

The Many Faces of *Borrelia burgdorferi*

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Abstract

In this review we describe several genetic regulatory mechanisms adopted by the agent of Lyme disease, *Borrelia burgdorferi*, to sense and adapt to different host and environmental conditions either *in vitro* or *in vivo*. This regulation results in the increased or decreased synthesis of several proteins whose levels are believed to play key roles in the ability of *B. burgdorferi* to cycle between both arthropod and mammalian hosts. Moreover, the differential synthesis of these proteins serves to modulate the response of *B. burgdorferi* to signals in the requisite host and may also, in some cases, function as virulence determinants of this spirochete. Elucidation of these mechanisms will help in the understanding of the pathogenicity of *B. burgdorferi* as well as aid in identifying proteins that are important during different stages of infection.

Introduction

Lyme disease was first reported as an outbreak of juvenile rheumatoid arthritis (Steere *et al.*, 1977) and is a multi-systemic, zoonotic illness that is present in most temperate parts of the Northern hemisphere. The etiologic agent is now referred to as the *Borrelia burgdorferi* sensu lato complex and comprises an expanding group of closely related *Borrelia* spp. (Barbour and Fish, 1993; Postic *et al.*, 1994). While the isolates from North America are predominantly grouped as *B. burgdorferi* sensu stricto, European and Asian isolates also include two additional species, *B. garinii* and *B. afzelii* (Baranton *et al.*, 1992). These are members of a growing cluster of closely related species that presently include *B. andersonii*, *B. japonica*, *B. valaisiana*, *B. lusitanae*, *B. turdae*, *B. tanukii*, and *B. bissettii* sp. nov. (Casjens *et al.*, 1995; Casjens *et al.*, 2000; Wang *et al.*, 1999). For this review most studies are restricted to *B. burgdorferi* sensu stricto, designated as *B. burgdorferi*, unless otherwise indicated.

Lyme disease is endemic in areas where there is a close ecological interaction between competent enzootic vectors, the spirochete and reservoir hosts. Transmission to humans is predominantly by *Ixodes scapularis* in the Eastern United States, *Ixodes pacificus* in Western United States, while *Ixodes ricinus* and *Ixodes persulcatus* are the primary

vectors in Europe and Eurasia, respectively. Since all borrelial species are host-propagated bacteria (*i.e.*, they do not survive naturally in water, soil and are not transmitted by aerosol or fecal contamination) that shuttle between a vertebrate and an arthropod host, these spirochetes have developed strategies to sense and survive in these diverse environments (Barbour and Hayes, 1986). This is achieved by altering the level of gene expression in response to changes in temperature, pH, salts, and other host dependent factors. *B. burgdorferi* has a unique genome composed of a 910 kb linear chromosome and 21 different plasmids (9 circular and 12 linear) (Casjens *et al.*, 2000) some of which may function as mini-chromosomes as seen in segmented genomes (Barbour, 1993). Some of the plasmids have been shown to be non-essential for *in vitro* propagation, yet required for normal infectivity, indicating a significant plasticity of its genome and implying that such a dynamic arrangement may be related to the ability of *B. burgdorferi* to occupy environments as disparate as a tick and a warm-blooded mammal (Casjens *et al.*, 2000). In addition, *B. burgdorferi* contains multiple related genes termed paralogues (Fraser *et al.*, 1997) encoding proteins that share significant similarity at the amino acid level with presumably similar functions that may help to compensate for the loss of some plasmids due to selective pressures encountered during infection. Alternatively, the genetic redundancy observed may provide a battery of genes that encode antigenically variable proteins with compensatory functions which are sequentially expressed via reversible phase variation or modified further by recombination. By assessing the mechanism of gene expression in response to different growth and survival conditions encountered by *B. burgdorferi*, it may be possible to better understand the adaptive mechanisms of this spirochete and perhaps more clearly define molecules associated with the pathogenesis of Lyme disease.

Temperature

Many pathogens have been shown to exhibit a coordinately regulated synthesis of virulence determinants in response to environmental signals (Mekalanos, 1992; Miller *et al.*, 1989). This coordinate gene regulation allows for pathogens to survive, replicate and complete their life cycles in different environmental niches as well as in different intermediate and terminal hosts. In this section we review the effect of temperature on the ability of *B. burgdorferi* to survive and grow in the host and speculate as to how these spirochetes change according to the thermal homeostasis of its hosts.

Heat Shock Response

Temperature is an important environmental cue that requires a rapid response. Dramatic increases in temperature mediate a regulatory cascade known as the heat shock response which has been extensively studied and the subject of many detailed reviews (Yura *et al.*, 1993). In addition to the typical heat shock response, temperature changes are also known to regulate virulence determinants

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in a number of different pathogens (Konkel and Tilly, 2000). Regulation of virulence determinants in response to varying temperatures would presumably be crucial for a pathogen like *B. burgdorferi*, which cycles between ticks (ambient temperature) and mammalian hosts (greater than or equal to 37°C), to complete its life cycle. Sequence analysis of the genome of *B. burgdorferi* (Fraser *et al.*, 1997) has identified a set of homologues of heat shock response genes (*groES*, *groEL*, *grpE*, *dnaJ*, *dnaK*, *hslU*, *hslV*, and *hspG*) and several of them have been characterized (Cluss and Boothby, 1990). Although *B. burgdorferi* does not have a homologue of heat shock response regulator sigma 32 (σ -32), this spirochete does exhibit a heat shock response upon exposure to elevated temperature (Cluss and Boothby, 1990; Scopio *et al.*, 1994). Thermoregulation in *B. burgdorferi* was assessed using either one or two-dimensional gel electrophoresis following radiolabeling of

B. burgdorferi proteins (Carreiro *et al.*, 1990). Steady state level accumulation, as well as turnover of such proteins, was also determined (Carreiro *et al.*, 1990). Using this approach, Cluss *et al.* also showed that there was an up-regulation of DnaK (Hsp70; ~72 kilodalton [kDa]) and three additional heat shock proteins (Hsp's) of 39, 27 and 21 kDa in molecular mass (Cluss and Boothby, 1990; Cluss *et al.*, 1996). Heat shock proteins have also been shown to play a role as chaperonins in most living systems, and, in the case of *B. burgdorferi*, in the molecular processing of endoflagellin critical for motility (Scopio *et al.*, 1994). In this study, a 70 kDa heat shock protein (Hsp70 or DnaK) bound endoflagellin at all temperatures tested between 33 and 41°C in the presence of ATP; however, the binding of a 60 kDa heat shock protein (Hsp60 or GroEL) to endoflagellin was independent of ATP levels at all temperatures studied. Though these interactions were

Table 1. *In vivo* Differential Gene Expression in *Borrelia*¹

Genetic Modulation ²	Signal ³	Host	<i>Borrelia</i> Strains ⁴	Description of Phenomenon	References
OspC ↑	Tick blood meal	Mice	B31	Antibodies to OspC in Lyme disease patients.	Schwan <i>et al.</i> , (1995)
Osp A ↑, OspB ↑	Pre-engorgement	Tick	B31	Absence of antibodies to OspAB in initial stages of human infection.	Schwan <i>et al.</i> , (1995)
OspC ↓				Antibodies to OspC absent in mice infected with spirochetes from unfed ticks.	
OspC ↑	Post-engorgement	Tick	B31	OspC upregulation mimicked with increased temperature/blood meal.	Schwan <i>et al.</i> , (1995);
Dbp A/B ↑	Active infection	Mice	B31	OspC upregulation mimicked by pH ↓.	Carroll <i>et al.</i> , (2000).
<i>p21</i> ↑	Active infection	Mice	N40	Antisera from DbpA immunized mice borrelidical against blood-phase spirochetes.	Hanson <i>et al.</i> , (1998); Cassatt <i>et al.</i> , (1998).
<i>pG</i> ↑	Active and experimental infection	Mice	ZS7	Antibodies to P21 observed at 3 weeks post infection in mice and in sera of Lyme arthritis patients. Antibodies to P21 not seen on injection with inactivated <i>B. burgdorferi</i> .	Suk <i>et al.</i> , (1995); Das <i>et al.</i> , (1997).
Expressed genes: <i>flaB</i> , <i>dbpA</i> , <i>p21</i> , <i>erpD</i> , <i>gene1</i> , <i>gene2</i> , <i>bba64</i> , <i>bba65</i> , <i>bba66</i>	Active infection	Mice	cN40	Antibodies to pG in sera of Lyme disease patients; anti-pG antibodies will not react with <i>in vitro</i> -propagated spirochetes.	Wallich <i>et al.</i> , (1995).
Except for <i>flaB</i> all other genes listed for cN40 not expressed.	Active infection	Mice	cN40-75	Differential expression of genes under <i>in vivo</i> growth of cN40 (low passage) analyzed by RT-PCR.	Anguita <i>et al.</i> , (2000).
<i>p35</i> ↑, <i>p37</i> ↑	Active infection	Mice	cN40	Passage 75 of cN40 (high passage) is infectious but not pathogenic as several genes are repressed under <i>in vivo</i> growth. Correlation of pathogenicity to <i>in vivo</i> expression of genes.	Anguita <i>et al.</i> , (2000).
OspE/F-related, (Erp) ↑	Active infection	Mice	B31	Antibodies to P35, P37 only in active infections in mice; in early and late stage Lyme disease patients.	Fikrig <i>et al.</i> , (1997).
<i>eppA</i> ↑	Active infection	Rabbits	B31	Antibodies to OspE/F-related (Erp) proteins in mice after infection with tick bite, OspE/F antibodies in a subset of Lyme disease patient sera.	Nguyen <i>et al.</i> , (1994), Stevenson <i>et al.</i> , (1998a)
<i>lp6.6</i> ↓	Chronic infection	Mice, Monkey	297	Antibodies to EppA in patients with Lyme disease.	Champion <i>et al.</i> , (1994).
OspC ↑, P21 ↑	Dialysis membrane chamber (DMC)	Rat	297	Mice and rhesus monkeys do not develop antibodies against lp6.6 after chronic infection.	Lahdenne <i>et al.</i> , (1997).
OspE/F ↑				Antibodies against OspC, P21, OspE/F in several hosts after active infection; no antibody response to lp6.6. DMC mimics host-adapted state and up-regulated determinants indicated are in response to growth in DMC. For response to other signals, refer to text.	Akins <i>et al.</i> , (1998).
2.9-7LpA/B ↑					
P22 ↓					
<i>lp6.6</i> ↓					
OspA/B ↓, Bbk2.10 ↓					

¹Genes or proteins expressed differentially on cultivation of *B. burgdorferi* strains under *in vivo* growth (in comparison to *in vitro* growth) in different hosts or host-adapted conditions. The list does not include genes and proteins expressed differentially in response to varying environmental *in vitro* growth conditions such as temperature, pH, or nutrients. Refer to the text for additional information.

²The upward and downward arrows indicate increased or decreased expression or synthesis of the corresponding genes or proteins, respectively.

³Only broadly defined experimental conditions are mentioned. Infection of mice by ticks or with infectious *B. burgdorferi* is referred to as active infection. Refer to the text for details.

⁴Designations are given for strains belonging to the *B. burgdorferi* sensu stricto group.

analyzed under *in vitro* conditions, the role of chaperonins in the processing of borrelial endoflagellins is likely to be similar to what has been observed with other bacterial systems and the molecular interactions of Hsp60/Hsp70-endoflagellin may facilitate export of endoflagellin subunits across the cytoplasmic membrane as well as other protein species associated with *B. burgdorferi* pathogenesis.

OspA/OspC Switch

Heat shock proteins modulate the host's response to a rapid increase in temperature, however they are not the only class of proteins up-regulated under these conditions. In *B. burgdorferi*, for example, outer surface protein A (OspA), but not OspC (Schwan *et al.*, 1995), is expressed in the tick midgut prior to feeding. However, once ticks have fed to repletion there is an up-regulation of OspC and a down regulation of OspA (see Table 1). Even though this differential expression of OspC was partly mimicked in response to *in vitro* growth of *B. burgdorferi* at 32 or 37°C instead of 24°C, the lower levels of OspC induced by temperature induction alone demonstrated that other factors in the blood are required for maximal OspC expression (Schwan *et al.*, 1995). These studies revealed that different environmental cues, like tick midgut temperature and their contents following feeding, could serve as signals to alter gene expression in *B. burgdorferi*. The derepression of *ospC* and concomitant repression of *ospA* was also observed upon co-cultivation of *B. burgdorferi* with mammalian cells at different ambient temperatures (Obonyo *et al.*, 1999). The down regulation of *ospA* was not observed when the spirochetes were cultivated in axenic medium at 37°C indicating that a combination of additional host factors that are not present in the *in vitro* cell culture, presumably limited metal ion availability, contribute to the differential expression of *ospA* and *ospC*. The presence of antibodies specific to OspC during the initial stages of human infection indicated that OspC is expressed during transmission from the tick vector and the resulting primary infection (Aguero-Rosenfeld *et al.*, 1993; Engstrom *et al.*, 1995; Fung *et al.*, 1994; Padula *et al.*, 1993). Moreover, *Borrelia* extracted from the unfed ticks are noninfectious, indicating that there is a correlation of *ospC* expression and mammalian infection (Piesman, 1993). Recent studies on temporal changes in the levels of OspA and OspC indicated that OspC synthesis occurred during the first 48 hours of attachment in feeding *Ixodes scapularis* ticks (Schwan and Piesman, 2000). In contrast, tick larvae and uninfected nymphs that fed to repletion on infected mice acquired *B. burgdorferi* cells with higher levels of OspA and little or no OspC. These results, taken together, suggest that the OspA/OspC switch in *B. burgdorferi* correlates with an increase in spirochetal infectivity for mammalian hosts and implies that the OspC switch observed is involved in the transmission from ticks to mammals, but is not involved in the movement of *B. burgdorferi* from the reservoir host to the naïve tick vector.

OspE/F-Related (Erp) Proteins

In addition to OspC, immunoblotting of bacterial lysates with sera from infected mice indicated that several additional proteins were induced when *B. burgdorferi* cultures were shifted from 23°C to 35°C (see Table 1; Stevenson *et al.*, 1995). A couple of these proteins were identified as OspE (a 19 kDa, surface-exposed lipoprotein)

and OspF (a 27 kDa lipoprotein) which are co-expressed in an operon (Lam *et al.*, 1994). Antibodies against the aforementioned temperature-regulated antigens were prevalent in the serum of patients with late stage Lyme disease. Furthermore, *B. burgdorferi* were substantially destroyed in ticks that engorged on either OspE- or OspF-immunized mice indicating that these antigens were expressed in the ticks and could be useful targets for spirochete killing (Nguyen *et al.*, 1994). Apart from OspE/F, additional homologous antigens in *B. burgdorferi* strain B31 were also identified that mapped to the 32 kilobase circular plasmids (cp32 plasmids) and a closely related 56 kb linear plasmid (lp56) (Casjens *et al.*, 1997; Stevenson *et al.*, 1998a; Stevenson *et al.*, 1996).

Low passage, infectious *B. burgdorferi* strain B31 contain cp32 in seven copies and all, along with lp56, contain at least one *ospE/F*-related or *erp* family gene (Casjens *et al.*, 2000; Casjens *et al.*, 1997). This degree of plasmid-associated genetic redundancy is unique and implies that there could be an evolutionary advantage in having several copies of related plasmids in low copy number as opposed to having a single plasmid in high copy number. One possible explanation for this may be that OspE/F-related (Erp's) proteins are antigenically distinct yet retain an as yet unknown compensatory function(s) that is (are) required for normal transmission and infectivity. Additional studies indicated that homologues of the OspE/F family (Erp proteins) are up-regulated both transcriptionally and translationally when low passage *B. burgdorferi* strain B31 cultures are shifted from 23°C to 35°C (Stevenson *et al.*, 1998a). Several of the OspE/F related (Erp) proteins from B31 isolates were recognized by both sera from Lyme disease patients and tick-infected mice (Stevenson *et al.*, 1998a). Since the OspE/F family (Erp) of proteins are induced in response to a temperature increase and appear to be antigenic during the early stages of mammalian infection, these proteins may also play a role in the transmission of *B. burgdorferi* from ticks to mammals. Several authors have speculated that the presence of numerous *ospE/F*-related (*erp*) genes on the different cp32 plasmids and lp56 may provide a mechanism to avoid immune clearance (Das *et al.*, 1997; Marconi *et al.*, 1996) akin to variable membrane proteins (Vmp proteins) of the relapsing fever *Borrelia* (Barbour *et al.*, 1982). The presence of cross-reactive OspE/F-related protein (Erp) antibodies during the early stages of infection that may neutralize these proteins expressed at later stages of infection, coupled with the lack of structurally distinct promoter elements that may aid in the temporal expression of *ospE/F*-related (*erp*) genes, would seem to contradict the aforementioned hypothesis regarding immune evasion. Nevertheless, the ubiquity of the plasmids carrying the *ospE/F*-related (*erp*) genes suggest that they may be important in the natural life cycle of *B. burgdorferi* (Casjens *et al.*, 1997). Further, the evidence of past recombination events at *ospE/F* loci (*erp*) suggests that there are possibly other subtle but unknown mechanisms that allow the spirochete to exploit the heterogeneity of the *ospE/F* (*erp*) paralogues (Stevenson *et al.*, 1998a; Stevenson *et al.*, 1998b). Along these lines, Sung *et al.*, have recently shown that *ospE/F*-related genes are subject to antigenic variation during infection in the mouse (Sung *et al.*, 2000), not unlike the other *B. burgdorferi* antigenic variant *vlsE* (Zhang *et al.*, 1997).

Other Infection Associated Antigens

More recently, it has been shown that the infection-associated antigen VraA (virulent strain associated repetitive antigen A), whose gene (*bbi16*) maps to the linear plasmid 28-4 (lp28-4) (Skare *et al.*, 1999), was up-regulated *in vitro* at 37°C relative to growth at either 32°C or 23°C in *B. burgdorferi* strain B31 (M. Labandeira-Rey and J. T. Skare, unpublished observations). Interestingly, VraA contains a unique 9 amino acid structure repeated 21 consecutive times that may confer adhesin-like properties with increased avidity for its target due to its repetitive structure. However, this hypothesis remains to be determined.

Another set of temperature regulated proteins are the decorin binding proteins A and B (DbpA and DbpB) (Cassatt *et al.*, 1998). These borrelial proteins are adhesins that bind decorin, a proteoglycan associated with type III collagen (Guo *et al.*, 1995). The skin is the primary site of infection and since collagen is abundant at this locale, the up-regulation of these adhesins in response to temperature would presumably increase the avidity of *B. burgdorferi* to decorin, aiding in colonization at this site. It remains to be seen if individual mutations in *dbpA* or *dbpB*, or a double mutant in *dbpAB*, will result in the loss of adherence of *B. burgdorferi* to decorin and whether this loss of binding correlates with a reduction in infectivity.

Even though it is possible to observe the *de novo* synthesis of some *B. burgdorferi* proteins under controlled experimental conditions (like temperature), the mechanism(s) by which these genes are regulated is not yet known. Moreover, the regulatory mechanism(s) involved in the OspA/OspC switch may either be mediated by a global effector or sensitive to many different environmental signals independent of temperature. As the genetic tools become available for making knockout mutations in infectious isolates, it will be critical to use isogenic mutants to assess the requirement of a defined gene (or genes) for infectivity in experimental models of Lyme borreliosis. Furthermore, the global effects of temperature on gene expression seen in other bacteria (Richmond *et al.*, 1999) will be facilitated when DNA microarrays of the entire *B. burgdorferi* genome become readily available.

pH and Nutrients

In addition to temperature, there are additional environmental signals that are unique to both the tick and mammalian hosts that *B. burgdorferi* must adapt to transiently. These include differences in the pH, osmolarity and availability of nutrients within these diverse microenvironments that *B. burgdorferi* inhabits. For example, prior to a blood meal, *B. burgdorferi* reside within the midgut of *Ixodes* ticks where the pH is alkaline (Munderloh and Kurtti, 1995). Upon exposure to the tick blood meal, *B. burgdorferi* is transmitted into the mammalian host where the pH encountered is approximately 7.4. Rapid adjustment to the approximate 100-fold change in proton concentration (*i.e.*, pH 9.5 to 7.4) would thus be a critical compensatory mechanism that provides an evolutionary advantage to *B. burgdorferi*. When the spirochetes are subjected to similar changes in the pH under *in vitro* growth conditions, there is either an up-regulation or exclusive expression of at least six membrane proteins at pH 6.0 or 7.0 but not at pH 8.0 (Carroll *et al.*,

1999). One of the major proteins that is down-regulated at a more alkaline pH (pH 8.0) is OspC, whose synthesis is decreased 10-fold under these conditions (Table 1). Conversely, there is an up-regulation of a 42 kDa protein at pH 8.0 when compared to pH 6.0 or 7.0. The reduction in the levels of OspC at alkaline pH is consistent with the previous observations that *ospC* is down regulated in the midgut of unfed ticks (Schwan *et al.*, 1995). It has also been suggested that the expression of *ospC* could be under coordinate regulation of both pH and temperature and, in part, explain the lack of *ospC* up-regulation in the midguts of infected unfed ticks exposed to higher temperature alone (Schwan *et al.*, 1995). A total of 37 changes to membrane protein profiles were observed by two-dimensional nonequilibrium pH gradient gel electrophoresis (2D-NEPHGE) analysis when *B. burgdorferi* cells were incubated at pH 6.0, 7.0 or 8.0. (Carroll *et al.*, 1999), suggesting that there is an extensive adaptive mechanism in *B. burgdorferi* in response to changes in pH that accompanies mobilization of the spirochetes from the midgut to the salivary glands and ultimately to the mammalian host. In addition to pH, there may be other uncharacterized factors that aid in the migration of *B. burgdorferi* from ticks to mammals. The migration of spirochetes within the tick vector is blocked if the ticks are fed on mice immunized with OspC, suggesting that OspC is involved in movement of *B. burgdorferi* from the midgut to the salivary glands (Gilmore and Piesman, 2000). Anti-OspC antibodies in the blood meal in conjunction with other unknown factors/nutrients were able to down regulate *ospC* expression even though these same OspC antibodies were not borrelidical under *in vitro* conditions (Gilmore and Piesman, 2000). It is tempting to speculate that *B. burgdorferi* may be able to sense the status of the mammalian host based on the contents of the blood meal and alter its gene expression in order to either escape killing in the midgut or migrate to the salivary gland for transmission to the mammalian host.

In addition to the ability of spirochetes to rapidly adapt to changes in temperature and pH, the differences in the nutrient contents of the tick and mammalian hosts may also play a role in borrelial survival in these disparate environments. Interestingly, the levels of purines in the tick may be sufficient for DNA synthesis in *B. burgdorferi* through the use of purine salvage pathway (Munderloh and Kurtti, 1995). Unlike other prokaryotes, *Borrelia* species carry genes involved in purine synthesis (*guaA* and *guaB*) on plasmids (the 26 kb circular plasmid [cp26] in *B. burgdorferi*) instead of the chromosome (Margolis *et al.*, 1994). The presence of these genes would allow for *de novo* purine biosynthesis and as such, would presumably be beneficial in terms of survival within mammalian host inasmuch as the levels of extracellular purines and pyrimidines are extremely low (Margolis *et al.*, 1994). Although it is not known if there is a preferential up-regulation of *gua* genes when *B. burgdorferi* shuttles between ticks and mammals, there is evidence that the copy number of a *B. hermsii* linear plasmid is lower in rich medium rather than in mice (Kitten and Barbour, 1992). Environmental levels of guanine may regulate *guaA* and *guaB* genes and the requirement of these genes for *de novo* purine synthesis may be in part responsible for the maintenance of the cp26 plasmid despite extensive *in vitro* cultivation and passage of *B. burgdorferi* (Barbour, 1988; Hinnebusch and Barbour, 1992; Schwan *et al.*, 1988).

In vivo Versus in vitro* Gene Expression in *B. burgdorferi

The fate of pathogenic bacteria to infect and adapt to the demanding conditions imposed by eukaryotic hosts is dependent on the ability of the bacterium to modulate gene expression in a manner appropriate for colonization and survival. Numerous studies have shown that gene expression *in vitro* is distinct from gene expression *in vivo* in a wide variety of pathogenic bacteria (Chiang *et al.*, 1999; Mahan *et al.*, 1993; Shea *et al.*, 1996; Valdivia and Falkow, 1997). Over the past several years, various studies have shown that *B. burgdorferi* is also subject to differential gene expression *in vivo* relative to *in vitro* cultivation, indicating that *B. burgdorferi* responds to host specific factors to express genes accordingly (Akins *et al.*, 1998; Schwan *et al.*, 1995; Suk *et al.*, 1995). Along these lines, Barthold and colleagues demonstrated that mice immunized with OspA, a protein known to be down-regulated in infected mammals, were resistant to needle inoculated *B. burgdorferi* yet were sensitive to challenge with transplanted skin from mice infected with *B. burgdorferi* (Barthold *et al.*, 1995). This result indicated that *B. burgdorferi* within the host did not express *ospA* and was consistent with the previous observation that infected humans have little or no antibody titer to OspA during early infection (Barthold *et al.*, 1995). Golde and co-workers also demonstrated that a passage 6 isolate of *B. burgdorferi* strain B31, when maintained in the natural zoonotic cycle of transmission between laboratory mice and laboratory-reared *Ixodes* ticks, exhibited limited genetic and antigenic variation after 5 cycles of transmission (Golde and Dolan, 1995). This study, along with others, (Barbour, 1988; Norris *et al.*, 1995; Schwan *et al.*, 1988), revealed that changes in the plasmid profiles of *B. burgdorferi* correlate with a change in infectivity in animal models of Lyme borreliosis. These results suggest that genes on plasmids contribute either directly or indirectly to the infectious phenotype and indicate that there is a tolerance for a dynamic genome, at least *in vitro*. Recent studies have shown that plasmid content also varies from clonal isolates obtained from infected mouse tissue, indicating that plasmid heterogeneity is also tolerated *in vivo* (M. Labandeira-Rey and J. T. Skare, unpublished observations; R. T. Marconi, personal communication).

Selection in the Arthropod and Mammalian Hosts

Sequential passage of *B. burgdorferi* between either an arthropod vector or a mammalian host indicated that selective pressures imposed by each eukaryotic host results in a population of spirochetes with a defined, unique phenotype (Ryan *et al.*, 1998). The transition of *B. burgdorferi* from one host to another produced a striking series of alternating phenotypic signatures resulting in the reciprocal expression of different antigenic forms of OspB and OspC. Sequence analysis of the *ospB* and *ospC* genes derived from both mammal and tick clonal isolates revealed two allelic forms of *ospC*. Interestingly, one form of *ospC* was specific for mammals and the other for ticks. Though there was no dramatic change in the amino acid sequence encoded by the *ospB* alleles, there was a marked difference in the antigenicity of the OspC variants as determined by differential reactivity with an OspC monoclonal antibody. These observations suggest the possibility of

recombinatorial activation, reminiscent of *Neisseria gonorrhoeae* pilin antigenic variation (Seifert, 1996), to generate a full-length allelic variant of *ospC*. In addition to these reciprocal antigenic variations, Ryan and co-workers also observed a differential selection for the 9.0 kilobase supercoiled plasmid in the tick isolates (Ryan *et al.*, 1998). Taken together, these data indicate that there is a selection for specific *B. burgdorferi* populations during *in vivo* propagation of this spirochete through the arthropod vector and mammalian host resulting in distinct antigenic changes. These changes could contribute to functions selected for within the different hosts, via recombinational mechanisms, similar to what is seen in several other eukaryotic and prokaryotic pathogens (Pays, 1991; Reeder and Brown, 1996; Restrepo and Barbour, 1994; Seifert, 1996).

***In vivo* Expressed Antigens**

An exported plasmid protein A (EppA) encoded on the 9.0 kb circular plasmid (cp9 according to TIGR; Fraser *et al.*, 1997) of *B. burgdorferi* was the first protein purported to be induced preferentially during the infection of the mammalian host (Champion *et al.*, 1994). This interpretation was based on the paradoxical observation that EppA was antigenic in some patients with Lyme disease and in rabbits experimentally infected with *B. burgdorferi* strain B31, but did not appear to be expressed by *in vitro* grown *B. burgdorferi* (see Table 1). Similar results were obtained for a genetic locus designated Ip6.6, encoding a 6.6 kDa lipoprotein, which was not antigenic in either chronically infected rhesus monkeys or mice, implying that this gene was not expressed in either warm-blooded species (Lahdenne *et al.*, 1997), not unlike *ospA* (Montgomery *et al.*, 1996; Schwan *et al.*, 1995). Based on these observations, the authors speculated that Ip6.6 was expressed preferentially in the tick vector; however, to date, no tick-specific transcript or protein species for Ip6.6 has been detected.

Subsequently, Suk *et al.* used sera from mice infected with *B. burgdorferi* to identify genes expressed only in the host (Suk *et al.*, 1995). Several genes (*p21*, *p35* and *p37*) that were specifically expressed *in vivo* were identified in this screen (Table 1). Further characterization of these genes revealed that *p21* encoded a 20.7 kDa protein that was closely related to, but distinct from, *ospE* while *p35* and *p37* each encoded for *B. burgdorferi* lipoproteins. Later studies indicated that P35 and P37 (now designated as BBK32 and BBK50 by TIGR, respectively (Fraser *et al.*, 1997)), elicited protective immunity in mice (Fikrig *et al.*, 1997). Additional experimentation has shown that *p21* is specifically derepressed *in vivo* and under host-adapted conditions described below (Akins *et al.*, 1998). Studies on the temporal expression of *p21* in mice infected with *B. burgdorferi* indicated that *p21* mRNA and anti-P21 antibodies were detectable at 21-28 days post infection, whereas expression of *ospE* mRNA and anti-OspE antibodies were present at day 7 (Das *et al.*, 1997). Even though *p21* and *ospE* are homologous genes, there are well defined signals, as yet unknown, that regulate their expression. While *ospE* is expressed in both the mammalian host and arthropod vector, *p21* is expressed only in the mammalian host and, moreover, appears to be constitutively expressed for several weeks following infection (Das *et al.*, 1997). Therefore, spirochetes from engorged ticks appear to differ from spirochetes in

vertebrate hosts, and the differential expression of related homologues in different hosts and at different times of infection may allow for successful colonization and dissemination of *B. burgdorferi*. A differential immunoscreening strategy was also used to identify a 22 kDa lipoprotein related to OspF, designated pG, induced *in vivo* in *B. burgdorferi* strain ZS7 (Table 1; Wallich *et al.*, 1995). Inasmuch as pG is an *ospE/F*-related locus, like p21, pG may also exhibit delayed expression. More importantly, Sung *et al.* have recently demonstrated that OspE/F-related proteins (Erp's) are subject to antigenic variation only *in vivo* (Sung *et al.*, 2000), implying that these additional homologues (*i.e.*, P21, pG) may contribute to immune evasion (see below for details).

Additional evidence for tissue specific differential gene expression comes from the study of Fikrig *et al.*, who showed that p35 and p37 specific mRNA were detected in the erythema migrans biopsy specimens from 2 human patients and in the synovium of patients with Lyme arthritis (Fikrig *et al.*, 1998). In contrast, mRNA encoding *ospA* was undetectable in these tissues, consistent with the known repression of *ospA* in mammalian infection (Akins *et al.*, 1998; Barthold *et al.*, 1995; Montgomery *et al.*, 1996; Schwan *et al.*, 1995).

Another strategy to identify genes uniquely expressed in infectious *B. burgdorferi* involved using antiserum from infection immune rabbits that was adsorbed against non-infectious *B. burgdorferi* strain B31 (Skare *et al.*, 1999). A total of 18 different immunoreactive phage clones from an expression library containing DNA from infectious *B. burgdorferi* were characterized. Sequence analysis indicated that these 18 clones were defined by 9 genetic loci. All of the 9 genes mapped to plasmids and included decorin binding proteins A and B (*dbpAB*), a *rev* homologue present on the 9 kb circular plasmid (cp9), a *rev* homologue from 32-kb circular plasmid (cp32-6), *erpLM*, *erpX* and 4 previously uncharacterized loci designated *bbi16*, *bbk19*, *bbj34* and *bbk45* by TIGR (Fraser *et al.*, 1997). Since these antigens were identified using serum from infection immune rabbits (Foley *et al.*, 1995), these antigens may represent targets for killing antibody that are preferentially expressed in the mammalian host (*i.e.*, a rabbit), and by analogy to *dbpAB* identified in this screen, may be involved in pathogenic mechanisms. Along these lines, *dbpAB* and *vraA* (*bbi16*) appear to be temperature inducible loci (see above section, "Other Infection Associated Antigens"). Furthermore, VraA, like DbpA, provides protection against infectious challenge in the mouse model of Lyme borreliosis (Cassatt *et al.*, 1998) (M. Labandeira-Rey and J. T. Skare, unpublished observations) and, in the case of DbpA, can confer passive immunity (Table 1; Hanson *et al.*, 1998) confirming that *dbpA* is expressed during infection.

Dialysis Membrane Chambers (DMC's): Host-Adapted Spirochetes

Determination and comparison of mRNA and protein levels induced under *in vitro* and *in vivo* conditions has also been used in several infectious disease models to identify genes and their products that are critical for conferring virulence (Finlay and Falkow, 1997). In an attempt to apply such an approach to *B. burgdorferi*, dialysis membrane chambers (DMC's) containing strain 297 spirochetes were implanted into the peritoneal cavities of rats (Akins *et al.*, 1998). This approach eliminates the limitations inherent to studying

host-adapted *B. burgdorferi*, notably the inability to recover spirochetes from infected tissues in appreciable numbers. These host-adapted spirochetes exhibited profound and reversible alterations when compared with *in vitro* cultivated *B. burgdorferi* grown at either 23°C, 34°C, or 37°C (see Table 1). While OspA and Lp6.6 were not expressed in host-adapted spirochetes, there was a dramatic increase in levels of OspC and P21 consistent with previous results indicating that these proteins are preferentially repressed or derepressed during mammalian infection, respectively (Lahdenne *et al.*, 1997; Schwan *et al.*, 1995; Suk *et al.*, 1995). OspE, OspF, and an OspF-related protein termed Bbk2.11, were produced at similar levels in either *in vitro* or host-adapted organisms. A considerably lower amount of these proteins were made when *B. burgdorferi* was cultivated at 23°C as opposed to 34°C indicating that, as seen in previous *in vivo* studies, OspE, OspF, or Bbk2.11 were not synthesized to the level observed for P21. These observations are difficult to reconcile inasmuch as p21 and related *ospE/F* loci (*erp* genes) contain nearly identical sequences upstream from their translational start codon, termed upstream homology boxes (UHB). As such, one would predict that these loci would be coordinately regulated. Clearly other factors, perhaps involving transcriptional activators that bring unlinked upstream regions of DNA into apposition via DNA bending, like the AraC protein in *Escherichia coli* (Lee and Schleif, 1989), may explain this apparent regulatory dilemma.

Differences in gene expression between *in vitro* grown and DMC grown spirochetes were further analyzed by differential display RT-PCR and this analysis revealed that two additional borrelial lipoproteins, designated 2.9-7lpA and 2.9-7lpB, are transcriptionally linked and induced in chamber grown spirochetes (Akins *et al.*, 1998). These related lipoproteins were previously shown to belong to a paralogous family of differentially expressed genes located at a loci termed 2.9 that is found on homologous 32 kilobase circular plasmids of *B. burgdorferi* strain 297 (Porcella *et al.*, 1996). These observations indicated that differential gene expression of *B. burgdorferi* within a mammalian host could be simulated in implanted DMC's. These studies have provided a foundation for assessing *in vivo* expression in mammalian hosts, yet have some clear limitations.

By analogy with other pathogens, it is conceivable that differential gene expression may involve contact dependent interaction of *B. burgdorferi* with host cells, something that cannot be mimicked by this procedure. Furthermore, detection of proteins (or transcripts) low in abundance that may be important in the regulation of the observed response have yet to be identified using this methodology. Clearly once the genetic tools become available to make stable isogenic mutants in infectious *B. burgdorferi*, identification of the genes and their products that are essential for normal infectivity will no longer be limited to issues pertaining to biochemical sensitivity.

Antigenic Variation, Diversity and Cloaking

Antigenic variation of virulence determinants has been shown to be an important, rapid adaptation strategy that pathogens have evolved to escape and survive detrimental host immune responses. Antigenic variation could be confined to regions of protein(s) that are critical for host-pathogen interactions and changes acquired through

several genetic mechanisms may result in hypervariable regions in these molecules. A complex mechanism of antigenic variation was first characterized in *Borrelia* species using the relapsing fever spirochete *Borrelia hermsii* as a model (Barbour, 1990; Barbour *et al.*, 1983; Barbour *et al.*, 1982; Kitten and Barbour, 1990; Plasterk *et al.*, 1985). Surface exposed lipoproteins termed variable major proteins (Vmp proteins) are encoded by homologous genes located on the 28-32 kb linear plasmid with covalently closed telomere-like sequences (Kitten and Barbour, 1990). Each organism has at least 40 *vmp* genes most of which are located in the storage plasmids in the silent or unexpressed form (Hinnebusch *et al.*, 1998). Only one *vmp* is expressed in each organism (Barbour *et al.*, 1991; Restrepo and Barbour, 1994; Restrepo *et al.*, 1994) and antigenic variation is generated when the expressed *vmp* is replaced partially or completely by one of the silent *vmp* genes. Several genetic mechanisms, including interplasmic recombination (Barbour *et al.*, 1991; Plasterk *et al.*, 1985), intraplasmic recombination (Restrepo *et al.*, 1994), and post-switch rearrangement (Restrepo and Barbour, 1994), have been attributed to the antigenic variation. Antigenic variation in relapsing fever *Borrelia* occurs at a frequency of 10^{-3} to 10^{-4} per generation in *B. hermsii* (Stoenner *et al.*, 1982) during both *in vitro* cultivation and *in vivo* indicating that immune selection does not mediate *vmp* recombination.

The VlsE Antigenic Variant

A similar yet distinct genetic system that promotes antigenic variation was reported for *B. burgdorferi* at a genetic loci called *vls* for *vmp*-like sequence that closely resembles the *vmp* system of *B. hermsii* (Zhang *et al.*, 1997). A *vls* expression site (*vlsE*) and 15 additional silent *vls* cassettes were identified on a 28 kilobase linear plasmid, subsequently designated as lp28-1 (Fraser *et al.*, 1997). The presence of lp28-1 correlates with a high infectivity phenotype in *B. burgdorferi* and the *vlsE* locus, located near a telomere of lp28-1, encodes a surface-exposed lipoprotein which exhibits promiscuous recombination upon infection of mice (Norris *et al.*, 1995; Zhang *et al.*, 1997). VlsE variants exhibited differential reactivity to antiserum generated against the parental *vls1* cassette region, providing evidence of antigenic variation at this loci in a mammalian host which could facilitate evasion of the host immune response. This mechanism is not unique to *B. burgdorferi* in that several pathogens utilize genetic variation in multi-gene families as a way to escape the host response due to the resulting antigenic variation (Borst *et al.*, 1995). The genetic variation at the *vlsE* site in *B. burgdorferi* is similar to the combinatorial recombination of the pilin-encoding genes of *Neisseria gonorrhoeae* (Seifert and So, 1988) inasmuch as it involves recombination to generate antigenic variants. However, unlike most antigenically variable loci, *vlsE* only exhibits variation *in vivo* indicating that the signal for recombination is host specific (Zhang *et al.*, 1997).

The mechanism of genetic variation induced at the *vlsE* locus has been shown to occur through an unidirectional process where segments of the silent *vls* cassettes recombine in the *vlsE* cassette region without affecting the sequence and organization of the silent *vls* loci (Zhang *et al.*, 1997; Zhang and Norris, 1998). This strategy allows *B. burgdorferi* to have a nearly inexhaustible

reservoir of *vlsE* sequence variation much like the repertoire of mammalian germline immunoglobulin and T cell receptor loci. The observation that *vlsE* has been found in numerous human and tick isolates suggests that antigenic variation at the *vlsE* locus is important, in part, for *B. burgdorferi* pathogenesis (Iyer *et al.*, 2000). It should be re-emphasized that although both *B. burgdorferi* and *B. hermsii* exhibit antigenic variation, the *vlsE* variation in *B. burgdorferi* occurs only under *in vivo* selection.

The OspE/F-Related (Erp) Antigenic Variants

Recently, the *ospEF*-related gene family of *B. burgdorferi* has also been shown to be antigenically variable (Sung *et al.*, 2000). The antigenic variation is presumably mediated by DNA repeats flanking the hypervariable domain (Sung *et al.*, 2000). As with *vlsE*, recombination at the *ospEF* alleles occurs only during infection and not during *in vitro* cultivation, suggesting again that the mammalian environment leads to or selects for the accumulation of the observed genetic changes. The genetic redundancy of the plasmid components of the *Borrelia* genome (Fraser *et al.*, 1997) could serve as an excellent substrate for recombination and rearrangements and this, coupled with differential gene expression strategies, may allow *B. burgdorferi* to escape the host immune clearance mechanisms leading to chronic, persistent infection associated with Lyme borreliosis. It is tempting to speculate that additional mechanisms, such as phase variation, may be operative in *B. burgdorferi* to evade host defenses and would partly explain the retention of genetic redundancy one observes within the *B. burgdorferi* genome sequence. The plethora of paralogous gene families identified in *B. burgdorferi* may be a gene organization pattern where individual members are sequentially expressed in order to not only maintain a compensatory function but to also provide a unique antigenic target that prevents clearance of the spirochetes by the host immune response. Further studies to address this hypothesis appear to be warranted and may explain the advantage of the redundancy and plasticity of the borrelial genome.

Plasticity of the B. burgdorferi Genome

In order to establish a link between pathogenicity and infectivity of *B. burgdorferi*, the role of several known differentially expressed determinants were examined after infection of C3H/HeN mice with either an attenuated or infectious isolate of *B. burgdorferi* strain N40. Anguita *et al.*, showed that strain N40 passage 75 (N40-75) isolate was capable of infecting C3H/HeN mice but did not cause arthritis and carditis as seen with infection by a low-passage clonal isolate of strain N40 (designated cN40) (Anguita *et al.*, 2000). N40-75 did not express *dbpAB* (*bba24/25*; *bb* designations assigned by TIGR (Fraser *et al.*, 1997)), *bba64*, *bba65*, *bba66*, *p21*, *erpD*, and loci designated *gene-1* and *gene-2*, whereas cN40 expressed all of these loci except *p21*, consistent with its delayed expression *in vivo* (Das *et al.*, 1997). Presumably N40-75 lacks a genetic component (*i.e.*, plasmid(s)?) that precludes this isolate from expressing these *in vivo* genes, thereby adapting to the mammalian host in a manner commensurate with the infectious cN40 isolate. Although the authors determined that the protein and antigenic profiles were essentially the same for cN40 and N40-75, they did not exhaustively account for the loss of plasmid species which may

contribute to the defect in adaptation observed. Along these lines, several investigators have shown that the loss of plasmids in *B. burgdorferi* correlates with a loss in infectivity (Schwan *et al.*, 1988; Barbour, 1988; Norris *et al.*, 1995), suggesting that a subset of genes on the lost plasmids contribute to pathogenesis. With the advent of the *B. burgdorferi* genome sequence, it is now possible to identify which plasmids are present in clonal isolates through the use of oligonucleotide primers specific for each plasmid coupled with PCR. Subsequently, these clones can then be tested for infectivity deficits in animal models of Lyme borreliosis. It is possible that the loss of plasmid(s) not only results in the direct loss of genes and their products, but may also lead to more indirect effects that manifest as differential expression defects, *i.e.*, inability to express a subset of antigens and/or ectopic expression of antigens in mutant (plasmidless) isolates, due to the loss of a global regulatory locus. To date, no studies pertaining to *B. burgdorferi* either support or refute this hypothesis.

Plasminogen Binding/Activation

In addition to changing its antigenic face, *B. burgdorferi* also cloaks itself with host factors. *B. burgdorferi* binds to these host factors at the primary site of infection, namely the skin, and uses them to penetrate normal tissue barriers such as vascular basement membranes and other organized extracellular matrices. Specifically, *B. burgdorferi* uses endogenous or host derived factors, particularly proteinases, to penetrate to other tissues of the mammalian hosts (Coleman *et al.*, 1995; Fuchs *et al.*, 1994; Klempner *et al.*, 1995). A 70 kDa protein and, to a lesser extent, OspA of *B. burgdorferi* bind to plasminogen resulting in the activation of plasmin in the presence of host-derived plasminogen activator (Hu *et al.*, 1995; Hu *et al.*, 1997). The binding of this host derived proteinase (plasminogen) has been proposed to aid in the dissemination of the spirochete (Coleman *et al.*, 1995; Fuchs *et al.*, 1996; Klempner *et al.*, 1995) and could contribute to cloaking *B. burgdorferi* such that it would be hidden from the host immune system. As such, this interaction could potentially contribute to the pathogenesis of *B. burgdorferi* and facilitate persistent infection due to immune evasion mechanisms.

Concluding Remarks

The survival and transmission of *B. burgdorferi* in tick and mammalian hosts impose conditions that require these spirochetes to express genes in accordance with their new microenvironments. The ability of these spirochetes to quickly adapt is essential for survival of *B. burgdorferi* and highlights their dynamic nature. Although details pertaining to the biochemical and genetic regulatory pathways that allow for this differential gene expression are unknown, a number of determinants that are either de-repressed or repressed have been identified in conjunction with the signal(s) that is (are) most predominant in inducing these proteins. In addition to variations in environmental conditions encountered by the spirochetes relative to the arthropod and mammalian hosts, it is becoming apparent that tissue tropism and the eventual pathogenesis of *B. burgdorferi* may very well depend on the temporal expression of host-adapted genes. It is tempting to speculate that the two-component regulatory systems

identified in the genome sequence may be involved in sensing and modulating gene expression in order to adapt to the host. The role of antigenic variation in *B. burgdorferi* has been established, however the potential for phase variation and the mechanisms mediating such a response have yet to be determined. Identification of such variable determinants may shed light on additional escape variants selected for by the host and would help explain the chronic infection associated with Lyme borreliosis. Recent advances in gene knockout systems for *B. burgdorferi* (Bono *et al.*, 2000; Elias *et al.*, 2000; Rosa *et al.*, 1996; Tilly *et al.*, 1997), as well as the wealth of genome sequence information available, should aid in defining the host-spirochete interactions at the molecular level and define which loci are involved in the pathogenesis of *B. burgdorferi*.

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