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Allelic Variation of the Lyme Disease Spirochete Adhesin DbpA Influences Spirochetal Binding to Decorin, Dermatan Sulfate, and Mammalian Cells∇

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After transmission by an infected tick, the Lyme disease spirochete, *Borrelia burgdorferi* sensu lato, colonizes the mammalian skin and may disseminate systemically. The three major species of Lyme disease spirochete—*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*—are associated with different chronic disease manifestations. Colonization is likely promoted by the ability to bind to target tissues, and Lyme disease spirochetes utilize multiple adhesive molecules to interact with diverse mammalian components. The allelic variable surface lipoprotein decorin binding protein A (DbpA) promotes bacterial binding to the proteoglycan decorin and to the glycosaminoglycan (GAG) dermatan sulfate. To assess allelic variation of DbpA in GAG-, decorin-, and cell-binding activities, we expressed *dbpA* alleles derived from diverse Lyme disease spirochetes in *B. burgdorferi* strain B314, a noninfectious and nonadherent strain that lacks *dbpA*. Each DbpA allele conferred upon *B. burgdorferi* strain B314 the ability to bind to cultured kidney epithelial (but not glial or endothelial) cells, as well as to purified decorin and dermatan sulfate. Nevertheless, allelic variation of DbpA was associated with dramatic differences in substrate binding activity. In most cases, decorin and dermatan sulfate binding correlated well, but DbpA of *B. afzelii* strain VS461 promoted differential binding to decorin and dermatan sulfate, indicating that the two activities are separable. DbpA from a clone of *B. burgdorferi* strain N40 that can cause disseminated infection in mice displayed relatively low adhesive activity, indicating that robust DbpA-mediated adhesive activity is not required for spread in the mammalian host.

Lyme disease spirochetes of the genus *Borrelia* are transmitted to humans from a bite by an infected *Ixodes* tick. The first stage of Lyme disease is local infection of the skin, typically giving rise to a characteristic rash termed erythema migrans. In the absence of antibiotic therapy, some strains may disseminate from the skin, via the blood, to multiple secondary sites including the joints, heart, and brain, resulting in the varied clinical manifestations of Lyme disease such as arthritis, carditis, and neuroborreliosis (for a review, see reference 49).

At least seven *Borrelia* species are associated with Lyme disease (11, 20, 45, 47, 49) and are collectively referred to as *B. burgdorferi* sensu lato. The three most clinically important Lyme disease spirochetes are *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. *B. burgdorferi* sensu stricto, referred to here simply as *B. burgdorferi*, is the most prevalent Lyme disease spirochete in the United States. In Europe all three species are associated with Lyme disease, with *B. garinii* and *B. afzelii* being the more prevalent (49). The three species are each capable of causing long-term infection in humans but are associated with different chronic manifestations: *B. burgdorferi* with Lyme arthritis, *B. garinii* with neuroborreliosis, and *B. afzelii* with the chronic skin lesion acrodermatitis (55). In addition, within a species, there is apparent strain-to-strain variation in the ability to cause disseminated infection (54, 59). *B. burgdorferi* strains, typed on the basis of polymorphisms in the rRNA operon or the highly polymorphic *ospC* gene, have been shown to vary in their association with bloodstream infection in humans (14, 56, 58). Although OspC, a surface lipoprotein, is required for experimental infection of the mouse (22, 37), allelic variation of OspC cannot fully account for observed differences in the capacity to disseminate within the mammalian host by various *B. burgdorferi* strains (1). Thus, other virulence factors likely contribute to the apparent differences in tissue colonization and clinical manifestations exhibited by diverse Lyme disease spirochetes.

Attachment to host tissues is thought to be a critical step in the ability of many pathogens to disseminate within the mammalian host. *B. burgdorferi* attaches to a wide variety of cells *in vitro*, including epithelial, endothelial, and glial cells, platelets, and lymphocytes (12, 13, 18, 19, 51, 52). In addition, multiple host cell and extracellular matrix molecules are recognized by *B. burgdorferi*, such as integrins (10), fibronectin (6, 21, 43), laminin (7, 53), collagen (61), and proteoglycans (24, 30, 33). Proteoglycans consist of a protein core covalently linked to one or more glycosaminoglycan (GAG) chains, which are long linear repeating disaccharides (for a review, see reference 34). GAGs are typically quite heterogeneous in size and charge and vary structurally between tissues and host species (42). Different GAGs can be segregated into classes based on epimerization of the

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glycan chain, the location and degree of sulfation, and sensitivity to enzymatic cleavage by different lyases.

We previously showed that diverse spirochetes recognize distinct classes of GAGs, and differences in GAG-binding specificity can lead to differences in the types of mammalian cells recognized (40). For example, *B. burgdorferi* strain N40, which recognizes heparan sulfate and dermatan sulfate GAGs, binds efficiently to glial and endothelial cells *in vitro*, while *B. afzelii* strain VS461, which predominantly recognizes dermatan sulfate, binds selectively to glial cells (40). These findings suggest that strain-specific differences in GAG recognition by Lyme disease spirochetes might influence the specificity of host cell attachment and thus tissue colonization during infection.

B. burgdorferi encodes multiple GAG binding proteins, including the highly related Dbp (decorin binding protein) A and DbpB, which comprise a single operon and were first identified on the basis of their ability to bind decorin, a chondroitin/dermatan sulfate proteoglycan that "decorates" collagen fibers in mammalian tissues (23–25). DbpA has been shown to be expressed by diverse *B. burgdorferi* sensu lato strains during murine infection (3, 25, 26) and, on the basis of ubiquitous seroreactivity among Lyme patients, is likely to be expressed during human Lyme disease as well (for a review, see reference 57). DbpA and DbpB, when expressed in a noninfectious and otherwise nonadherent *B. burgdorferi* strain, were shown to be sufficient to promote spirochetal attachment to isolated dermatan sulfate GAGs as well (16). The *dbpA* (and presumably *dbpB*) gene is not expressed in the unfed tick, but transcription is induced upon tick feeding (28). In addition, DbpA and DbpB are required for maximal infectivity and dissemination after intradermal inoculation of either immunodeficient or immunocompetent mice (3, 45, 54). These findings are consistent with the hypothesis that spirochetal binding to extracellular matrix mediated by DbpA and DbpB promotes colonization of the mammalian host.

DbpA and DbpB are related proteins that each bind to dermatan sulfate but differ in their GAG and mammalian cell type binding specificities (16). In addition, whereas DbpB is highly conserved among Lyme disease spirochete strains, DbpA is highly polymorphic (44), and recombinant derivatives of DbpA allelic variants differ in decorin binding (41). In the present study, we found that allelic variation of DbpA is associated with dramatic differences in the ability of this adhesin to promote bacterial attachment to decorin, GAGs, and mammalian cells.

MATERIALS AND METHODS

Bacterial strains and cell lines. The high-passage *B. burgdorferi* strain B314 (46) was a generous gift from Tom Schwan (Rocky Mountain Labs, Hamilton, MT). *B. burgdorferi* strains N40 clone D10/E9 ($N40_{D10/E9}$), B356, B31, and 297, *B. garinii* strain PBr, and *B. afzelii* strain VS461 have been described previously (10, 17). B314 and its derivatives were cultured at 33°C in BSKII complete medium (2), supplemented with 200 μg of kanamycin/ml where appropriate. For a list of bacterial strains used in the present study, see Table 1. 293 (human kidney epithelial) cells, C6 (rat glioma) cells, and EA-Hy926 (human umbilical vein endothelial) cells were cultured as described previously (40).

Plasmids and cloning. For initial sequence analysis, *dbpA* from (i) *B. burgdorferi* strains $N40_{D10/E9}$, B356, B31, and 297, (ii) *B. afzelii* strain VS461, and (iii) *B. garinii* strain PBr were amplified by PCR from genomic DNA using primers and reaction conditions described previously (44). Briefly, the forward primer, 10F4 5′-GTGGTTAAGGAAAAACAAAA-3′, is homologous to a sequence in the highly conserved *dbpB* gene (44), and the reverse primer, 5R1 5′-CCAAATAA CATCAAAAAGGA-3′, is homologous to a sequence downstream of *dbpA*. PCR with these primers resulted in the generation of amplicons ~1 kb in size, which were inserted into pCR-XL-TOPO vector (Invitrogen) and then sequenced. Nucleotide sequence analysis showed that *dbpA* cloned from strains B31, 297, PBr, and VS461 were identical to previously published sequences (44, 60). DbpA from strain $N40_{D10/E9}$ was found to be 95% identical to DbpA from an independent clone of N40 (44) and is, for clarity, referred to as $DbpA_{N40-D10/E9}$ in the present study.

TABLE 1. Bacterial strains used in this study

Strain	Species	Origin and/or notes[a]	Source or reference
N40 (clone D10/E9)	*B. burgdorferi*	Tick, United States	9
B356	*B. burgdorferi*	Skin	54
B31	*B. burgdorferi*	Tick, Switzerland	2, 17
297	*B. burgdorferi*	Human, CSF, United States	31
PBr	*B. garinii*	Human, CSF, Germany	9
VS461	*B. afzelii*	Tick, Switzerland	35
B314/pJF21	*B. burgdorferi*	B314 clone JF5 (harboring vector control)	15
B314/$DbpA_{N40}$	*B. burgdorferi*	B314 clone VB2-7	This study
B314/$DbpA_{B356}$	*B. burgdorferi*	B314 clone JF12	This study
B314/$DbpA_{B31}$	*B. burgdorferi*	B314 clone YL1	This study
B314/$DbpA_{297}$	*B. burgdorferi*	B314 clone JF11	This study
B314/$DbpA_{PBr}$	*B. burgdorferi*	B314 clone JF8	This study
B314/$DbpA_{VS461}$	*B. burgdorferi*	B314 clone VB1-7	This study

[a] CSF, cerebrospinal fluid.

For expression in B314, the entire coding sequence of each *dbpA* allele was amplified from genomic DNA by PCR using the primers listed in Table 2. These amplicons were then cloned into a modified shuttle vector, pJF21 (15), using engineered SalI and BamHI sites at the 5′ and 3′ ends, respectively, and sequenced with the M13F and M13R primers. For production of recombinant polyhistidine-tagged proteins, *dbpA* genes from strains B31, $N40_{D10/E9}$, PBr, and VS461 without their lipoprotein signal sequences were amplified by PCR using the primers described in Table 2 and cloned into pET15b (Novagen, Madison, WI).

Purification of human decorin. Recombinant human decorin, a generous gift from David Mann (MedImmune, Inc.), was purified from stably transfected Chinese hamster ovary cells as described previously (29).

Generation of recombinant proteins and antisera. Plasmids encoding recombinant His-tagged DbpA proteins were transformed into *Escherichia coli* strain BL21, and protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacteria were lysed, and proteins were purified by nickel affinity chromatography as described in the manufacturer's instructions (Novagen). The homogeneity of recombinant proteins was confirmed by SDS-PAGE and Coomassie blue staining. Five-week-old BALB/c mice were immunized with 100 μg of His-$DbpA_{B31}$, His-$DbpA_{N40-D10/E9}$, His-$DbpA_{PBr}$, or His-$DbpA_{VS461}$ in complete Freund adjuvant. The animals were boosted twice with 100 μg of the same proteins in incomplete Freund adjuvant at 2-week intervals, and antisera were collected from blood after terminal cardiac puncture.

Attachment of immobilized recombinant DbpA proteins to biotinylated glycosaminoglycans. Dermatan sulfate (Calbiochem), heparin, and chondroitin-6-sulfate (Sigma) were biotinylated using EZ-Link Biocytin Hydrazide (Pierce) and then dialyzed in phosphate-buffered saline (PBS) using a Slide-A-Lyzer cassette (Pierce) with 10,000-kDa molecular mass cutoff. Microtiter plates (96 well; Linbro) were coated overnight at 4°C with 1 μg of His-tagged recombinant DbpA in PBS. The next day, proteins were removed, and the wells were washed twice with PBS and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The blocking buffer was removed, and 50 μl of each biotinylated GAG, diluted to a final concentration of 100 μg of 1% BSA/ml in PBS, was added to the wells, followed by incubation for 2 h. After a washing step, bound GAGs were detected by enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase-tagged anti-biotin antibody, followed by TMB substrate.

Transformation of *B. burgdorferi* B314. Electrocompetent B314 spirochetes were prepared and transformed as described previously (36). Briefly, 100-ml portions of mid-log-phase cultured spirochetes were harvested and washed twice in electroporation solution (EPS; 15% [vol/vol] glycerol, 0.27 M sucrose) and resuspended in 100 μl of EPS. Then, 30 to 40 μg of plasmid DNA was added to the suspension, and the mixtures were electroporated at 2,200 V in a 0.2-cm cuvette and cultured in BSKII complete medium at 33°C for 24 h. The transfor-

TABLE 2. Primers used in this study

Purpose	Primer	Sequence (5′–3′)[a]	Nucleotide position[b]
B314/pDbpA$_{N40-D10/E9}$	AN40F	ACGCGTCGACATGAATAAATATCAAAAAACTTTC	1–24
	AN40R	CGCGGATCCTTAGTTATTTTTGCATTTTTCATCAGT	559–582
B314/pDbpA$_{B356}$	A356F	ACGCGTCGACATGAATAAATATCAAAAAACTTTC	1–24
	A356R	CGCGGATCCTTAGTTATTTTTGCATTTTTCATC	562–585
B314/pDbpA$_{B31}$	AB31F	CGGTCGACATGATTAAATGTAAT	1–15
	AB31R	CGGGATCCTTAGTTATTTTTGCA	562–576
B314/pDbpA$_{297}$	A297F	ACGCGTCGACATGATTAAATGTAATAATAAAACT	1–24
	A97R	CGCGGATCCTTACGATTTAGCAGTGCTGTCTTC	541–564
B314/pDbpA$_{PBr}$	APBrF	ACGCGTCGACATGATTAAATATAATAAAATATTG	1–24
	APBrR	CGCGGATCCTTATGTAGTAGTAGCAGTTTTGGC	535–558
B314/pDbpA$_{VS461}$	AVS461F	ACGCGTCGACATGATTAAATATAATAAAATTATA	1–24
	AVS461R	CGGGATCCTTATTTTTGATTTTTAGTTTGTTTTTCTTTAATGTTTTCC	487–510
BL21/pHis-DbpA$_{B31}$	B31 HisDbpAF	GCGGATCCGGACTAACAGGAGCAACA	76–93
	B31 HisDbpAR	CGCTCGAGTTAGTTATTTTTGCATTT	559–576
BL21/pHis-DbpA$_{PBr}$	PBrHisDbpAF	GCGGATCCGGCTTAACAGGAGAAACT	64–81
	PBrHisDbpAR	CGCTCGAGTTATGTAGTAGTAGCAGT	541–558
BL21/pHis-DbpA$_{VS461}$	5VS461DBPA	GGAATTCCATATGAGTTTAACAGGAAAAGCTAGATTGGAA	64–90
	AVS461R	CGCGGATCCTTATTTTTGATTTTTAGTTTGTTTTTCTTTAATGTTTTCC	487–510

[a] Underlining denotes the restriction sites.
[b] The nucleotide position is given relative to the first nucleotide of the initiation codon. Note that to generate recombinant DbpA proteins, the open reading frame of only the mature proteins (i.e., those lacking a signal sequence) was amplified.

mation mixture was added to 1.7% analytical-grade agarose (Invitrogen) and plated onto a 1.5× BSKII/agarose bottom layer in a sterile tissue culture dish (100 mm × 20 mm; Corning) in the presence of kanamycin (200 μg/ml), and the plates were incubated at 34°C in a 2% CO_2 atmosphere for 2 weeks. Colonies were picked and cultured at 33°C to mid-log-phase density in BSKII complete medium containing kanamycin (200 μg/ml).

Proteinase K treatment, SDS-PAGE, and Western blotting. To detect DbpA proteins, lysates from 5×10^7 spirochetes were separated by SDS–15% PAGE. DbpA and FlaB were identified by immunoblotting with polyclonal antibodies against the appropriate DbpA (diluted 1:5,000) or a monoclonal antibody, CB1 (a gift from J. Benach, Stony Brook University, Stony Brook, NY), against FlaB (diluted 1:500), respectively. Surface localization of DbpA proteins in recombinant B314 strains was determined as previously described (43). Briefly, 5×10^7 spirochetes were centrifuged, and the pellets were washed twice in PBS plus 0.2% BSA. After the final wash, pellets were gently lifted with 5 mM $MgCl_2$ in PBS supplemented with 4 mg of proteinase K (Sigma)/ml or buffer only and incubated at room temperature for 30 min. To inactivate the proteinase K, 150 μg of phenylmethylsulfonyl fluoride (PMSF) was added to each pellet. Pellets were washed twice with PBS plus 0.2% BSA, lysed, and separated by SDS–15% PAGE. The DbpA and FlaB proteins were identified by immunoblotting as described above.

Triton X-114 fractionation. Membrane fractions of B314 expressing DbpA were prepared as described previously (5). Briefly, *Borrelia* cultures were grown to log phase as determined by counting spirochetes by dark-field microscopy, and 10^9 spirochetes were harvested and washed twice with 0.2% BSA in PBS. Spirochetes were then resuspended in 2% Triton X-114 and allowed to incubate overnight with rocking at 4°C. The following day, insoluble material was removed by centrifugation, and the supernatants were phase separated as follows. The supernatants were warmed to 37°C for 15 min and then centrifuged at 13,000 rpm for 15 min to separate the aqueous and detergent fractions. The aqueous phase was discarded, and the detergent phase was washed three times by adding cold PBS to the original volume and rewarming and recentrifuging the samples as described above in the phase separation step. After the final wash, the proteins were precipitated by adding 9 volumes of cold 100% ethanol, incubated overnight at −20°C, and recovered by centrifugation. After a washing with 90% ethanol, the proteins were resuspended in PBS. The protein concentration was determined by a BCA assay (Pierce). Then, 15 μg of total protein from the outer membrane (detergent) fraction was separated on a 15% SDS-PAGE gel and stained with Coomassie blue.

Attachment of spirochetes to mammalian cells. Radiolabeled bacteria were prepared by growing spirochetes at 33°C in BSKII complete medium supplemented with 60 μCi of [^{35}S]methionine/ml. When the cultures achieved mid-log phase (approximately 5×10^7/ml), bacteria were harvested at 10,000 × *g*, and the pellets were washed twice with 0.2% BSA in PBS. Labeled spirochetes were then stored as aliquots at −80°C in BSK-H (Sigma) containing 20% glycerol.

One day before each assay, mammalian cells were lifted with 0.5% trypsin–0.5 mM EDTA (Invitrogen) and plated in 96-well break-apart microtiter plates (Nunc) which were previously UV sterilized and coated with MBP-Inv497, a maltose-binding protein fusion containing the cell-binding domain of the invasin protein from *Yersinia pseudotuberculosis* (32). Frozen aliquots of radiolabeled *B. burgdorferi* were thawed and resuspended at 10^8cells/ml in BSK-H, followed by incubation at room temperature for 2 h. Prior to the addition of radiolabeled spirochetes, the cell monolayers were washed twice with PBS. Radiolabeled spirochetes were then diluted 1:3 into GHS buffer (10 mM glucose, 10 mM HEPES, 50 mM NaCl [pH 7.0]) and added to quadruplicate wells at 10^6 spirochetes/well. To enhance spirochete-cell contact, the plates were centrifuged at 1,000 rpm for 5 min and then rocked at room temperature for 1 h. Unbound spirochetes were removed by washing wells four times with 0.2% BSA in PBS. The plates were then air-dried, and the percentage of bound bacteria in each well was determined by liquid scintillation. Each strain was tested for cell binding in three to five independent experiments.

Enzymatic removal of specific classes of GAGs. Monolayers were incubated for 2 h with 0.5 U of heparinase I, heparitinase, or chondroitinase ABC (Sigma)/ml at 37°C in RPMI supplemented with 1% BSA, 10^{-2} U of aprotinin/ml, and 165 μg of PMSF/ml. After the monolayers were washed with PBS, radiolabeled spirochetes were added to the pretreated monolayers as described above.

Inhibition of binding with exogenous GAGs. Radiolabeled spirochetes were prepared as described above and incubated for 30 min at room temperature in BSK-H supplemented with between 80 ng and 6.25 mg of the GAGs/ml. After incubation, the spirochetes were diluted 1:3 in GHS buffer before addition to the cell monolayers.

Attachment of radiolabeled bacteria to purified GAGs and decorin. Prior to each assay, wells from Nunc 96-well break-apart microtiter plates were coated with either a titration of purified human decorin (1.25 to 0.156 μg/ml) or dermatan sulfate (2.5 to 0.625 mg/ml) in PBS at 4°C overnight. Wells were washed three times with 0.05% Tween 20 in PBS. The wells were then blocked with 1% BSA in PBS for 1 h at room temperature. After removal of the blocking buffer, radiolabeled *B. burgdorferi* were added to the wells as described above.

RESULTS

Ectopic expression of diverse DbpA alleles on the surface of a nonadherent *B. burgdorferi* strain. We previously showed that recombinant DbpA from *B. burgdorferi* strain N40 clone D10/E9 (N40$_{D10/E9}$) bound to purified GAGs (16, 31). To determine whether GAG binding activity is a common property of diverse DbpA alleles, we measured GAG binding by

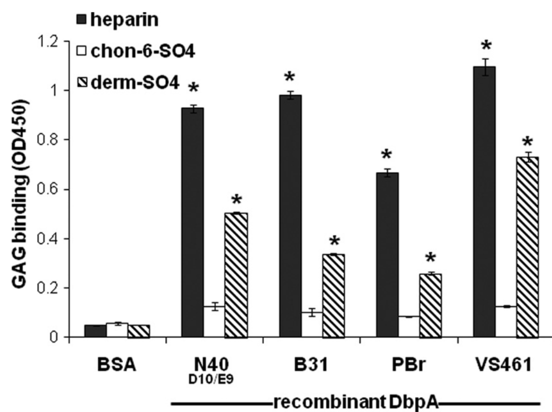

FIG. 1. Recombinant DbpA proteins bind to biotinylated GAGs. Microtiter wells were mock coated (with PBS) or coated with His-tagged $DbpA_{N40}$,$DbpA_{B31}$, $DbpA_{PBr}$, or $DbpA_{VS461}$ and probed with biotinylated heparin, dermatan sulfate, or chondroitin-6-sulfate (see Materials and Methods). After a washing step, bound GAGs were detected by ELISA, as indicated by the absorbance at 450 nm. Each bar represents the mean of four independent determinations ± the standard errors. Asterisks indicate that GAG bound to DbpA-coated wells significantly ($P < 0.05$ [Student *t* test]) better than to mock-coated wells.

recombinant DbpA from strain $N40_{D10/E9}$ and three additional Lyme disease spirochetes. *B. burgdorferi* strain B31 is the type strain for this organism and is highly infectious in the mouse model (17), *B. garinii* strain PBr displays high levels of the hemagglutination activity that is associated with GAG binding (33), and strain VS461 is a representative of *B. afzelii* (35). DbpA from strain $N40_{D10/E9}$ was found to be 95% identical to DbpA from an independent clone of N40 (44) and for clarity is referred to here as $DbpA_{N40\text{-}D10/E9}$. Immobilized recombinant His-tagged DbpA alleles were tested for their ability to bind to biotinylated GAGs. DbpA from each strain bound to heparin, as well as to dermatan sulfate, but not to a control GAG, chondroitin-6-sulfate (Fig. 1). Biotinylated heparin and dermatan sulfate did not bind to BSA-coated wells in this experiment (Fig. 1) or to a control His-tagged protein in previously described studies (38).

Allelic differences of DbpA could result in differences in binding activity. Although the initial analysis of recombinant DbpA proteins revealed potential differences in GAG binding (Fig. 1), given that it is not clear how faithfully the binding activities of recombinant DbpA reflect its activities when expressed on the spirochetal outer membrane, we tested the adhesive function of DbpA alleles on the surface of an otherwise nonadherent *B. burgdorferi* strain (16, 46). *B. burgdorferi* strain B314 is a high-passage strain that lacks all discernible linear plasmids, including lp54 (46), which encodes the *dbpBA* operon, and appears to be incapable of binding to GAGs or cultured mammalian cells. Alleles of *dbpA* were cloned into the expression shuttle vector pJF21 downstream of the *ospC* promoter (16, 50), which is highly active in strain B314 and is predicted to facilitate high-level transcription (46). Recombinant plasmids were transformed into *B. burgdorferi* strain B314. In addition to the alleles of *B. garinii* PBr, *B. afzelii* VS461, and *B. burgdorferi* $N40_{D10/E9}$ and B31, we also analyzed *dbpA* from *B. burgdorferi* 297, a commonly studied strain, and from *B. burgdorferi* B356, a skin biopsy isolate that is incapable of causing persistent disseminated infection in mice (54). The set of six DbpA alleles share as little as 56% identify (Table 3). To confirm that these DbpA alleles were expressed in strain B314, bacterial outer membrane proteins were isolated by Triton X-114 extraction and immunoblotted using mouse anti-DbpA antisera. As expected, each recombinant strain harboring a recombinant plasmid encoding *dbpA* was found to produce DbpA, whereas B314 harboring the pJF21 vector control did not (Fig. 2A). Proteinase K digestion of intact spirochetes resulted in complete (e.g., Fig. 2B, PBr) or near-complete (e.g., Fig. 2B, B31) loss of reactivity to DbpA antisera in immunoblot analysis, indicating that all DbpA alleles were largely exported to the surface of strain B314. As predicted, the periplasmic protein FlaB remained intact, indicating that the integrity of the outer membrane during proteinase K digestion was maintained (Fig. 2B).

DbpA displays allelic variation in the recognition of dermatan sulfate on the surface of cultured epithelial cells. $DbpA_{N40\text{-}D10/E9}$ was previously shown to promote attachment to cultured epithelial cells but not to endothelial nor glial cells (16). Therefore, recombinant B314 strains expressing different DbpA alleles were tested for gain-of-function in attachment to 293 epithelial, EA-Hy926 endothelial, and C6 glial cells. Similar to our previous analysis of $DbpA_{N40\text{-}D10/E9}$, all DbpA alleles promoted spirochete attachment to cultured epithelial cells (Fig. 3) but not glial or endothelial cells (data not shown). $DbpA_{PBr}$ promoted levels of epithelial cell attachment significantly ($P < 0.05$) higher than other DbpA alleles (Fig. 3). $DbpA_{297}$ and $DbpA_{B31}$ mediated spirochetal binding less efficiently than $DbpA_{PBr}$, but more efficiently than $DbpA_{N40\text{-}D10/E9}$, $DbpA_{B356}$, and $DbpA_{VS461}$ (Fig. 3). Complementation of the nonadherent strain B314 gave reproducible results across multiple assays, and independently derived clones expressing the same allele displayed similar binding to 293 epithelial cells, purified decorin, and dermatan sulfate (Fig. 4). These results suggested that the binding phenotype measured in this way reflects intrinsic properties of the allele rather than the particular clone analyzed.

We previously showed that spirochetal binding to 293 cells mediated by $DbpA_{N40\text{-}D10/E9}$ required dermatan sulfate (16). To determine whether GAGs promoted binding of the recombinant B314 strains to 293 cells, monolayers were digested with

TABLE 3. Homology of DbpA alleles used in this study[a]

DbpA allele	Homology (%)					
	B. burgdorferi				*B. garinii* PBr	*B. afzelii* VS461
	$N40_{D10/E9}$	B356	B31	297		
B. burgdorferi						
$N40_{D10/E9}$	100	99	78	75	70	62
B356		100	78	72	67	56
B31			100	90	69	58
297				100	71	59
B. garinii PBr					100	63
B. afzelii VS461						100

[a] A pairwise comparison of amino acid identities between DbpA alleles is shown. Values for the two groups of alleles that share ≥90% homology are shaded.

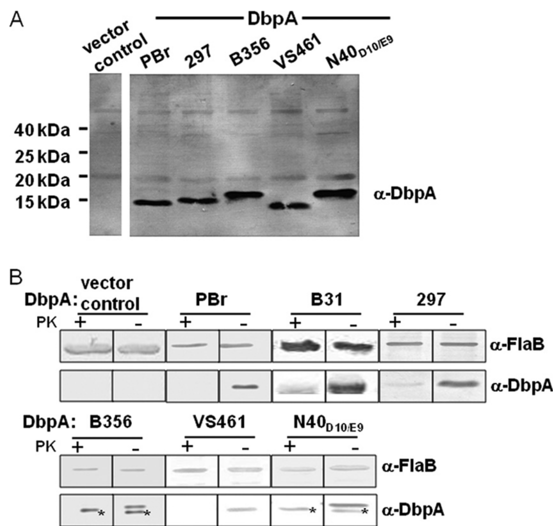

FIG. 2. DbpA alleles from diverse Lyme disease spirochetes are expressed on the surface of a nonadherent *B. burgdorferi* strain. (A) Triton X-114 extractions of *B. burgdorferi* strain B314 expressing the indicated DbpA allele were subjected to SDS–15% PAGE and immunoblotted with a mixture of antisera raised against $DbpA_{B31}$, $DbpA_{N40\text{-}D10/E9}$, $DbpA_{PBr}$, and $DbpA_{VS461}$. (B) Spirochetes expressing DbpA protein were digested with proteinase K (PK+) or incubated in PBS (PK−). Lysates from 5×10^7 treated bacteria were separated by SDS–15% PAGE, and DbpA and FlaB proteins were identified by Western blotting with an individual antiserum against $DbpA_{N40\text{-}D10/E9}$, $DbpA_{B31}$, $DbpA_{PBr}$, or $DbpA_{VS461}$. The proteinase K sensitivity experiments were performed several times and Fig. 2 is representative of such studies. Proteinase K-resistant species in lysates of B314/pDbpA$_{B356}$ and B314/pDbpA$_{N40\text{-}D10/E9}$, indicated by asterisks, were consistently observed and may represent intracellular nonlipidated DbpA protein. Flagellin, a periplasmic protein, was immunoblotted as a control for the retention of outer membrane integrity. *B. burgdorferi* strain B314 expressing $DbpA_{B31}$ was analyzed in a separate experiment.

specific lyases to remove different classes of GAGs from the mammalian cell surface. Consistent with this, the removal of dermatan or chondroitin sulfates with chondroitinase ABC virtually eliminated attachment promoted by all DbpA alleles, whereas the removal of heparin or heparan sulfates with heparinase or heparitinase had no significant effect (Table 4). In addition, exogenous dermatan sulfate, but not heparin or chondroitin-6-sulfate, significantly impaired cell binding by recombinant B314 expressing DbpA from strains B356, 297, or PBr (Table 5), suggesting that cell attachment was associated with GAG binding activity.

DbpA displays allelic variation in the ability to promote spirochetal attachment to purified decorin or dermatan sulfate. We found previously that $DbpA_{N40\text{-}D10/E9}$ was able to confer binding to purified dermatan sulfate to strain B314 (16). To determine whether the variation in the ability of different DbpA alleles is associated with differences in their ability to promote binding to purified GAGs, we tested for the binding of the recombinant B314 strains to microtiter wells coated with increasing concentrations of dermatan sulfate. Of the DbpA-expressing strains, B314 expressing $DbpA_{PBr}$ bound significantly ($P < 0.05$) better to dermatan sulfate than all of the other B314 strains at each of the concentrations tested (Fig. 5, left panel). $DbpA_{297}$ and $DbpA_{B31}$ promoted binding somewhat less efficiently than $DbpA_{PBr}$ but significantly more efficiently than $DbpA_{VS461}$, $DbpA_{B356}$, or $DbpA_{N40\text{-}D10/E9}$ (Fig. 5, left panel). Binding mediated by the latter three alleles, while not robust, was nevertheless considerably (and significantly) above the background levels observed for strain B314 harboring the vector control. Thus, variation in the ability of the DbpA alleles to promote binding to purified dermatan sulfate correlated with their ability to promote binding to 293 cells

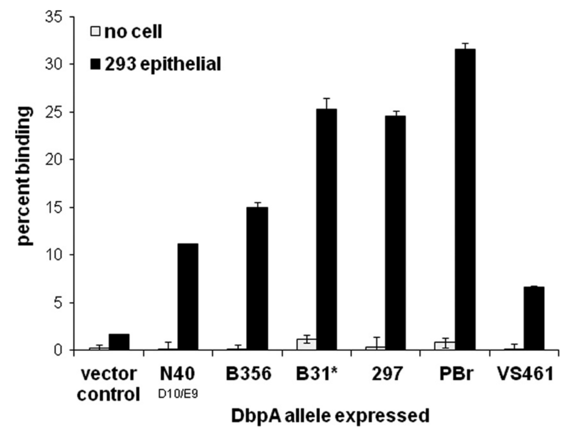

FIG. 3. DbpA-mediated binding to cultured mammalian cells is cell type specific and variable among alleles. Radiolabeled *B. burgdorferi* strain B314 spirochetes expressing the indicated DbpA allele were added to wells containing 293 epithelial cells or to empty wells (media). Each bar represents the mean (± the standard error) of four independent determinations. *B. burgdorferi* strain B314 expressing $DbpA_{B31}$ was analyzed in a separate experiment in which the binding by B314 strains expressing $DbpA_{PBr}$ or no DbpA (vector control), analyzed in parallel as controls, bound with an efficiency similar to that in the depicted experiment.

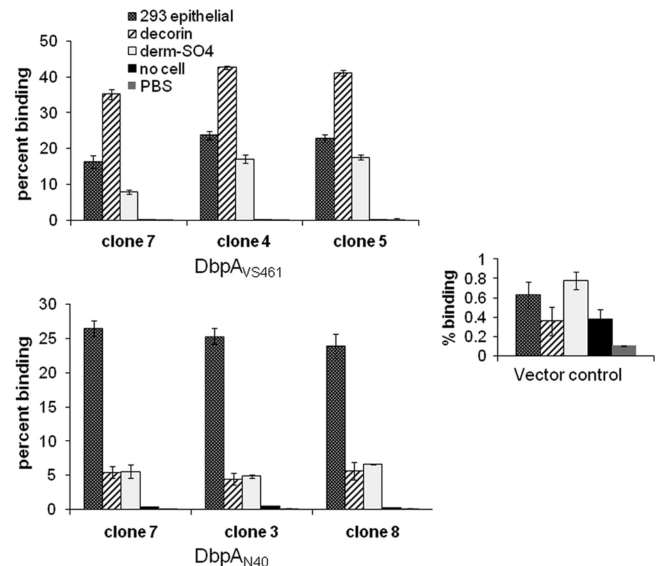

FIG. 4. Independently derived clones expressing the same allele of DbpA display similar *in vitro* binding phenotypes. Radiolabeled *B. burgdorferi* strain B314 spirochetes expressing either $DbpA_{VS461}$ (top panel), $DbpA_{N40}$ (bottom panel), or vector control (inset panel) were added to wells containing 293 epithelial cells or to wells containing either no cells (media) or decorin, dermatan sulfate, or PBS (mock coated). Each bar represents the mean (± the standard error) of four independent determinations.

TABLE 4. Removal of chondroitin sulfate GAGs from the surfaces of epithelial cells diminishes binding by spirochetes expressing diverse DbpA alleles

Plasmid	Mean binding (%) ± SE[a]			
	Mock digestion	Heparinase	Heparatinase	ChonABC
Vector control	2.9 ± 0.2	NA	NA	NA
pDbpA$_{N40\text{-}D10/E9}$	10.3 ± 0.7	8.3 ± 1.3	10.2 ± 0.9	0.6 ± 0.2*
pDbpA$_{B356}$	17.0 ± 2.4	17.6 ± 0.5	23.6 ± 2.8	0.2 ± 0.1*
pDbpA$_{297}$	33.7 ± 3.3	34.8 ± 3.3	42.3 ± 2.3	2.0 ± 0.6*
pDbpA$_{PBr}$	42.3 ± 1.3	38.4 ± 3.0	42.6 ± 0.6	1.1 ± 1.0*
pDbpA$_{VS461}$	6.6 ± 0.4	4.6 ± 0.8	5.5 ± 0.3	0.5 ± 0.2*

[a] Binding of radiolabeled transformants to 293 epithelial monolayers was determined after pretreatment of cells with the indicated lyase, as described previously (15, 16). ChonABC, chondroitinase ABC. Each value represents the mean of four independent determinations ± SE. For all strains, <2% of bacteria bound to identically treated wells without mammalian cells (data not shown). *, Significant ($P < 0.05$) difference in binding to mock-treated versus lyase-treated monolayers as determined by Student *t* test analysis. NA, not applicable.

TABLE 5. Dermatan sulfate inhibits cell binding by *B. burgdorferi* strain B314 expressing DbpA alleles from diverse Lyme disease spirochetes

Plasmid	Mean binding (%) ± SE[a]			
	No inhibitor	Heparin	C6S	DS
Vector control	2.9 ± 0.5	NA	NA	NA
pDbpA$_{B356}$	15.6 ± 1.9	22.6 ± 0.7	18.4 ± 2.1	3.8 ± 0.3*
pDbpA$_{297}$	42.5 ± 2.8	33.0 ± 2.2	47.6 ± 3.5	26.9 ± 1.5*
pDbpA$_{PBr}$	57.5 ± 4.2	59.1 ± 1.9	58.8 ± 3.7	32.9 ± 4.3*

[a] Binding of radiolabeled transformants to 293 epithelial monolayers was determined after pretreatment of bacteria with the indicated soluble GAG, as described previously (15, 16). DS, dermatan sulfate; C6S, chondroitin-6-sulfate. Each point represents the mean of four independent determinations ± SE. For all strains, <2% of bacteria bound to identically treated wells without mammalian cells (data not shown). *, Significant ($P < 0.05$) difference in binding to mock-treated versus GAG-treated bacteria as determined by Student *t* test analysis. NA, not applicable.

(compare Fig. 3 and the left panel of Fig. 5), suggesting that 293 cell attachment reflects dermatan sulfate binding activity.

DbpA was originally identified by its ability to bind to decorin, a dermatan sulfate proteoglycan (23). To test whether allelic variation of DbpA in binding to decorin correlates with binding to dermatan sulfate and 293 cells, the B314 recombinants expressing different DbpA alleles were similarly tested for the ability to bind to increasing concentrations of immobilized decorin. Again, DbpA$_{PBr}$ promoted decorin binding significantly better than did other alleles, and DbpA$_{297}$ and DbpA$_{B31}$ promoted attachment to decorin better than DbpA$_{B356}$ or DbpA$_{N40\text{-}D10/E9}$ (Fig. 5, right panel). Notably, DbpA$_{VS461}$ promoted relatively efficient binding to decorin, in spite of promoting only low level binding to dermatan sulfate and epithelial cells (compare to Fig. 5, left and right panels, and Fig. 3). These results indicate that DbpA-mediated bacterial attachment to decorin and dermatan sulfate are distinguishable, with binding to purified dermatan sulfate correlating better with 293 epithelial cell binding.

The C terminus of DbpA$_{VS461}$ is required for spirochetal attachment to mammalian cells and to purified decorin or dermatan sulfate. Basic amino acids are often critical for the ability of GAG-binding proteins to bind their substrate (27), and three lysine residues of DbpA$_{297}$—K82, K163, and K170—were previously implicated in decorin binding (8, 41). The sequences flanking K82 of DbpA$_{B31}$ or DbpA$_{N40\text{-}D10/E9}$, which differed significantly in their ability to promote binding to epithelial cells, dermatan sulfate and decorin (Fig. 3 and 5), are nearly identical (44), suggesting that other segments of the proteins may contribute to GAG, decorin, and cell binding. A comparison of DbpA sequences shows that C terminal to K170, all alleles contain a lysine-rich segment of amino acids (Fig. 6A). Among the alleles from different Lyme disease species, this region is not highly conserved in specific amino acid sequence, but its basic nature could promote binding to GAGs and/or decorin. In fact, analysis of a fortuitous PCR-generated deletion-substitution mutant of DbpA$_{VS461}$ that lacks this segment did not bind to decorin, dermatan sulfate, or 293 epithelial cells (Fig. 6C and D, ΔC11+13). The defect in binding was apparently due to the loss of the 11-residue highly basic C-terminal DbpA sequence rather than the acquisition of 13 residues of exogenous sequence because simple deletion of the DbpA$_{VS461}$ C-terminal 11 amino acids also eliminated the ability of DbpA$_{VS461}$ to promote bacterial attachment (Fig. 6, ΔC11). The defect in binding by either mutant was not due to alteration in surface localization of DbpA (Fig. 6B), suggesting that the extreme C-terminal region of this DbpA allele is critical for decorin, dermatan sulfate, and 293 cell binding.

DISCUSSION

The bacterial factors that contribute to the ability of some Lyme disease spirochete strains to cause disseminated infection are not fully characterized, and may include surface proteins that mediate attachment to mammalian cells or extracellular matrix (ECM) in target tissues. The Lyme disease spirochete exhibits strain-specific variation in GAG-binding specificity that corresponds to variation in the mammalian cell types that are recognized *in vitro* (40). *B. burgdorferi* encodes multiple GAG-binding proteins, including DbpA, DbpB (16), Bgp (39), and BBK32 (15), and the expression pattern of these adhesins might vary with strain. In addition, *B. burgdorferi* may encode allelic-variable GAG-binding adhesins that exhibit differences in GAG recognition. DbpA has long been known to be highly variable among Lyme disease spirochetes (44) and could contribute to such strain-specific variation. In the present study, we analyzed DbpA alleles from each of the three major species of Lyme disease spirochete, as well as representatives of *B. burgdorferi* sensu stricto that were associated with different abilities to disseminate in the mammalian host.

After confirming that different recombinant DbpA alleles possessed GAG-binding activity, we utilized an *ospC* promoter-based vector to ectopically express different DbpA alleles on the surface of the high-passage, noninfectious strain *B. burgdorferi* B314. This strain lacks the *dbpA*-encoding Lp54, as well as any other linear plasmid (46; unpublished observations), and is not known to adhere to any cell line (16). The outer membrane of *B. burgdorferi* strain B314 lacks many lipoproteins (46) and undoubtedly differs from that typical of infectious *B. burgdorferi* strains. Nevertheless, this strain provides one means to analyze the ability of an adhesin to promote spirochetal binding in the absence of confounding alternate

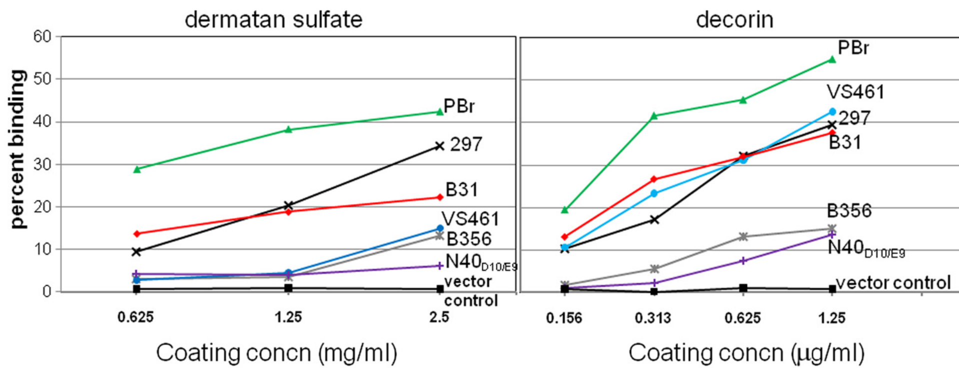

FIG. 5. Decorin- and GAG-binding levels vary among DbpA alleles. Radiolabeled B314 spirochetes expressing the indicated DbpA allele were added to wells coated with increasing concentrations of decorin or dermatan sulfate, and the percentage of bacteria stably bound was determined. Analysis of B314 expressing $DbpA_{B31}$ was performed in a separate experiment in which the binding by B314 strains expressing $DbpA_{PBr}$ or no DbpA, analyzed in parallel to the controls, bound with an efficiency similar to that in the depicted experiment. Each point represents the mean of four independent determinations, with the standard error omitted for clarity. At a concentration of 1.25 mg of dermatan sulfate/ml, $DbpA_{PBr}$ bound to this substrate at a significantly higher percentage ($P < 0.05$) than both $DbpA_{297}$ and $DbpA_{B31}$, and the latter two strains bound at a significantly higher percentage than $DbpA_{B356}$, $DbpA_{N40}$, and $DbpA_{VS461}$. At a concentration of 1.25 μg of decorin/ml, $DbpA_{PBr}$ bound at a significantly higher percentage ($P < 0.05$) than $DbpA_{297}$,$DbpA_{B31}$, and $DbpA_{VS461}$, and the latter three bound at a significantly higher percentage ($P < 0.05$) than $DbpA_{B356}$ and $DbpA_{N40}$.

binding pathways. In addition, a preliminary survey of Lyme disease strains corresponding to several of the DbpA alleles analyzed in the present study indicates that *B. garinii* strain PBr, which we show here encodes a DbpA allele that mediates efficient spirochetal attachment to 293 cells, also binds with high efficiency to this cell line (N. Parveen and D. Robbins, unpublished observations).

For each DbpA allele, ectopic expression conferred the ability to attach to epithelial (but not endothelial or glial) cells and to purified dermatan sulfate. In all cases, DbpA-mediated mammalian cell attachment was eliminated upon enzymatic removal of this class of GAGs, indicating that diverse DbpA alleles are capable of promoting epithelial cell attachment through the recognition of dermatan sulfate GAGs.

Each DbpA-expressing B314 derivative also bound to decorin significantly above background levels. Interestingly, upon a recent survey of allelic variants of DbpA (and DbpB), J. Salo and coworkers found that several alleles, including all *B. afzelii* DbpA alleles, were devoid of detectable decorin-binding activity (47a). The apparent discrepancy in the two studies could be due to technical differences or to the analysis of different alleles, given that the sets of DbpA alleles analyzed

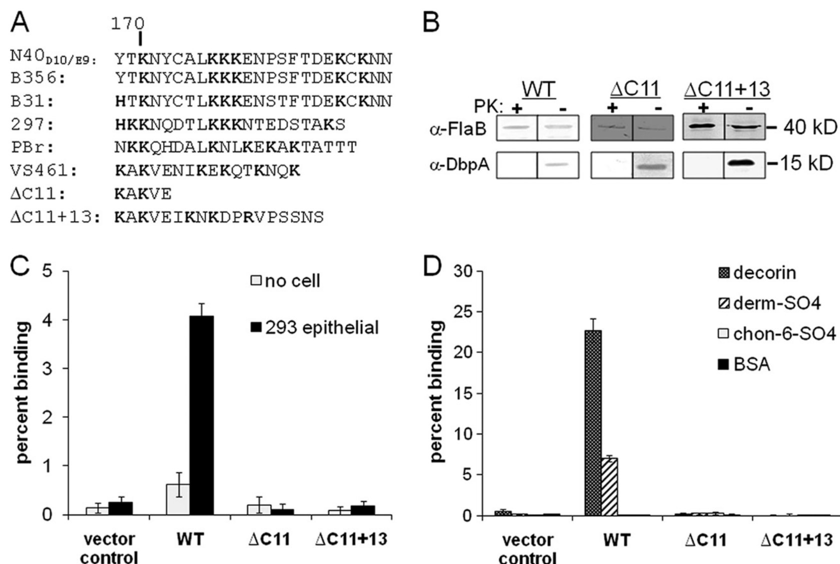

FIG. 6. The C-terminal region of $DbpA_{VS461}$ is required for binding to GAGs and mammalian cells. (A) The C-terminal amino acid sequences of DbpA proteins analyzed in the present study. (B) Strain B314 expressing wild- type (WT) or mutant $DbpA_{VS461}$ were subjected to proteinase K (PK+) or mock (PK−) digestion and immunoblotted for DbpA or flagellin, as described for Fig. 1. Radiolabeled B314 expressing the vector control or the indicated $DbpA_{VS461}$ derivative was added to wells with 293 epithelial cells or no cells (C), or dermatan sulfate, decorin, chondroitin-6-sulfate, or BSA (D). The percentage of bacteria bound was determined by scintillation counting. Each bar represents the mean (± the standard errors) of four independent determinations.

in the two studies were nonoverlapping. Clarification of the reason(s) behind the different findings will require further investigation.

In the present study, although we detected common binding activities for each DbpA allele, we also observed allelic differences in the level of adhesiveness. For example, $DbpA_{PBr}$ promoted >4-fold more efficient epithelial cell attachment than $DbpA_{VS461}$ and >20-fold more efficient dermatan sulfate binding. These differences were reproducible and were a property of the allele expressed because multiple clones of a given allele were associated with similar binding activities. The differences in binding could not be attributed to the levels of expression of different alleles. First, no dramatic differences in expression levels were detected upon immunoblotting of outer membrane preparations with pooled antisera that collectively recognize the entire collection of DbpA alleles. Second, to the degree that SDS-PAGE and Coomassie blue staining revealed minor potential differences in DbpA levels (data not shown), we found no correlation between expression level and substrate binding. For example, $DbpA_{PBr}$ and $DbpA_{VS461}$, which as noted above differed dramatically in cell and GAG binding, were expressed at roughly equivalent levels.

As predicted, highly homologous alleles displayed similar binding activities. For example, the dermatan sulfate, decorin and 293 cell binding profiles of $DbpA_{N40\text{-}D10/E9}$ and $DbpA_{B356}$, which are 99% identical in sequence, were virtually indistinguishable. Similarly, $DbpA_{297}$ and $DbpA_{B31}$, which are 90% identical, also mediated roughly equivalent levels of cell, decorin, and dermatan sulfate binding. Consistent with the previously documented sequence diversity of DbpA alleles, we observed considerable intraspecies variation in adhesive activities: DbpA alleles derived from *B. burgdorferi* sensu stricto that varied considerably in sequence, e.g., $DbpA_{297}$ and $DbpA_{N40\text{-}D10/E9}$, also varied in attachment activities. Interestingly, given that strain $N40_{D10/E9}$ causes disseminated infection in mice (9), the observation that $DbpA_{N40\text{-}D10/E9}$ promoted levels of adhesiveness lower than most other alleles indicates that robust DbpA-mediated *in vitro* adhesiveness is not a prerequisite for disseminated infection. Related to this, $DbpA_{N40\text{-}D10/E9}$ and $DbpA_{B356}$ displayed similar binding activities in spite of the fact that *B. burgdorferi* $N40_{D10/E9}$ and B356 differ considerably in their ability to disseminate in mice (9, 54).

Decorin contains a chondroitin or dermatan sulfate GAG chain that is critical for recognition by DbpA (24) and, for five of the six DbpA alleles analyzed here, we observed a strong correlation between decorin and dermatan sulfate binding. However, one allele, $DbpA_{VS461}$, promoted relatively high-level spirochetal attachment to decorin but low-level binding to purified dermatan sulfate. Therefore, although the dermatan sulfate GAG chain of decorin is critical for recognition by DbpA (24), the dermatan sulfate and decorin binding properties are not identical. Consistent with this observation, the protein core of decorin was previously shown to be required for detectable binding by DbpA in a radiometric assay (24). We found here that $DbpA_{VS461}$ displayed a relatively poor activity in promoting 293 cell attachment, suggesting that, at least in this instance, cell attachment correlates more closely with dermatan sulfate binding than decorin binding. Definitive determination of the relative contribution of decorin and dermatan sulfate binding in cell attachment mediated by DbpA is complicated by the close structural relationship of these ligands and awaits further study.

Previous work showed that the lysine residues K163 and K170 in the C terminus of $DbpA_{297}$, along with the centrally located lysine residue K82, were required for binding to decorin (8, 41). The sequence of the DbpA C terminus is highly divergent, and in particular the $DbpA_{VS461}$ C terminus is quite dissimilar from that of $DbpA_{297}$ (44). Nevertheless, the C termini of all of the alleles tested are highly basic and thus might contribute to GAG binding. We found that the 11-residue C-terminal segment of VS461 just C-terminal to K170 is required for the ability of $DbpA_{VS461}$ to promote spirochetal binding to cells, decorin, and dermatan sulfate. This and other relatively variable regions of DbpA might contribute to the allelic diversity of attachment activity.

B. burgdorferi mutants that lack DbpA demonstrate a colonization defect in mice (4, 48). In the present study, we showed that the adhesive activity of DbpA is subject to considerable allelic variation. A detailed understanding of the molecular basis of this variation, combined with experimental infection by isogenic infectious strains that express different alleles of DbpA, may provide insight into the adhesive activities of this protein that contribute to infectivity and colonization in the mammalian host.

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The authors declare they have no competing financial interests.

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