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Borrelia burgdorferi Lacking DbpBA Exhibits an Early Survival Defect during Experimental Infection▽

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Several *Borrelia burgdorferi* genes induced under mammalian host conditions have been purported to be important in Lyme disease pathogenesis based on their binding to host structures. These genes include the *dbpBA* locus, whose products bind host decorin and glycosoaminoglycans. Recently, the *dbpBA* genes were reported to be involved in borrelial infectivity. Here we extended the previous observations by using culture and quantitative PCR to evaluate low- and high-dose murine infection by a Δ*dbpBA*::Gent^r derivative of *B. burgdorferi* strain B31. The results indicate that the Δ*dbpBA*::Gent^r mutant is attenuated in the ability to initially colonize and then persist in multiple tissues. The mutant exhibited a colonization defect as early as 3 days postinfection, before the development of an adaptive immune response, and after low-dose infection of SCID mice, which are deficient in adaptive immunity. These findings suggest that the inability to adhere to host decorin may promote clearance of *B. burgdorferi*, presumably via innate immune mechanisms. In a high-dose infection, the mutant disseminated to several tissues, particularly joint tissue, but it was generally cleared from these tissues by 3 weeks postinfection. Finally, following high-dose infection of SCID mice, the *dbpBA* mutant exhibited only a mild colonization defect, suggesting that the adaptive response is involved in the clearance of the mutant in immunocompetent mice. Taken together, these results suggest that the DbpBA proteins facilitate the colonization of multiple tissues by *B. burgdorferi* and are required for optimal resistance to both innate and adaptive immune mechanisms following needle inoculation.

Borrelia burgdorferi is the etiologic agent of Lyme disease and traffics within an enzootic cycle that involves an arthropod vector and rodent mammalian reservoirs, but it can also infect other mammalian species, including humans. In humans, the bite of an infected *Ixodes* tick usually results in a red skin lesion, designated erythema migrans, and the illness is accompanied by general malaise and, in some cases, cardiac and neurologic sequela (for reviews, see references 39 and 58). Individuals who do not seek antibiotic therapy at this stage of the infection are at risk for developing manifestations associated with late Lyme disease, which in the United States are usually arthritis. In Europe, a neurologic pathology and an inflammatory skin condition known as acrodermatitis chronica atrophicans can occur in chronically infected individuals. As such, in areas where it is endemic, Lyme disease contributes to significant morbidity.

By virtue of its ability to transition between ticks and mammals, *B. burgdorferi* must modify gene expression quickly to adapt to such disparate environments. Previous studies using transcriptional profiling demonstrated that *B. burgdorferi* gene expression changes in response to pH, temperature, redox status, exposure to blood, and as-yet-uncharacterized mammal-specific factors (1, 2, 5, 10, 11, 15, 17, 26, 36, 40, 48, 51, 52, 59, 60, 63). One set of genes transcribed under conditions that mimic host-adapted conditions (either increased temperature, lower pH, redox status, or blood exposure) includes the *dbpA* and *dbpB* genes (5, 26, 40, 48, 52, 60), which are apparently cotranscribed from *dbpB* to *dbpA* (i.e., *dbpBA*) (22) and encode adhesins that bind to mammalian decorin (20, 21). Decorin is abundant in the extracellular matrix (ECM) of the dermal skin layer, as well as in joint tissue (7, 34, 43). The induction of *dbpBA* under simulated host conditions is consistent with the hypothesis that these genes have a role in borrelial pathogenesis, and the DbpBA proteins, along with other adhesins, are presumed to assist in cell and ECM attachment of *B. burgdorferi* to mediate colonization and initiate dissemination within the mammalian host (7, 20, 21, 34). To investigate this hypothesis, the *dbpBA* genes were deleted, and the resulting mutant was tested using BALB/c mice (56). In their initial study, Shi et al. concluded that DbpA and DbpB were not essential for mouse infection, although only 58% of the tissues were culture positive when a single large inoculum (10^5 *B. burgdorferi* cells) was used and no genetic complementation of intact *dbpBA* was evaluated (56). Another study indicated that the constitutive overexpression of *dbpBA* makes *B. burgdorferi* more infectious but impairs its ability to disseminate, suggesting that DbpBA has an important role in experimental infections (62). A more recent study by Shi et al. indicated that the *dbpBA* genes are required for virulence in mice following needle inoculation (55), a finding that was corroborated re-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Reference(s) or source
B. burgdorferi strains		
ML23	Missing lp25	30, 31
ML23/pBBE22	Missing lp25, complemented with BBE22	54
JF105	ML23 Δ*dbpBA*::Gent^r	This study
JF105/pBBE22	ML23 Δ*dbpBA*::Gent^r, complemented with BBE22	This study
JF105/pJBF17	ML23 Δ*dbpBA*::Gent^r, complemented with BBE22 and *dbpBA*	This study
E. coli strains		
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
ccdB survival T1R	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG tonA*::P_{trc}-*ccdA*	Invitrogen
Plasmids		
pCR2.1-TOPO	Amp^r Kan^r; PCR cloning vector	Invitrogen
pCR-XL-TOPO	Kan^r; PCR cloning vector	Invitrogen
pCR8/GW/TOPO	Spc^r; Gateway PCR cloning/entry vector	Invitrogen
pDEST17	Cam^r Amp^r; Gateway destination vector	Invitrogen
pBSV2G	Gent^r; borrelial shuttle vector	16
pBBE22	Kan^r; borrelial shuttle vector carrying BBE22	46
pBBE22gate	Cam^r Kan^r; pBBE22 modified to be a Gateway destination vector containing *attR* sites[a]	This study
pNP3	Gent^r Kan^r; Δ*dbpBA*::Gent^r construct containing region of lp54 that contains 1.5 kb upstream and downstream of the end of *dbpA* (BBA24) and the start of *dbpB* (BBA25)	This study
pJBF12	Spc^r; intact *dbpBA* cloned into pCR8/GW/TOPO flanked by *attL* sites	This study
pJBF17	Kan^r; intact *dbpBA* recombined via Gateway system into pBBE22gate	This study

[a] See Materials and Methods.

cently by Blevins et al. (4). Due to the differences in the studies mentioned above, the role of DbpBA in borrelial pathogenesis was independently evaluated and extended in the current study.

In this report, we demonstrate that deletion of *dbpBA* results in marked attenuation of *B. burgdorferi* in immunocompetent C3H mice following needle inoculation and we suggest that binding to host decorin early in the infectious process may be important in preventing clearance of *B. burgdorferi* via the innate immune response. The ability of the *dbpBA* mutant to colonize immunodeficient mice at a high-dose inoculum with which immunocompetent mice are effectively not infected suggests that, in addition to the early defect in survival observed, adaptive immunity further reduces the numbers of *dbpBA* mutant cells. These results suggest that binding to decorin within the ECM represents an important early step in the colonization of *B. burgdorferi* that promotes the establishment and persistence of this spirochete during experimental infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. All *B. burgdorferi* strains were grown in complete BSK-II medium as described previously (57). For selective pressure, *B. burgdorferi* was grown in BSK-II medium with antibiotics, where appropriate, at the following concentrations: kanamycin, 300 μg/ml; streptomycin, 50 μg/ml; and gentamicin, 50 μg/ml. All *Escherichia coli* strains were grown with aeration in LB media at 37°C. For experiments involving *E. coli*, antibiotics were used at the following concentrations: carbenicillin, 100 μg/ml; ampicillin, 100 μg/ml; spectinomycin, 100 μg/ml; gentamicin, 5 μg/ml; chloramphenicol, 15 μg/ml; and kanamycin, 50 μg/ml.

PCR. A PCR was conducted using either Herculase polymerase (Stratagene, La Jolla, CA) or *Taq* polymerase (Supermix; Invitrogen, Carlsbad, CA) as previously described (26, 52, 54). The oligonucleotide primers used in this study are listed in Table 2.

Construction of the Δ*dbpBA*::Gent^r plasmid. To delete the *dbpBA* operon in *B. burgdorferi*, a 1.7-kb fragment that included the 3′ end of *dbpA* (BBA24) and sequences downstream, including part of BBA21, was amplified using the BBA21F and dbpAsalmut primers and borrelial genomic DNA as the template (Table 2). Next, a 2.8-kb fragment containing the start of *dbpB* (BBA25) and its upstream region, including a portion of BBA36, was amplified similarly using the BBA31com and dbpBsalmut primers (Table 2). Both fragments were cloned separately into the TA cloning vector pCR-XL-TOPO (Invitrogen), and transformants were selected for with kanamycin. After the sequences of the 1.7- and 2.8-kb fragments were confirmed, the 1.7-kb BBA21-*dbpA*-SalI fragment was purified following digestion with BamHI and SalI. The resulting product was then cloned into the SalI-BBA31-*dbpB* SalI fragment construct mentioned above that also was digested with BamHI and SalI. The resulting construct was missing most of the *dbpB* and *dbpA* sequences, and a single unique SalI site was generated in place of these sequences. The gentamicin resistance cassette was amplified using pBVS2G (generously provided by Patricia Rosa [16]) as the template, which contained the *aacC1* gentamicin resistance (Gent^r) gene linked to the strong borrelial *flgB* promoter, using 5flgGentsal and 3flgGentsal as the primers (Table 2). The resulting amplified product was TA cloned into the pCR-XL-TOPO vector, and *E. coli* transformants were selected based on coresistance to kanamycin and gentamicin. The resulting clone was sequenced, digested with SalI, and cloned into the unique SalI site engineered in the *dbpBA* deletion construct. *E. coli* transformants containing the Gent^r *dbpBA* deletion construct were selected based on coresistance to kanamycin and gentamicin. The final construct containing the P_{flgB}-Gent^r cassette in place of *dbpBA* (Δ*dbpBA*::Gent^r) was screened by digestion with SalI, confirmed by sequencing, and subsequently designated pNP3.

Gateway vector-borrelial shuttle vector construction. To isolate a borrelial shuttle vector that could be easily modified using Invitrogen's Gateway recombination-based cloning system, the *attR1*-Cam^r-*ccdB*-*attR2* region from pDEST17 (Invitrogen) that confers resistance to chloramphenicol was PCR amplified with SphI linkers using oligonucleotide primers ATTR1F and ATTR1R (Table 2). The amplified product was cloned into pCR2.1-TOPO (Invitrogen) and transformed into *ccdB* survival T1R cells. The resulting construct was digested with SphI, and the SphI-*attR1*-Cam^r-*ccdB*-*attR2*-SphI-containing fragment was cloned into pBBE22 (generously provided by Steve Norris [46]) cut with SphI, ligated, and transformed into *ccdB* survival T1R cells (Invitrogen). Transformants were selected with kanamycin and chloramphenicol,

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)	Description
BBA21F dbpAsalmut	GATAAATAAAAGAAATTTATCTGAAA ACGCGTCGACATCCTTCTTTTACTGATGA	Primer pair that amplifies a 1.7-kb fragment containing *bba21* to sequences downstream of *bba24* (*dbpA*)
BBA31com dbpBsalmut	CGCATTTGCAAGAGAATCAAGCGCATCGTCTT ACGCGTCGACCAAGACCATAACTATTGAATT	Primer pair that amplifies a 2.8-kb fragment containing *bba31* to sequences upstream of *bba25* (*dbpB*)
5pflgGentSal 3pflgGentSal	ACGCGTCGACGAACGAATTGTTAGGTGGCGGTACT ACGCGTCGACCCCGAGCTTCAAGGAAGATTTCCT	Primer pair that amplifies a 0.9-kb fragment containing the *aacC1* allele (Gent^r) fused to the borrelial *flgB* promoter (P_{flgB}-Gent^r) in the Δ*dbpBA*::Gent^r mutant only
5ABMut	TTTATGTCTTGATTATCGGGCGAAGAGTTTA	When used with 3ABMut, amplifies 1.6- and 1.4-kb bands in the parent and Δ*dbpBA*::Gent^r mutant, respectively; when used with 5pflgGentSal, amplifies a 1.1-kb fragment in the Δ*dbpBA*::Gent^r mutant only
3ABMut	AAGCCAGATTGCATAGCAAGCTTGAATTCCAA	When used with 3pflgGentSal, amplifies a 1.2-kb fragment in the Δ*dbpBA*::Gent^r mutant only
ATTR1F ATTR1R	GCATGCCCTCGAATCAACAAGTTTG GCATGCCGCAGCCTCGAATCAACCAC	Primer pair used to amplify the *attR1*-Cam^r-*ccdB*-*attR2* region from pDEST17 for cloning into borrelial shuttle vectors to convert them to Gateway amenable vectors
DbpBA-F-XmaNco DbpBA-R-XmaNde	CCCGGGCCATGGCTTGATTATCGGGCGAAGAG CCCGGGCATATGGCAAACTGGAAACAAGTC	Primer pair that amplifies a 1.7-kb fragment containing the intact *dbpBA* locus with 289 bp upstream and 131 bp downstream for cloning into pCR8/GW/TOPO
nTM17FrecA nTM17RrecA	GTGGATCTATTGTATTAGATGAGGCTCTCG GCCAAAGTTCTGCAACATTAACACCTAAAG	Primer pair used for real-time quantitative PCR detection of *B. burgdorferi recA* gene[a]
bactinF bactinR	CAAGTCATCACTATTGGCAACGA CCAAGAAGGAAGGCTGGAAAA	Primer pair used for real-time quantitative PCR detection of the murine β-actin gene[b]

[a] See reference 35.
[b] See reference 37.

and the resulting plasmid construct was designated pBBE22gate. Subsequently, the *dbpBA* genes with 289 bp upstream and 131 bp downstream of the open reading frame was PCR amplified with oligonucleotide primers DbpBA-F-XmaNco and DbpBA-R-XmaNde (Table 2), and the resulting 1,689-bp band was cloned into the entry vector pCR8/GW/TOPO (Spc^r; Invitrogen) that contains *attL* sites that flank the inserted fragment. Clones that conferred resistance to spectinomycin were screened, and the desired construct was designated pJBF12. An LR reaction, which involved the LR recombinase that promotes recombination with constructs containing *attL* and *attR* sites, was performed with pJBF12 and pBBE22gate, respectively, by using the manufacturer's specifications, except that both plasmids were incubated at 55°C for 10 min to relax supercoiling prior to addition of the LR recombinase. The LR reaction mixture was then incubated at 25°C for 1 h and then overnight at 15°C. Then the LR reaction mixture was transformed into DH5α cells, and transformants were selected with kanamycin and screened for sensitivity to chloramphenicol. The resulting plasmid with *dbpBA* recombined into pBBE22gate (resulting in concomitant loss of Cam^r and *ccdB*) was designated pJBF17.

Genetic manipulation of *B. burgdorferi*. The *B. burgdorferi* strain B31 derivative ML23 (Table 1) was made electrocompetent as previously described (54) and transformed with pNP3 that contained the Δ*dbpBA*::Gent^r construct. Transformants were selected by limiting dilution in liquid BSK-II containing gentamicin using 96-well plates as described previously (64) to obtain strain JF105 (ML23 Δ*dbpBA*::Gent^r) (Table 1). Strain JF105 was then made electrocompetent and transformed with either pBBE22 (Kan^r) to complement the infectivity defect inherent in the lp25-deficient ML23 strain background or with pJBF17 (Kan^r) to complement the infectivity defect and provide intact *dbpBA* (Table 1).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analyses were performed as previously described (26, 52–54). Antiserum to DbpA was used in conjunction with appropriate substrates for chemiluminescent detection as described previously (26, 52–54).

Mouse infectivity: ID_{50} analysis. The 50% infective doses (ID_{50}) of ML23/pBBE22, JF105/pBBE22 (Δ*dbpBA*::Gent^r), and JF105/pJBF17 were determined as previously described (31, 54). C3H/HeN mice were infected for 21 days, sacrificed, and processed to determine whether organs (skin at the site on inoculation, spleen, heart, bladder, lymph nodes, and tibiotarsal joint) were colonized by examining growth in BSK-II medium for 1 month after the animals were sacrificed. Each of the infectivity experiments was conducted two separate times over a 5-month period to assess reproducibility.

Mouse infectivity: kinetics-based analysis. C3H/HeN mice were infected intradermally with either 10^3 or 10^5 cells of ML23/pBBE22, JF105/pBBE22 (Δ*dbpBA*::Gent^r), or JF105/pJBF17, and 12 h later the mice were sacrificed and skin samples from the site of inoculation were cultured in BSK-II medium (31, 54). Additional kinetics-based infectivity analyses were conducted essentially as described above on days 3, 5, 7, 14, and 21 (30). Groups of mice were infected using inocula containing 10^3 and 10^5 cells for each *B. burgdorferi* strain tested (except ML23/pBBE22, for which only an inoculum containing 10^3 cells was used) in two separate infectivity experiments. Following sacrifice at the time points indicated above, the tissues described above for the ID_{50} analysis were removed and cultured in BSK-II medium.

Mouse infectivity: SCID mouse infectivity. C3H-SCID mice (Harlan Laboratories, Philadelphia, PA) were infected with 10^3 and 10^5 cells of ML23/pBBE22, JF105/pBBE22 (Δ*dbpBA*::Gent^r), and JF105/pJBF17 as previously described (30), except that the mice were sacrificed after 21 days.

Extraction of DNA from tissue for quantitative PCR analysis. Joint and skin samples were aseptically removed and placed in 200 μl of cold phosphate-buffered saline. Total genomic DNA was extracted using a Roche High Pure PCR template preparation kit as described previously (37) except that a 2%

collagenase solution (Sigma Aldrich) was added prior to the overnight incubation at 55°C.

Quantification of *B. burgdorferi* in infected tissues. *B. burgdorferi* genomic equivalents in infected tissues were enumerated using an Applied Biosystems ABI 7500 fast real-time PCR system (Applied Biosystems Corp., Foster City, CA) in conjunction with Sybr green PCR Mastermix (Applied Biosystems). Approximately 100 ng of DNA was added to each reaction mixture. *B. burgdorferi* genome copies were detected using oligonucleotide primers nTM17FrecA and nTM17RrecA (Table 2) (35) at a final concentration of 0.3 μM to specifically detect the *B. burgdorferi recA* gene. The numbers of borrelial genomic copies were calculated by comparing the threshold cycle (C_T) values with the values for serial dilutions of known amounts of *B. burgdorferi* genomic DNA, which were used as standards. Mouse genomic copies were detected using oligonucleotide primers bactinF and bactinR, which are specific for the mouse β-actin gene, at a final concentration of 0.3 μM. The numbers of β-actin copies were calculated by comparing the C_T values with the values for serial dilutions of known amounts of the β-actin gene, which were used as standards (37). Triplicate measurements were obtained for all samples, and the values shown below are the numbers of *B. burgdorferi recA* copies per 10^6 mouse β-actin copies.

Statistical analysis. Quantitative PCR data were compared by transforming the number of *B. burgdorferi* genome copies (N_c) (normalized using 10^6 copies of mouse β-actin) to $\log_{10}(N_c + 0.1)$ prior to one-factor (see Fig. 2) or two-factor (see Fig. 3A and 3B) analysis of variance. Parent, mutant, and complement copy numbers were compared by constructing appropriate orthogonal contrasts using the analysis of variance model coefficients. Calculations were performed using S-PLUS 7 for Windows (Insightful Corporation, Seattle, WA).

RESULTS

Isolation of Δ*dbpBA*::Gent^r mutant of *B. burgdorferi*. To isolate a *dbpBA* deletion in *B. burgdorferi*, the region of the 54-kb linear plasmid (lp54) that encodes *dbpBA* (BBA24 [*dbpA*] and BBA25 [*dbpB*]) was amplified by PCR such that the genes were eliminated and a unique restriction site was generated simultaneously. Subsequently, a borrelial promoter-gentamicin resistance cassette was cloned into the engineered site to generate the Δ*dbpBA*::Gent^r construct pNP3 (Fig. 1A). In order to isolate a Δ*dbpBA*::Gent^r strain, pNP3 was transformed into strain ML23, which lacks the 25-kb linear plasmid (lp25) and, due to the absence of a restriction/modification system, is more readily transformable (54). Gentamicin-resistant isolates were obtained and analyzed by PCR (Fig. 1B) and Southern blotting (data not shown) to confirm the desired deletion. PCR analysis indicated that a 1.4-kb product was amplified from the Δ*dbpBA*::Gent^r strain and the genetically complemented strain when primers 5ABmut and 3ABmut were used, whereas a 1.6-kb fragment was obtained when the same primers were used to amplify the native *B. burgdorferi dbpBA* region of lp54 (Fig. 1A and 1B). A smaller faint band was observed when the parent strain was used as the template, but it was slightly below the 1.4-kb band obtained for the Δ*dbpBA*::Gent^r strain. For the genetically complemented strain both the 1.4- and 1.6-kb fragments were amplified, consistent with the presence of Δ*dbpBA*::Gent^r and wild-type *dbpBA*, respectively (Fig. 1A and 1B). As expected, primers 3pflgGentSal and 5pflgGentSal amplified a product only from the mutant and genetically complemented strain, as did other primer combinations that amplified sequences only from strains carrying the Δ*dbpBA*::Gent^r allele (i.e., either primers 5ABmut and 5pflgGentSal or primers 3ABmut and 3pflgGentSal), and the parent was negative for all of the PCRs when these three primer pairs were used (Fig. 1A and 1B). Western blot analysis confirmed that DbpA was not produced in the isolates screened (Fig. 1C, compare lane 1 [parent] with lane 2 [Δ*dbpBA*::Gent^r]). Subsequent plasmid profile analysis indicated that all of the Δ*dbpBA*::Gent^r clones obtained did not contain the 9-kb circular plasmid (cp9) and lp25, like the parent strain ML23 (31; data not shown). One clone, designated JF105, was used for all subsequent analyses.

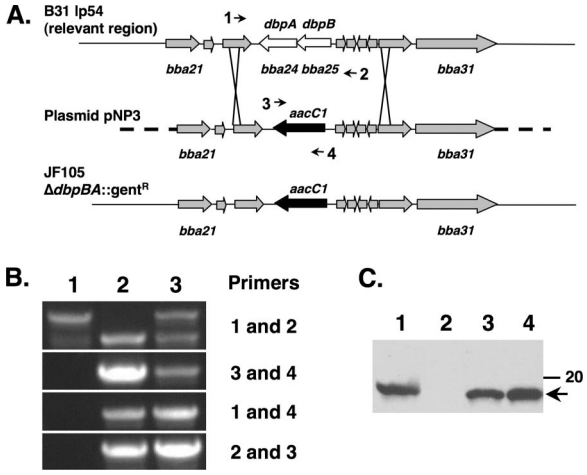

FIG. 1. Isolation and confirmation of *dbpBA* deletion mutants of infectious *B. burgdorferi*. (A) Strategy for deleting *dbpBA*. Plasmid pNP3, in which the BBA24 (*dbpA*) and BBA25 (*dbpB*) genes from lp54 were replaced by the gentamicin resistance gene *aacC1*, was used to transform *B. burgdorferi* strain B31 isolate ML23. Transformants were screened by PCR using primers shown to confirm the *dbpBA* deletion. Arrows 1 through 4 represent oligonucleotide primers 5ABMut, 3ABMut, 3pflgGentSal, and 5pflgGentSal, respectively (Table 2). One isolate that retained all plasmids (except cp9 and lp25) was obtained and designated JF105. (B) PCR analysis of the *dbpBA* deletion mutant and complemented strain. Lane 1, ML23/pBBE22 (parental strain); lane 2, JF105/pBBE22 (Δ*dbpBA*::Gent^r); lane 3, JF105/pJBF17 (Δ*dbpBA*::Gent^r with intact *dbpBA*). The primers used are indicated on the right (see panel A). (C) Western blot analysis of the Δ*dbpBA*::Gent^r mutant and the genetically complemented strain. Samples were immunoblotted using antiserum specific for DbpA. Lane 1, ML23/pBBE22 (parental strain); lane 2, JF105/pBBE22 (Δ*dbpBA*::Gent^r); lanes 3 and 4, JF105/pJBF17 (Δ*dbpBA*::Gent^r with intact *dbpBA*). Lanes 1 and 2 contained protein from 1×10^7 borrelial cells, while lanes 3 and 4 contained whole-cell equivalents from 1.25×10^6 and 2.5×10^6 organisms, respectively. The arrow indicates the location of DbpA.

Genetic complementation of the Δ*dbpBA*::Gent^r mutant. The genetic background used for isolation of Δ*dbpBA*::Gent^r strain JF105 was ML23, which is a strain B31 derivative that is missing lp25 (54). The lp25 plasmid is absolutely required for survival in mammals due to the presence of the BBE22 gene, which encodes a nicotinamidase essential for growth in these hosts (46). In order to use this genetic background to determine how the loss of a specific gene affects *B. burgdorferi* pathogenesis, the BBE22-containing region must be provided in *trans*, as was done previously for the BBK32::Str^r strain JS315 (54). In addition, the genes that are inactivated or deleted need to be provided back for genetic complementation. To facilitate this, the borrelial shuttle vector pBBE22, which carries BBE22 and BBE23 (46), was modified with Invitrogen's Gateway system, which resulted in a plasmid backbone that allowed efficient recombination of target genes in conjunction with commercially available Gateway entry vectors. The resulting construct, designated pBBE22gate, was used to construct plasmids carrying *dbpBA* with its native promoter region,

TABLE 3. ID_{50} of the Δ*dbpBA*::Gent^r strain, JF105/pBBE22, in immunocompetent C3H mice

Isolate	Inoculum (cells)	No. of cultures positive/total no.							No. of mice positive/total no.	Calculated ID_{50}
		Lymph node	Skin[d]	Heart	Spleen	Bladder	Joint	All sites		
ML23/pBBE22[a]	10^4	2/2	2/2	2/2	2/2	2/2	2/2	12/12	2/2	~150[e]
	10^3	4/4	4/4	4/4	4/4	4/4	4/4	24/24	4/4	
	10^2	1/3	1/3	1/3	1/3	1/3	1/3	6/18	1/3	
JF105/pBBE22[b]	10^6	3/3	0/3	0/3	0/3	1/3	1/3	5/18	3/3	$>10^6$
	10^5	2/5	1/5	0/5	0/5	0/5	0/5	3/30	2/5	
	10^4	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3	
	10^3	0/6	0/6	0/6	0/6	0/6	0/6	0/36	0/6	
	10^2	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3	
JF105/pJBF17[c]	10^5	2/3	3/3	3/3	2/3	3/3	3/3	16/18	3/3	316
	10^4	3/3	2/3	3/3	0/3	3/3	3/3	14/18	3/3	
	10^3	4/5	4/5	4/5	0/5	4/5	4/5	20/30	4/5	
	10^2	2/3	0/3	1/3	1/3	1/3	1/3	6/18	2/3	

[a] Parental strain B31 derivative which provides BBE22 in *trans* and is infectious (46).
[b] Δ*dbpBA*::Gent^r derivative of ML23/pBBE22.
[c] Δ*dbpBA*::Gent^r derivative of ML23/pBBE22 complemented with intact and expressed *dbpBA*.
[d] The skin tested was skin from the site of inoculation.
[e] The ID_{50} of ML23/pBBE22 is based on the results of numerous reproducible infectivity studies (31).

which yielded plasmid pJBF17. Plasmids pBBE22 and pJBF17 were then separately transformed into JF105. Following selection, the transformants were screened by PCR, Southern blotting, and plasmid profiling to ensure that the desired constructs were obtained and that no additional loss of plasmids had occurred (Fig. 1B and data not shown). The strains obtained had the same plasmid profiles as their genetic parent (data not shown). Furthermore, all the strains tested had similar growth kinetics in BSK-II medium (data not shown). Subsequently, immunoblot analysis was conducted to assess DbpA production (Fig. 1C). Protein lysates were prepared for ML23/pBBE22 (parental strain) (Fig. 1C, lane 1), JF105/pBBE22 (Δ*dbpBA*::Gent^r) (lane 2), and JF105/pJBF17 (Δ*dbpBA*::Gent^r with intact *dbpBA*) (lanes 3 and 4). Lanes 1 and 2 contained protein from 1×10^7 borrelial cells, while lanes 3 and 4 contained whole-cell equivalents from 1.25×10^6 and 2.5×10^6 organisms, respectively, and were immunoblotted and probed with antiserum specific for DbpA (Fig. 1C). The intensities of the bands in lanes 1 and 3 are approximately the same, indicating that genetic complementation of *dbpBA* on a borrelial shuttle vector resulted in an approximately eightfold increase in DbpA production. Serial dilutions of the *dbpBA*-complemented samples indicated that the amounts of protein in these samples were between four- and eightfold greater than the amount of protein obtained with genomically encoded *dbpA* (not shown), perhaps due to the increased copy number of the shuttle vectors. Alternatively, the high copy number may have altered the regulation of *dbpBA* by titrating out regulatory proteins, resulting in the overproduction of DbpA. The latter explanation seems unlikely given that the *dbpBA* genes are regulated in a positive manner by borrelial RpoS (9, 25).

Deletion of *dbpBA* attenuates the infectivity potential of *B. burgdorferi*. To determine how the *dbpBA* deletion affected *B. burgdorferi* pathogenesis, immunocompetent C3H mice were infected with 10-fold serial dilutions of JF105/pBBE22 (with the Δ*dbpBA*::Gent^r deletion) and JF105/pJBF17 (the *dbpBA*-complemented isolate), and the results were compared with the results for mice infected with ML23/pBBE22 (the infectious parent) in order to estimate the ID_{50} following 21 days of infection (Table 3). The data indicated that the infectious parent, ML23/pBBE22, which had an estimated ID_{50} of 150 organisms, exhibited infectivity similar to that of wild-type infectious *B. burgdorferi* (31, 47). The ID_{50} for Δ*dbpBA*::Gent^r strain JF105/pBBE22 was more than 10^6 organisms, since no tissues were culture positive for any of the mice infected with 10^2, 10^3, or 10^4 cells of the mutant and only 10 and 28% of the tissues were culture positive for mice infected with 10^5 and 10^6 Δ*dbpBA*::Gent^r *B. burgdorferi* cells, respectively (Table 3). In contrast, complementation with *dbpBA* restored the infectivity, and the calculated ID_{50} was 316 organisms (Table 3). This value is approximately twofold greater than the value for wild-type infectious *B. burgdorferi* (Table 3). Interestingly, whereas the positive-culture values for nearly all tissues were similar for the parental and complemented strains, the complemented strain exhibited an apparent defect in colonization of the spleen (Table 3). Nevertheless, the results obtained clearly indicate that the dramatic infectivity defect observed for the Δ*dbpBA*::Gent^r *B. burgdorferi* strain is due to the lack of DbpBA and not the result of polar effects, a secondary mutation, or loss of an infectivity-associated plasmid. The infectivity defect of the Δ*dbpBA*::Gent^r mutant observed in this study following needle inoculation is in contrast to the results of the initial study of Shi et al. (56), but it is in good agreement with the results reported more recently by Shi et al. (55) and Blevins et al. (4). Taken together, these results provide independent corroboration that *dbpBA* is required for maximal infectivity in the mouse model of experimental Lyme borreliosis following needle inoculation.

To obtain a quantitative assessment of colonization, real-time quantitative PCR was conducted. The results obtained support the ID_{50} findings since the ability of *B. burgdorferi* lacking *dbpBA* to colonize joint tissue following 21 days of infection was significantly impaired compared to the abilities of the infectious parent and complemented strain ($P < 0.0001$

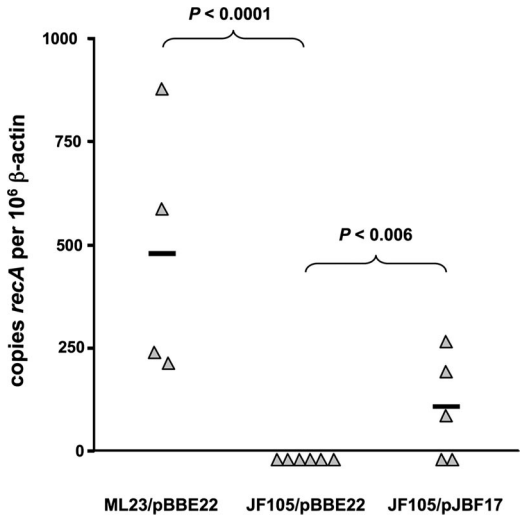

FIG. 2. Quantitative real-time PCR analysis of the *B. burgdorferi* parental (ML23/pBBE22), Δ*dbpBA*::Gent^r (JF105/pBBE22), and genetically complemented (JF105/pJBF17) strains in joint tissue following 21 days of infection in C3H mice. The absolute numbers of *B. burgdorferi* cells obtained from joint tissue were determined using mice infected with all three strains at a dose of 10^3 organisms. To normalize for mouse tissue in each sample tested, the number of β-actin copies was also determined. The results are expressed as the number of *B. burgdorferi* genome copies per 10^6 mouse β-actin copies. *P* values are indicated above the data.

and $P < 0.006$, respectively) (Fig. 2). Specifically, all of the joint samples from mice infected with 10^3 Δ*dbpBA*::Gent^r *B. burgdorferi* cells were culture negative, and the bacteria were not detected by quantitative PCR (Fig. 2). The number of *B. burgdorferi* genomes obtained for the genetically complemented strain in joint tissue was less than the number obtained for the ML23/pBBE22 parent, and this may have reflected incomplete complementation of the phenotype in this assay (Table 3). Nevertheless, the quantitative PCR data support the notion that the infectious potential of the Δ*dbpBA*::Gent^r mutant is significantly reduced following 21 days of infection.

Infection of C3H-SCID mice with Δ*dbpBA*::Gent^r *B. burgdorferi*. A possible explanation for the clearance of the Δ*dbpBA*::Gent^r mutant observed is that the adaptive immune response results in rapid sterilization of the tissues coinciding with the appearance of the immunoglobulin M response to *B. burgdorferi* infection, as has been observed previously for a mutant missing lp28-1 (30). Previously, Shi et al. reported that SCID mice infected with 10^5 cells of *B. burgdorferi* having a *dbpBA* deletion were completely infectious, supporting the hypothesis that adaptive immunity is required for clearance of this mutant (56). To determine how the absence of an adaptive immune response impacted infectivity, we infected C3H-SCID mice with either 10^3 or 10^5 cells of the infectious parent, the Δ*dbpBA*::Gent^r mutant, or the *dbpBA*-complemented strain. After 21 days the animals were sacrificed, and the tissues were scored for the ability of *B. burgdorferi* to grow in BSK-II medium. The results showed that after inoculation of 10^5 organisms, there was effectively no difference in infectivity among mice infected with the three strains, with the possible notable exception of the results for the skin, which appeared to be somewhat less efficiently colonized by the Δ*dbpBA*::Gent^r mutant (Table 4). In contrast, when C3H-SCID mice were infected with 10^3 spirochetes, the Δ*dbpBA*::Gent^r mutant could not colonize the C3H-SCID mice in any tissue tested, whereas the parent and genetically complemented strain colonized every tissue (Table 4). These observations suggest that following low-level infection (i.e., 10^3 spirochetes), the ability of the Δ*dbpBA*::Gent^r mutant to survive in SCID mice is impaired in a manner similar to that observed with immunocompetent mice (Table 3) due to an inability to bind to host decorin and/or to evade innate killing (compare the data for the 10^3-cell inocula in Tables 3 and 4). Furthermore, adaptive immunity is needed to effectively clear the Δ*dbpBA*::Gent^r mutant following inoculation of large doses, as shown by the differential infectivity observed for immunocompetent and SCID mice (Tables 3 and 4, respectively).

To quantify the *B. burgdorferi* present in C3H-SCID mouse tissues, samples of the skin and joint tissue were analyzed using quantitative PCR. The results indicate that the spirochetal loads in the joints of C3H-SCID mice infected with infectious *B. burgdorferi* cells were greater (approximately 10-fold greater) (Fig. 3) than the spirochetal loads in the joints of immunocompetent mice (Fig. 2), consistent with the idea that an adaptive immune response is able to keep the infection in check but does not clear *B. burgdorferi* from the tissues (Fig. 2). Furthermore, the values obtained for the Δ*dbpBA*::Gent^r mutant are consistent with the culture data obtained, because all mice infected with 10^3 Δ*dbpBA*::Gent^r spirochetes were cultivation negative (Table 4) and based on quantitative PCR the numbers of organisms were significantly less than the numbers of cells of the parent and complemented strain ($P < 4 \times 10^{-14}$

TABLE 4. Infectivity of the Δ*dbpBA*::Gent^r strain in C3H-SCID mice

Isolate	Inoculum (cells)	No. of cultures positive/total no.							No. of mice positive/total no.
		Lymph nodes	Skin[a]	Heart	Spleen	Bladder	Joint	All sites	
ML23/pBBE22	10^5	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
	10^3	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
JF105/pBBE22	10^5	5/5	3/5	5/5	5/5	5/5	5/5	28/30	5/5
	10^3	0/5	0/5	0/5	0/5	0/5	0/5	0/30	0/5
JF105/pJBF17	10^5	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
	10^3	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3

[a] The skin tested was skin from the site of inoculation.

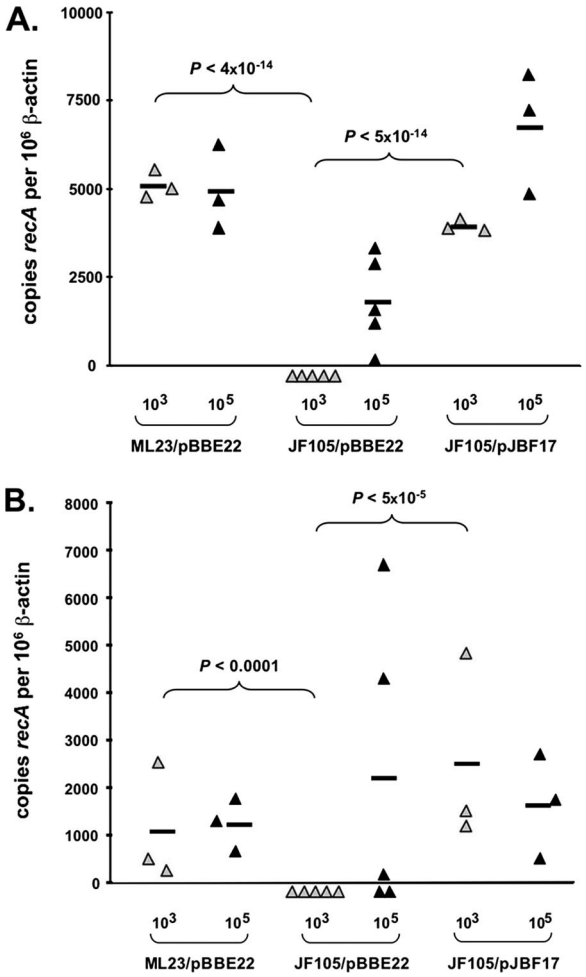

FIG. 3. Quantitative PCR to determine the absolute numbers of spirochetes in joint tissue (A) and skin tissue (B) after 21 days of infection of C3H-SCID mice by the *B. burgdorferi* parental, Δ*dbpBA*::Gent^r, and genetically complemented strains at the inocula indicated. The results are expressed as the number of *B. burgdorferi* genome copies per 10^6 mouse β-actin copies. *P* values are indicated above the data sets that are being compared.

and $P < 5 \times 10^{-14}$, respectively) (Fig. 3A). In contrast, C3H-SCID mice infected with 10^5 Δ*dbpBA*::Gent^r *B. burgdorferi* cells were culture positive (Table 4), and spirochete genomes were detectable in all five joint samples tested, although the levels were lower than those of the infectious parent (however, the difference was not statistically significant [$P < 0.4$]) (Fig. 3A). As predicted, the colonization of the joint tissue of C3H-SCID mice by the complemented strain was indistinguishable from the colonization of the joint tissue of C3H-SCID mice by the parent strain after inoculation of either a low or high dose ($P \sim 0.65$ and $P \sim 0.6$, respectively) (Fig. 3A).

The efficiencies of colonization of the skin of C3H-SCID mice by the parent and complemented strains were statistically indistinguishable both when inocula containing 10^3 cells were used and when inocula containing 10^5 cells were used ($P \sim 0.65$ and $P \sim 0.95$, respectively) (Fig. 3B). As predicted by our inability to culture Δ*dbpBA*::Gent^r *B. burgdorferi* from the skin (or any other tissue) after injection of 10^3 organisms, quantitative PCR of skin samples detected no bacteria, and the differences were significant when the data were compared to data for both the parent and complemented strains ($P < 1 \times 10^{-4}$ and $P < 5 \times 10^{-5}$, respectively) (Fig. 3B). After inoculation of 10^5 mutant bacteria, spirochete genomes were detectable in the skin of three of five mice (Fig. 3B), which is in good agreement with the culture data obtained (Table 4). Two of the three positive skin samples contained inordinately high numbers of *B. burgdorferi* cells, but the reason for the variability is not clear. Nevertheless, with the exception of these two samples, all quantitative PCR values obtained for the Δ*dbpBA*::Gent^r *B. burgdorferi* strain in joint tissue or skin, with low or high doses, were lower than the values for the parent and complemented strains, providing compelling evidence that the ability of the Δ*dbpBA*::Gent^r strain to establish an infection is impaired even in immunodeficient mice.

Kinetics of infection for Δ*dbpBA*::Gent^r *B. burgdorferi*. Since the loss of *dbpBA* compromised the ability of *B. burgdorferi* to colonize mice following 21 days of infection of immunocompetent mice, we wanted to determine both the temporal basis and spatial basis of clearance. Also, since we determined that adaptive immunity played a part in the clearance of the Δ*dbpBA*::Gent^r mutant (Table 4), we were interested in assessing colonization at earlier time points, when the innate immune response may play a role in reducing the colonization of the mutant. To this end, we performed a kinetics-based infectivity experiment with either 10^3 or 10^5 cells of the parent, the Δ*dbpBA*::Gent^r mutant, and the genetically complemented strain using immunocompetent C3H mice. Following 12 h of infection of immunocompetent C3H mice, lymph node tissue was positive for one-half of the animals tested that were infected with 10^3 *B. burgdorferi* cells irrespective of the strain used; all other tissue sites tested (skin and spleen) were culture negative for the 10^3-cell dose independent of the *dbpBA* status of the samples tested (Table 5). For mice infected with 10^5 spirochetes, all strains tested colonized the site of intradermal inoculation in all animals tested, and a disseminated infection was also observed in most mice (50 to 70% of distant sites depending on the strain tested [Table 5]). Thus, when culture assays are used, the loss of *dbpBA* does not completely eliminate the ability of *B. burgdorferi* to survive in mouse skin, as well as other tissues, early in infection. However, at this point we cannot exclude the possibility that the absolute numbers of

TABLE 5. Infectivity of the *B. burgdorferi* parental, Δ*dbpBA*::Gent^r, and genetically complemented strains following 12 h of infection in immunocompetent C3H mice

Isolate	Inoculum (cells)	No. of cultures positive/total no.				No. of mice positive/ total no.
		Lymph nodes	Skin[a]	Spleen	All sites	
ML23/pBBE22	10^5	4/5	5/5	3/5	12/15	5/5
	10^3	3/5	0/5	0/5	3/15	3/5
JF105/pBBE22	10^5	4/5	5/5	3/5	12/15	5/5
	10^3	2/5	0/5	0/5	2/15	2/5
JF105/pJBF17	10^5	3/5	5/5	2/5	10/15	5/5
	10^3	2/4	0/4	0/4	2/12	2/4

[a] The skin tested was skin from the site of inoculation.

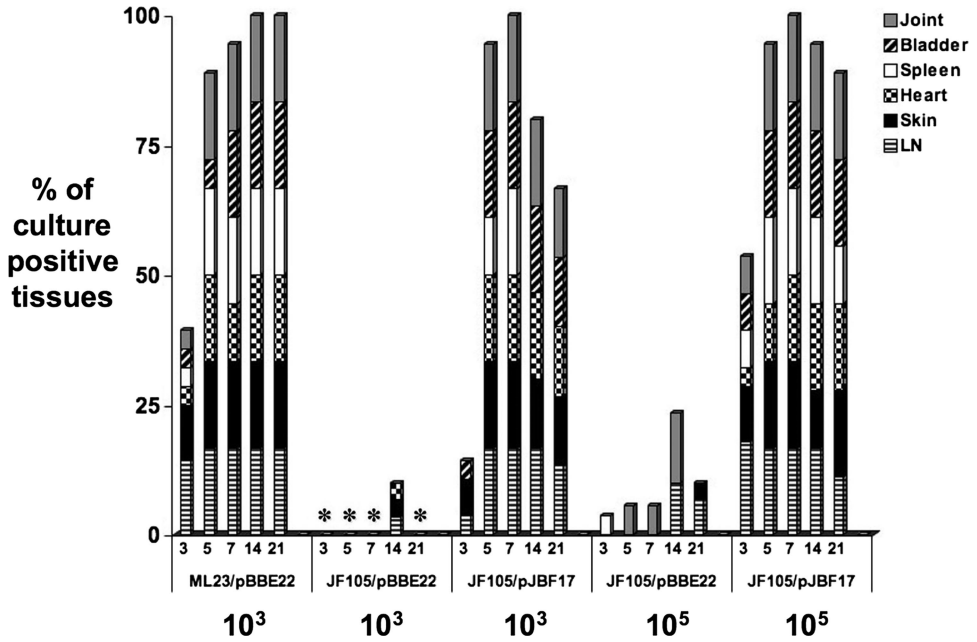

FIG. 4. Kinetics of *B. burgdorferi* dissemination in C3H mice. C3H mice were infected with the *B. burgdorferi* parental, Δ*dbpBA*::Gent^r, and genetically complemented strains using 10^3 spirochetes, and the Δ*dbpBA*::Gent^r and genetically complemented strains were also infected using 10^5 organisms. Mice were sacrificed on days 3, 5, 7, 14, and 21, and the tissues were cultured to determine *B. burgdorferi* growth. The strains and doses tested are indicated below the *x* axis. The percentages of culture-positive samples are indicated on the *y* axis. The results are expressed as percentages of culture-positive samples for the strains and concentrations of spirochetes tested. The values represent data for three to six mice depending on the strain and time point evaluated. An asterisk indicates that all samples tested were culture negative. LN, lymph nodes.

Δ*dbpBA*::Gent^r strain cells are much less than the absolute numbers of cells of the infectious parent and the genetically complemented strain.

When assays were performed over 21 days after inoculation of 10^3 or 10^5 cells, the parent strain was capable of disseminating and persisting in all tissues tested. In contrast, at the low dose (10^3 spirochetes), the Δ*dbpBA*::Gent^r mutant colonized only one of five mice (in only three tissues) after 14 days. At the higher dose (10^5 spirochetes), the mutant was capable of dissemination, albeit impaired, in some of the mice, as shown by the ability to culture borrelial cells lacking *dbpBA* from a small minority of the tissues tested throughout the duration of the kinetics experiment (Fig. 4). Specifically, the heart and bladder were negative for all infections and at all time points tested with the Δ*dbpBA*::Gent^r *B. burgdorferi* strain. Moderate dissemination was observed in the spleen (1/5 sites positive only at day 3), in the skin at the inoculation site (1/5 sites positive only at day 21), and in the lymph nodes (3/5 and 2/5 sites positive at days 14 and 21) (Fig. 4). Interestingly, the joint tissue was the tissue colonized best by the mutant, with 1/3, 1/3, and 4/5 sites positive at days 5, 7, and 14, respectively. Although the joint tissue of four of five mice was infected at day 14, none of these mice was still infected at day 21, indicating that the Δ*dbpBA*::Gent^r *B. burgdorferi* strain that could colonize this tissue was not able to persist, so no tissues were culture positive by day 21 after low-dose infection and only a small percentage of tissues were infected at this time point following high-dose infection. Since our study ended at 21 days, we do not know if the organism in the sole skin sample that became culture positive between days 14 and 21 could persist or whether additional sites might become culture positive at a later time.

The colonization defects revealed in this kinetic study were due to the *dbpBA* mutation, because the genetically complemented strain (JF105/pJBF17) was, with few exceptions, capable of wild-type levels of colonization. In mice infected with 10^5 cells of the complemented strain there were dissemination and nearly wild-type levels of persistence (Fig. 4). After administration of a dose containing 10^3 cells, there was an apparent decrease in colonization by the *dbpBA*-complemented strain (JF105/pJBF17) at day 21, but this was due mostly to the complete lack of infectivity in one of the five mice tested (Fig. 4). In addition, this kinetics experiment revealed that this strain was cleared from the spleen between days 7 and 14, consistent with our previous analysis of splenic colonization at day 21 (Table 3). It is possible that the increased amount of DbpBA produced by the genetically complemented strain resulted in clearance due to enhanced binding of antiserum against DbpA and DbpB, as proposed by Xu et al. (62). The slight differences between the parental and complemented strains aside, taken together, the results indicate that a *B. burgdorferi* strain lacking DbpBA was able to disseminate in some of the mice infected, but to a markedly reduced extent, and, more importantly, that the ability of this strain to persistently infect immunocompetent mice was greatly impaired.

DISCUSSION

As the first step in successful infection of a host, a pathogen must bind to host structures and either replicate and dissemi-

nate from the binding site or subsequently invade host cells. For either extracellular or intracellular pathogens, successful colonization requires an ability to replicate and disseminate to peripheral sites or to infect adjacent cells or tissues, after which a secondary colonization step can occur. In the case of *B. burgdorferi*, deposition in the dermis of a mammal resulting from the bite of an infected tick introduces the pathogen to a number of potential binding substrates in this locale, most notably ECM proteoglycan ligands.

Previous biochemical studies demonstrated that *B. burgdorferi* can bind to several host structures found in the ECM, including decorin and fibronectin, and on host cells, including integrin receptors, glycosoaminoglycans, and surface-associated factor H, via the activity of DbpA, DbpB, BBK32, P66, Bgp, and factor H binding proteins (6, 12, 14, 19–21, 23, 27, 28, 33, 38, 41, 42, 44, 45, 49, 54). Accordingly, it has long been argued that adherence to these sites is a key step in borrelial pathogenesis (for reviews, see references 8 and 13). However, until recently, it was difficult to assess the role of a particular gene or its product in borrelial pathogenesis due to an inability to genetically manipulate *B. burgdorferi*. Recently, many different investigators have independently developed methodologies to isolate and characterize isogenic mutants of infectious *B. burgdorferi* strains, and, with the advent of these advances (for a review, see reference 50), several borrelial adhesins have been inactivated and their effects on the resulting virulence have been examined. Specifically, isogenic *dbpBA*, BBK32, and *bgp* mutants of infectious *B. burgdorferi* strains have been isolated (42, 54–56). A *p66* mutant has been obtained only in a noninfectious background (12), so its role in borrelial pathogenesis is not known yet. Knockout of *bgp* had no effect on *B. burgdorferi* virulence when the organism was assayed using immunodeficient mice (42), whereas the BBK32 mutant exhibited only a slight infectivity defect (54). The lack of a dramatic phenotype for either the *bgp* or BBK32 mutant implies that other adhesins may compensate for the known binding properties of the *bgp* and BBK32 proteins or that these molecules play a subtle role in the pathogenesis of *B. burgdorferi*. With regard to compensatory function, the ability to bind to glycosoaminoglycans has been reported for Dbp proteins, Bgp, and BBK32 (8, 13, 18, 19, 41, 42). Thus, if the binding to a given adhesin is directed primarily against a glycosoaminoglycan structure, then inactivation of such a gene (e.g., *bgp*) may have a more subtle effect on borrelial pathogenesis.

For *dbpBA*, recent publications from one laboratory suggested (i) that DbpA and DbpB were not essential for infection in immunocompetent mice, (ii) that overexpression of *dbpA* increased the infectivity of *B. burgdorferi*, and (iii) that DbpB and DbpBA were required for full virulence of *B. burgdorferi* following needle inoculation (55, 56, 62). To reconcile these apparently disparate observations, we examined how the loss of DbpBA affects the ability of *B. burgdorferi* to colonize C3H mice by calculating an ID_{50} and performing a kinetics-based infectivity analysis.

Our results clearly showed that deletion of *dbpBA* attenuates the pathogenic potential of *B. burgdorferi* based on ID_{50} analyses after 21 days of infection following needle inoculation (Table 3), which independently corroborated the results most recently reported by Shi et al. (55) and Blevins et al. (4). Specifically, the Δ*dbpBA*::Gent^r strain was isolated from only 10 and 28% of the sites when inocula containing 10^5 and 10^6 spirochetes, respectively, were used and from no tissue sample when inocula containing fewer spirochetes were used (Table 3). In addition, genetic complementation of the Δ*dbpBA*::Gent^r strain (JF105/pBBE22) with intact *dbpBA* (JF105/pJBF17) restored infectivity in mice in most instances, indicating that the infectivity defect observed in the Δ*dbpBA*::Gent^r strain was due to the deletion of *dbpBA* and not the result of polar effects, an unlinked mutation, or loss of an infectivity-associated plasmid from *B. burgdorferi* (Table 3). Finally, the quantitative real-time PCR analysis corroborated the infectivity data for immunocompetent mice as the Δ*dbpBA*::Gent^r *B. burgdorferi* strain was not detected in joints when an inoculum containing 10^3 organisms was used. Conversely, analysis of the parent and *dbpBA*-complemented strain samples showed that there was ample *B. burgdorferi* in the joint tissues tested, although the levels for the complemented strain samples were lower than the levels for the parent strain samples (Fig. 2). Taken together, these results indicate that DbpA and/or DbpB is required for maximal infectivity in the mouse model following 21 days of infection.

Because the Δ*dbpBA*::Gent^r strain has such a dramatic phenotype, we were interested in determining the temporal and spatial basis of clearance of this strain. The kinetics-based analysis indicated that, particularly after a high-dose intradermal inoculum was used, the Δ*dbpBA*::Gent^r strain was able to disseminate to several tissues, including the joint tissue, in four of five of the mice infected after 14 days but was unable to maintain a persistent infection (Fig. 4). The inability of the Δ*dbpBA*::Gent^r strain to maintain an infection might be explained by two possible mechanisms that are not mutually exclusive. First, the inability of the Δ*dbpBA*::Gent^r strain to bind to host decorin could result in more efficient clearance of the mutant from the host. Although unproven, it is conceivable that the lack of binding to host decorin results in free-floating Δ*dbpBA*::Gent^r cells in interstitial fluid in the skin that are more readily phagocytosed by innate immune cells. Previously, Liang et al. reported that the interaction of *B. burgdorferi* with host decorin protected the spirochete from clearance, presumably due to the inability of antibodies directed against the borrelial Dbp proteins to bind to and eliminate the spirochete (34). This may be an important factor in the inability of the Δ*dbpBA*::Gent^r mutant to persist, but it does not explain the rapid clearance of the mutant from the infected mice before adaptive immune mechanisms clear the organism. Thus, the second possibility is that the Dbp proteins provide a mechanism to override the initial innate immune response, which allows the spirochete to disseminate and remain infective. The exact mechanism used by the Dbp proteins is not known, but it may involve interactions with other host proteins that alter the immunological response to favor borrelial colonization and persistence.

Previously, Brown et al. reported that decorin-deficient mice ($Dcn^{-/-}$) exhibited a reduction in *B. burgdorferi* colonization, particularly at low doses (7). In the subsequent study of Liang et al., when the size of the *B. burgdorferi* inoculum was increased, few differences between wild-type and decorin-deficient mice were observed except for the joint and skin, where in the decorin-deficient mice there was reduced colonization following 2 weeks of infection (34). Interestingly, the numbers for the skin and joint tissue were significantly reduced follow-

ing 2 months of infection compared to the data for the $Dcn^{+/+}$ mice, suggesting that the organisms might localize to different sites in wild-type and $Dcn^{-/-}$ mice and/or that the adaptive immune response is able to process *B. burgdorferi* more efficiently in the $Dcn^{-/-}$ mice (34).

Although the infectivity of *B. burgdorferi* was reduced in the $Dcn^{-/-}$ mice, *B. burgdorferi* was able to colonize the decorin-deficient mice with most of the inocula tested (7). The striking difference in infectivity observed for the Δ*dbpBA*::Gent^r strain reported here compared to the results for the decorin-deficient mice implies that DbpA and DbpB may have additional roles in borrelial pathogenesis over and above their adherence activity with host decorin. If adherence to decorin were the only function attributable to DbpA and DbpB, then the infectivity phenotype for decorin-deficient mice and wild-type *B. burgdorferi* and the infectivity phenotype for wild-type mice and the Δ*dbpBA*::Gent^r *B. burgdorferi* strain should be comparable. The difference observed suggests that Dbp proteins may affect the host response to *B. burgdorferi* infection. Alternatively, as indicated above, it is possible that the inability of the Δ*dbpBA*::Gent^r strain to bind decorin puts the *B. burgdorferi* cells at greater risk of being phagocytosed by innate immune cells. Studies to examine these possibilities are under way.

It is highly likely that the innate and adaptive immune responses are both involved in the clearance of the Δ*dbpBA*::Gent^r isolate, as shown by the SCID mouse infectivity data (Table 4 and Fig. 3). Specifically, whereas the low-dose inoculum of the Δ*dbpBA*::Gent^r strain tested (10^3 organisms) was cleared in SCID mice, infection of SCID mice by the parent strain and by the genetically complemented strain was indistinguishable from infection of immunocompetent mice by these strains based on the culture-positive phenotype (Table 4). This is in stark contrast to the results for complete infection of SCID mice with 10^5 organisms independent of the status of the *dbpBA* genes, suggesting that clearance of the Δ*dbpBA*::Gent^r strain in immunocompetent mice is also dependent on the adaptive immune response (Table 4). Previously, Shi et al. showed that SCID mice infected by the *dbpBA* mutant were indistinguishable from SCID mice infected by the infectious parent, but these workers evaluated only infections with 10^5 *B. burgdorferi* cells (56). With only data for this high dose, one might erroneously conclude that one function of DbpBA is to avoid clearance by adaptive immunity. If adaptive immunity were the sole immune clearance mechanism, then the Δ*dbpBA*::Gent^r mutant would disseminate to all tissues, with clearance initiated when borrelia-specific antibodies are generated, as was observed for *B. burgdorferi* cells lacking lp28-1 (30). The enhanced clearance of the Δ*dbpBA*::Gent^r strain suggests that decorin binding and/or the binding to other host factors via the Dbp proteins protects the spirochetes from rapid clearance via innate immune cells, presumably resident macrophages, langerhans cells, and/or neutrophils. Alternatively, the loss of Dbp proteins from the surface of *B. burgdorferi* may put the spirochetes at risk since high levels of DbpA and DbpB would likely be produced during infection. Thus, the loss of an abundant surface protein may compromise the overall integrity of the borrelial outer membrane and lead to enhanced innate immune clearance, as recently proposed by Xu et al. (61).

Recently, Blevins et al. reported that a *dbpBA* deletion mutant of strain 297 was severely defective for infectivity following needle inoculation of immunocompetent mice (4), similar to the infectivity results presented here. Interestingly, the strain 297 mutant did not exhibit as dramatic a phenotype when the infection was initiated using infected ticks. While one possible interpretation of these findings is that the DbpA and DbpB proteins do not play an important role during mammalian infection (contrary to conclusions based on needle inoculation studies), it is also possible that some defect in the infectivity of a *dbpBA* mutant after tick inoculation might be revealed upon further investigation. For example, whereas 100% (two of two) of mice that were infected using 5 ticks containing the parental strain became infected, 50% (one of two) and 67% (four of six) of mice that were infected using 5 and 10 ticks containing the *dbpBA* mutant, respectively, became infected. It is also not clear whether the 297 *dbpBA* mutant was capable of disseminated infection following tick infection since the infectivity of tick-infected mice was evaluated only by testing ear skin at a site presumably adjacent to where the tick feeding occurred. Finally, if in fact DbpA and DbpB play a less critical role after tick inoculation than after needle inoculation, the results may reflect the ability of tick saliva to locally immunosuppress the host immune response (3, 24, 29, 32) such that the *B. burgdorferi dbpBA* deletion strain deposited in the skin can migrate to immunoprotected niches in the skin, which are then protected against subsequent clearance by the adaptive immune response. This explanation is consistent with the data that we obtained with SCID mice, which showed that a *dbpBA* deletion mutant can colonize immunodeficient mice when higher doses are used (Table 4).

In summary, we showed that loss of the *dbpBA* genes results in a dramatic loss of infectivity in the mouse model of experimental Lyme disease. Furthermore, kinetics analyses indicated that *B. burgdorferi* cells that do not synthesize DbpBA exhibit muted dissemination when high-dose inocula are used and are unable to persist. The ability of SCID mice to clear the *B. burgdorferi* Δ*dbpBA*::Gent^r strain when low doses were used suggests that the DbpBA proteins are required for early survival due to binding to host decorin, which may provide a microenvironment that prevents innate immune clearance in a manner reminiscent of tick-based infectivity, where the tick saliva may locally immunosuppress the host response (3, 24, 29, 32). As indicated above, this may explain the difference between the infectivity potential observed for ticks and the infectivity potential observed for needle inoculation, where the *dbpBA* mutant was able to colonize mice following a blood meal (4). Nevertheless, the results presented here extend previously reported data by demonstrating that Δ*dbpBA*::Gent^r cells are cleared early during the infectious process in immunocompetent and SCID mice. The number of cells that are capable of surviving in immunocompetent mice is then greatly reduced when the host mounts a borrelia-specific adaptive immune response. In addition, these studies provided independent corroboration of the conclusion that the *dbpBA* genes are important for the maximum pathogenic potential of *B. burgdorferi* following needle inoculation (55, 56). Finally, the large difference between the infection potential of wild-type *B. burgdorferi* in decorin-deficient mice (7) and the infection potential of the Δ*dbpBA*::Gent^r deletion strain in wild-type mice shown here suggests that, in addition to their role in binding host decorin, the Dbp proteins may modulate the host immune response through interaction with additional host structures in

a manner that allows *B. burgdorferi* to establish and maintain an infection.

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