Morb Mortal Wkly Rep **62:**993–996. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6249a1.htm.
2. **Forrester JD, Meiman J, Mullins J, Nelson R, Ertel SH, Cartter M, Brown CM, Lijewski V, Schiffman E, Neitzel D, Daly ER, Mathewson AA, Howe W, Lowe LA, Kratz NR, Semple S, Backenson PB, White JL, Kurpiel PM, Rockwell R, Waller K, Johnson DH, Steward C, Batten B, Blau D, DeLeon-Carnes M, Drew C, Muehlenbachs A, Ritter J, Sanders J, Zaki SR, Molins C, Schriefer M, Perea A, Kugeler K, Nelson C, Hinckley A, Mead P.** 2014. Notes from the field: update on Lyme carditis, groups at high risk, and frequency of associated sudden cardiac death—United States. MMWR Morb Mortal Wkly Rep **63:**982–983. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6343a4.htm.
3. **Mead PS.** 2015. Epidemiology of Lyme disease. Infect Dis Clin North Am **29:**187–210. http://dx.doi.org/10.1016/j.idc.2015.02.010.
4. **Johnson RC, Kodner C, Russell M.** 1986. Active immunization of hamsters against experimental infection with *Borrelia burgdorferi*. Infect Immun **54:**897–898.
5. **Johnson RC, Kodner CL, Russell ME.** 1986. Vaccination of hamsters against experimental infection with Borrelia burgdorferi. Zentralbl Bakteriol Mikrobiol Hyg A **263:**45–48.
6. **Earnhart CG, Marconi RT.** 2007. Construction and analysis of variants of a polyvalent Lyme disease vaccine: approaches for improving the immune response to chimeric vaccinogens. Vaccine **25:**3419–3427. http://dx.doi.org/10.1016/j.vaccine.2006.12.051.
7. **Earnhart CG, Marconi RT.** 2007. OspC phylogenetic analyses support the feasibility of a broadly protective polyvalent chimeric Lyme disease vaccine. Clin Vaccine Immunol **14:**628–634. http://dx.doi.org/10.1128/CVI.00409-06.
8. **Earnhart CG, Marconi RT.** 2007. An octavalent Lyme disease vaccine induces antibodies that recognize all incorporated OspC type-specific sequences. Hum Vaccin **3:**281–289. http://dx.doi.org/10.4161/hv.4661.
9. **Sadziene A, Thompson PA, Barbour AG.** 1996. A flagella-less mutant of Borrelia burgdorferi as a live attenuated vaccine in the murine model of Lyme disease. J Infect Dis **173:**1184–1193. http://dx.doi.org/10.1093/infdis/173.5.1184.
10. **Sigal LH, Zahradnik JM, Lavin P, Patella SJ, Bryant G, Haselby R, Hilton E, Kunkel M, Adler-Klein D, Doherty T, Evans J, Molloy PJ, Seidner AL, Sabetta JR, Simon HJ, Klempner MS, Mays J, Marks D, Malawista SE.** 1998. A vaccine consisting of recombinant Borrelia burgdorferi outer-surface protein A to prevent Lyme disease. Recombinant Outer-Surface Protein A Lyme Disease Vaccine Study Consortium. N Engl J Med **339:**216–222.
11. **Steere AC, Drouin EE, Glickstein LJ.** 2011. Relationship between immunity to Borrelia burgdorferi outer-surface protein A (OspA) and Lyme arthritis. Clin Infect Dis **52**(Suppl 3)**:**s259–s265. http://dx.doi.org/10.1093/cid/ciq117.
12. **Hodzic E, Feng S, Freet KJ, Borjesson DL, Barthold SW.** 2002. *Borrelia burgdorferi* population kinetics and selected gene expression at the host-vector interface. Infect Immun **70:**3382–3388. http://dx.doi.org/10.1128/IAI.70.7.3382-3388.2002.
13. **Schwan TG, Piesman J, Golde WT, Dolan MC, Rosa PA.** 1995. Induction of an outer surface protein on Borrelia burgdorferi during tick feeding. Proc Natl Acad Sci U S A **92:**2909–2913. http://dx.doi.org/10.1073/pnas.92.7.2909.
14. **Yang XF, Pal U, Alani SM, Fikrig E, Norgard MV.** 2004. Essential role for OspA/B in the life cycle of the Lyme disease spirochete. J Exp Med **199:**641–648. http://dx.doi.org/10.1084/jem.20031960.
15. **Akin E, McHugh GL, Flavell RA, Fikrig E, Steere AC.** 1999. The immunoglobulin (IgG) antibody response to OspA and OspB correlates with severe and prolonged Lyme arthritis and the IgG response to P35 correlates with mild and brief arthritis. Infect Immun **67:**173–181.
16. **Drouin EE, Glickstein L, Kwok WW, Nepom GT, Steere AC.** 2008. Searching for borrelial T cell epitopes associated with antibiotic-refractory Lyme arthritis. Mol Immunol **45:**2323–2332. http://dx.doi.org/10.1016/j.molimm.2007.11.010.
17. **Kalish RA, Leong JM, Steere AC.** 1993. Association of treatment-resistant chronic Lyme arthritis with HLA-DR4 and antibody reactivity to OspA and OspB of *Borrelia burgdorferi*. Infect Immun **61:**2774–2779.
18. **Kamradt T, Lengl-Janssen B, Strauss AF, Bansal G, Steere AC.** 1996. Dominant recognition of a *Borrelia burgdorferi* outer surface protein A peptide by T helper cells in patients with treatment-resistant Lyme arthritis. Infect Immun **64:**1284–1289.
19. **Chen J, Field JA, Glickstein L, Molloy PJ, Huber BT, Steere AC.** 1999. Association of antibiotic treatment-resistant Lyme arthritis with T cell responses to dominant epitopes of outer surface protein A of Borrelia burgdorferi. Arthritis Rheum **42:**1813–1822. http://dx.doi.org/10.1002/1529-0131(199909)42:9<1813::AID-ANR4>3.0.CO;2-0.
20. **Wressnigg N, Barrett PN, Pollabauer EM, O'Rourke M, Portsmouth D, Schwendinger MG, Crowe BA, Livey I, Dvorak T, Schmitt B, Zeitlinger M, Kollaritsch H, Esen M, Kremsner PG, Jelinek T, Aschoff R, Weisser R, Naudts IF, Aichinger G.** 2014. A novel multivalent OspA vaccine against Lyme borreliosis is safe and immunogenic in an adult population previously infected with *Borrelia burgdorferi sensu lato*. Clin Vaccine Immunol **21:**1490–1499. http://dx.doi.org/10.1128/CVI.00406-14.
21. **Comstedt P, Hanner M, Schuler W, Meinke A, Lundberg U.** 2014. Design and development of a novel vaccine for protection against Lyme borreliosis. PLoS One **9:**e113294. http://dx.doi.org/10.1371/journal.pone.0113294.

22. **Meirelles Richer L, Aroso M, Contente-Cuomo T, Ivanova L, Gomes-Solecki M.** 2011. Reservoir targeted vaccine for Lyme borreliosis induces a yearlong, neutralizing antibody response to OspA in white-footed mice. Clin Vaccine Immunol **18:**1809–1816. http://dx.doi.org/10.1128/CVI.05226-11.
23. **Richer LM, Brisson D, Melo R, Ostfeld RS, Zeidner N, Gomes-Solecki M.** 2014. Reservoir targeted vaccine against Borrelia burgdorferi: a new strategy to prevent Lyme disease transmission. J Infect Dis **209:**1972–1980. http://dx.doi.org/10.1093/infdis/jiu005.
24. **Bensaci M, Bhattacharya D, Clark R, Hu LT.** 2012. Oral vaccination with vaccinia virus expressing the tick antigen subolesin inhibits tick feeding and transmission of Borrelia burgdorferi. Vaccine **30:**6040–6046. http://dx.doi.org/10.1016/j.vaccine.2012.07.053.
25. **Bhattacharya D, Bensaci M, Luker KE, Luker G, Wisdom S, Telford SR, Hu LT.** 2011. Development of a baited oral vaccine for use in reservoir-targeted strategies against Lyme disease. Vaccine **29:**7818–7825. http://dx.doi.org/10.1016/j.vaccine.2011.07.100.
26. **Scheckelhoff MR, Telford SR, Hu LT.** 2006. Protective efficacy of an oral vaccine to reduce carriage of Borrelia burgdorferi (strain N40) in mouse and tick reservoirs. Vaccine **24:**1949–1957. http://dx.doi.org/10.1016/j.vaccine.2005.10.044.
27. **Tsao JI, Wootton JT, Bunikis J, Luna MG, Fish D, Barbour AG.** 2004. An ecological approach to preventing human infection: vaccinating wild mouse reservoirs intervenes in the Lyme disease cycle. Proc Natl Acad Sci U S A **101:**18159–18164. http://dx.doi.org/10.1073/pnas.0405763102.
28. **Coburn J, Chege W, Magoun L, Bodary SC, Leong JM.** 1999. Characterization of a candidate *Borrelia burgdorferi* β3-chain integrin ligand identified using a phage display library. Mol Microbiol **34:**926–940. http://dx.doi.org/10.1046/j.1365-2958.1999.01654.x.
29. **Coburn J, Cugini C.** 2003. Targeted mutation of the outer membrane protein P66 disrupts attachment of the Lyme disease spirochete, Borrelia burgdorferi, to integrin alphavbeta3. Proc Natl Acad Sci U S A **100:**7301–7306. http://dx.doi.org/10.1073/pnas.1131117100.
30. **Exner MM, Wu X, Blanco DR, Miller JN, Lovett MA.** 2000. Protection elicited by native outer membrane protein Oms66 (p66) against host-adapted *Borrelia burgdorferi*: conformational nature of bactericidal epitopes. Infect Immun **68:**2647–2654. http://dx.doi.org/10.1128/IAI.68.5.2647-2654.2000.
31. **Ristow LC, Miller HE, Padmore LJ, Chettri R, Salzman N, Caimano MJ, Rosa PA, Coburn J.** 2012. The β_3-integrin ligand of Borrelia burgdorferi is critical for infection of mice but not ticks. Mol Microbiol **85:**1105–1118. http://dx.doi.org/10.1111/j.1365-2958.2012.08160.x.
32. **Barbour AG.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med **57:**521–525.
33. **Bunikis I, Kutschan-Bunikis S, Bonde M, Bergstrom S.** 2011. Multiplex PCR as a tool for validating plasmid content of Borrelia burgdorferi. J Microbiol Methods **86:**243–247. http://dx.doi.org/10.1016/j.mimet.2011.05.004.
34. **Policastro PF, Schwan TG.** 2003. Experimental infection of Ixodes scapularis larvae (Acari: Ixodidae) by immersion in low passage cultures of Borrelia burgdorferi. J Med Entomol **40:**364–370. http://dx.doi.org/10.1603/0022-2585-40.3.364.
35. **Chan K, Casjens S, Parveen N.** 2012. Detection of established virulence genes and plasmids to differentiate *Borrelia burgdorferi* strains. Infect Immun **80:**1519–1529. http://dx.doi.org/10.1128/IAI.06326-11.
36. **Barthold SW, de Souza MS, Janotka JL, Smith AL, Persing DH.** 1993. Chronic Lyme borreliosis in the laboratory mouse. Am J Pathol **143:**959–971.
37. **Wilske B, Preac-Mursic V, Jauris S, Hofmann A, Pradel I, Soutschek E, Schwab E, Will G, Wanner G.** 1993. Immunological and molecular polymoprhisms of OspC, an immunodominant major outer surface protein of *Borrelia burgdorferi*. Infect Immun **61:**2182–2191.
38. **Aronowitz RA.** 2012. The rise and fall of the Lyme disease vaccines: a cautionary tale for risk interventions in American medicine and public health. Milbank Q **90:**250–277. http://dx.doi.org/10.1111/j.1468-0009.2012.00663.x.
39. **Auwaerter PG, Bakken JS, Dattwyler RJ, Dumler JS, Halperin JJ, McSweegan E, Nadelman RB, O'Connell S, Shapiro ED, Sood SK, Steere AC, Weinstein A, Wormser GP.** 2011. Antiscience and ethical concerns associated with advocacy of Lyme disease. Lancet Infect Dis **11:**713–719. http://dx.doi.org/10.1016/S1473-3099(11)70034-2.
40. **Poland GA.** 2011. Vaccines against Lyme disease: what happened and what lessons can we learn? Clin Infect Dis **52**(Suppl 3)**:**s253–s258. http://dx.doi.org/10.1093/cid/ciq116.
41. **Shen AK, Mead PS, Beard CB.** 2011. The Lyme disease vaccine—a public health perspective. Clin Infect Dis **52**(Suppl 3)**:**s247–s252. http://dx.doi.org/10.1093/cid/ciq115.
42. **Brown EL, Kim JH, Reisenbichler ES, Hook M.** 2005. Multicomponent Lyme vaccine: three is not a crowd. Vaccine **23:**3687–3696. http://dx.doi.org/10.1016/j.vaccine.2005.02.006.
43. **Coughlin RT, Fish D, Mather TN, Ma J, Pavia C, Bulger P.** 1995. Protection of dogs from Lyme disease with a vaccine containing outer surface protein (Osp) A, OspB, and the saponin adjuvant QS21. J Infect Dis **171:**1049–1052. http://dx.doi.org/10.1093/infdis/171.4.1049.
44. **Earnhart CG, Buckles EL, Dumler JS, Marconi RT.** 2005. Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response. Infect Immun **73:**7869–7877. http://dx.doi.org/10.1128/IAI.73.12.7869-7877.2005.
45. **Earnhart CG, Buckles EL, Marconi RT.** 2007. Development of an OspC-based tetravalent, recombinant, chimeric vaccinogen that elicits bactericidal antibody against diverse Lyme disease spirochete strains. Vaccine **25:**466–480. http://dx.doi.org/10.1016/j.vaccine.2006.07.052.
46. **Hagman KE, Yang X, Wikel SK, Schoeler GB, Caimano MJ, Radolf JD, Norgard MV.** 2000. Decorin-binding protein A (DbpA) of *Borrelia burgdorferi* is not protective when immunized mice are challenged via tick infestation and correlates with the lack of DbpA expression by *B. burgdorferi* in ticks. Infect Immun **68:**4759–4764. http://dx.doi.org/10.1128/IAI.68.8.4759-4764.2000.
47. **Hanson MS, Cassatt DR, Guo BP, Patel NK, McCarthy MP, Dorward DW, Hook M.** 1998. Active and passive immunity against *Borrelia burgdorferi* decorin binding protein A (DbpA) protects against infection. Infect Immun **66:**2143–2153.
48. **Scheiblhofer S, Weiss R, Durnberger H, Mostbock S, Breitenbach M, Livey I, Thalhamer J.** 2003. A DNA vaccine encoding the outer surface protein C from Borrelia burgdorferi is able to induce protective immune responses. Microbes Infect **5:**939–946. http://dx.doi.org/10.1016/S1286-4579(03)00182-5.
49. **Elias AF, Stewart PE, Grimm D, Caimano MJ, Eggers CH, Tilly K, Bono JL, Akins DR, Radolf JD, Schwan TG, Rosa P.** 2002. Clonal polymorphism of *Borrelia burgdorferi* strain B31 MI: implications for mutagenesis in an infectious strain background. Infect Immun **70:**2139–2150. http://dx.doi.org/10.1128/IAI.70.4.2139-2150.2002.
50. **Leong JM, Moitoso de Vargas L, Isberg RR.** 1992. Binding of cultured mammalian cells to immobilized bacteria. Infect Immun **60:**683–686.