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Borrelia burgdorferi Gene Expression In Vivo and Spirochete Pathogenicity

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Borrelia burgdorferi **spirochetes that do not cause arthritis or carditis were developed and used to investigate Lyme disease pathogenesis. A clonal isolate of** *B. burgdorferi* **N40 (cN40), which induces disease in C3H/HeN (C3H) mice, was repeatedly passaged in vitro to generate nonpathogenic spirochetes. The passage 75 isolate (N40-75) was infectious for C3H mice but did not cause arthritis or carditis, and spirochetes were at low levels or absent in the joints or hearts, respectively. N40-75 could, however, cause disease in severe combined immunodeficient (SCID) mice, suggesting that the response in immunocompetent mice prevented effective spirochete dissemination and the subsequent development of arthritis and carditis. Administration of immune sera at 4 days after spirochete challenge aborted N40-75, but not cN40, infection in SCID mice. A** *B. burgdorferi* **genomic expression library was differentially probed with sera from cN40- and N40-75-infected mice, to identify genes that may not be effectively expressed by N40-75 in vivo. N40-75 was defective in the up-regulation of several genes that are preferentially expressed during mammalian infection, including** *dbpAB, bba64,* **and genes that map to the cp32 family of plasmids. These data suggest that adaptation and gene expression may be required for** *B. burgdorferi* **to effectively colonize the host, evade humoral responses, and cause disease.**

Borrelia burgdorferi, the causative agent of Lyme disease, is the most common vector-borne pathogen in the United States (15). *B. burgdorferi*-infected mice develop arthritis and carditis, thereby partially mimicking the human illness (3, 5, 8, 10, 19, 43). The pathogenesis of murine Lyme borreliosis is multifactorial. The severity of disease has been associated with the number of *B. burgdorferi* in the affected organs (57), the virulence of specific spirochete isolates (12, 32, 40), and the host immune response to this organism (2, 11, 33, 35, 37, 53, 56). The severity of murine Lyme arthritis is genotype dependent. For example, C3H/HeN (C3H) mice develop more severe arthritis and have greater numbers of spirochetes in the joints than BALB/c mice (5, 36, 44).

B. burgdorferi spirochetes adapt to different environments during their life cycle in *Ixodes scapularis* ticks and the reservoir host. Within engorging ticks, the *B. burgdorferi* protein expression dramatically changes, with the synthesis of OspC and down-regulation of OspA (21, 46). These changes are likely to occur in response to the incoming blood meal, temperature shifts, and alterations in spirochete density (21, 42, 49). In the mammalian host, *B. burgdorferi* must evade host antibodies that arise in response to infection, and selective gene expression and recombination events may facilitate spirochete survival (21, 58–60). Several genes, including *eppA*, *ospE/F* paralogues (*erps*), *bbk32*, *bbk50*, and the decorin-binding protein gene *dbpA*, are not expressed, or are expressed at low levels, by *B. burgdorferi* cultured in laboratory medium but are apparently up-regulated during infection (1, 16, 25, 51, 54).

The function of most of these conditionally expressed gene products is not known. OspA may be one of several plasminogen-binding proteins (28), DbpA adheres to decorin (30), and BBK32 mediates the attachment of *B. burgdorferi* to fibronectin (41).

The examination of spirochetes continuously cultured in vitro has enhanced our understanding of *B. burgdorferi* pathogenesis. The sequential passage of uncloned *B. burgdorferi* in Barbour-Stoenner-Kelly (BSK) II medium results in selection of noninfectious subpopulations with variable plasmid and protein profiles (4, 34, 39, 45, 47). This suggests that specific genes are required for infection. A correlation between plasmid content, or specific *B. burgdorferi* genes, and infectivity has more recently been demonstrated using cloned spirochetes. Xu and colleagues have shown that specific plasmids are associated with the infectivity of *B. burgdorferi* B31 (55). Zhang and associates (58) used subtractive hybridization to identify a plasmid-encoded gene family, designated the *vmp*-like sequence locus (*vls*), which undergo antigenic variation by promiscuous recombination. This gene family is present in an infectious, clonal isolate of *B. burgdorferi* B31 but not in clonal in vitro-passaged organisms, suggesting that it is necessary for spirochete infectivity (58).

A clonal isolate of *B. burgdorferi* N40 (cN40) which causes severe arthritis and carditis in C3H mice has now been passaged in BSK II medium. Spirochetes passaged 75 times in vitro, designated N40-75, were investigated for infectivity and pathogenicity and compared to the parental isolate. The lack of arthritis and carditis was then correlated to a defect in differential N40-75 gene expression in vivo, resulting in an inability of N40-75 to rapidly adapt to the murine *B. burgdorferi*-specific antibody response.

MATERIALS AND METHODS

Mice. C3H, C57BL/6 (B6), C3H severe combined immunodeficient (C3H-*scid*), and C.B-17-*scid* mice were purchased from the Frederick Cancer Research

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Center, Frederick, Md. Mice were kept in filter-framed cages and euthanized with CO_2.

B. burgdorferi and infections. cN40 is a clonal isolate of *B. burgdorferi* N40 that is infectious and pathogenic in mice (9). Spirochetes were grown in BSK II medium at 33°C. To obtain derivatives of cN40, *B. burgdorferi* spirochetes were serially passaged in BSK II medium every 3 to 5 days by inoculating 50 μl of grown cultures into 7 ml of fresh medium. Clones of N40-75 were obtained by subsurface culture in solid BSK medium, as reported previously (58, 60).

Every 5 to 10 passages, spirochetes were assessed for infectivity and the ability to induce carditis and arthritis in 3- to 6-week-old C3H mice. Individual mice in groups of five were inoculated with 10^4 or 10^7 spirochetes in the midline of the back by intradermal injection (26). The animals were necropsied at 21 and 60 days, time points that represent the peak of acute disease and the regression phase of inflammation (10), respectively. Mice were assessed for infection by culturing specimens of the blood, urinary bladder, and skin (at the inoculation site) in BSK II medium at 33°C (24). Cultures were read after 14 days, a time period sufficient to allow a single spirochete to grow to stationary phase.

Quantitation of spirochetes in vitro. Quantitation of the spirochetes grown in vitro was performed as follows. Cultures were spun down and washed twice with phosphate-buffered saline (PBS). The spirochetes were resuspended in PBS, and the absorbance at 600 nm was determined. The absorbance at 600 nm was shown in parallel experiments to correlate with the number of spirochetes, as counted by dark-field microscopy, in a linear manner. One unit of absorbance at 600 nm was determined to equal 1.9×10^9 spirochetes/ml.

Histopathology of murine Lyme disease. Formalin-fixed, paraffin-embedded hearts and joints (both knees and tibiotarsi) were examined microscopically for evidence of disease (23). Arthritis and carditis were assessed as previously described (3, 5). All histopathologic assessments were made in a blinded fashion.

In vivo serum treatments. Relative susceptibility of cN40 and N40-75 spirochetes to immune serum in the murine host was studied in C3H-*scid* mice. Mice were administered 50 μl of immune sera at the time of spirochete inoculation and 4 and 8 days after infection (7, 20). Immune sera were obtained from immunocompetent C3H mice that had been infected with cN40 or N40-75 for 21 days. C3H-*scid* mice were necropsied 14 days after challenge and assessed for infection.

DNA extraction and PCR. Cultured *B. burgdorferi* DNA was obtained by proteinase K digestion (100 μg/ml, overnight at 55°C) followed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation. PCR was performed on DNA samples using primers for *flaB* and *p39* (chromosomal targets), *ospA* (lp54 plasmid), *erpT* (lp28-1), *bbk50* (lp36), *ospD* (lp38), *ospC* (cp26) and *erp*s (cp32 family of plasmids [52]).

Detection and quantitation of B. burgdorferi in infected tissues. DNA was extracted from ears, joints, and hearts of cN40- and N40-75-infected mice, using the QIAamp tissue kit (Qiagen Inc., Chatsworth, Calif.). Quantitation of *B. burgdorferi* was performed by PCR of twofold serial dilutions of the purified DNA. Primers corresponded to the *flaB* gene. The number of spirochetes was calculated per milligram of DNA, using the following formula: reciprocal of the last dilution with a positive PCR × 200/milligram of DNA used in the first dilution. The sensitivity of the assay was determined to be approximately 200 spirochetes in parallel experiments, and it did not depend on the source of the DNA (not shown).

Immunoscreening of a genomic expression library. An N40 genomic expression library in lambda ZAP II (51) was differentially screened using immune sera from mice infected with cN40 or N40-75 for 21 days. Duplicate nitrocellulose filters were obtained for each plate, which contained approximately 10^4 plaques. Duplicate filters were screened with cN40- and N40-75-immune sera, and cN40-specific reactive plaques were rescued for further screening. Potential specific clones were subjected to three rounds of screening. Filters were blocked for 2 h using PBS with 3% bovine serum albumin (PBS-BSA) and incubated for 1 h with sera (1:100 dilution). Membrane filters were then washed three times using PBS with 0.05% Tween 20 and incubated for 30 min with goat anti-mouse immunoglobulin G in PBS-BSA (1:1,000 dilution), conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.). After washing, the filters were incubated with tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Positive clones were recovered in SM buffer with chloroform. After three rounds of screening, positive clones were further tested for possible reactivity with low titers of antibodies using N40-75-immune sera at a dilution of 1:50. Clones 2, 10, and 16 were then excised and sequenced using standard protocols. DNA homology comparisons were performed using BLAST search and the *B. burgdorferi* genome database (27).

RNA purification and RT-PCR. RNA was extracted from the spleens of cN40- or N40-75-infected C3H mice by the guanidinium thiocyanate method (17), using a Micro RNA isolation kit (Stratagene, La Jolla, Calif.). To check for DNA contamination, RNA samples were subjected to PCR without further processing, using 100 ng of RNA and primers corresponding to *flaB*, *ospA*, *bba64* (27), *dbpA*, *dbpB*, *bba65* (27), *bba66* (27), *p21*, *erpD*, *gene-1*, and *gene-2*. Forty cycles were performed, with denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min) steps. If necessary, the samples were treated with DNase (Boehringer Mannheim, Indianapolis, Ind.) for 2 h at 37°C, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and ethanol precipitated. Aliquots of 5 to 10 μg were then used to obtain cDNA with a reverse transcription

TABLE 1. Primers used

Primer (reference)	Sequence
flaB	5′-AGA ATT ACT TCA AAG GCT-3′
	5′-TGT AGT TGT AAC ATT AAC-3′
ospA	5′-GGT CAA ACC ACA CTT GAA GTT-3′
	5′-GTC AGT GTC ATT AAG TTC-3′
erpT	5′-TTC ACA AGT AGC ACA ACA TGC TCC-3′
	5′-GCT ATC ACC ACC AAT TTC AAG TCC-3′
bbk50	5′-CAG CTT CTT CTA AAG AGG TAG AAG C-3′
	5′-TAA ATC GCA ACA AGC TTT AGC-3′
ospD	5′-TAT CTT GTG TTC ATG ATA AAC-3′
	5′-ATC CAA TGT CTC TTT GAC TG-3′
ospC	5′-GCC GTG AAA GAA GTT GAG ACC TTA-3′
	5′-TAA GAT TGT CCA GAC CAA GCA CTG-3′
*erp*s	
E(13) (52)	5′-ATG TTT ATT ATT TGT GCT GTT T-3′
E (310) (52)	5′-AAA CTA GTT TTA AAT GAT CC-3′
dbpA	5′-GCT ACT GTA GTA GCT CGC A-3′
	5′-TTG AAT CGT CCT CTA AGG-3′
dbpB	5′-CTG GAA ACA GTG GTC AAT TC-3′
	5′-TCT CTC CAC CAT TTT CAA T-3′
bba64	5′-TTC CTT CGT TGA AAT TTA AAG ATG-3′
	5′-TGA TTT CTT GCT CTC TGG AAG TC-3′
bba65	5′-AGC GGA TCT AAT TGA CAA GC-3′
	5′-TCC ATG TTG ATT GCT AAA TC-3′
bba66	5′-GAT TGT TTT TAT TTT CTT GCA CG-3′
	5′-TGA AAC GGC TTG AAT AGG TAT TC-3′
p21	5′-TTC TAA CTT CTA TTT TAA ATT-3′
	5′-AAT AAT GAG TTA AAA GTT AAG CAA-3′
erpD	5′-GCG ATG ATC CTA ATG GCA-3′
	5′-CCA AGT TGT ACG TAT AGA GCT-3′
gene-1	5′-AAC AAG CTC TAT TTA ACT AGT C-3′
	5′-GAT TCT TAC TGG TGA AAA G-3′
gene-2	5′-GCA TTT AAG CAA TAT TCT TAG-3′
	5′-TCA CTT GAG CCC TTT GCT ATT-3′

(RT)-PCR kit (Stratagene) utilizing random primers. PCR were then performed using the same primers and conditions and 2 to 5 μl of the cDNA samples.

Primers. Sequences of the primers used are shown in Table 1.

RESULTS

B. burgdorferi N40 loses pathogenicity, but not infectivity, upon in vitro passage. A clonal isolate of *B. burgdorferi* N40 (cN40) that is highly infectious and pathogenic in C3H mice was continuously passaged in vitro to determine whether the spirochete would lose the ability to infect mice or cause disease. Despite being passaged 75 times in vitro, N40 retained its capacity to infect C3H mice, as demonstrated by recovery of the spirochete from selected organs at 2 to 3 weeks following inoculation with 10^4 organisms (Table 2). *B. burgdorferi* started to lose pathogenicity (the ability to cause arthritis and carditis) after 22 passages in vitro (Table 2). At passage 75, the spirochetes no longer elicited joint or cardiac inflammation in immunocompetent mice, regardless of their genetic (C3H or B6) background or dose (up to 10^7) of inoculum (Table 2). Arthritis and carditis were also not evident when N40-75-infected animals were examined for evidence of inflammation after 60 days (Table 2), indicating that the appearance of disease was not merely delayed.

Characterization of N40-75. To understand why N40-75 was not pathogenic, we compared N40-75 and cN40 for antigenic and genetic differences. The protein profiles of cultured cN40 and N40-75 were similar except for a lower level of OspA/B and increased level of OspC expression by N40-75 (Fig. 1A).

TABLE 2. Loss of *B. burgdorferi* pathogenicity with in vitro passage in immunocompetent mice[a]

Mouse	Isolate	Day of sacrifice	Infection (no. of specimens positive/no. examined)			Arthritis (no. of mice with arthritis/no. examined)	Carditis (no. of mice with carditis/no. examined)
			Blood	Inoculation site	Bladder		
C3H	cN40	21	1/5	4/5	5/5	5/5	5/5
	p22	21	2/5	2/5	5/5	3/5	3/5
	p51	21	0/5	2/3	4/5	0/5	1/5
	N40-75	21	1/5	4/5	3/5	0/5	0/5
	N40-75	60	0/5	4/5	4/4	0/5	0/5
	N40-75 (10^7)	21	0/5	3/4	2/3	0/5	0/5
B6	cN40	21	0/5	5/5	5/5	0/5	5/5
	N40-75	21	0/5	3/5	4/5	0/5	0/5

[a] C3H and B6 mice were infected with cN40 or its derivatives (p22, p51, and N40-75) obtained by in vitro culturing. Mice were infected with 10^4 spirochetes, unless otherwise indicated, and necropsied at 21 or 60 days after infection. Arthritis and carditis were assessed in a blinded fashion.

Nevertheless, the level of expression of OspA/B varied among different clonal N40-75 spirochetes (Fig. 1A). Moreover, the analysis of N40 and N40-75 extracts by Western blotting using rabbit cN40 hyperimmune sera showed no differences between the two passages (Fig. 1B). Western blot analysis of cN40 and N40-75 with cN40- and N40-75-infected mouse sera revealed some differences in the proteins recognized by both sera (Fig. 1C). The differences were more notable in the range of 19 to 25 kDa, as well as 30 to 32 kDa (Fig. 1C, arrows), indicating that infection with both passages induced differentiated antibody responses.

DNA PCR targeted at genes expressed in the linear chromosome, as well as several plasmids, indicated the presence of these genes both in cN40 and N40-75 (Fig. 2), as well as several N40-75 clones (Fig. 2). The analysis of the amplification products obtained with cN40 and N40-75 DNA when using primers E(13) and E(310) (52) indicated the presence of *ospE* and *p21*, but no other *erp*s, in both passages (not shown), in agreement with the fact that only these two genes have been reported to be sequenced from this isolate (N40) (52). The presence of *erpT* by PCR (Fig. 2) and the *vls* locus by Southern blotting (not shown) in both spirochetes indicated that plasmid lp28-1 had not been lost during in vitro passage. These data indicate that no readily apparent loss of DNA could be associated with the loss of pathogenicity resulting from in vitro passage.

To determine whether pathogenicity was related to spirochete growth, cN40 and N40-75 multiplication rates in vitro and bacterial burden in vivo were examined. When equal numbers of cN40 and N40-75 (1.4×10^4) were incubated in BSK II medium, N40-75 grew more rapidly than cN40 (Fig. 3). Differences were evident as early as 48 h (doubling times of 5.2 and 3.7 h for cN40 and N40-75, respectively) and continued throughout the culture period (t test, $P < 0.05$). Once stationary-phase growth had been reached, the density of N40-75 was greater than that of cN40 (Fig. 3). In contrast, N40-75 was less abundant than cN40 during infection of immunocompetent mice. Two weeks after inoculation into mice, the number of N40-75 in the skin (ear tissues) was significantly ($P < 0.05$) less than the number of cN40 (average ± standard error [SE] per milligram of DNA, 27,000 ± 15,000 for N40-75 and 360,000 ± 120,000 for cN40 [Fig. 4]). The number of N40-75 spirochetes in the joints of the infected mice was extremely low (6,000 ± 6,000) compared with cN40 (340,000 ± 88,000) (Fig. 4). N40-75 could not be detected by PCR in the heart tissues, whereas large numbers of cN40 (1,100,000 ± 500,000) were apparent (Fig. 4).

N40-75 causes arthritis and carditis in SCID mice. Pathogenicity and bacterial burden in five cN40- and five N40-75-infected C3H-*scid* mice were then examined to assess whether spirochete growth was influenced by the *B. burgdorferi*-specific response. All of the N40-75-infected SCID mice developed arthritis and carditis that was indistinguishable from cN40-induced disease. Moreover, the severity of the inflammation was comparable in cN40- and N40-75-infected SCID mice (not shown). Quantitative PCR revealed similar number of spirochetes in ear (N40-75, 200,000 ± 10,000; cN40, 400,000 ± 200,000), joint (N40-75, 50,000 ± 10,000; cN40, 60,000 ± 20,000), or heart (N40-75, 180,000 ± 110,000; cN40, 70,000 ± 60,000) tissues in N40-75- and cN40-infected SCID mice (Fig. 5).

N40-75 does not evade immune clearance as rapidly as cN40. The SCID mouse studies suggest that the inability of N40-75 to efficiently colonize murine tissues may be related to the host immune response. Previous reports showed that *B. burgdorferi*-specific antibodies protect against infection (6, 7). Passive transfer experiments also demonstrated that *B. burgdorferi* spirochetes become resistant to immune clearance within 4 to 5 days following syringe inoculation (7, 20). We therefore determined whether *B. burgdorferi*-specific humoral responses were responsible for the decreased number of spirochetes in N40-75-infected immunocompetent mice. Antisera from *B. burgdorferi*-infected C3H mice were administered to cN40- or N40-75-infected SCID mice. As expected (20), administration of immune sera at the time of challenge with cN40 resulted in protection of the mice (Table 3). The protective effect of immune sera was not apparent at 4 days after cN40 inoculation (20), suggesting that syringe-administered cN40 spirochetes adapt to their new environment by this interval (Table 3). In contrast, when N40-75-infected SCID mice were administered immune sera from *B. burgdorferi*-infected immunocompetent mice, protection against spirochetal challenge was evident when the sera were administered at 4 days after infection (Table 3). Sera from either cN40- or N40-75-infected immunocompetent mice were equally protective (Table 3), suggesting that the protective antibody specificities in the two sera were similar. At day 8 of infection, immune sera were no longer protective (Table 3). These data suggest that N40-75 requires a longer period of time than cN40 to adapt and evade antibodies present in sera from *B. burgdorferi*-infected immunocompetent mice.

N40-75 is deficient in in vivo gene expression. After entering the mammalian host, several *B. burgdorferi* genes are differentially expressed (21). We examined whether the delayed ability of N40-75 to host adapt was associated with alterations in the up- or down-regulation of in vivo-expressed genes or genes coding for antigens recognized by antibodies in immune sera.
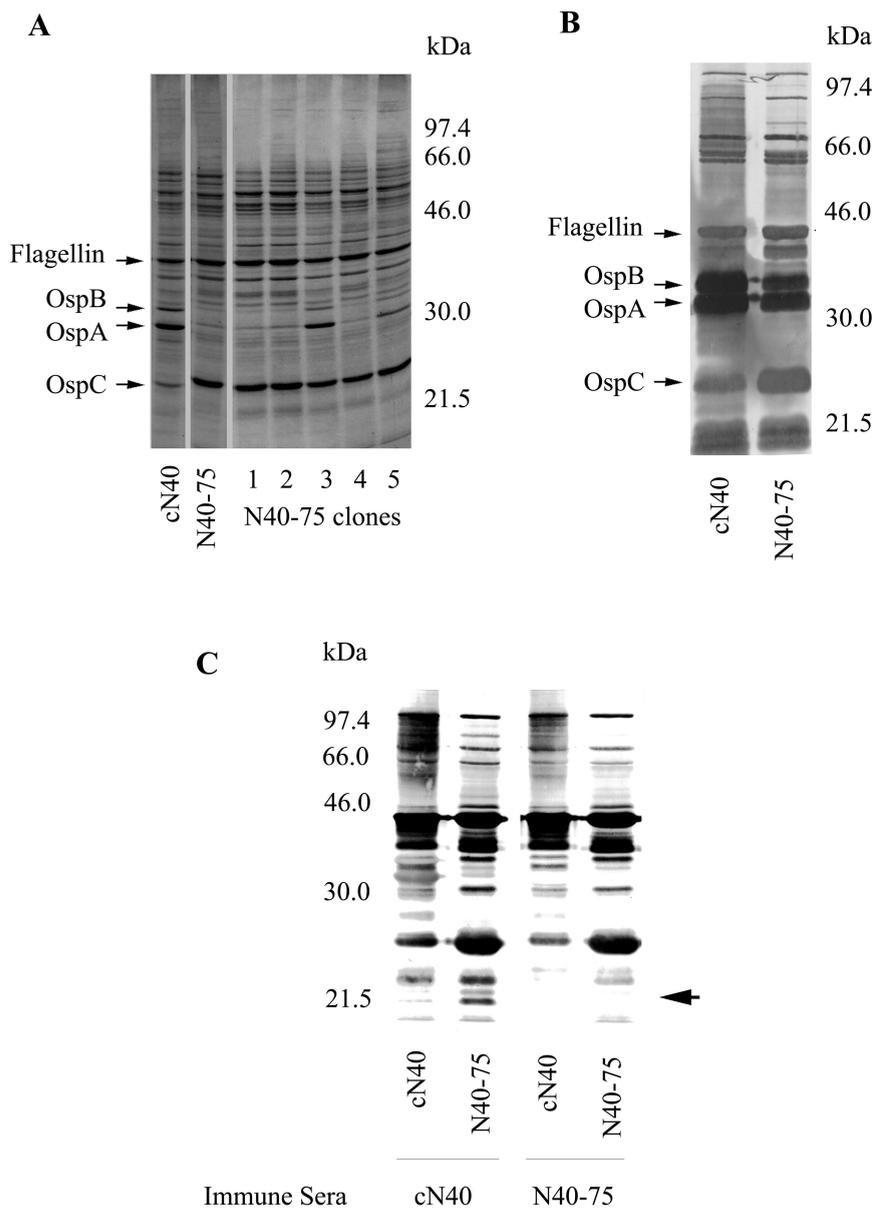
FIG. 1. cN40 and N40-75 do not differ substantially when grown in vitro. cN40, N40-75, and several N40-75 clones were grown in vitro to stationary phase and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to compare protein contents (A). Western blot analysis with cN40 rabbit hyperimmune sera was also performed to test the presence of OspA, -B, and -C in both passages (B). The immune response to cN40 and N40-75 was also tested by Western blotting (C), by using 21-day-infected mouse sera with cN40 and N40-75.

To identify antigens selectively recognized by sera from cN40-infected mice, a genomic expression library was differentially screened with immune sera from cN40- and N40-75-infected mice (51). Immune sera from *B. burgdorferi*-infected mice contain antibodies to antigens synthesized during infection. If the antigenic profiles of N40-75 and cN40 are different during infection, then the antibodies raised during infection may differ. Using this strategy, we isolated three clones that were recognized by cN40- but not N40-75-infected murine sera (Table 4). Clone 2 had an insert of 2,570 bp, containing the genes *bba64* (a *p35* homologue [29] in the published B31 genome sequence [27]), *bba65*, and *bba66* (yet another *p35* homologue) (Table 4). Clone 10 had an insert of 2,032 bp and included

dbpA (incomplete) and *dbpB* (*bba24* and *bba25*, respectively), which form an operon (22, 31). It also contained three small open reading frames (*bba26*, *bba27*, and *bba28*) (27) and the incomplete sequence for *bba29*. Clone 16 had an insert of 3,360 bp and contained sequences related to the *ospEF* gene family: p21 (51), *erpD* (13, 50), and two unknown genes (Table 4). Both clones 2 and 10 mapped within the linear plasmid of 54 kb, while clone 16 contained sequences not present in the published B31 sequence (27). The presence of p21 and ErpD suggests that this clone may belong to the cp32 family of plasmids.

N40-75 does not express *bba64*, *bba65*, *bba66*, and *dbpAB*. To directly determine whether these specific genes, which were
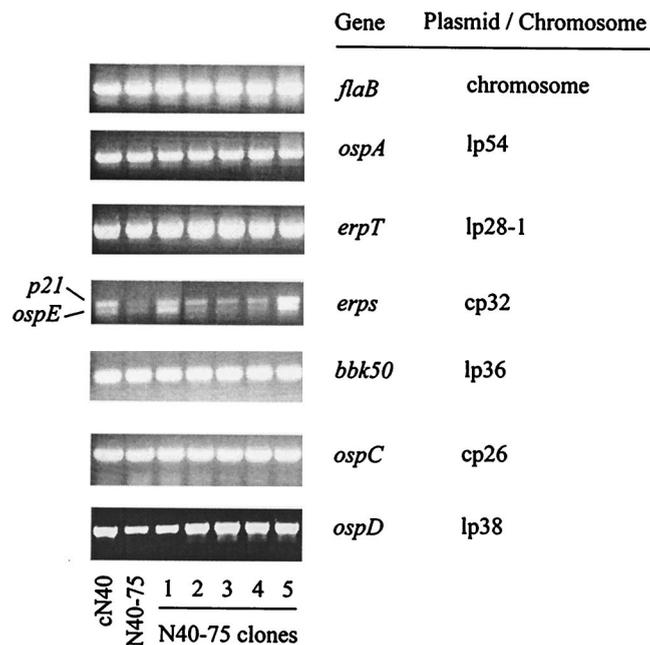
FIG. 2. DNA contents of cN40 and N40-75, as well as of several N40-75 clones. DNA PCR for several genes was performed to detect any possible loss of DNA as a result of in vitro passage. The plasmids in which the amplified genes are present are indicated.

identified by differential immunoscreening, were expressed by cN40 but not N40-75 during infection, we performed RT-PCR analysis of spleens from cN40- and N40-75-infected mice. *dbpAB*, *bba64*, *bba65*, *bba66*, *erpD*, and *gene-2* mRNA could be readily detected in spleens of cN40-infected mice at 2 weeks of infection (Fig. 6). In contrast, N40-75-infected spleens lacked mRNA for these genes. As expected, the *flaB* gene (control) could be detected in cN40- or N40-75-infected animals, indicating that the spirochetes were present in these tissues. Neither cN40 or N40-75 expressed detectable levels of p21 mRNA (Fig. 6), consistent with previous reports for this time period of
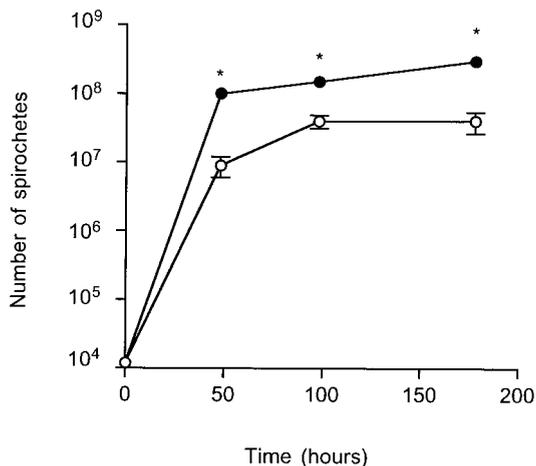

FIG. 3. N40-75 grows faster than cN40 in vitro. Equal amounts of cN40 and N40-75 were grown in BSK II medium and analyzed at different time points over 1 week. Data represent the average of five individual experiments ±SE. An asterisk indicates significant difference between cN40 and N40-75 at that particular time point (t test, $P < 0.05$).
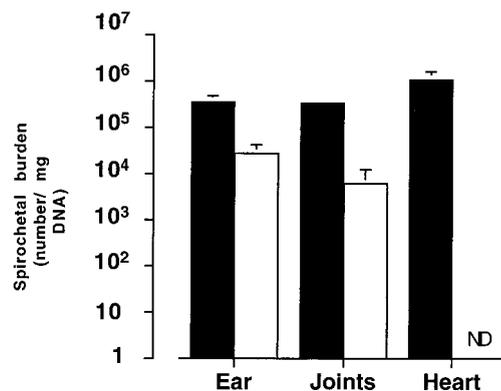

FIG. 4. N40-75 does not effectively disseminate in the murine host. Ear, joint, and heart tissue was used to quantitate *B. burgdorferi* by DNA PCR using *flaB* targets in mice infected for 3 weeks. Data represent the average ± SE of five individual mice. ■, cN40; □, N40-75.

infection (18). Finally, *gene-1* mRNA was lacking in cN40 or N40-75 upon infection during 2 weeks (Fig. 6).

DISCUSSION

We characterized the pathogenicity of derivatives of a clonal population of *B. burgdorferi* cN40. Previous researchers have shown that *B. burgdorferi* passaged in vitro rapidly loses infectivity (40, 58). Our results differ in that cloned N40 spirochetes remained infectious in mice for up to 75 passages, suggesting that the infectivity of cN40 is relatively stable compared with other spirochetes passaged in vitro. Alternatively, infectivity could be maintained becaused short subculture intervals were used. It may be that prolonged growth under stationary-phase conditions leads to plasmid loss and more rapid reduction in infectivity. Indeed, both cN40 and N40-75 contained the plasmid expressing *vls* genes, supporting the contention that *vls* genes are important for infectivity and are not lost by cN40 spirochetes. Although N40-75 is infectious in C3H mice, these spirochetes do not cause arthritis or carditis in immunocompetent mice. The lack of disease in N40-75-infected mice correlated with a reduction, or lack, of spirochetal DNA in the joints and hearts. N40-75 could be readily cultured, or amplified by PCR, from the bladder, skin, and spleen, suggesting that the reduction in pathogenesis is due to an inability of N40-75 to effectively colonize specific tissues in sufficient numbers to cause disease. Lack of pathogenicity of N40-75 cannot be explained by assuming that the majority of cells in the uncloned N40-75 population are not infectious, since infection
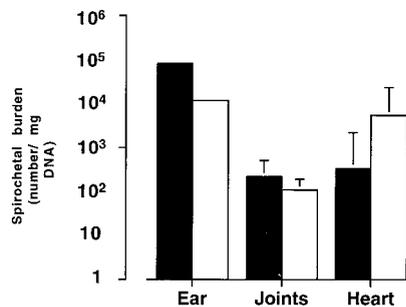

FIG. 5. N40-75 disseminates efficiently in SCID mice. cN40 and N40-75 were quantitated in ears, joints, and hearts of C.B-17-*scid* mice after 2 weeks of infection as for Fig. 3. ■, cN40; □, N40-75. Data represent five individual mice.

TABLE 3. Effect of immune sera on N40-75- or cN40-infected SCID mice[a]

Spirochete challenge	Immune serum	Day of serum administration	Infection (no. of mice infected/no. examined)		
			Blood	Inoculation site	Bladder
cN40	NMS	0	1/5	5/5	5/5
	cN40 immune	0	0/5	0/5	0/5
		4	2/5	4/5	5/5
		8	1/4	4/4	4/4
	N40-75 immune	0	0/5	0/5	0/5
		4	0/5	3/5	3/4
N40-75	NMS	0	2/5	3/5	5/5
	cN40 immune	0	0/5	0/5	0/5
		4	0/5	0/5	0/5
		8	1/4	4/4	3/4
	N40-75 immune	0	0/5	0/5	0/5
		4	0/5	0/5	0/5

[a] C3H-*scid* mice were inoculated with 10^4 spirochetes and then treated with immune sera from *B. burgdorferi*-infected mice. Immune sera were administered within 1 h of spirochete inoculation (day 0) or 4 or 8 days after inoculation. Immune sera from immunocompetent C3H mice that had been infected with cN40 or N40-75 for 21 days were used. NMS, normal mouse serum (control).

of mice with 10^4 spirochetes rendered culture positive organs that were not significantly different between cN40- and N40-75-infected animals (Table 2). Moreover, infection with 1,000 times more N40-75 spirochetes (10^7) did not change the non-pathogenic phenotype of high-passaged N40, arguing against this explanation. This reasoning is supported by the obtention and characterization at the DNA level by PCR of several clones of N40-75, which showed no loss of different pieces of DNA tested. These clones were infectious in C3H mice, but they did not cause arthritis or carditis.

The analysis of the proteins profiles of cN40 and N40-75 indicated differences in OspA/B and OspC. OspA/B appeared to be down-regulated in in vitro-grown N40-75, although the analysis of both passages by Western blotting using cN40-hyperimmune sera revealed that both contained the proteins. Moreover, the analysis of N40-75 clones revealed variability in the amount of OspA/B expressed. In contrast, OspC was up-regulated in high-passage N40. The down-regulation in vivo of *ospAB* and the up-regulation of *ospC* gene expression after syringe inoculation have been demonstrated (18, 38). Therefore, these differences are not likely to be related with the lack of N40-75 pathogenicity.

In order to survive and efficiently colonize its hosts, *B. burgdorferi* must adapt to different environments during its life cycle. It is well known that when infected *I. scapularis* engorge, the spirochete protein expression alters before migrating to the tick salivary glands and entering the vertebrate host (4, 21, 45, 47, 55). These changes include the up-regulation of *ospC* and the down-regulation of *ospA* (21, 46). The adaptation to invade the mammalian host is associated with changes in several

genes, including *bbk32*, *bbk50*, *ospEF* homologues, and *dbpA/B*. There is a time period (around 4 days after syringe inoculation) in which cN40 is no longer vulnerable to the passive administration of infected mouse sera (7, 20). The process of adaptation and immune evasion probably involves changes in gene expression or recombination events in genes like *vls* (60). Zhang and collaborators have demonstrated that recombination of the *vls* gene occurs in vivo, after experimental infection of mice and as soon as 4 days after the initiation of the infection (60). The evasion of the *B. burgdorferi*-specific humoral responses is probably due to a combination of selective gene expression and recombination, although the relative contribution of each remains to be elucidated.

These data provide evidence that long-term-cultured *B. burgdorferi* spirochetes are more susceptible to host immunity. Susceptibility of N40-75-infected SCID mice to the development of arthritis and carditis suggests that efficient colonization and dissemination in the host involves immune evasion. Passive transfer of immune sera to N40-75-infected SCID mice at 8 days following challenge did not influence the establishment of infection, indicating the N40-75 can adapt within this interval. The delayed adaptation period contribute to the lower number of N40-75 in immunocompetent mice.

Spirochete genes selectively expressed during infection are likely to be important for the maintenance of *B. burgdorferi* in the mammalian host and, ultimately, the completion of the vector-host-vector cycle. Initial characterization of antibody responses arising against cN40 and N40-75 in vivo (Fig. 1C) indicated that cN40-immune sera reacted with a wider range of proteins. Our immunoscreening procedure resulted in the detection of three clones that represented genes expressed exclusively by cN40 and not N40-75 upon infection. These expression units included genes that are preferentially expressed in the vertebrate host, including *dbpAB* (14) and *ospEF* homologues (21). Our results also showed that these genes were not merely lost by N40-75, since we could readily amplify these sequences when using N40-75 DNA. Moreover, DNA subtraction techniques indicated that a loss of DNA contents had not occurred during in vitro passage (data not shown). Interestingly, other researchers have reported a low level of expression of both DbpA and -B in high (>30)-passage *B. burgdorferi* (30, 31), consistent with our findings for N40-75. Clone 2 contained three genes that could have expressed an-

TABLE 4. cN40-specific clones selected by immunoscreening of an N40 genomic library with cN40- and N40-75-immune sera[a]

Clone no.	Size of insert (bp)	Gene(s) present	Location
2	2,570	*bba64*,[a] *bba65*, *bba66*	lp54
10	2,032	*dbpAB*[a]	lp54
16	3,360	*p21*, *erpD*, *gene-1*,[b] *gene-2*[c]	cp32 (?)

[a] cN40-specific clones reacted with antibodies in immune sera from cN40-infected mice but not N40-75-infected mice.
[b] Incomplete sequence; 3′ sequence missing.
[c] GenBank accession number AF023852. GenBank accession number: U19754.

sion or recombination events, temperature has been the most extensively studied. Stevenson and collaborators (48, 49) have examined the effect of temperature shifting on the expression of *ospC* (49) and the *erp*s (48). The temporal expression of *p21*, an *ospE* homologue, cannot be explained solely by temperature alterations, since this gene is not expressed until 21 days after initiation of the infection (18). Moreover, Zhang and collaborators have recently excluded temperature as the factor initiating recombination at the *vls* locus, speculating about the necessity of other host factors triggering this phenomenon (60). A detailed study of the changes in gene expression occurring after infection should provide insight into host factors that influence those changes.

Our results indicate that *B. burgdorferi* has to adapt to the mammalian host in order to be able to reach a certain number and induce inflammation. Our data also suggest that the adaptation process implies at least two phenomena: the evasion of immune responses arising during infection and the up-regulation of genes that are needed for new functions that need to be performed in the new environment. To comply with the first task, there are two possibilities that could explain our results: N40-75 has impaired the ability to induce recombination (i.e., at the *vls* locus), and/or the down-regulation of highly immunogenic genes that do not need to be expressed in the new environment is impaired in N40-75. The adaptation phenomenon would then be a complex process in which both immune evasion and up-regulation of certain genes need to be accomplished. This is true for immunocompetent mice, although our results with SCID mice suggest that immune evasion in the absence of an acquired immune response is not required for pathogenicity. However, until a good system is provided to study in vivo function of the genes obtained in the differential immunoscreening (i.e., a transformation system that works in infectious and pathogenic *B. burgdorferi*), no direct evidence will be obtained about the exact roles of these genes during infection.

In conclusion, we have generated a derivative of cN40, designated N40-75, which do not cause arthritis or carditis in immunocompetent mice. The inability of N40-75 to adapt to the mammalian host correlates with its inability to induce disease. This defect in differential gene expression results in an increase and prolonged susceptibility of N40-75 to the antibodies in immune sera and an inability of these N40-75 spirochetes to effectively colonize and disseminate within the murine host due to that immune-based vulnerability.
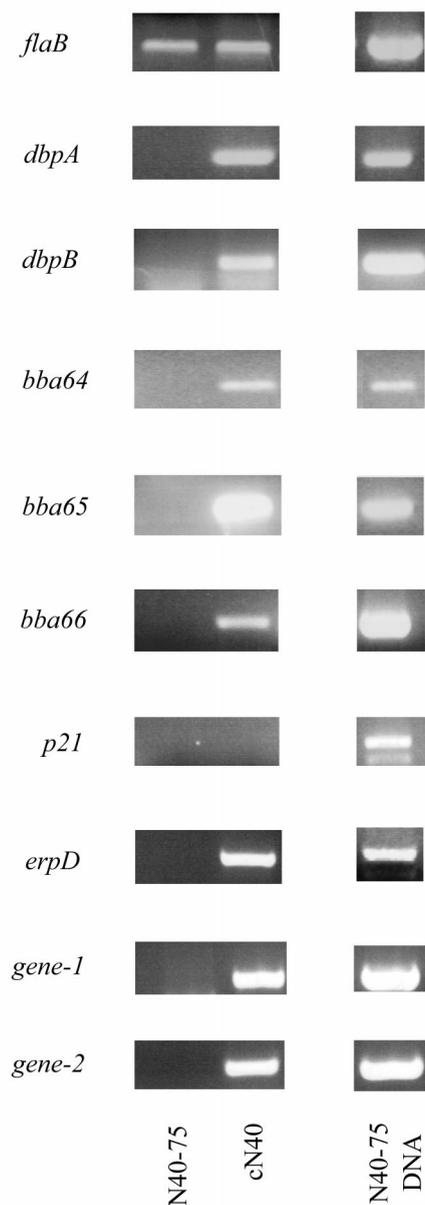
FIG. 6. N40-75 does not up-regulate in vivo genes. cN40- and N40-75-infected mouse spleens were used to extract total RNA, and RT-PCR for *B. burgdorferi dbpA*, *dbpB*, *bba64*, *bba65*, *bba66*, *p21*, *erpD*, *gene-1*, and *gene-2* was performed. As a control, *flaB* was used to identify spirochetes in spleens of the infected animals. N40-75 DNA was used as a positive control, demonstrating the presence of the genes in high-passage N40.

tigens that were only recognized by the immune sera from cN40-infected mice. The three genes, *bba64*, *bba65*, and *bba66*, were expressed exclusively by cN40 in infected mice. Clone 16 contained four genes: *p21* (18), *erpD*, and two unknown genes. Of them, *p21* was not detected in cN40- or N40-75-infected mice, consistent with a later time point expression during infection (18), *erpD* and *gene-2* were specifically expressed by cN40 at 2 weeks of infection.

The exact functions of most of the in vivo genes, as well as the signals that trigger the initiation of their expression once *B. burgdorferi* enters the mammalian host, remain unknown. Among the factors that could trigger changes in gene expres-

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