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Association of Linear Plasmid 28-1 with an Arthritic Phenotype of *Borrelia burgdorferi*

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***Borrelia burgdorferi*, the Lyme disease spirochete, has a genome comprised of a linear chromosome and up to 21 plasmids. Loss of plasmids is associated with decreased infectivity and pathogenicity. Sixteen transformants were generated by transforming the noninfectious clone 5A13 with the recombinant plasmid pBBE22. The transformants were classified into nine groups based on plasmid content analysis. An infectivity study revealed that all nine transformants examined, each of which represented one of the plasmid patterns, were infectious in mice with severe combined immunodeficiency (SCID) regardless of their genomic compositions. Tissue bacterial quantification revealed that the loss of plasmids significantly reduced the spirochete burden in the heart and joint tissues, not in the skin, suggesting virulence factors may be tissue specific. Four transformants containing lp28-1 induced severe arthritis in SCID mice, in contrast to the five transformants lacking lp28-1. These pathogenicity studies associated lp28-1 with an arthritic phenotype and further studies may identify factors that contribute to arthritic pathology.**

Lyme disease is a multisystem disorder that can result in arthritis, neurological abnormalities, carditis, and cutaneous lesions such as erythema migrans and acrodermatitis chronica atrophicans (56). The causative agent, *Borrelia burgdorferi*, has an unusual genome, including a chromosome and up to 21 linear and circular plasmids (12, 20). Its genomic composition can be well maintained during the enzootic life cycle involving the tick vector *Ixodes scapularis* and a mammal, probably because of various selection pressures it encounters. However, in vitro propagation of *B. burgdorferi* results in plasmid loss (43, 52). It eventually survives well even after the removal of most of the plasmids (33). To date, only cp26 has been clearly associated with in vitro multiplication, as the plasmid harbors an essential gene, *resT*, that encodes a telomere resolvase (10, 28).

Loss of plasmids is coupled with decreased infectivity and pathogenicity (43, 52). The lack of lp25 completely abolishes infectivity since this plasmid encodes a nicotinamidase gene, BBE22, that is essential for the basic survival of *B. burgdorferi* in the mammalian environment (31, 32, 47, 48). *B. burgdorferi* expresses plenty of outer surface protein A (OspA) but very little OspC in the unfed tick (14, 44, 53); a fresh blood meal down-regulates OspA and up-regulates OspC, consistent with the central roles of OspA in tick colonization and of OspC in salivary invasion and initial mammalian infection (22, 45, 64). OspA and OspC are encoded on plasmids lp54 and cp26, respectively (20).

To avoid innate immunity, *B. burgdorferi* may have to express OspE/F-related proteins (Erps) and other complement regulator-acquiring surface proteins (CRASPs) (24, 29, 30, 41, 57). Erps and CRASP-1 interact with complement regulatory proteins such as factor H and factor H-like protein 1, and are encoded on the cp32 family of plasmids and lp54, respectively (12, 20). To evade adaptive immunity, *B. burgdorferi* dramatically up-regulates immune evasion genes such as *vlsE* (13, 39, 65), consistent with the well-defined role of lp28-1, which carries the gene, in the establishment of chronic infection in immunocompetent mice (32, 48, 59, 65). In addition, ligand-binding lipoproteins such as decorin-binding proteins A and B, encoded on lp54, and fibronectin-binding protein, carried by lp36, may help the spirochete colonize the host tissues and establish chronic infection in the immune environment (9, 23, 35, 46).

Several features that parallel human Lyme disease are consistently induced in a murine model, including carditis and arthritis. In mice with severe combined immunodeficiency (SCID), *B. burgdorferi* infection maintains high tissue spirochete burdens and causes destructive arthritis (2). Specific antisera dramatically reduce tissue spirochete loads, alter surface antigenic expression, and resolve arthritis in this model (3, 7, 39). In immunocompetent mice, arthritis peaks within 2 to 3 weeks postinoculation and infection remains as the immune response against *B. burgdorferi* develops (4). In this study, we generated infectious transformants with various plasmid contents; some of the transformants caused arthritis but others did not in the murine model. This allowed us to identify an arthritis-related plasmid.

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MATERIALS AND METHODS

Spirochete and mouse strains. *B. burgdorferi* B31 clones 5A11 and 5A13 (gifts from Steven Norris, University of Texas, Houston, TX) were cultivated in Bar-

TABLE 1. PCR primers for examination of plasmid content in *B. burgdorferi*

Plasmid	Sequence	
	Forward	Reverse
cp9	5′-AGCAACAATTACAG-3′	5′-CGTATTTTGAGTTTG-3′
lp28-1	5′-TTTCATTATAAGGAGAC GATGA-3′	5′-CGTCGTACTACTTATAT CGC-3′
lp28-2	5′-TTCTAGTATCTACTAGA CCG-3′	5′-AGCCAAGTATTTCTTCT GCA-3′
lp28-4	5′-GTGTCGCTTAATTTTAG AGTTA-3′	5′-ATAGAGATCCTCTATTA GAGA-3′
lp56	5′-CAAGGGTGCATGAATG GA-3′	5′-TAGACATATCATTAGC GACG-3′
cp32-3	5′-CTGTTTAATCCACCACC AGTA-3′	5′-ACTTTCTCAGTACTTTC TCC-3′
lp5	5′-ACGATACACTGGTCGC AA-3′	5′-CTTCTCCTTACAATTAT GGTG-3′
lp21	5′-GTAGTGTACAGTGTGA ATCATG-3′	5′-TCTATTGTTCTAAATTC GGCT-3′

bour-Stoenner-Kelly H (BSK-H) complete medium at 33°C (Sigma Chemical Co., St. Louis, MO). BALB/c mice were bred at the Division of Laboratory Animal Medicine at Louisiana State University. BALB/c background SCID mice were purchased from Harlan (Indianapolis, IN). Mice were 4 to 8 weeks old when they were inoculated with spirochetes.

Generation of infectious transformants. B31 5A13 spirochetes were grown in 50 ml of BSK-H medium at densities of 5×10^7 to 1×10^8 cells/ml (mid- to late exponential phase), harvested, washed, and transformed with 2.0 μg of plasmid pBBE22 DNA (a gift from Steven Norris) under standard electroporation conditions (33, 58). pBBE22 was derived from the shuttle vector pBSV2 by inserting gene BBE22 (47, 58). The cells were allowed to recover in 20 ml of BSK-H complete medium at 33°C for 18 h. After kanamycin was added at a concentration of 200 μg/ml, the suspension was transferred into 96 PCR tubes (200 μl/tube). The limited dilution assay has been widely used in the screening of transformants (6, 11, 50, 64). Aliquots were incubated at 33°C for 10 days and live spirochetes were examined under a dark-field microscope. Viable spirochetes were found in 23 of the 96 tubes. Approximately 30 μl of the kanamycin resistance culture was transferred to 1.4 ml of BSK-H medium in a 1.5-ml microcentrifuge tube and grown to stationary phase at 33°C. Spirochetes were harvested from 500 μl of culture by centrifugation at $11,000 \times g$ for 5 min at room temperature, and washed twice with excess volumes of phosphate-buffered saline (pH 7.3) to remove residual pBBE22 DNA and resuspended in 500 μl of deionized H_2O. One microliter of suspension was used as a DNA template source for the examination of recombinant plasmid pBBE22 by PCR. The specific kanamycin primers (forward: 5′-ATGAGCCATATTCAACGGGAAACGT-3′; reverse: 5′-TCAGCGTAATGCTCTGCCAGTGT-3′) were used and the PCR conditions are described below.

Examination of plasmid content by PCR. The presence of cp9, lp28-1, lp28-2, lp28-4, lp56, cp32-3, lp5, and lp21 was surveyed by PCR with primer pairs specific for each of the eight plasmids (Table 1). Approximately 1 μl of spirochete culture was used as a DNA template source in a PCR volume of 25 μl. *Taq* polymerase was purchased from Takara Mirus Bio Inc. (Madison, WI). The PCR conditions included: 94°C for 1 min; 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 min, 35 cycles; and 72°C for 10 min. PCR products were separated on an ethidium bromide-agarose gel.

Confirmation of plasmid content by microarray hybridization. A microarray was developed for plasmid content analysis. On this array were printed, including two DNA sequences specific for each of 19 plasmids: A (lp54), B (cp26), C (cp9), D (lp17), E (lp25), F (lp28-1), G (lp28-2), H (lp28-3), I (lp28-4), J (lp38), K (lp36), L (cp32-8), M (cp32-6), N (cp32-9), O (cp32-7), P (cp32-1), Q (lp56), R (cp32-4), S (cp32-3), two sequences shared by both plasmids, T (lp5) and U (lp21), and one sequence unique for lp21. In addition to these 41 sequences, four chromosomal sequences were also included as controls. These 45 DNA fragments were printed in triplicate in the order plasmids A to U followed by the four chromosomal sequences, producing a total of 135 spots. Since the sequence of lp5 is essentially contained within lp21, no sequences specific for this plasmid can be selected for microarray hybridization. Thus, lp5 could be specifically detected only in the absence of lp21 when this array was used. However, we were able to design a PCR primer pair to specifically amplify lp5 in the presence of lp21 (Table 1). DNA samples were prepared from cultured spirochetes, fluorescently labeled, and hybridized to the array as described previously (37, 38). The array was examined with an AlphaArray 7000 scanner (Alpha Innotech, San Leandro, CA).

Isolation of single clones from a spirochete population. The cell density of a culture was estimated by counting spirochetes under a dark-field microscope. Approximately 0.2, 2.0, 20 and 200 spirochetes were suspended in volumes of 10 ml BSK-H medium and transferred into four 96-well plates (100 μl/well). Plates were incubated at 33°C in a 5% CO_2 atmosphere for 2 weeks. Only the plate that had spirochetes growing in less than 20 wells was selected for further analysis. Thus, the chance for one aliquot to have two or more clones was less than 4.5%. Plasmid content was assessed by PCR as described above.

Mouse infection study. Wild-type and SCID mice were given a single intradermal/subcutaneous injection of 10^4 spirochetes. For the infectivity study, SCID and wild-type mice were sacrificed at 2 and 3 weeks postinoculation, respectively. Heart, tibiotarsal joint, and skin specimens were aseptically collected for spirochete culture. Small pieces of specimens were suspended in 1.4 ml of BSK-H medium in a 1.5-ml microcentrifuge tube and incubated at 33°C. The medium was supplemented with kanamycin at a concentration of 200 μg/ml or with phosphomycin (300 μg/ml) and rifampin (500 μg/ml). Cultures were examined for viable spirochetes under a dark-field microscope every week for 3 weeks.

For the pathogenicity study, mice were examined for the development of arthritis at intervals of 2 days, starting at 2 weeks, and sacrificed at 1 month postinoculation. One tibiotarsal joint from each mouse was used for histopathological study. Tissues were fixed in 4% paraformaldehyde for 24 h, decalcified in Decalcifier B (Fisher Scientific) for 48 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin under standard conditions. The second joint, the heart, and a piece of skin (not from the inoculation site) were frozen for DNA preparation.

ELISA. The immune response in wild-type mice was monitored by an enzyme-linked immunosorbent assay (ELISA). The 5A11 spirochetes were grown to stationary phase, harvested by centrifugation, washed with excess volumes of phosphate-buffered saline, and sonicated. The protein content was measured by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). An antigen preparation with a protein concentration at 2.0 μg/ml was used to coat 96-well plates (Corning Inc., Corning, NY). Serum samples were diluted 1/200. The ELISA was performed as described previously (34).

Tissue DNA preparation. Frozen heart and joint specimens were transferred in liquid nitrogen and ground thoroughly with a mortar and pestle. DNA was extracted by using the DNeasy mini kit following the manufacturer's instructions (QIAGEN Inc., Valencia, CA). Skin samples were directly digested with proteinase K for DNA extraction.

Preparation of *flaB*-actin fusion standard. To better normalize *flaB* and actin concentration standards for quantitative PCR (qPCR), we cloned an internal sequence from both the actin and *flaB* genes into TA vector pNCO1T (15). A primer pair (forward: 5′-AAGGATCCATGAGACCACTTTCAACT-3′; reverse: 5′-GGACAGTGAGGCCAGAATGGA-3′) was designed to amplify a 231-bp internal fragment of the actin gene using murine DNA as a template. A second primer pair (forward: 5′-CAGCTGAAGAGCTTGGAATGCA-3′; reverse: 5′-AAGGATCCGCCTTGAGAAGGTGCT-3′) was used to generate a 260-bp internal fragment of *flaB* with spirochete DNA as a template. The underlined sequences were BamHI sites.

After digestion with the restriction enzyme and purification, the two amplicons were ligated and then amplified by nested PCR with use of a third primer pair (forward: 5′-CCTCTAGAGTTCATGTTGGAGCAAAC-3′; reverse: 5′-ATTC TAGAATGGAGCCACCGATCCA-3′). The PCR product was cloned into TA-vector pNCO1T as described previously (15). The *flaB*-actin insert was confirmed by DNA sequencing. DNA concentrations were determined by measuring the optical density at 260 nm wavelength and converted to copy numbers. The recombinant plasmid was digested with restriction enzyme EcoRI to release its potential supercoiled structure that might affect PCR efficiency.

qPCR. qPCR analyses were performed by using the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The Platinum *Taq* DNA polymerase high fidelity kit was purchased from Invitrogen Life Technologies (Carlsbad, CA). The sequences of primers and internal probes were described previously (26, 35) for *flaB* (forward: 5′-GCAGCTAATGTTGCAAAT CTTTTC-3′; reverse: 5′-GCAGGTGCTGGCTGTTGA-3′; probe: 5′-AAACTG CTCAGGCTGCACCGGTTC-3′) and actin (forward: 5′-CCATGTACCCAGG CATTGC-3′; reverse: 5′-CCAGACTGAGTACTTGCGTTC-3′; probe: 5′-TGC AGAAGGAGATCACAGCCCTAGCACC-3′).

Taqman 6-carboxytetramethylrhodamine (TAMRA) probes were ordered from Applied Biosystems. Amplification was performed in a final volume of 10 μl in the ABI PRISM 384-well clear optical reaction plate (Applied Biosystems). Two sets of 12 wells on each plate were assigned as DNA standards for actin or *flaB*. The standard concentrations ranged from 10^2 to 10^7 copies/well for actin

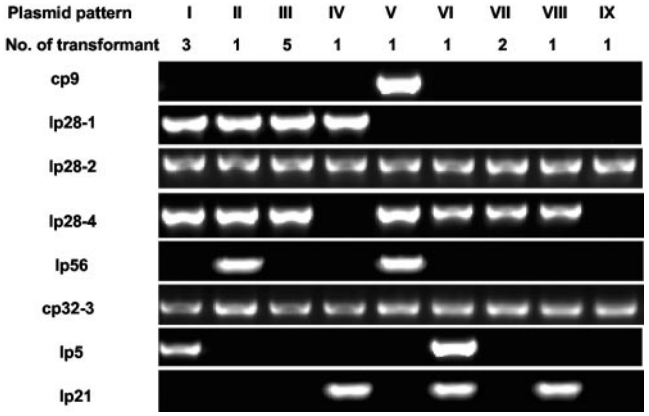

FIG. 1. Plasmid patterns of transformants. Sixteen transformants were analyzed by PCR for the presence of cp9, lp28-1, lp28-2, lp28-4, lp56, cp32-3, lp5, and lp21 and classified into nine plasmid patterns, I to IX. Patterns II, IV, V, VI, VIII, and IX each include one transformant. The rest have two to five.

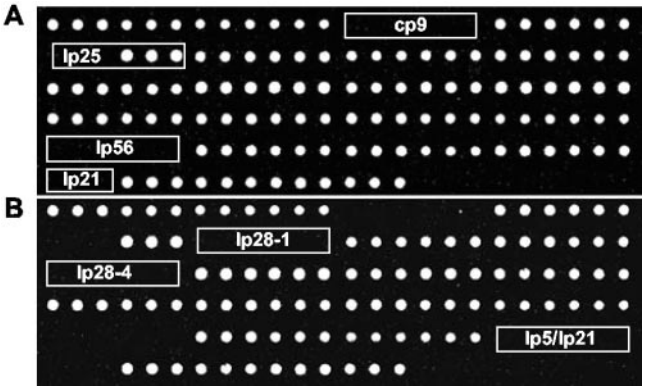

FIG. 2. Microarray confirmation of plasmid content. DNA samples were prepared from transformants IA (A) and IXA (B), fluorescently labeled, and hybridized to the plasmid microarray. Like the parental clone 5A13, neither transformants carried lp25. Three spots representative of one sequence specific for lp25 were fluorescent because of the presence of gene BBE22 introduced by plasmid pBBE22. Transformant IA also lost cp9, lp56, and lp21. In addition to these four plasmids, IXA lacks lp28-1, lp28-4, and lp5.

DNA quantification, and from 10^0 to 10^5 copies/well for *flaB*. Both standards and samples were amplified in duplicate wells. A PCR program with the following parameters was used: 50°C for 30 seconds; 95°C for 5 min; 50 cycles of 95°C for 20 seconds; and 60°C for 1 min. The mean DNA copy numbers of *flaB* and actin of each DNA sample were calculated from duplicate wells. Tissue spirochete burdens were converted to *flaB* DNA copy number per 2×10^6 actin DNA copies (spirochete number per 10^6 host cells).

Statistical analysis. Data were analyzed by a one-way analysis of variance, followed by a post hoc test (Scheffe's test). Calculated P values of ≤ 0.05 were considered significant.

RESULTS

Generation of transformants with various plasmid contents. Twenty-three kanamycin-resistant clones were generated from electroporation of 1.5×10^9 5A13 spirochetes. Sixteen of them were found to contain plasmid pBBE22. A transformation frequency of 1.1×10^{-8} was achieved. Resistance to the antibiotic noted in the rest of the clones was most likely due to spontaneous mutation. The plasmid content of the 16 transformants was surveyed by PCR for the presence of cp9, lp28-1, lp28-2, lp28-4, lp56, cp32-3, lp5, and lp21. These eight plasmids were selectively examined because they tend to be spontaneously lost during in vitro propagation (48). None of the transformants lost lp28-2 or cp32-3 (Fig. 1). Fourteen of the clones retained lp28-4 and 10 had lp28-1. cp9 was found in one; lp56 in two; lp21 in three; and lp5 in four clones. The 16 transformants were designated IA, IB, IC, IIA, IIIA, IIIB, IIIC, IIID, IIIE, IVA, VA, VIA, VIIA, VIIB, VIIIA, and IXA and represented nine plasmid patterns (I to IX). Six of the patterns, including II, IV, V, VI, VIII, and IX, were each represented by one clone. Patterns I, III, and VII were detected in three, five, and two clones, respectively.

To confirm the PCR results, DNA samples were prepared from transformants IA, IIA, IIIA, IVA, VA, VIA, VIIA, VIIIA, and IXA, each of which represented one of the plasmid patterns, fluorescently labeled, and hybridized to the microarray. The array hybridization not only confirmed the PCR results, but also showed that there was no additional plasmid loss in any of these selected clones. The results obtained for IA and IXA are presented in Fig. 2.

To determine whether the extensive plasmid loss noted in the transformants occurred before transformation, the plasmid profiles of the parental spirochetes were investigated. Fifteen clones were isolated from a 5A13 culture that was used for the preparation of electrocompetent cells via limited dilution. PCR survey revealed that all 15 clones contained lp28-2, lp28-4, cp32-3, and lp56 (data not shown). Eleven, 10, 13, and 11 clones also retained cp9, lp28-1, lp5, and lp21, respectively. These data indicated that transformation caused loss of cp9, lp56, lp5, and lp21. The rest of the plasmids, including lp28-1, were maintained during the process. Loss of cp9 and lp56 resulting from transformation has been reported previously (33, 58).

Transformants are infectious in SCID mice regardless of plasmid content. Loss of plasmids has been associated with decreased infectivity. Numerous reports demonstrated diminished infectivity of infectious strains resulting from genetic manipulation (16, 17, 45, 58, 63). To address these concerns, we examined infectivity of selected transformants in SCID mice. Groups of three SCID mice were challenged with clone IA, IIA, IIIA, IVA, VA, VIA, VIIA, VIIIA, or IXA and sacrificed 2 weeks postinoculation. Spirochetes were successfully isolated from the heart, joint, and skin specimens in all 27 mice (Table 2), indicating that each of the nine transformants is infectious in SCID mice.

To investigate infectivity in immunocompetent mice, groups of three BALB/c mice were challenged with the transformants. Five animals were inoculated with 5A13 spirochetes as a control. At 3 weeks postinoculation, *B. burgdorferi* was grown from the heart, joint, and skin specimens from each of the 12 mice that had been inoculated with IA, IIA, IIIA, and IVA, but not from the specimens of the 15 mice that had been given VA, VIA, VIIA, VIIIA, and IXA (Table 3). The result was consistent with previous studies demonstrating that lp28-1 is essential for the establishment of *B. burgdorferi* infection in immunocompetent mice (31, 47). Although the five transformants that lost lp28-1 could not be recovered, they were able to

TABLE 2. Infectivity of transformants in SCID mice[a]

Transformant	No. of cultures positive/no. examined			
	Heart	Joint	Skin	All sites
IA	3/3	3/3	3/3	9/9
IIA	3/3	3/3	3/3	9/9
IIIA	3/3	3/3	3/3	9/9
IVA	3/3	3/3	3/3	9/9
VA	3/3	3/3	3/3	9/9
VIA	3/3	3/3	3/3	9/9
VIIA	3/3	3/3	3/3	9/9
VIIIA	3/3	3/3	3/3	9/9
IXA	3/3	3/3	3/3	9/9

[a] Groups of three SCID mice were inoculated with transformants IA, IIA, IIIA, IVA, VA, VIA, VIIA, VIIIA, and IXA and sacrificed 2 weeks postinoculation. Heart, tibiotarsal joint, and skin specimens were harvested and cultured for spirochetes in BSK-H medium.

induce serum conversion (Fig. 3), suggesting that significant replication could occur during initial infection. As expected, the parental clone 5A13 was not able to induce serum conversion.

Transformant IXA lost seven plasmids, including lp25, but was infectious in SCID mice. This was the infectious clone with the least number of plasmids that had been identified. To examine whether it could lose further plasmids and retain infectivity, we isolated a variant with fewer plasmids. Spirochetes were recovered from a mouse that had been infected with IXA and cultured in BSK-H medium for 1 month (approximately 100 generations). Fourteen subclones were obtained via limiting dilution. The PCR plasmid survey revealed that only cp32-3 was lost from one of the subclones. It was designated IXA/cp32-3$^-$ and inoculated into three SCID mice. Spirochetes were successfully isolated from the heart, joint, and skin specimens, indicating that the derivative remains infectious in SCID mice (data not shown).

lp28-1 is an arthritis-related plasmid in SCID mice. Next, we selectively investigated the pathogenicity of transformants in terms of arthritis virulence and tissue spirochete burdens. Groups of 10 SCID mice were inoculated with clones IA, VA, and IXA and with 5A11 spirochetes as a control. As expected, the group of 10 mice that were infected with 5A11 spirochetes

TABLE 3. Infectivity of transformants in immunocompetent mice[a]

Transformant	No. of cultures positive/no. examined			
	Heart	Joint	Skin	All sites
IA	3/3	3/3	3/3	9/9
IIA	3/3	3/3	3/3	9/9
IIIA	3/3	3/3	3/3	9/9
IVA	3/3	3/3	3/3	9/9
VA	0/3	0/3	0/3	0/9
VIA	0/3	0/3	0/3	0/9
VIIA	0/3	0/3	0/3	0/9
VIIIA	0/3	0/3	0/3	0/9
IXA	0/3	0/3	0/3	0/9
5A13	0/5	0/5	0/5	0/15

[a] Groups of three BALB/c mice were inoculated with transformants IA, IIA, IIIA, IVA, VA, VIA, VIIA, VIIIA, and IXA and sacrificed 3 weeks postinoculation. Five mice were given 5A13 spirochetes as a control. Heart, tibiotarsal joint, and skin specimens were harvested and cultured for spirochetes in BSK-H medium.

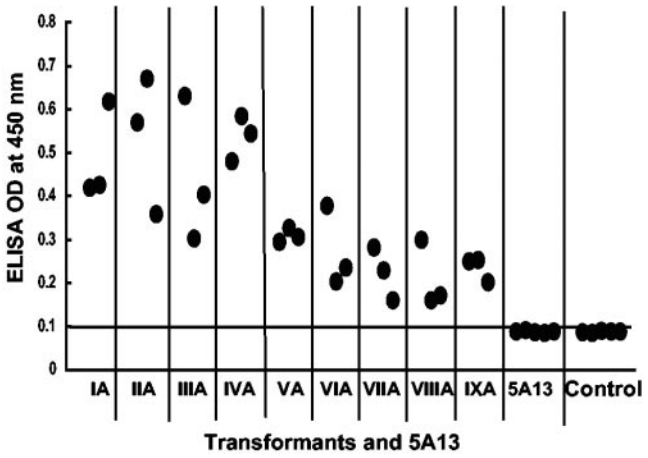

FIG. 3. Antibody response induced by inoculation with transformants. Groups of three BALB/c mice were given 10^4 cultured spirochetes of transformant IA, IIA, IIIA, IVA, VA, VIA, VIIA, VIIIA, or IXA, and five mice were challenged with the same dose of 5A13. Animals were sacrificed 3 weeks postinoculation. The specific immune response was assessed by ELISA using spirochetal lysate as an antigen. Sera collected from five mice preinoculation were used as controls. The cutoff value (line) was defined as the mean optical density (OD) plus 3 standard deviations of the five control samples.

all developed severe joint swelling 2 to 3 weeks postinoculation (Fig. 4A). Following the same course, arthritis evolved in all the 10 mice of the group that had received transformant IA but not in the remaining groups, indicating that clones VA and IXA had lost pathogenicity. Histopathological examination confirmed that intensive tissue lesions appeared in the joints of mice receiving the 5A11 and IA spirochetes but not in those that were inoculated with either clone VA or IXA (Fig. 4B).

Since both transformants VA and IXA had been shown to be infectious in SCID mice, diminished arthritis virulence could be due to their inability to cause high tissue spirochete burdens. The bacterial burden in the heart, joint, and skin tissues was analyzed by qPCR (Fig. 5). In the heart tissue, the spirochete burden caused by clone 5A11 was 161 to 217% higher than by the three transformants (P values ranging from 3.0×10^{-6} to 6.3×10^{-7}). Transformant IA generated 28 and 18% higher spirochete numbers than its counterparts VA and IXA, respectively ($P = 0.005$ and 0.02, respectively). There was no significant difference between the VA and IXA groups ($P > 0.16$). In the joint tissue, the spirochete burden caused by 5A11 was 230 to 702% higher than those by the three transformants (P values ranging from 3.1×10^{-5} to 1.3×10^{-6}). Among the three clones, IXA generated the lowest spirochete burden, 59 and 56% lower than those caused by IA and VA, respectively ($P = 1.6 \times 10^{-4}$ and 0.003, respectively). No significant difference was noted in the joint tissue spirochete burden between clones IA and VA ($P = 0.72$), suggesting that a high spirochete burden may not cause arthritis.

A different trend was observed in the skin. The spirochete burden in this tissue caused by IXA and VA was 70 and 56% higher, respectively, than that by 5A11 ($P = 0.007$ and 0.04, respectively), although there were no significant differences among the three transformants (P values ranging from 0.21 to

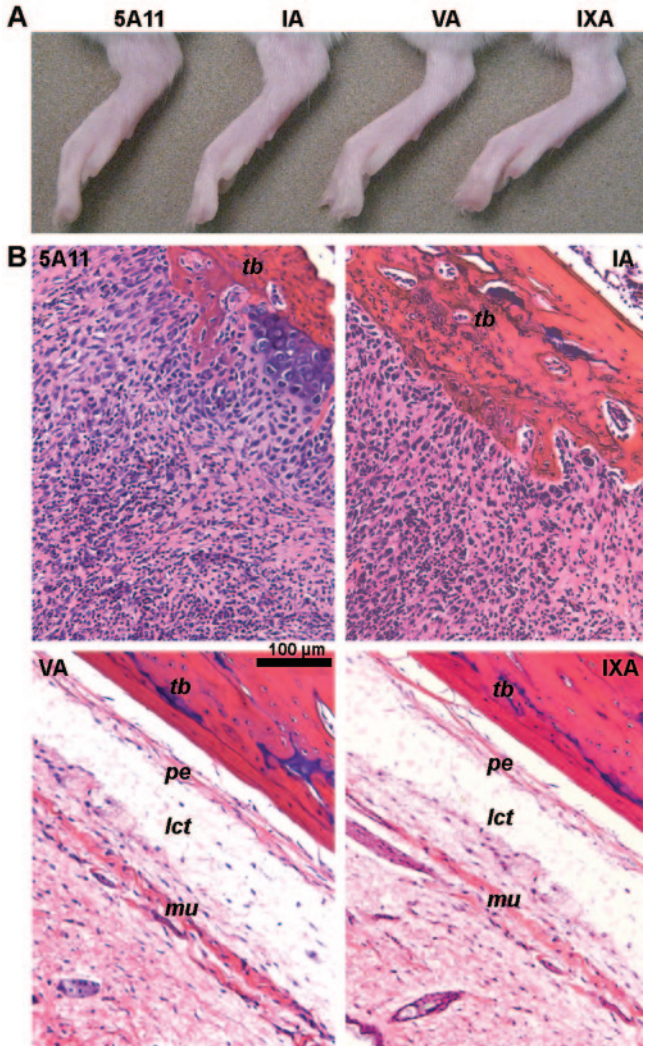

FIG. 4. Severe joint swelling (A) and massive inflammatory infiltration (B). SCID mice were inoculated with spirochetes 5A11, IA, VA, and IXA and sacrificed 1 month later. Severe joint swelling (A) was noted in mice challenged with clones 5A11 and IA but not with VA or IXA. This is consistent with histopathological examination (B), showing abundant infiltration of macrophages and neutrophils adjacent to the tibial bone of mice infected with clones 5A11 and IA but not with VA or IXA. *tb*: tibial bone; *pe*: periostium; *lct*: loose connective tissue; *mu*: muscle.

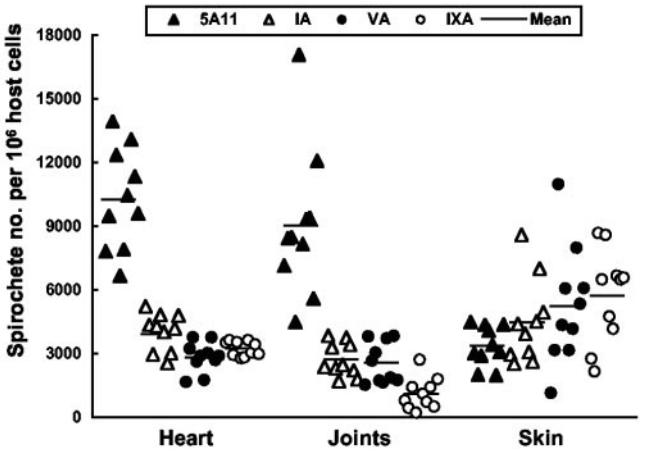

FIG. 5. Influence of plasmid content on tissue spirochete burdens. DNA samples were prepared from the heart, joints, and skin of SCID mice that were inoculated with 5A11 spirochetes as a control and transformants IA, VA, and IXA. Spirochetal *flaB* and murine actin DNAs were quantified by qPCR. The data are expressed as spirochete (*flaB* DNA copy) numbers per 10^6 host cells (2×10^6 actin DNA copies).

0.72). There was no significant difference between 5A11 and IA ($P > 0.16$).

5A11 and IA, both of which contained lp28-1, caused arthritis, while VA and IXA, which lacked the plasmid, failed to induce pathology. This prompted us to investigate whether lp28-1 is essential for arthritis virulence in all the transformants. Groups of three SCID mice were inoculated with IIA, IIIA, IVA, VIA, VIIA, or VIIIA. Severe joint swelling appeared in all nine mice that were infected with the first three transformants (Table 4). No arthritis was noted in any of the mice that were challenged with the other transformants. Our observations that IA, IIA, IIIA, and IVA all contain lp28-1, combined with the observed loss of lp28-1 in VA, VIA, VIIA, VIIIA, and IXA, indicate that cells containing lp28-1 are associated with the induction of Lyme arthritis.

DISCUSSION

The noninfectious clone 5A13 contains 20 of the 21 *B. burgdorferi* plasmids (48). The lack of lp25 abolishes the infectivity of *B. burgdorferi* because it carries the nicotinamidase gene BBE22 that is essential for the basic survival of *B. burgdorferi* in the mammalian environment (47). Transformation with recombinant plasmid pBBE22 restores infectivity and pathogenicity in 5A13, as the vector contains a copy of the BBE22 gene. Sixteen transformants with nine different plasmid contents were generated with use of the 5A13/pBBE22 system, providing an opportunity for the study of plasmid content and infectivity in genetically manipulated *B. burgdorferi*. Such a correlation has been extensively investigated with spirochete isolates without genetic manipulation (31, 32, 47, 48).

We used a SCID mouse model because it allowed us to examine the roles of plasmids in infectivity and pathogenicity in the absence of lp28-1. The study showed that transformants were infectious even after extensive plasmid loss. Transformant IXA had only 14 of the 21 plasmids but remained infec-

TABLE 4. Arthritis virulence of transformants in SCID mice[a]

Transformant	No. of mice with severe arthritis/no. infected
IIA	3/3
IIIA	3/3
IVA	3/3
VIA	0/3
VIIA	0/3
VIIIA	0/3

[a] Groups of three SCID mice were inoculated with transformants IIA, IIIA, IVA, VIA, VIIA, and VIIIA. The development of arthritis was assessed at intervals of 2 days, starting at 2 weeks. Mice were sacrificed at 1 month postinoculation.

tious in SCID mice. We were able to isolate a variant that had lost cp32-3 while maintaining its infectivity. It remains possible to remove more plasmids from the derivative without changing its infectivity in SCID mice.

Naïvity and immunity constitute remarkably different environments for spirochete persistence. To survive in the fundamental mammalian environment, *B. burgdorferi* must synthesize building blocks that cannot be acquired from the host. One well-defined example is a nicotinamidase involved in NAD synthesis, which is encoded by BBE22. Lacking this gene, 5A13 is unable to multiply in the dialysis membrane chamber implanted within the peritoneal cavities of rats, a mammalian host-adapted state (1, 47). In the case of our transformants, this function was complemented by the recombinant plasmid pBBE22. To persist in the naïve environment, *B. burgdorferi* must be able to evade innate immunity. This ability may, in part, be due to the expression of Erps and other CRASPs that interact with complement regulators (12, 20). All the transformants examined and the subclone IXA/cp32-3$^-$ had plasmids that encode these CRASPs and were isolated from SCID mice.

To establish infection in the immune environment, *B. burgdorferi* must be able to evade adaptive immunity. This capacity is carried by lp28-1 (31, 32, 47, 48), which is consistent with our results showing transformants with lp28-1 were isolated from wild-type mice, and those lacking lp28-1 were replicated only during early infection. Thus, SCID mice can serve as a unique model for the study of virulence factors by using variants that are unable to escape the adaptive immune response.

Loss of plasmids significantly affects virulence, in terms of tissue spirochete loads. In both heart and joint tissues, clone 5A11, which lacks only lp5 (48), generated significantly higher bacterial loads than transformant IA, VA, or IXA. These dramatic reductions could be caused by the absence of lp25, as all the transformants lack the plasmid. Norgard and colleagues have recently shown that the disruption of gene BBE16, which is located on lp25, significantly increases the 50% infectious dose values of variants in a mouse model (50). Among the three transformants, IA produced significantly higher spirochete numbers in the heart tissues than its two counterparts. In the joint tissues, both IA and VA produced higher loads than IXA. These observations can be explained by the fact that IXA has the fewest plasmids.

Unlike the heart and joint tissues, the skin harbors similar numbers of the three transformants, indicating that the loss of plasmids does not reduce bacterial load in this specific tissue. Interestingly, 5A11 caused significantly lower spirochete burdens in the skin than transformants VA and IXA. To explain these observations, we hypothesize that *B. burgdorferi* may have a broad spectrum of virulence factors. They may direct *B. burgdorferi* to colonize selective tissues. For instance, some of them may facilitate colonization of heart or joint tissues, while others may help in invading the skin. Plasmids that encode products responsible for heart or joint colonization may be lost from the transformants, leading to dramatic reductions of the spirochete burdens in these tissues. In contrast, plasmids that carry virulence genes responsible for skin colonization may be retained, causing a higher bacterial burden in this tissue. Further studies may identify tissue tropism-specific factors of *B. burgdorferi*.

Loss of plasmids significantly affects the pathogenicity of *B. burgdorferi*. In the present study, only the four transformants that contained lp28-1 induced arthritis, allowing us to associate lp28-1 with an arthritic phenotype. The contributions of lp28-1 to the induction of arthritis could include increasing the tissue spirochete burden and encoding virulence factors. Spirochete quantification ruled out the first possibility because both IA and VA generated similar bacterial loads in joint tissue.

The role of lp28-1 is most likely to carry arthritis-related factors. It is also the plasmid that is required for *B. burgdorferi* to evade adaptive immunity and establish chronic infection in the immunocompetent animal (31, 32, 47, 48). Therefore, it is impossible to study arthritis virulence genes in such an animal model. There is no evidence that murine Lyme arthritis is immune mediated. In fact, infection of wild-type mice stimulates vigorous immune responses that dramatically reduce the spirochete burden and resolve arthritis, making it difficult to dissect the roles of the bacterial density and virulence factors. By using immunodeficient mice, we showed that *B. burgdorferi* may have to express virulence factors to cause pathology.

Although lp28-1 carries 32 open reading frames, most of them are either paralogues found on other borrelial genetic elements, frameshifted genes or pseudogenes, or encode genes of less than 100 amino acids (20). BBF01 and *vlsE* may be the only unique, functional genes. Interestingly, both of them code for a lipoprotein. Extensive in vitro studies have demonstrated that lipoprotein triggers a robust production of proinflammatory cytokines via the CD14 Toll-like receptor 2 (TLR2) signaling pathway (21, 25, 42, 49, 54, 55, 61, 62). Moreover, Feng and colleagues showed that BBF01 antibody reduces the severity of arthritis in infected SCID mice, and designated the antigen arthritis-related protein (Arp) (18). A second study by Feng et al. confirmed their earlier findings but also reported that specific Arp immunity is unable to protect against an initial infection or reduce the tissue spirochete load in immunocompetent mice (19).

Another possible explanation for the activity of Arp antibody in resolving arthritis could be that the specific antibody reduces the antigen's expression as observed in the OspC immune response where the antigen is selectively down-regulated (13, 36, 39). Our previous study has ruled out this possibility, as BBF01 gene expression is not reduced by the adaptive immune response (39). Most recently, Steere and colleagues have shown that there is no correlation between the anti-BBF01 humoral response and the severity or duration of human Lyme arthritis (51). Although there may be differences between what is observed in an animal model and what occurs in humans, the role of BBF01 in the induction of arthritis remains to be confirmed.

It has been reported that myeloid differentiation factor 88 (MyD88) deficiency reduces the host ability to control the tissue spirochete burden but does not affect the inflammatory response (8, 40). Given the fact that MyD88 is the common adaptor for the TLR signaling pathway, the studies strongly suggest that the lipoprotein-CD14-TLR2 signaling pathway is not required for *B. burgdorferi* to cause pathology (8, 40). This is consistent with a previous report by Wooten et al., whose study suggests that the TLR2 signaling is not required for the inflammatory response to *B. burgdorferi* (60). Most recently, Sellati and colleagues have shown that a CD14-independent pathway, not the CD14-TLR2 signaling pathway, plays a dom-

inant role in the development of destructive pathology in Lyme disease (5). In fact, the CD14-TLR2 pathway attenuates the inflammatory response to *B. burgdorferi* infection (5). Unlike the CD14-TLR2 pathway, which can be triggered by purified lipoprotein or spirochete lysates (21, 25, 55, 62), the CD14-independent signaling pathway is effectively activated only by intact spirochetes (5).

The spectrum of stimulants for the inflammatory response in Lyme disease may be broad. Lipoprotein may not be the only factor that contributes to the induction of pathology. The current study has associated lp28-1 with an arthritic phenotype. The plasmid may encode virulence factors that are directly involved in the induction of pathology or regulators that facilitate the expression of arthritis-related genes harbored on other genetic elements.

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