Published in final edited form as:

Microbes Infect. 2017 ; 19(4-5): 267–276. doi:10.1016/j.micinf.2017.01.001.

Correlation between Antigenicity and Variability in the *vls* Antigenic Variation System of *Borrelia burgdorferi*

Wei Zhou^{a,*} and Dustin Brisson^a

^aUniversity of Pennsylvania, 3451 Walnut Street, Philadelphia, PA 19104, United States of America

Abstract

Many parasites have evolved antigenic variation systems that alter surface proteins in order to evade recognition by presently expressed antibodies and subsequent death. Although the amino acid positions in antigens to which antibodies most commonly target are expected to be the most variable, this assumption has not been investigated. Using the *vls* antigenic variation system of *Borrelia burgdorferi* as a model, we first investigated this assumption computationally and then developed a sensitive immunoassay to experimentally validate the computational results. There was a strong correlation between variability at an amino acid position and each of the computational metrics associated with antibody reactivity. However, empirical measures of antibody reactivity were not consistently greater at the variable amino acid positions than at the invariant amino acid positions. The inconsistent experimental support for this hypothesis suggests that the biological effect of variability at an amino acid position is obfuscated by other factors.

Keywords

Antigenic variation; VlsE; *Borrelia burgdorferi*; epitope mapping

1. Introduction

Survival within a vertebrate host, despite the lethal and rapidly changing immune environment, is essential to the evolutionary success of most microbial parasites [1–4]. Many parasites have evolved antigenic variation systems to alter surface antigens [1, 3, 5, 6, 4] in order to survive the adaptive immune responses that targeted previously expressed antigens [7]. Antigenic variation systems can elevate within-host density and increase infection duration of the parasites, both of which increase the probability of transmission to new hosts [3, 6], but often at a cost of exacerbating pathogenesis and decreasing host fitness

*Corresponding author: Wei Zhou, Department of Biology, University of Pennsylvania, Leidy Laboratories, 327, 433 South University Avenue, Philadelphia PA 19104-6018, Phone: (215) 746-1732, zhouwei2@sas.upenn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

6. Competing interests

The authors declare that they have no competing interest

[8–11]. The continual arms race between host immune responses and antigenic variation of microbial parasites results in strong natural selection shaping the molecular interactions at the antigen-antibody interface. Here we present an investigation of the molecular interactions and selective forces that shape antigenic variation systems, using the *vls* antigenic variation system in the Lyme disease bacterium, *Borrelia burgdorferi*, as a model. The *vls* antigenic variation system provides a suitable model to investigate both molecular interactions and selective forces because the amino acids that are altered during antigenic variation have been well-characterized and the crystal structure of the antigen has been solved, thus allowing an association of molecular interactions to structural features of the antigen. We tested the hypotheses that naturally variable amino acid sites are more commonly targeted by antibody responses and that the potential for evading the presently expressed antibodies is correlated with the degree of exposure of the variable amino acid sites on the surface of the protein.

Continuous alteration of the immunodominant surface antigen, VlsE, is required for *B. burgdorferi* to escape antibody recognition and establish long-term infections in vertebrate hosts [12–17]. Although the exact function of VlsE is unclear, it has been suggested that VlsE attracts host antibodies and therefore protects other cell surface antigens due to its immunodominance [18], high expression level in vertebrate hosts [19, 20] and co-localization with other cell surface proteins [12]. Throughout vertebrate infections, *B. burgdorferi* generate novel variants of VlsE that have changed sequences and can potentially evade recognition of expressed antibodies. Novel variants of VlsE are generated by unidirectional recombination of a random segment from one of several unexpressed, paralogous *vls* cassettes into the *vlsE* expression site [17, 21]. The probability of evading detection by antibodies is expected to correlate with the sequence variability of VlsE [22, 23]. The sequence variability of VlsE is correlated with the diversity among the unexpressed *vls* cassettes [22, 23]. Sequence differences among the unexpressed *vls* cassettes are concentrated in six regions that correspond to the six antibody-accessible, surface-loop structures of the VlsE protein [17, 24]. To increase the probability of evading the expressed antibodies, natural selection should maximize among-cassette diversity in order to maximize alterations in the VlsE sequence [22]. However, only a subset of the sites within the loop structures are variable [17, 23]. The absence of variability at the remaining sites in the antigenically-important loop structures suggests that these sites are either rarely targeted by antibodies or are constrained to be invariant by other selective forces such as maintenance of protein structure or function.

The degree to which an antigen must change to escape binding by an antibody depends largely on the physicochemical properties of the amino acids that are recognized by the antibody. Antigen-antibody contact regions consist of an average of 18 amino acids in the antigen [25], each of which has a quantitatively different effect on antibody-antigen reactivity. Variation among amino acid residues in their reactivity to antibodies is most strongly correlated with the solvent accessible surface areas of the amino acid residues [26, 27]. Other physicochemical or statistical properties of amino acid residues including hydrophilicity [28, 29] and statistical epitope propensity [30] are also correlated with reactivity between residues and antibodies. Thus, the amino acid sites in VlsE that have large solvent-accessible surface areas, are hydrophilic, and have high epitope propensity scores

are likely to have the largest effect on antibody evasion when mutated, and are thus the sites that are expected to be variable among *vls* cassettes.

In this study, we investigated the structural and physicochemical properties of amino acids in the antigenically-important surface loops of VlsE to assess if naturally variable amino acid sites have a larger effect on antibody reactivity than naturally invariant sites. We approached this question first through computational analyses of the properties of the amino acid residues in the loop regions of VlsE. The reactivity of the amino acid residues to host antibodies were then assessed in an exploratory experimental investigation.

2. Materials and methods

2.1. Correlations between amino acid variability and metrics of antibody reactivity

The published VlsE crystal structure from *B. burgdorferi* clone 5A3 ([PDB:1L8W], chain B) [24] was used to computationally investigate the hypothesis that amino acid residues that are more reactive to antibodies are more likely to be altered during antigenic variation. Variable and invariant amino acid sites in all six of the surface exposed loops of VlsE were identified as previously described [17, 23]. We used three metrics to assess reactivity of antibodies to each amino acid: 1. solvent accessible surface area, 2. hydrophilicity, and 3. statistical epitope propensity. The three metrics have been widely and successfully used to predict antibody binding sites in protein antigens [26–30]. Solvent accessible surface areas of each amino acid residue in all six surface loops in VlsE were calculated based on the published VlsE crystal structure using the POPS algorithms [31]. The hydrophilicity of each amino acid was estimated using the Parker hydrophilicity scale [28]. Statistical epitope propensities (the log-odds ratio of the frequencies of a type of amino acid appearing in known epitopes) were estimated as previously described [30]. We then asked if the solvent accessibility, hydrophilicity, and epitope propensities of invariant VlsE amino acids – those that are never altered by recombination with any *vls* cassette – were significantly lower than the solvent accessibility, hydrophilicity, and epitope propensities of variable amino acids.

2.2. Effect of experimentally altering amino acids on antibody reactivity

2.2.1. Overview of experimental methods—To assess the antigenic importance of single amino acid residues, 28 full-length recombinant VlsE proteins were generated, each of which differs from the wild-type VlsE protein at one amino acid site. Each of the 28 amino acid sites chosen for mutation is either found at naturally variable or naturally invariant sites in one of two surface exposed variable loops that interact with antibodies (Fig. 1)[14]. Polyclonal antisera were raised in rabbits using purified wild-type VlsE recombinant protein. Reactivity of the antisera to the wild-type VlsE was compared to the reactivity of the antisera to each of the 28 mutant VlsE proteins. The difference in antibody reactivity reflects the antigenic influence of altering each residue. However, the differences between the reactivity of the antibodies with the wild-type and with the mutant VlsE proteins are expected to be small because most of the antibodies in the polyclonal antiserum target protein surface areas common to both the mutant and wild-type VlsE protein. The cross-reactive antibodies can result in substantial background noise that obscures true differences in reactivity among the proteins. We developed a negative purification ELISA technique that

removes antibodies targeting the common areas in different VlsE proteins, thus allowing detection of changes in reactivity due to the antibodies that target the mutated amino acid residue. We then compared the effects of mutations at naturally variable and naturally invariant amino acid sites on antibody reactivity.

2.2.2. Cloning, expression, and purification of the wild-type VlsE and VlsE mutants—The DNA sequence of the full-length wild-type *vlsE* without the lipid moiety region was amplified from *B. burgdorferi* (clone 5A3) using primers 5A3F (CGGGGATCCCAGCCAAGTTGCTGATAAGGACGACCC) and 5A3R (CGGAAGCTTCAATCATGAGGGCATAGTCGTGTCCATACA). The ends of the amplicon were digested with BamHI and HindIII prior to ligation into expression vector pET45(b) (Novagen). The wild-type VlsE protein was expressed in *E. coli* BL21 (DE3), purified using Ni-NTA Superflow Resin (Qiagen), and dialyzed overnight (100mM NaCl, 50mM Tris-8 and 0.1% Tween-20). Polyclonal antibodies against the wild-type VlsE protein were generated in two rabbits at Anaspec, Inc. (Fremont, CA).

Twenty-eight full-length VlsE mutant proteins were generated using alanine scanning mutagenesis [32] (Fig. 1B). To generate each VlsE mutant protein, a single amino acid in the full-length VlsE was changed to an alanine using the Quikchange mutagenesis kit (Agilent Technologies). Among the 28 full-length VlsE mutant proteins, 18 have a mutated residue in loop 1 and 10 mutated residues are in loop 4 (Fig. 1A and B). Loop 1 and loop 4 were chosen because approximately half of the amino acid sites are polymorphic, and therefore variable, among the silent cassettes in each of the two loops, optimizing the power of comparison between the variable and invariant amino acid sites. Recombination events between *vlsE* and the unexpressed cassettes are not biased in terms of the choice of variable regions or unexpressed cassettes [33], suggesting loop1 and loop 4 are likely to be representative of the six variable loops. VlsE mutant proteins were expressed and purified as described for the wild-type VlsE protein.

2.2.3. ELISA using negatively purified antisera—We compared changes in antibody reactivity caused by each individual mutation using ELISA. However, the reduction in antibody reactivity caused by a single amino acid mutation is expected to be small as the majority of antibodies in polyclonal antisera bind to areas common to the wild-type and to the mutant VlsE. This background binding limits the ability to detect true differences in antibody binding caused by the mutated residue. We used negative purification of the antisera to remove antibodies that target both the wild-type and mutant VlsE variants (Fig. 2).

Negative purification of the polyclonal antisera was performed independently for each mutant VlsE protein. That is, each VlsE mutant was used to remove antibodies from different aliquots of each antiserum. Negative purification was performed by coating wells of standard ELISA plates with one VlsE mutant (50µg/mL) diluted in PBS (4°C overnight), blocking with BSA diluent/blocking solution (KPL; 4°C overnight), and then incubating antiserum from one rabbit in wells for 90 minutes at room temperature. The antibodies generated against the wild-type VlsE that bind to the VlsE mutant, and are thus cross-reactive with the wild-type VlsE, were removed by repeating this process six times, each

time transferring the antisera into a new well coated with the same VlsE mutant and incubating for 90 minutes (Fig. 2A). After six rounds of negative purification, only antibodies that bind to the wild-type VlsE but not to the mutant VlsE are expected to remain in the antisera.

Negatively-purified antisera aliquots were used in a standard indirect ELISA using wild-type VlsE as the target antigen under standard procedures recommended by Abcam. The final wild-type VlsE concentration of 7.5µg/mL was used to coat ELISA wells. Goat-anti-rabbit HRP was used as the secondary antibody and Turbo TMB was used as the chromogen. ELISA signals were measured by reading absorbance at 450nm (A450). The ELISA signals reflect the amount of antibodies in the negatively purified antiserum that did not bind to the VlsE mutant, which corresponds to the proportion of antibodies that do not bind to the mutant VlsE due to the mutation (Fig. 2A). That is, the quantity of the antibodies remaining after negative-purification demonstrates the effect of the amino acid substitution on antibody reactivity (Fig. 2B). All absorbance readings were normalized by the absorbance reading from a control well on each ELISA plate to account for among-plate variability. The control well used antiserum negatively purified using the wild-type VlsE. Thus, mutations that do not affect antibody reactivity result in a normalized score ($A450(\text{mutant})/A450(\text{wt})$) equal to 1.

The negative purification protocol successfully reduced background binding in ELISA resulting in more precise and detectable measures of changes in antibody reactivity. For example, negative purification of the antisera from rabbit II using the wild-type VlsE or mutant VlsE variants (L1M160i or L4M253i) increased the ability to detect differences in the antibody reactivity to each protein by decreasing standard error in ELISA readings (Fig. 2B). Negative purification with wild-type VlsE exhausted VlsE-specific antibodies from antisera raised against wild-type VlsE, resulting in a weak signal in the subsequent ELISA using wild-type VlsE as the target antigen. By contrast, considerable wild-type VlsE-specific antibodies remained in the same antiserum after negative purification with mutant L1M160i, resulting in a relatively strong ELISA signal when using wild-type VlsE as the target antigen.

3. Results

3.1. Amino acid residues with greater solvent accessible surface areas, greater hydrophilicity, and greater epitope propensity scores are more likely to be altered during antigenic variation

Amino acid sites that are variable during antigenic variation in all surface loops have significantly larger solvent accessible surface areas, which is strongly associated with antibody reactivity, than the invariant amino acid sites in the surface loops ($p<0.05$) (Fig. 3A). Further, surface accessibility is greater at variable amino acid sites than at invariant sites that use the same amino acid residue (Fig. 3B). Amino acid sites that are variable during antigenic variation also have significantly larger hydrophilicity and epitope propensity scores ($p<0.05$) (Fig. 3C, D), both of which are associated with greater targeting by antibodies. Unlike solvent accessible surface area, the hydrophilicity index and epitope propensity score are not conditional on the three-dimensional structure of the protein.

3.2. Empirical correlations between variability at an amino acid site and antibody reactivity are inconsistent

The primary hypothesis that mutations at naturally variable amino acid positions will cause greater changes in antibody reactivity than mutations at naturally invariant sites was not supported by the empirical data. Although the amino acids that are altered during antigenic variation have greater solvent accessibilities, are more hydrophilic, and have greater epitope propensities than invariant sites, experimentally mutating one residue at a variable site does not consistently result in a greater reduction in antibody reactivity than experimentally mutating a residue at an invariant site (Fig. 4A). In fact, several invariant amino acid residues have large impacts on antibody reactivity when mutated (Table 1).

Changes in antibody reactivity caused by each experimentally-introduced mutation varied considerably between the antisera from different rabbits, potentially resulting in the inconsistent correlations between antibody reactivity and solvent accessibility (Fig. 5). That is, a VlsE mutant that resulted in a large reduction in antibody reactivity in the antiserum from one rabbit might have only a moderate impact on antibody reactivity in the antiserum from a different rabbit (for example, L1M146i, Table 1). Nevertheless, the variation among technical replicates in which multiple aliquots of antiserum from the same rabbit were negatively purified using the same VlsE mutant protein was low (Table 1). These data suggest that even genetically identical animals develop considerably different immune responses to the same protein.

The hypothesis that the accessibility of an amino acid site for antibodies is positively correlated with antibody reactivity, which was supported by the computational analyses, was not statistically supported even among the naturally variable sites. Among variable sites, there is a positive but statistically insignificant correlation between accessible surface area and changes in antibody reactivity due to a mutation (Fig. 4B). This slight positive relationship between amino acid accessibility and antibody reactivity is apparent when pooling the data from antisera from both rabbits.

4. Discussion

Antigenic variation systems in pathogens generate divergent antigens in order to evade recognition by presently expressed host antibodies. Thus, naturally occurring changes in antigens are expected to occur at amino acid positions that are commonly and effectively targeted by antibodies in order to effectively reduce antibody reactivity [22, 23, 34, 35]. We found that the amino acid residues that are variable in the VlsE surface antigen of *B. burgdorferi* are those with properties associated with high antibody reactivity including larger accessible surface areas, higher hydrophilicity, and higher epitope propensity scores (Fig. 3). However, experimentally altering one variable amino acid residue in VlsE does not consistently cause a greater reduction in antibody reactivity than altering an invariant amino acid residue. Although the mechanism causing this discrepancy cannot be deduced from this exploratory study, our analyses suggest several biological and technical explanations.

The hypothesis that variable amino acids in VlsE have a greater effect on antibody reactivity is not statistically supported in the experimental data (Fig. 4). Despite limited surface

exposure and no potential to vary in the face of antibody pressure, many invariant amino acids have as great an effect on antibody reactivity as do variable sites (Table 1). Although contrary to the original hypothesis, these data could be explained by the structural changes that commonly occur due to mutations of buried amino acid residues. Altering naturally invariant amino acids, which have less surface exposure and are more hydrophobic, are expected to destabilize the protein structure [36] resulting in conformational changes that strongly affect antibody binding [37]. However, the effect of mutations at invariant amino acid sites on the conformational stability and function of VlsE is likely deleterious and will be selected against during the bacteria's natural transmission cycle [38], despite the potential advantages for antigenic escape. Therefore, the location of naturally variable sites may be driven more by functional constraints than by evasion of antibody recognition. Future investigations into the structural and molecular changes caused by mutating variable and invariant sites may identify targets of natural selection that have organized the locations and composition of residues at sites that are variable among the *vls* cassettes and may aid in determining the natural functions of the VlsE protein.

Among the variable amino acid sites, surface accessibility was weakly correlated with impacts on antibody reactivity, which is consistent with, and weakly supportive of, the hypothesis that exposed amino acids are more likely to be variable because they are more reactive to antibodies (Fig. 4B). The inconsistent experimental patterns in antibody reactivity changes may be caused by the stochasticity in immune development among animals (Fig. 5) or the limited effect of changing only one amino acid on antibody reactivity. Among individuals, variation in epitope preference is common (for example see [39]), suggesting that antisera from a large number of experimental animals may be necessary to empirically address this hypothesis.

Investigating changes in antibody reactivity by altering individual amino acids may have resulted in empirical patterns that were less consistent than computational predictions due to the limited impact of altering individual amino acid residues. An average of 5 to 89 *vlsE* nucleotides are altered during each recombination event during natural *B. burgdorferi* infections [33], suggesting that changing more than one amino acid residue may be necessary to achieve consequential reductions in antibody reactivity. In addition, replacing wild-type amino acids with the biochemically and structurally conservative residue, alanine, may have resulted in the insignificant patterns. Experimentally focal amino acids with the residues that are found in the naturally occurring VlsE variants may reveal a clearer pattern. The invariant sites, however, do not have alternative residues in naturally occurring VlsE variants, which restricted the choice of replacement amino acids to structurally conservative ones such as alanine. Further, the combination of altered amino acids often has an epistatic effect on antibody reactivity that would not be detected when single amino acids are altered [40]. Although changes in antibody reactivity caused by one amino acid substitution may be difficult to detect empirically, removal of the background noise by negative purification of antisera did enable precise estimates of changes in antibody reactivity (Fig. 2). Negative purification is a useful tool to increase the sensitivity of ELISA and other antibody-based immunoassays that can be coupled with semi-rational mutagenesis methods for high-throughput mapping of polyclonal epitopes. To account for these limitations, future studies should 1) use a larger number of animals for antibody production to account for the

stochasticity in immune development; 2) use a larger array of amino acid residues to replace the wild-type residues in order to increase the sensitivity of the experimental assays; and 3) use VlsE variants with mutations at multiple, adjacent amino acid sites to increase the effect size of the analyses. In addition, immunoassays using *B. burgdorferi* cells with mutated *vlsE* genes, instead of recombinantly expressed VlsE proteins, will improve the interpretation of the biochemical results in a physiological context.

Acknowledgments

We are very thankful to Steven Norris and Roland Dunbrack for assistance with the project, and to Junhyong Kim, Paul Sniegowski, Rahul Kohli and Timothy Linksvayer for helpful suggestions. This research was supported by Burroughs Wellcome Fund (1012376), an NIH training grant (T32 AI055400), and grants from the National Institute of Allergy and Infectious Diseases (AI076342 and AI097137).

References

1. Brunham RC, Plummer FA, Stephens RS. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. *Infect Immun*. 1993; 61:2273–6. [PubMed: 8500868]
2. Combes C. Fitness of parasites: pathology and selection. *Int J Parasitol*. 1997; 27:1–10. [PubMed: 9076524]
3. Deitsch KW, Moxon ER, Welles TE. Shared themes of antigenic variation and virulence in bacterial, protozoal, and fungal infections. *Microbiol Mol Biol Rev*. 1997; 61:281–93. [PubMed: 9293182]
4. Schmid-Hempel P. Immune defence, parasite evasion strategies and their relevance for ‘macroscopic phenomena’ such as virulence. *Philos Trans R Soc Lond B Biol Sci*. 2009; 364:85–98. [PubMed: 18930879]
5. Frank, SA. *Immunology and Evolution of Infectious Disease*. Princeton; (NJ): 2002.
6. Moxon ER, Rainey PB, Nowak MA, Lenski RE. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr Biol*. 1994; 4:24–33. [PubMed: 7922307]
7. van der Woude MW, Baumlér AJ. Phase and antigenic variation in bacteria. *Clin Microbiol Rev*. 2004; 17:581–611. table of contents. [PubMed: 15258095]
8. Frank SA. Models of parasite virulence. *Q Rev Biol*. 1996; 71:37–78. [PubMed: 8919665]
9. Levin BR, Bull JJ. Short-sighted evolution and the virulence of pathogenic microorganisms. *Trends Microbiol*. 1994; 2:76–81. [PubMed: 8156275]
10. Froissart R, Doumayrou J, Vuillaume F, Alizon S, Michalakakis Y. The virulence-transmission trade-off in vector-borne plant viruses: a review of (non-)existing studies. *Philos Trans R Soc Lond B Biol Sci*. 2010; 365:1907–18. [PubMed: 20478886]
11. Frank SA, Schmid-Hempel P. Mechanisms of pathogenesis and the evolution of parasite virulence. *J Evol Biol*. 2008; 21:396–404. [PubMed: 18179516]
12. Bankhead T, Chaconas G. The role of VlsE antigenic variation in the Lyme disease spirochete: persistence through a mechanism that differs from other pathogens. *Mol Microbiol*. 2007; 65:1547–58. [PubMed: 17714442]
13. Labandeira-Rey M, Skare JT. Decreased infectivity in *Borrelia burgdorferi* strain B31 is associated with loss of linear plasmid 25 or 28-I. *Infect Immun*. 2001; 69:446–55. [PubMed: 11119536]
14. McDowell JV, Sung SY, Hu LT, Marconi RT. Evidence that the variable regions of the central domain of VlsE are antigenic during infection with lyme disease spirochetes. *Infect Immun*. 2002; 70:4196–203. [PubMed: 12117928]
15. Purser JE, Norris SJ. Correlation between plasmid content and infectivity in *Borrelia burgdorferi*. *Proc Natl Acad Sci U S A*. 2000; 97:13865–70. [PubMed: 11106398]
16. Rogovskyy AS, Bankhead T. Variable VlsE is critical for host reinfection by the Lyme disease spirochete. *PLoS One*. 2013; 8:e61226. [PubMed: 23593438]

17. Zhang JR, Hardham JM, Barbour AG, Norris SJ. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. *Cell*. 1997; 89:275–85. [PubMed: 9108482]
18. Philipp MT, Bowers LC, Fawcett PT, Jacobs MB, Liang FT, Marques AR, et al. Antibody response to IR6, a conserved immunodominant region of the VlsE lipoprotein, wanes rapidly after antibiotic treatment of *Borrelia burgdorferi* infection in experimental animals and in humans. *J Infect Dis*. 2001; 184:870–8. [PubMed: 11550127]
19. Crother TR, Champion CI, Whitelegge JP, Aguilera R, Wu XY, Blanco DR, et al. Temporal analysis of the antigenic composition of *Borrelia burgdorferi* during infection in rabbit skin. *Infect Immun*. 2004; 72:5063–72. [PubMed: 15321999]
20. Liang FT, Yan J, Mbow ML, Sviat SL, Gilmore RD, Mamula M, et al. *Borrelia burgdorferi* changes its surface antigenic expression in response to host immune responses. *Infect Immun*. 2004; 72:5759–67. [PubMed: 15385475]
21. Zhang JR, Norris SJ. Genetic variation of the *Borrelia burgdorferi* gene *vlsE* involves cassette-specific, segmental gene conversion. *Infect Immun*. 1998; 66:3698–704. [PubMed: 9673251]
22. Graves CJ, Ros VI, Stevenson B, Sniegowski PD, Brisson D. Natural selection promotes antigenic evolvability. *PLoS Pathog*. 2013; 9:e1003766. [PubMed: 24244173]
23. Zhou W, Brisson D. Potentially conflicting selective forces that shape the *vls* antigenic variation system in *Borrelia burgdorferi*. *Infect Genet Evol*. 2014
24. Eicken C, Sharma V, Klabunde T, Lawrenz MB, Hardham JM, Norris SJ, et al. Crystal structure of Lyme disease variable surface antigen VlsE of *Borrelia burgdorferi*. *J Biol Chem*. 2002; 277:21691–6. [PubMed: 11923306]
25. Stave JW, Lindpaintner K. Antibody and antigen contact residues define epitope and paratope size and structure. *J Immunol*. 2013; 191:1428–35. [PubMed: 23797669]
26. Novotny J, Handschumacher M, Haber E, Brucoleri RE, Carlson WB, Fanning DW, et al. Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). *Proc Natl Acad Sci U S A*. 1986; 83:226–30. [PubMed: 2417241]
27. Thornton JM, Edwards MS, Taylor WR, Barlow DJ. Location of ‘continuous’ antigenic determinants in the protruding regions of proteins. *EMBO J*. 1986; 5:409–13. [PubMed: 2423325]
28. Parker JM, Guo D, Hodges RS. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. *Biochemistry*. 1986; 25:5425–32. [PubMed: 2430611]
29. Sun J, Xu T, Wang S, Li G, Wu D, Cao Z. Does difference exist between epitope and non-epitope residues? Analysis of the physicochemical and structural properties on conformational epitopes from B-cell protein antigens. *Immunome Research*. 2011; 7:11.
30. Haste Andersen P, Nielsen M, Lund O. Prediction of residues in discontinuous B-cell epitopes using protein 3D structures. *Protein Sci*. 2006; 15:2558–67. [PubMed: 17001032]
31. Cavallo L, Kleinjung J, Fraternali F. POPS: A fast algorithm for solvent accessible surface areas at atomic and residue level. *Nucleic Acids Res*. 2003; 31:3364–6. [PubMed: 12824328]
32. Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science*. 1989; 244:1081–5. [PubMed: 2471267]
33. Coutte L, Botkin DJ, Gao L, Norris SJ. Detailed analysis of sequence changes occurring during *vlsE* antigenic variation in the mouse model of *Borrelia burgdorferi* infection. *PLoS Pathog*. 2009; 5:e1000293. [PubMed: 19214205]
34. Ye J, Xu Y, Harris J, Sun H, Bowman AS, Cunningham F, et al. Mutation from arginine to lysine at the position 189 of hemagglutinin contributes to the antigenic drift in H3N2 swine influenza viruses. *Virology*. 2013; 446:225–9. [PubMed: 24074585]
35. Air GM, Laver WG, Webster RG. Mechanism of antigenic variation in an individual epitope on influenza virus N9 neuraminidase. *J Virol*. 1990; 64:5797–803. [PubMed: 1700825]
36. Pace CN, Fu H, Fryar KL, Landua J, Trevino SR, Shirley BA, et al. Contribution of hydrophobic interactions to protein stability. *J Mol Biol*. 2011; 408:514–28. [PubMed: 21377472]
37. Collawn JF, Wallace CJ, Proudfoot AE, Paterson Y. Monoclonal antibodies as probes of conformational changes in protein-engineered cytochrome c. *J Biol Chem*. 1988; 263:8625–34. [PubMed: 2454225]

38. Parsell DA, Sauer RT. The structural stability of a protein is an important determinant of its proteolytic susceptibility in *Escherichia coli*. J Biol Chem. 1989; 264:7590–5. [PubMed: 2651442]
39. Muhle C, Schulz-Drost S, Khrenov AV, Saenko EL, Klinge J, Schneider H. Epitope mapping of polyclonal clotting factor VIII-inhibitory antibodies using phage display. Thromb Haemost. 2004; 91:619–25. [PubMed: 14983240]
40. Mateu MG, Andreu D, Carreno C, Roig X, Cairo JJ, Camarero JA, et al. Non-additive effects of multiple amino acid substitutions on antigen-antibody recognition. Eur J Immunol. 1992; 22:1385–9. [PubMed: 1376255]

Figure 1. The 28 VlsE mutant proteins used in this study

A) Surface exposed Loop 1 and Loop 4 are highlighted in the structure of VlsE. B) The amino acids replaced by alanine in each of the mutant VlsE are shown in red. Sites coding for alanine in the wild-type protein were not altered. The amino acids that are naturally variable in surface exposed Loops 1 and 4 are shaded in the wild-type (WT) amino acid sequences. Mutant VlsE proteins are named after the location of the mutation in the wild-type protein. For example, the **M**utation in mutant protein L1M140i is at the 140th amino acid residue which is naturally invariable and falls in **L**oop 1.

Figure 2. Negative purification of antisera for high-sensitivity ELISA by adsorption with a VlsE mutant

A) Cross-reactive antibodies are removed by adsorption to the mutant VlsE. The quantities of remaining antibodies, which are not cross-reactive, are measured by ELISA using the wild-type antigen. B) Negative purification of antisera increases sensitivity in ELISA immunoassays. In the figure, antisera generated by rabbit II were progressively adsorbed with the wild-type or one of two VlsE mutants. Without negative purification (“0 Rounds”), antisera have high binding capacity to wild-type VlsE, the protein used to generate the polyclonal antisera. Negative purification with wild-type VlsE removes VlsE-specific

antibodies such that using wild-type VlsE as the target antigen in an indirect ELISA results in low reactivity (blue line). Negative purification of different aliquots of the same antisera using mutant L1M160i (red line) or L4M253i (yellow line) also removes many VlsE-specific antibodies. However, antibodies that do not bind to the mutant protein due to the single amino acid difference between the mutant and wild-type protein remain in the antiserum. Indirect ELISA using these negatively purified antisera results in much greater antibody reactivity with the wild-type VlsE. Repeated rounds of negative purification (“3 Rounds” and “6 Rounds”) result in progressively reduced standard error of the mean (error bars) in immunoassays, resulting in highly precise data.

Figure 3. Metrics of antigenicity are significantly greater at variable amino acid sites than at invariant sites

A) Solvent accessible surface areas of the variable amino acid sites are significantly greater than at the invariant sites in all six surface loops ($p < 0.05$). B) Even among sites that use the same amino acid, the solvent accessible surface area is greater at variable sites than invariant sites. The seven types of amino acids that are found in both the variable and invariant sites are shown. Hydrophilicity (C) and statistical epitope propensity (D) are also significantly greater at variable sites than at the invariant sites in all six surface loops ($p < 0.05$).

Figure 4. Changes in antibody reactivity caused by mutating a variable or an invariant amino acid residue

The hypothesis that mutations at naturally variable amino acids will cause greater changes in antibody reactivity than mutations at naturally invariant sites was not supported by the empirical data. Each mutant protein was used to negatively purify aliquots of antisera from each rabbit which was subsequently used in indirect ELISA assays with wild-type VlsE as the target antigen. The ELISA data for each mutant are normalized by data from ELISA assays that used antisera that was negatively purified by wild-type VlsE. Measurements greater than 1 indicate that the single amino acid mutation in the mutant protein reduces the

ability of some of the antibodies in the antisera to bind to the mutant. These changes could result from the antibodies not recognizing the new protein sequence, as expected due to mutations at the variable sites, or because the mutation caused a conformational change, as expected due to mutations at invariant sites. (A) The reduction in antibody reactivity due to a mutation at a naturally variable amino acid is not consistently greater than the reduction due to a mutation at a naturally invariant amino acid. (B) Change in antibody reactivity caused by changing a single residue at a variable site is weakly and insignificantly correlated with the solvent accessible surface area of the amino acid residue. Solvent accessible surface area of the mutated amino acid was calculated based on the published VlsE crystal structure using the POPS algorithm.

Figure 5. Immune reactivity to the same VlsE mutants was inconsistent among antisera raised in different rabbits

ELISA results are shown for each VlsE mutant reacting with negatively purified antisera generated from rabbit I and rabbit II. Each mutant protein was used to negatively purify aliquots of antisera from each rabbit which was subsequently used in indirect ELISA assays with wild-type VlsE as the target antigen, normalized by ELISA data using antisera negatively purified with wild-type VlsE.

Table 1

Antibody reactivity of mutated amino acid residues in 28 VlsE mutants.

Mutant	Solvent accessible surface area of the mutated residue (Å ²)	A450(mutant)/A450(wt) Rabbit I	A450(mutant)/A450(wt) Rabbit II
L1M140i	21.58	1.116±0.066	1.202±0.097
L1M141v	55.93	1.200±0.096	1.222±0.087
L1M142i	19.53	1.390±0.097	1.490±0.059
L1M143i	114.28	1.818±0.094	1.967±0.055
L1M146i	17.91	3.086±0.148	1.380±0.037
L1M147i	22.84	1.929±0.093	2.055±0.046
L1M148i	43.95	1.683±0.205	1.982±0.128
L1M149v	24.52	1.342±0.026	1.513±0.121
L1M150i	24.17	1.800±0.229	1.436±0.086
L1M152v	97.37	1.822±0.128	1.362±0.077
L1M154v	147.16	1.815±0.034	1.427±0.079
L1M157v	105.54	1.066±0.029	1.240±0.073
L1M158v	56.74	1.590±0.064	1.382±0.033
L1M160i	35.15	3.208±0.155	1.502±0.047
L1M161v	125.25	2.801±0.120	1.670±0.094
L1M163i	11.86	1.204±0.025	1.362±0.062
L1M164i	13.54	2.760±0.142	1.340±0.096
L1M165v	62.07	1.476±0.126	1.806±0.180
L4M243v	151.12	1.101±0.116	1.667±0.254
L4M244i	42.81	1.034±0.128	1.559±0.216
L4M245v	77.87	1.104±0.063	1.494±0.185
L4M246i	21.14	1.208±0.042	1.570±0.097
L4M247v	68.7	1.769±0.088	1.344±0.022
L4M248i	43.6	1.315±0.048	1.657±0.153
L4M249i	13.01	1.692±0.069	1.842±0.161
L4M250v	36.63	1.268±0.052	1.396±0.071
L4M251v	83.14	2.863±0.269	1.502±0.085
L4M253i	76.93	1.373±0.147	1.415±0.079