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CHAPTER 4.3

The Genus *Borrelia*

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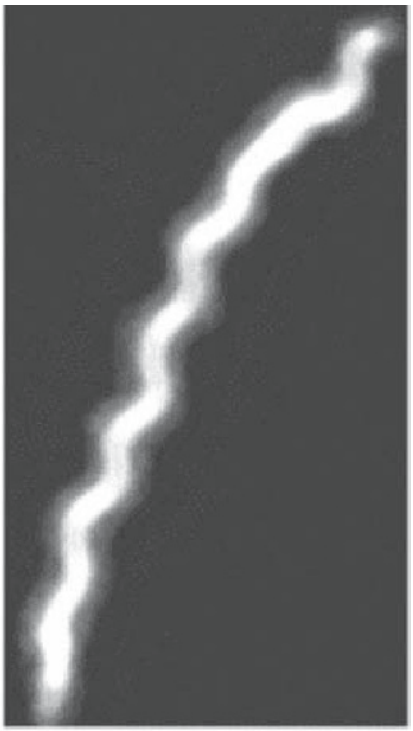
Phylogeny

The inability to cultivate many spirochetes, including the borreliae, at one time significantly hindered the placement of these organisms into a taxonomic scheme based on traditional biochemical methodologies. As a result, these organisms were initially grouped based primarily on their common helical or spiral shape (Fig. 1). The advent of contemporary ribosomal RNA cataloging has facilitated the division of the spirochetes into three phylogenetic families, the Spirochaetaceae (including the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, *Spironema* and *Treponema*), the Brachyspiraceae (including the genus *Brachyspira* [*Serpulina*]), and the Leptospiraceae (including the genera *Leptospira* and *Leptonema*; Thomas et al., 2001). All species in the genus *Borrelia* are transmitted to vertebrates by hematophagous (blood-feeding) arthropods. In most cases, the differentiation of borreliae species was based on 1) identification of the specific vector that transmits the spirochete, 2) the vertebrate host (humans, animals or birds), and 3) the variable infectivity of isolated borreliae for different species of laboratory animals (Davis, 1956; Felsenfeld, 1971; Burgdorfer, 1976a; Table 1). To date, thirty species of *Borrelia* have been recognized (Thomas et al., 2001). The principal species of *Borrelia* and their primary vectors are listed in Table 1. Additional information on *B. burgdorferi* sensu lato strains is also available: (http://www.pasteur.fr/recherche/borrelia/Bb_ strains_alphabetic.html.) With the exception of louse-borne relapsing fever, all of the borrelioses are zoonoses.

Borreliae, like most spirochetes, were historically considered to be Gram negative because of their double-membrane structure (Fig. 6), but as stated above, genetic analyses have placed them, along with the other spirochetes, into a separate eubacterial phylum (Paster et al., 1991). Ultrastructural (*Borrelia* Molecular Architecture), molecular, and biochemical studies also have emphasized the wide taxonomic gap between spirochetes and Gram-negative bacteria. Most notably, *Borrelia burgdorferi*, and presumably all *Borrelia* spp., does not contain lipopolysaccharide (LPS; Takayama et al., 1987). In addition to lacking LPS, borrelial outer membranes differ from those of Gram-negative bacteria in that they 1) exhibit considerably greater fluidity, 2) are more susceptible to physical manipulations (i.e., centrifugation, washing and resuspension; Brusca and Radolf, 1994; Cox et al., 1994; Cox et al., 1996), 3) are more easily solubilized in very dilute detergents than are the membranes of Gram-negative bacteria (Barbour and Hayes, 1986b; Brusca and Radolf, 1994; Cox et al., 1994; Cox et al., 1996), and 4) have a much lower density of transmembrane proteins (Walker et al., 1991; Radolf et al., 1994b; Radolf et al., 1995b). (For excellent reviews of *Borrelia* morphology and ultrastructure, see Hovind-Hougen, 1976, Barbour, 1989, Hayes and Burgdorfer, 1993, and Radolf, 1994a.)

Habitat

All known *Borrelia* species have an inherent requirement for a competent (often genospecies-specific) arthropod vector and vector-suitable mammalian host(s) to be maintained in their natural enzootic cycles (Table 1). Three types of arthropod vectors have been associated with transmission of *Borrelia*: the human body louse (Fig. 2), the argasid or soft tick (Fig. 3), and the ixodid or hard tick (Fig. 4). Numerous mammalian hosts appear to be involved in maintaining *Borrelia* within a population, with the primary reservoir usually being a small mammal. The coincident geographic distribution of both of these elements is thought to be foremost in establishing and perpetuating a given *Borrelia* species within a locale. This point is clearly demonstrated by Lyme borreliosis; the geographic distribution of its two arthropod vectors (Fig. 15), *Ixodes scapularis* and *I. pacificus*, closely mirrors that of the disease (Orloski et al., 2000; Fig. 16).



Darkfield Image of Spirochete

Fig. 1. *Borrelia burgdorferi* strain 297, a cerebrospinal isolate (Steere et al., 1983) visualized by darkfield microscopy. Micrograph image kindly provided by Ken Bourell, University of Connecticut Health Center, Center for Microbial Pathogenesis (cmp.uchc.edu).

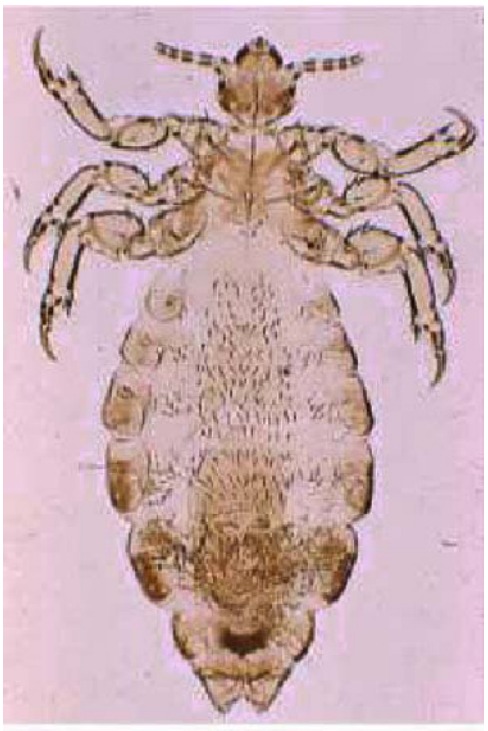


Image of a body louse

Fig. 2. Human body louse (*Pediculus humanus*). Image kindly provided by Stephen Wikel, Ph.D., University of Connecticut Health Center, Center for Microbial Pathogenesis (cmp.uchc.edu).

Presence in Lice

Borrelia recurrentis is the etiological agent of louse-borne (epidemic) relapsing fever and is the only species of borreliae that is louse-transmitted (Table 1). The human is the only known reservoir of this spirochete. The human body louse, *Pediculus humanus* subsp. *humanus* (Fig. 2), becomes infected after feeding on a spirochetemic individual and remains infected during its life span of approximately 10–61 days. The ingested borreliae enter the louse's midgut where, within several days, they penetrate the gut epithelium to gain entrance to the hemolymph where they multiply. Infection of the mammalian host is thought to occur when spirochetes, liberated when the lice are crushed on the skin by scratching, enter abraded skin or mucous membranes. Since body lice prefer normal body temperature to the higher temperatures of patients with acute relapsing fever, they are predisposed to seek an afebrile person after feeding on a febrile individual. This behavior results in the rapid transmission of infection during epidemics.

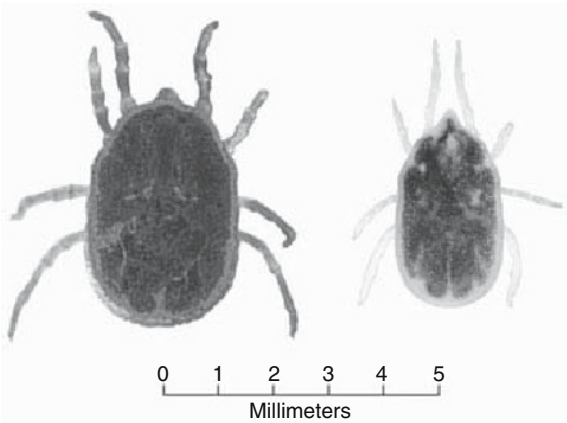


Fig. 3. Soft tick (*Ornithodoros* spp.). Image kindly provided by Julie Rawlings, MPH, Texas Department of Health (http://www.tdh.state.tx.us/yellow/r.htm).

Presence in Soft Ticks

All of the other pathogenic borreliae are parasites of either soft or hard ticks (Table 1). All tick-borne borrelial infections, with the possible exception of infections by *Borrelia duttonii*,

Table 1. Characteristics of *Borrelia* species of medical and veterinary significance.

Species	Vector	Animal Reservoir	Disease	Geographic distribution
B. recurrentis	*Pediculus humanus* subsp. *humanus*	Humans	Louse-borne relapsing fever	Worldwide
B. duttonii	*Ornithodoros moubata*	Humans	Old World tick-borne relapsing fever	Central, eastern and southern Africa
B. hispanica	*O. marocanus*	Rodents	Old World tick-borne relapsing fever	Spain, Portugal, Morocco, Algeria, Tunisia
B. crocidurae, *B. merionesi*, *B. microti*, *B. dipodilli*	*O. erraticus*	Rodents	Old World tick-borne relapsing fever	Northern and eastern Africa, Near and Middle East, southeastern Europe
B. persica	*O. tholozani*	Rodents	Old World tick-borne relapsing fever	Middle East, Greece, Central Asia, Russia, western China
B. caucasica	*O. verrucosus*	Rodents	Old World tick-borne relapsing fever	Iraq, eastern Europe
B. latyschewii	*O. tartakowskyi*	Rodents	Old World tick-borne relapsing fever	Iraq, Iran, Afghanistan, Russia
B. coriaceae	*O. coriaceus*	Rodents	Epizootic bovine abortion	California
	Alectorobius sonrai		Tick-borne relapsing fever	Western Africa
B. hermsii	*O. hermsi*	Rodents, chipmunks, tree squirrels	New World tick-borne relapsing fever	Canada, western USA
B. turicatae	*O. turicata*	Rodents	New World tick-borne relapsing fever	Southwestern USA
B. parkeri	*O. parkeri*	Rodents	New World tick-borne relapsing fever	Western USA
B. mazziottii	*O. talaje (O. dugesi)*	Rodents	New World tick-borne relapsing fever	Southwestern USA, Mexico, Central and South America
B. venezuelensis	*O. rudis*	Rodents	New World tick-borne relapsing fever	Central and South America
B. venezuelensis	*O. rudis*	Rodents	New World tick-borne relapsing fever	Central and South America
B. burgdorferi sensu lato	*Ixodes scapularis*	Rodents, Lagomorpha, Deer, Fowl	Lyme (borreliosis) disease	Midwestern and eastern USA
	I. pacificus			Western USA
	I. ricinus			Europe
	I. persulcanis			Asiatic Europe, China, Japan
B. lonestari	*Amblyomma americanum*	Rodents	Lyme disease-like illness	Southern USA
B. theileri	*Rhipicephalus evertsi*, *Boophilus microplus*, *B. annulatus*, *B. decoloratus*	Cattle, Horses, Sheep	Bovine borreliosis	South Africa, Australia, Brazil, Mexico
B. anserina	*Argas persicus* and other *Argas* spp.	Fowl	Avian borreliosis	Worldwide

Adapted from Barbour (1986), used with permission.

Image of Hard Ticks

Fig. 4. From left to right: The deer tick (*Ixodes scapularis*) adult female, adult male, nymph, and larva on a centimeter scale. Image taken from the Centers for Disease Control and Prevention (CDC) Lyme Disease Home Page (http://www.cdc.gov/ncidod/dvbid/lymeinfo.htm).

involve humans as an accidental host. In East Africa, *B. duttonii* is carried by the domestic soft tick, *Ornithodoros moubata*, and the reservoir for this spirochete appears to be the human population. The relapsing fever spirochetes are the primary borreliae transmitted by the soft tick (Fig. 3). The numerous species of *Borrelia* are responsible for the tick-borne relapsing fevers and are distributed worldwide in both tropical and temperate climates (Table 1). Soft ticks of the genus *Ornithodoros* serve as the primary vectors of these relapsing fever spirochetes, with wild rodents being the major reservoir. Occasionally, lizards, toads, turtles and owls can also serve as competent reservoirs for these spirochetes.

Soft ticks become infected while obtaining a blood meal from a spirochetemic animal. Following ingestion of a blood meal on an infected mammal, *Borrelia* rapidly multiply within the tick. Within hours, a generalized infection ensues such that spirochetes can be found in a variety of tissues, including the salivary glands, the coxal glands on the legs, and the ovaries. Consistent with the presence of spirochetes in the ovaries, the female *Ornithodoros* spp. tick, unlike the body louse, can transmit borreliae transovarially to their progeny. Humans and animals become infected while the tick is feeding, through infectious saliva, excrement, and/or coxal fluid entering via the bite wound or skin. The presence of the *Ornithodoros* spp. vector on humans often goes unnoticed since this vector 1) typically feeds at night, 2) usually completes its feeding in 5 to 20 minutes, and 3) does not cause pain. Soft ticks are particularly durable vectors as they are able to survive for months to years without a blood meal. The spirochetes are similarly able to survive in the ticks for extended periods of time. In addition, the spirochetes can be maintained within the vector during the transition from one developmental stage to another (e.g., larva to nymph to adult).

Presence of *Borrelia* in Hard Ticks

The Ixodidae (hard) ticks are the primary vectors of *B. burgdorferi*, the etiological agent of Lyme disease (Table 1). The association between *B. burgdorferi* and *I. scapularis*, the vector in the northeastern and north-central United States (Fig. 4), has been studied extensively (Burgdorfer et al., 1982; Burgdorfer et al., 1989b; Spielman et al., 1984; Benach et al., 1987; Ribeiro et al., 1987; Piesman, 1989; Dolan et al., 1998; Humair and Gern, 2000). This relationship is similar to that occurring between the European Lyme disease (Table 5) spirochetes and ticks of the *Ixodes ricinus* complex. The larval and nymphal forms of *I. scapularis* commonly become infected with *B. burgdorferi* when obtaining a blood meal from infected rodents, with the white-footed mouse, *Peromyscus leucopus*, being the primary reservoir (Table 2). A variety of birds, in addition to small mammals, may also serve as a reservoir for the Lyme disease spirochete (Anderson and Magnarelli, 1984). One recent study suggests that latent *Borrelia burgdorferi* sensu lato infection in migratory birds (i.e., redwing thrush) may be reactivated by the stress associated with migration. Many of these birds are infested with ixodid ticks and, subsequently, may serve as an efficient mechanism of spreading ticks carrying this pathogen over long distances (Gylfe et al., 2000).

Recent studies using *B. burgdorferi*-infected *I. pacificus* nymphs fed on the western fence lizard (*Sceloporus occidentalis*) suggest that lizard serum contains a thermolabile, borreliacidal factor, probably a protein, that destroys spirochetes in the midgut of feeding *I. pacificus* ticks. The presence of lizards in the enzootic cycle may help to reduce the force of transmission of *B. burgdorferi* by diverting a competent vector tick from an alternative reservoir-competent host (i.e., the dusky-footed wood rat or the California kangaroo rat; Lane and Quistad, 1998).

In contrast to the soft tick- (see Presence in Soft Ticks) and louse-borne (see Presence in Lice) relapsing fever *Borrelia* spp., *B. burgdorferi* remains in the midgut of the hard tick (Fig. 4) until the next blood meal (Ribeiro et al., 1987; Burgdorfer, 1989a), at which time it penetrates the basal membrane of the midgut, invades the hemolymph and various tissues, including the salivary glands, and is transmitted to the host via infectious saliva (Schwan, 1996). Recent studies suggest that the differential expression of lipo-

Table 2. *Borrelia* species associated with Lyme borreliosis, and their ecological and pathogenic characteristics, and their geographic distribution.

Taxon	Vector	Animal host	Human Disease	Distribution
B. burgdorferi sensu lato	*I. scapularis*	Mammals, birds	EM, **arthritis**, carditis, neuroborreliosis	United States
	I. pacificus			
	I. ricinus			Europe
	I. persulatus?			Asia?
B. garinii	*I. ricinus*	Birds, small	EM, arthritis, **neuroborreliosis**	Europe
	I. persulatus	mammals		Asia
B. afzelii	*I. ricinus*	Small mammals	EM, arthritis, neuroborreliosis, **ACA**	Europe
	I. persulatus			Asia
B. japonica	*I. ovatus*	Small mammals	No	Japan
B. miyamotoi	*I. persulatus*	Small mammals	Unclear	Japan
B. valaisiana	*I. ricinus*	Birds	Unclear	Europe
	I. granulatus			Asia
B. lusitaniae	*I. ricinus*	Unknown	No	Central Europe
B. andersonii	*I. dentatus*	Rabbit	No	United States
B. bissettii sp. nov.	*I. pacificus*	Rodents, birds	Unclear	United States
	I. neotomae			
	I. scapularis			
	I. ricinus		EM, lymphocytoma	Slovenia
B. takunii	*I. takunus*	Small mammals	No	Japan
B. turdi[b]	*I. turdus*	Small mammals	No	Japan

[a]The clinical syndrome associated with distinct *B. burgdorferi* sensu lato species are in boldface type.
[b]The name has been corrected from *B. turdae* (Fukunaga et al., 1996) by International Union of Microbiological Societies (1998).

proteins (see Differential Gene Expression) on the surface of the spirochete in response to the blood meal facilitates migration from the midgut to the salivary glands and subsequent transmission to the mammalian host (Schwan et al., 1995; de Silva et al., 1996; Ohnishi et al., 2001). Transovarial transmission by *Ixodes* spp. ticks is thought to occur infrequently. For a review comparing spirochete transmission by hard and soft ticks, see Schwan (1996).

Isolation

The study of *Borrelia* was greatly facilitated by the development of a culture medium by Kelly that would support the growth of the spirochetes (Kelly, 1971). In 1982, Stoenner enriched this formulation by adding yeast extract and tissue culture medium (referred to as "fortified Kellys medium"; Stoenner et al., 1982). Subsequent modifications by Barbour (1984a) resulted in Barbour-Stoenner-Kelly (BSK I) medium, which has allowed the isolation of borreliae from a variety of sources (Barbour, 1986a). For historical reviews of the cultivation of *Borrelia* spp., see Barbour and Hayes (1986b), Barbour (1984a), and Anderson and Magnarelli (1992). The successful cultivation of *Borrelia* spp. is usually dependent on the quality of the animal serum or albumin used in the preparation of the medium (Callister et al., 1990). These reagents are thought to provide necessary long-chain fatty acids, but other, as yet unidentified, trace components may also be important. A commercial source for this medium has now become available (http://www.sigma-aldrich.com, Sigma-Aldrich, BSK [catalog # B 8291]). Semisolid medium is prepared by the addition of 100 ml of sterile 1.89% agarose (dissolved in boiling water) to the BSK I recipe given above to yield BSK II. The agarose solution (cooled to 55°C) is added just prior to dispensing medium. Several methods have been described for plating *Borrelia* as a surface lawn (Kurtti et al., 1987) or by subsurface plating (Samuels et al., 1994a).

Isolation of *Borrelia* from Ticks

The hemolymph and most tissues of *Ornithodoros* spp. ticks can be used as a source of the relapsing fever borreliae and *B. coriaceae*. In contrast, *B. burgdorferi* is usually restricted to the midgut in flat (unfed) *Ixodes* ticks. *Borrelia burgdorferi* has been successfully cultured from the midguts of live, infected *Ixodes* nymphs and adults by crushing the entire contents of the midgut in a sterile Petri dish or on a glass slide, mixing the contents with a small amount (0.5 ml) of BSK II (Table 3) medium, transferring the contents to a sterile screwcap microcentrifuge tube containing an additional 1 ml of BSK II medium and then incubating at 30–35°C for 7–21 days. Cultures should be checked every sev-

Table 3. Liquid BSK II medium for growth of *Borrelia* spp. (Barbour, 1984a).

1. To 900 mls of glass-distilled water, add the following:	
HEPES (Sigma)	6.0 g
Neopeptone (Difco)	5.0 g
Sodium citrate	0.7 g
Glucose	5.0 g
Sodium bicarbonate	2.2 g
TC Yeasolate (Difco)	2.5 g
Sodium pyruvate	0.8 g
N-acetyl glucosamine (Sigma)	0.4 g
Bovine serum albumin, fraction V	50.0 g
2. Adjust pH of medium at room temperature to 7.6 with 1N NaOH.	
3. Sterilize by filtration (0.2 μm).	
4. Add 100 mls of sterile 10× CMRL 1066 without glutamine (Gibco/BRL).	
5. Add 200 mls of sterile 7% gelatin (Difco), which has been dissolved in boiling water.	
6. Add 64 mls of sterile, heat-inactivated rabbit serum (Pel-Freeze Biologicals, Inc).	
7. Dispense to glass or polystyrene tubes or bottles.	
8. Fill to 90% capacity and cap tightly.	
9. Store complete medium at 4°C.	

eral days for spirochetes by microscopy. Owing to their slender shape, they are best visualized by darkfield microscopy (Fig. 1). *Borrelia* also can be isolated from *Ornithodoros* spp. and *Ixodes* spp. tick tissues using BSK II (Table 3) medium.

Isolation of *Borrelia* from Patient Specimens

The *Borrelia* strains causing relapsing fever (Table 1) are typically present in high numbers circulating in the blood of patients during acute (febrile) phases of this disease. The tick-borne relapsing fever spirochete, *B. hermsii*, is readily cultivated from patient samples, primarily blood. Several reports also have described the cultivation of the louse-borne relapsing fever spirochete, *B. recurrentis* (Cutler et al., 1997; Cutler et al., 1999), and the tick-borne relapsing fever spirochete, *B. duttonii* (Cutler et al., 1999), from the blood of spirochetemic patients. Both of these types of relapsing fever spirochetes may be isolated from blood by inoculating one to two drops per 10 ml of liquid or semisolid BSK II (Table 3) media. Cutler et al. (1999) suggest that the critical factor in determining the success of cultivation of *B. duttonii* is the quality of the albumin used in preparation of the BSK II (Table 3) medium; over two-thirds of the batches of this critical medium ingredient tested were rejected because they failed to support growth of the relapsing fever spirochete. Larger tissue and organ samples (bladder, heart, spleen and joint) can be cultured by homogenization in liquid BSK II (Table 3) medium (10% wt/vol) with a Stomacher Lab Blender (Tekmar, Cincinnati, OH). Larger debris is allowed to settle and serial dilutions (10-fold) of the supernatant are made in the semisolid BSK II (Table 3) medium. Cultures are incubated at 30–35°C and examined by darkfield microscopy for spirochetes at weekly intervals.

In contrast to in relapsing fever patients, relatively low densities of spirochetes are present in the blood and tissues of Lyme disease patients. Nevertheless, numerous laboratories have reported the successful isolation of *B. burgdorferi* from skin, blood and other tissues obtained from Lyme disease patients. Spirochetes are most readily cultured from biopsy specimens of the skin lesion of erythema migrans (EM; Fig. 20) and acrodermatitis chronicum atrophicans (ACA; Berger et al., 1985; Berger et al., 1992; Schwartz et al., 1992; van Dam et al., 1993; Nadelman et al., 1996; Picken et al., 1998). *Borrelia burgdorferi*, however, has been isolated and subcultured from the blood and plasma of Lyme disease patients (Benach et al., 1983; Nadelman et al., 1989; Berger et al., 1994; Wormser et al., 1998; Wormser et al., 2000). Significantly improved rates of spirochete cultivation were obtained when plasma (instead of serum or blood) and/or large sample sizes (up to 9 ml of whole blood or 3 ml of serum) were used to inoculate BSK II (Table 3) medium (Wormser et al., 1998; Wormser et al., 2000). Although spirochetes have been isolated and cultured from the cerebrospinal fluid (CSF) of patients exhibiting neurological symptoms (Steere et al., 1983; Nocton and Steere, 1995), attempts to recover spirochetes from this tissue are usually not productive. Although this fastidious organism is clearly difficult to cultivate in vitro, the lack of successful cultivation of spirochetes from CSF may be more a reflection of the limited invasion of the central nervous system by *B. burgdorferi* (Steere et al., 1983; Barthold et al., 1991; Estanislao and Pachner, 1999; Cadavid et al., 2000).

Isolation of *Borrelia* from Animal Tissues

The most common method of cultivating *Borrelia* spp. from field-caught or experimentally infected animals is by inoculation of BSK II (Table 3) medium with several drops of blood (relapsing fever) or by ear punch biopsy (Lyme borreliosis; Sinsky and Piesman, 1989). For ear punch biopsy, a small sample of skin tissue (3 mm) may be taken from an infected animal's ear using a punch tool (available from most biological and veterinary suppliers) or by using fine point scissors to excise a 3-mm long "pie cut" from the animal's ear. The entire ear biopsy is placed in a small volume (1–2 ml) of liquid BSK

II (Table 3) medium. In this case, use of antibiotic additives (rifampicin [5 mg/ml], phosphomycin [2 mg/ml], and amphotericin B [250 mg/ml]), commercially available as a cocktail from Sigma-Aldrich Chemical Corp. (http://www.sigma-aldrich.com) (catalog # A 1956), is recommended to reduce the risk of contamination with animal and/or environmental flora. Although borreliae usually can be detected in culture medium within one to three weeks of incubation, some isolates may not be visible for several months. *Spirochetes* also may be cultured from other animal tissues, including heart, bladder and joint (Anderson et al., 1986; Schwan et al., 1988a; Sinsky and Piesman, 1989; Anderson and Magnarelli, 1992; Pachner et al., 1993).

Uncultivatable *Borrelia* spp.

Despite the successful use of artificial media in cultivating spirochetes from both ticks and mammalian tissues, several examples of uncultivatable *Borrelia* spp. have been reported. Anda et al. (1996) described the isolation of a new relapsing fever *Borrelia* following isolation of this agent from the blood of three febrile patients. Although refractory to in vitro cultivation attempts using numerous BSK II (Table 3) formulations and growth conditions, this organism can be passaged in mice. In addition to being visible in the blood of infected mice, spirochetes were also detected in liver and spleen by Dieterle stain 15 days post-inoculation (Anda et al., 1996). A second notable example of an uncultivatable *Borrelia* spp. is the *Amblyomma americanum* spirochete, *Borrelia lonestari* (*B. barbouri*). This spirochete is thought to be responsible for a tick-associated Lyme disease-like illness in the southern United States (Barbour et al., 1996a). Although immunological and molecular reagents have been developed to detect and partially characterize this spirochete using field-collected tick specimens (Barbour et al., 1996b; Rich et al., 2001), it has yet to be successfully cultivated in vitro using media that support the growth of *B. burgdorferi* (i.e., BSK; Table 3). The inability to cultivate this spirochete (Barbour et al., 1996a) from patient samples and distinguish it from the closely related *B. burgdorferi* may result in the true incidence of *B. lonestari* borreliosis being significantly underestimated. This is a particular concern in areas where the vectors of both spirochetes coexist and coassociate with the human population. In such areas, *B. lonestari*-associated borreliosis could result in physician-diagnosed or -suspected Lyme disease that could be both culture-negative and seronegative. In addition, coinfection with both species of spirochetes may also go undetected.

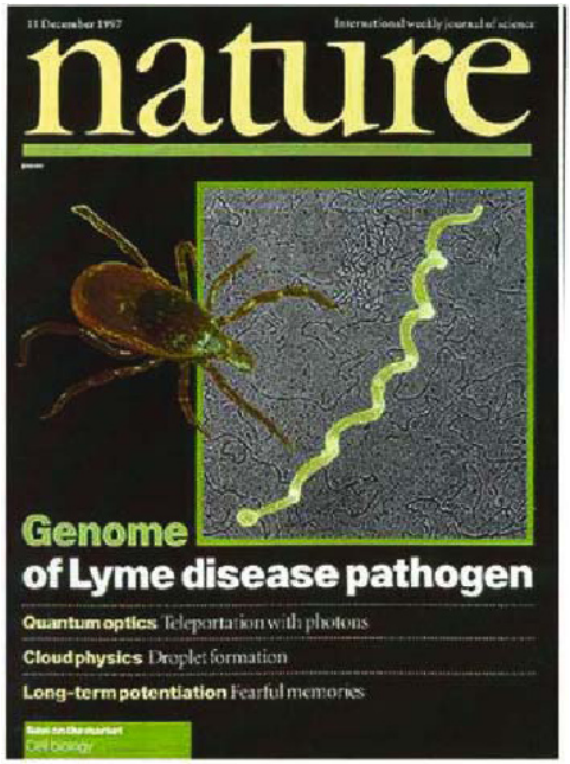
Use of PCR for Detection of *Borrelia* in Tissues

In addition to the complex media requirements and the fastidious nature of *Borrelia*, several additional factors, including the low density of spirochetes in blood and other tissues during infection, the large volume of sample required, and the expertise needed to biopsy skin and other tissues (i.e., cerebrospinal fluid [CSF] and synovial fluid [SF]) from Lyme disease patients, have made clinical diagnosis based on spirochete isolation unreliable. The development of assays based on the sensitive polymerase chain reaction (PCR), however, has made the detection of *Borrelia* within relatively small samples of blood and other tissues feasible. PCR-based assays have been used to detect spirochetes in EM skin lesions (Fig. 20) and lesion exudate (Melchers et al., 1991; Schwartz et al., 1992), ACA lesions (Moter et al., 1994), plasma (Goodman et al., 1995; von Stedingk et al., 1995), blood (Guy and Stanek, 1991), urine (Lebech and Hansen, 1992; Huppertz et al., 1993; Liebling et al., 1993; Priem et al., 1997), CSF (Keller et al., 1992; Lebech and Hansen, 1992; Huppertz et al., 1993; Liebling et al., 1993; Nocton et al., 1994; Nocton et al., 1996; Priem et al., 1997), and SF (Liebling et al., 1993; Bradley et al., 1994; Nocton et al., 1994; Jaulhac et al., 1996; Priem et al., 1997). Although these studies demonstrate that PCR-based methodologies can successfully detect *Borrelia* spp. from a variety of tissues, the clinical utility of these detection methods, however, has not been fully established. For a comprehensive review of PCR-based detection of *Borrelia*, see Schmidt (1997).

Culture Bias

The application of culture-independent techniques (i.e., direct PCR-amplification) has revealed that sequences retrieved from bacteria cultured from tick and patient samples often do not fully reflect the degree of sequence diversity present in the uncultured sample. This phenomenon, called "culture bias," is thought to result from the preferential growth of one genetically distinct subpopulation present within a sample such that it outgrows other members within the population. One of the first examples of borrelial culture bias was discovered during a comparative analysis of cultured and uncultured (i.e., PCR-amplified directly) tick samples collected from an area of the country (Colorado) where Lyme disease is not endemic (Fig. 16). In these studies, Norris et al. (1997) demonstrated that the genetic diversity of alleles at three different loci (*flaB* [see "Flagella"], *p66* [*Borrelia* Molecular Architecture], and *ospA* [see "Reciprocal

Expression of OspA and OspC"]) was consistently lower in cultured spirochetes, suggesting that culturing of *B. burgdorferi* in BSK-H medium may select for specific genotypes. In addition, some alleles were restricted to either cultured organisms or ticks alone (Norris et al., 1997). The potential clinical relevance of culture bias became evident when a similar study was performed using samples from Lyme disease patients (Liveris et al., 1999). In this study, DNA from in vitro-cultivated *B. burgdorferi* or from blood and tissue biopsy samples obtained from Lyme disease patients was characterized by restriction length polymorphism (RFLP) typing of the 16S-23S ribosomal DNA intergenic spacer region. The RFLP diversity seen by direct tissue analysis was significantly greater than that present in the cultured samples. These results are consistent with the conclusions drawn from the Colorado tick study cited above. The combined results of these studies suggest that the diversity of genotypes present within either tick or human populations may be significantly underestimated if based on in vitro cultivation or direct PCR-amplification alone.



Nature Cover

Fig. 5. From Fraser et al. (1997). (www.ncbi.nlm.nih.gov). Reprinted by permission from Nature (http://www.nature.com) © 1997 Macmillan Magazines Ltd.

Preservation

Borreliae are best preserved by the addition of glycerol (final concentration, 10–15%) to blood or plasma from infected animals (relapsing fever borreliae), or to culture medium, and stored at –70°C or in liquid nitrogen. Spirochetes may be recovered from glycerol stocks by the addition of a small amount (0.5 ml) of BSK II (Table 3) medium to the top layer of the frozen culture, pipetted several times, removed, and transferred to a sterile test tube containing an additional 1–3 ml of BSK II (Table 3) medium. The "resurrected" spirochetes should be visible by darkfield microscopy within 1–2 weeks.

Physiology

The publication of the *Borrelia burgdorferi* genome (The *B. burgdorferi* Genome Sequence) has vastly increased our understanding of the physiology of this *Borrelia* species. For a complete outline of the results of this project, visit The Institute for Genome Research (TIGR)). A survey of the *B. burgdorferi* genome revealed a paucity of genes associated with the biosynthesis of amino acids, fatty acids, enzyme cofactors, and nucleotides (Fraser et al., 1997). The absence of genes involved in fatty acid synthesis is in agreement with the biochemical evidence that 1) borreliae are unable to elongate or beta-oxidize fatty acids (Livermore and Johnson, 1978; Belisle et al., 1994) and 2) the fatty acid composition of borrelial cells reflects that of the growth medium (Livermore and Johnson, 1978; Belisle et al., 1994). These findings are thought to explain, at least in part, the fastidious nature of this organism and the requirement for serum-supplemented mammalian tissue culture medium (i.e., CMRL; see Table 3). Genes encoding all of the enzymes of the glycolytic pathway were identified (Fraser et al., 1997). Work by Gebbia et al. (1997) identified the glycolytic enzyme operons in *B. burgdorferi* and *B. hermsii*, and as expected, these loci exhibit very high sequence identity and similar orientations on the linear chromosomes of both organisms. Analysis of the metabolic pathways identified suggests that glucose serves as the major energy source (Fraser et al., 1997). Consistent with the microaerophilic nature of borreliae, genes involved in the conversion of pyruvate (pro-

duced by glycolysis) to lactate were identified (Fraser et al., 1997). This finding also is supported by biochemical studies that identified lactic acid as the predominant metabolic end product of glucose utilization (Kelly, 1976). None of the genes encoding proteins of the tricarboxylic acid cycle or oxidative phosphorylation were identified. These findings, along with the lack of the necessary components of a respiratory transport chain, may explain the slow growth rate (12–30-hour doubling time) of this organism. Analysis of the genome also revealed genes involved in the utilization of glycerol, glucosamine, fructose and maltose, suggesting that the bacterium may also use these substrates as alternative carbon sources (Fraser et al., 1997). Although primarily serving as a component of the cell wall, genes (*N*-acetylglucosamine-6-phosphate deacetylase [BB0151] and glucosamine-6-phosphate isomerase [BB0152]) were identified which would allow *N*-acetylglucosamine (NAG) to be used as an alternative carbon source. The NAG is a necessary additive to BSK II (Table 3) medium and is required for growth of *B. burgdorferi* (Kelly, 1971; Barbour and Hayes, 1986b). Additionally, a model has been proposed for the potential utilization of the tick cuticle component chitin (composed primarily of NAG) as an alternative carbon source for *Borrelia*, which may be contained within the midgut of the tick for extended periods of time (months to a year) between feeding and subsequent transmission to a new mammalian host (Fraser et al., 1997). Support for this hypothesis is provided by the identification of a phosphotransferase system (PTS) cellobiose transporter operon (BBB04, BBB05, and BBB06) that could possibly transport chitobiose (di-*N*-acetyl-D-glucosamine), a molecule with structural similarity to NAG, and a gene product with sequence similarity to chitobiase BBB02, which may convert chitobiose to NAG (Fraser et al., 1997).

The genes encoding essential transcription (sigma factors σ70, σ54, and *rpoS*, translation *nusA*, *nusB*, *nusG*, *rho*, and 31 tRNAs), and DNA replication and repair machinery (DNA pol III, ligase, gyrase, *uvrA-D*, and *recA*) were identified, along with the chromosomal origin of replication (*ori*; Fraser et al., 1997; Picardeau et al., 1999a). In addition, two independent studies have examined the effect of DNA replication and transcription on triplet codon usage for *B. burgdorferi* (McInerney, 1999; Lafay et al., 1999). These studies suggest that evolutionary pressures have resulted in an enrichment for highly expressed genes on the leading strand during the asymmetrical replication of this organism's linear chromosome.

Growth of *Borrelia burgdorferi*: No Iron Requirement

A common theme in microbial pathogenesis is a requirement for iron during growth within a mammalian host. For reviews, see Payne (1993), Litwin and Calderwood (1993), Vasil and Ochsner (1999), and Ratledge (2000). The level of free iron present within human serum and/or tissues is well below that required for growth of many microorganisms. This level is further limited by the production and secretion of the iron-binding protein lactoferrin by the human host during infection. To overcome this host limitation, many bacteria have evolved specialized systems for the acquisition and assimilation of iron in vivo. A recent survey of the *Borrelia* genome suggests that this bacterium has overcome iron limitation within the human host by dispensing with those proteins that utilize iron as a cofactor (i.e., catalase, peroxidase, cytochromes, respiratory proteins, and metalloenzymes; Fraser et al., 1997; Posey and Gherardini, 2000). In support of this hypothesis, Posey and Gherardini (2000) demonstrated that 1) *Borrelia* were able to grow normally in iron-limited medium and 2) the level of iron (either free or bound to proteins) detected in *B. burgdorferi* cell lysates was well below a physiologically relevant concentration. The genes encoding a number of metal-requiring proteins, however, were identified within the *Borrelia* genome, including superoxide dismutase, RNA polymerase and DNA polymerase (Fraser et al., 1997). Although the metal requirements of these putative borrelial metalloenzymes have not been determined, it has been suggested that these proteins may have evolved to utilize alternative metal ions (i.e., manganese and zinc; Posey and Gherardini, 2000).

Borrelia Molecular Architecture

Borrelia share the same basic ultrastructural features as other members of the Family Spirochaetaceae. These features include an outer membrane that encloses a protoplasmic cylinder encased within a cytoplasmic membrane (Fig. 6). Although similar to Gram-negative bacteria in possessing both cytoplasmic and outer membranes, several fundamental differences in the outer membranes of these two types of bacteria have been revealed using combined molecular, ultrastructural and immunological methods. The outer membranes of Gram-negative bacteria characteristically contain a high density of proteins with extensive β-pleated sheet structure (i.e., porins) and no or only rare surface-exposed lipoproteins (Lugtenberg and van Alphen, 1983; Pugsley, 1993). In addition to a

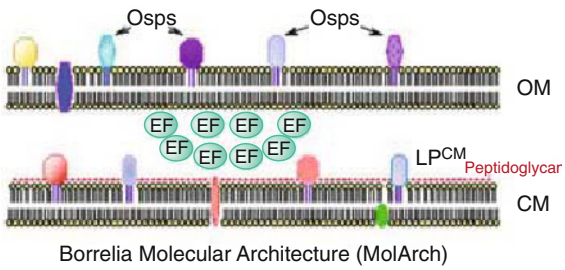


Fig. 6. *Borrelia burgdorferi* molecular architecture. The outer membrane (OM) of *B. burgdorferi* contains a limited number of integral membrane proteins (outer surface proteins or Osps). The majority of proteins within borrelial outer (OM) and inner cytoplasmic (CM) membranes are lipoproteins, anchored via associated lipid moieties. Lipoproteins (LP) anchored to the periplasmic leaflet of the CM are in close association to the peptidoglycan layer. Endoflagella (EF; see "Flagella") are located in the periplasmic space.

significantly lower protein-to-lipid ratio, *B. burgdorferi* outer membranes, in contrast to Gram-negative membranes, contain predominantly lipoproteins (many of which are lipid-anchored to the periplasmic face of the cytoplasmic membrane) and relatively small amounts of proteins with transmembrane (membrane-spanning) domains (Walker et al., 1991; Radolf et al., 1994b; Radolf et al., 1995b; Jones et al., 1995). In concert with the spirochete's ability to alter its outer membrane composition (see "Antigenic Variation") during the course of infection, these two characteristics of the borrelial membrane may help to explain the Lyme disease spirochete's ability to avoid host clearance mechanisms and establish persistent infection. In addition, *Borrelia* spp. have the potential to differentially express (see "Differential Gene Expression") (i.e., downregulate) many of their surface-exposed lipoproteins, thereby further limiting the ability of the host immune system to recognize these immunogens.

One of the most striking characteristics of spirochetal outer membranes, particularly those of *B. burgdorferi* and *Treponema pallidum* (see The Genus *Treponema* in this Volume), is their lability. As a result, the outer membranes of these organisms are often disrupted during routine experimental manipulations (i.e., centrifugation), initially leading some investigators to conclude (using immunofluorescence) that a number of borrelial lipoproteins (i.e., outer surface protein OspA [see "Reciprocal Expression of OspA and OspC"] and OspB) were exclusively surface-exposed (Barbour et al., 1983; Barbour et al., 1984b; Luft et al., 1989). Later studies, using techniques that do not disrupt the spirochetal outer membrane prior to analysis (immunocryoultramicrotomy, Brusca et al., 1991, and immunofluorescence using spirochetes encapsulated within agarose beads, Cox et al., 1996) demonstrated that most of the cell's complement of OspA and OspB is located in the periplasmic space. A similar localization pattern has now been established for a third lipoprotein, OspC (Cox et al., 1996; see "Reciprocal Expression of OspA and OspC"). The presence of some lipoproteins simultaneously in both the inner and outer membranes suggests that the content of borrelial membranes is dynamic (Brusca et al., 1991; Cox et al., 1996). This hypothesis is further supported by a recent study in which exogenously provided recombinant lipoproteins were shown to be incorporated into the outer membranes of viable spirochetes (Bunikis et al., 2001). It is tempting to speculate whether *Borrelia* spp. use the inner membrane as a "holding depot" for lipoproteins that may eventually be transferred to the outer membrane and bacterial surface as the organism encounters different environmental/growth conditions.

A second class of proteins within the outer membranes of *Borrelia* is the membrane-spanning proteins. To date, only a handful of such proteins have been identified. Of these, P66 has attracted the most attention. This 66-kDa protein was identified as a component of borrelial membrane-enriched cell fraction (Barbour et al., 1984b; Bunikis et al., 1995) and also, by an independent group, using a protease screening assay (Probert et al., 1995). Computer analyses predict that, in addition to having an N-terminal signal peptidase I cleavable leader peptide, P66 contains a C-terminal intramembranous domain (amino acid residues 498–597) composed of two transmembrane-spanning hydrophobic α-helices (Bunikis et al., 1998). This prediction is supported by experimental studies, immunoelectron microscopy (Bunikis et al., 1995) and protease (trypsin and proteinase K) sensitivity (Probert et al., 1995), which identified a small (~5 kDa) flexible loop fragment near the C-terminus. This 5-kDa loop domain appears to be the only portion of P66 that is exposed on the spirochetal surface (Bunikis et al., 1996; Bunikis and Barbour, 1999). Recent evidence suggests that P66 is physically associated with OspA and that OspA may shield P66 from proteolytic attack (Bunikis and Barbour, 1999). Consistent with its having surface-exposed epitopes, mice immunized with recombinant P66 were partially protected against *B. burgdorferi*-challenge (Exner et al., 2000). Antibodies against P66 also were readily detectable in immune sera from human Lyme disease patients (Sadziene et al., 1993; Bunikis et al., 1996; Ntchobo et al., 2001), confirming that this protein is expressed during infection.

Two different functions have been proposed for P66. Coburn et al. (1999) reported that recombinant P66 binds to β_3 chain integrins, whereas Skare and coworkers (Skare et al., 1997) reported that the recombinant protein has porin-like activity in planar lipid bilayers. While neither of these functions is mutually exclusive, they are problematic, however, when viewed against the topological analyses indicating that P66 has only limited surface exposure. The P66 integrin-binding domain was mapped to a region that does not appear to be surface-exposed (Coburn et al., 1999). Porins typically contain multiple membrane-spanning domains (Nikaido, 1996). Further work will be needed to resolve these ostensible discrepancies.

Two other membrane-spanning proteins identified in *B. burgdorferi* are *Borrelia* glycosaminoglycan (GAG)-binding protein (Bgp) and an outer membrane-spanning protein, Oms28, with an observed mass of 28 kDa. The Bgp protein was identified from membrane-enriched extracts of *B. burgdorferi* on the basis of its glycosaminoglycan-binding and hemagglutination activities (Parveen and Leong, 2000). Indirect immunofluorescence and Western blotting of purified outer membrane vesicles (both with anti-Bgp antiserum) were used to localize this protein to the spirochetal surface. Purified recombinant Bgp also was shown to inhibit attachment of *B. burgdorferi* to the surface of several different mammalian cell lines (293, Vero and C6), a result consistent with this protein being present on the surface of spirochetes (Parveen and Leong, 2000). The GAG-binding specificity of Bgp, however, has not been determined. The Oms28 protein was identified in *Borrelia burgdorferi* outer membrane preparations based on its porin activity; this activity was demonstrated in a planar lipid bilayer assay using *Escherichia coli* overexpressing recombinant Oms28 (Skare et al., 1996).

Peptidoglycan

The peptidoglycan of *Borrelia* spp. is responsible for imparting structural stability to the protoplasmic cylinder of the spirochete and is the primary component of the bacterial cell wall (Beck et al., 1990; Fig. 6). Additional data demonstrating a structural role of the borrelial peptidoglycan were reported by Charon and coworkers (Motaleb et al., 2000); spirochetes with their outer membranes removed were readily lysed by the addition of the peptidoglycan-degrading enzyme streptozyme (Motaleb et al., 2000). Muramic acid (Ginger, 1963) and ornithine (Klaviter and Johnson, 1979) have been identified as components of the peptidoglycan in relapsing fever *Borrelia*. Although biochemical analyses of the peptidoglycan of other *Borrelia* have not been performed to date, it is likely that both of these components are present in all borrelial cell walls.

Flagella

Spirochetes are unique in that their organelles of motility are located beneath their surface (Fig. 6). For detailed reviews of spirochetal and borrelial flagella ultrastructure, see Holt (1978) and Barbour and Hayes (1986b), respectively. For a comprehensive contemporary review of spirochete flagella and motility, see Li et al. (2000a). Encased between the outer and cytoplasmic (inner) membranes are the periplasmic flagella, also referred to as "endoflagella" (Fig. 6); in this respect, spirochetes are distinct from other bacteria in having their organelles for motility reside inside the cell within the periplasmic space. The number of periplasmic flagella in borreliae varies depending on the species, as well as within the same strain; the relapsing fever spirochetes generally have between 15 and 30 flagella per cell end, whereas Lyme spirochetes have from 7 to 11 per end (Hovind-Hougen, 1976; Barbour and Hayes, 1986b). The periplasmic flagella are composed of two classes of proteins, FlaB, core protein, flagellin, and FlaA (a possible sheath protein, with FlaB being the major constituent). Although the periplasmic flagella of other spirochete species (i.e., *Brachyspira*) have been shown to be composed of at least three core proteins (FlaB1, FlaB2 and FlaB3) and two sheath proteins (FlaA1 and FlaA2; Li et al., 2000b), the periplasmic flagella of *Borrelia burgdorferi* have been shown to be composed of only one each of these two types of proteins (Ge and Charon, 1997a; Fraser et al., 1997; Ge et al., 1998). In addition, the relatively low abundance of FlaA in *B. burgdorferi* suggests that it is unlikely to form a sheath along the entire flagellar length (Li et al., 2000a). A comparison of the periplasmic flagella proteins shows a high degree of conservation (immunological crossreactivity and sequence similarity) within the spirochete phylum (Limberger and Charon, 1986; Cockayne et al., 1987; Norris et al., 1988; Koopman et al., 1992; Trueba et al., 1992; Limberger et al., 1993; Ruby et al., 1997; Ge et al., 1998).

The flagella of *Borrelia* spp. have been shown to be 1) inserted subterminally and bipolarly, 2) wrapped around the protoplasmic cylinder, and 3) overlapping in the middle of the protoplasmic cylinder (Holt, 1978). Electron microscopy studies revealed that the periplasmic flagella form a bundle and are not dispersed around the cell in the periplasmic space (Hovind-Hougen, 1984). Purified periplasmic flagella have been shown to be left-handed with a defined helix pitch and

diameter. These dimensions, however, are dramatically altered when measured in situ (i.e., in association with the protoplasmic cylinder; Goldstein et al., 1996). The helical shape of the flagella, wrapped in a bundle around the peptidoglycan-encased protoplasmic cylinder, imparts to the spirochete a flat-wave morphology (Motaleb et al., 2000; Fig. 7). Charon and coworkers (Motaleb et al., 2000) hypothesize that the outer membrane juxtaposes the peptidoglycan and periplasmic flagella so that these structures can optimally interact with one another to promote cell motility. The dynamics of *Borrelia* spp. motility also have been examined (Goldstein et al., 1994). These studies have demonstrated that, in contrast to their helical shape, translating (i.e., moving) spirochetes swim with a planar waveform similar to that of eukaryotic flagella and propagate backward moving spiral waves. The waves on individual spirochetes were observed to gyrate in a counterclockwise direction (Goldstein et al., 1994; Fig. 7). In addition, periplasmic flagella, inserted at opposite ends of the protoplasmic cylinder, were shown to rotate in opposite directions around one another, causing the cell to bend or exhibit characteristic "flexing" morphology (Goldstein et al., 1994). This rotation, however, was not required for the spirochetes to maintain their helical morphology (Motaleb et al., 2000). The contribution of the periplasmic flagella to both motility and cell morphology has been confirmed using both a spontaneous *Borrelia* mutant (Sadziene et al., 1991) and a *flaB* mutant strain created by allelic exchange (Motaleb et al., 2000). In both studies, the spirochetes not only were nonmotile, but also exhibited a long, straight, rod-shaped morphology. The periplasmic flagella also help the cells maintain their motility in environments of relatively high viscosity (Berg and Turner, 1979; Kimsey and Spielman, 1990; Goldstein et al., 1994). Although borreliae are extracellular parasites, this vigorous form of motility has also been implicated in the spirochete's ability to invade and infect various tissues (including connective tissue and extracellular matrix) and to traverse endothelial cell junctions (Comstock and Thomas, 1989; Comstock and Thomas, 1991; Szczepanski et al., 1990; Sadziene et al., 1991; Klempner et al., 1993).

The 35 genes encoding the proteins required for the synthesis of the complete flagellar organelle are grouped into seven clusters (Fig. 8) on the *B. burgdorferi* chromosome (Li et al., 2000a). Promoter analyses of the *B. burgdorferi* *flaA* and *flaB* genes revealed a striking difference between the regulation of flagellin gene (*flaB*) expression in this spirochete and in other bacteria; transcription of the *flaB* gene in *B. burgdorferi* appears to be initiated by the housekeeping transcription factor, σ 70. This is in contrast to the flagellin genes, *flaB* and *fliC*, in other spirochetes and bacteria, respectively, which are known to be or thought to be controlled by the alternative σ factor, σ 28 (Li et al., 2000b). These data also are consistent with the absence of 1) σ 28 promoter recognition sequences, 2) a σ 28-encoding gene, and 3) a gene encoding the anti-σ factor, FlgM, within the *B. burgdorferi* genome sequence (Fraser et al., 1997; Li et al., 2000a). Interestingly, transcription of the *flaB* gene from *T. pallidum* (the only other sequenced spirochetal genome) does appear to be initiated by σ 28, and a gene encoding this σ factor was identified within this treponemal genome.

Lipoproteins

The study of borrelial lipoproteins has long been a focus of research involving this bacterium. For a recent review, see Haake (2000). Borrelial lipo-

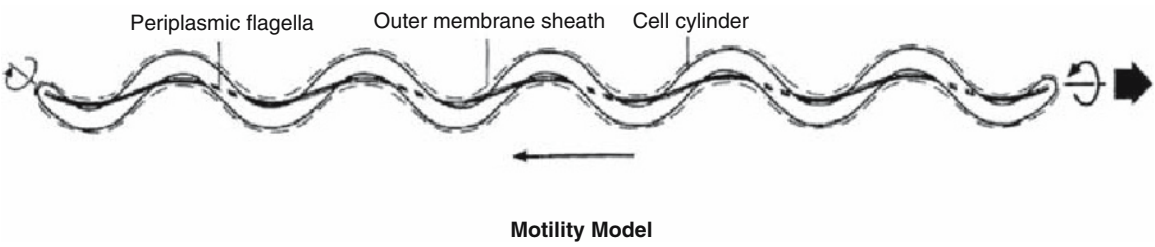


Fig. 7. Proposed model of *Borrelia burgdorferi* motility translating toward the right (wide arrow). The ridge, which is composed of the periplasmic flagella, extends down the length of the cell, and is left-handed with respect to the cell axis. The periplasmic flagella rotate counterclockwise (CCW) as viewed from the back of the cell (thin arrow at anterior end). This rotation causes waves to move from the anterior to the posterior ends of the cell (center arrow). Concomitant with this rotation, the cell rolls clockwise (CW) about the body axis (thin arrow at the posterior end). Image taken from Li et al. (2000b), with permission of Horizon Scientific Press.

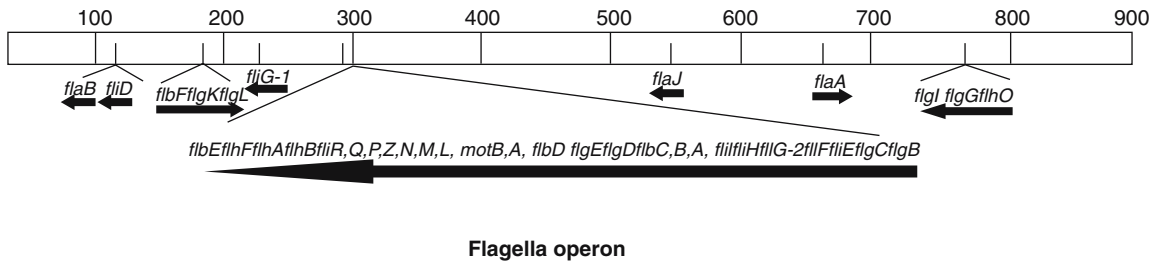


Fig. 8. Organization of the motility genes of *B. burgdorferi* (Fraser et al., 1997; Ge and Charon, 1997a; Ge and Charon, 1997b). Black arrows indicate direction of transcription of genes, and promoters have been identified by primer extension and operons by RT-PCR (Ge and Charon, 1997a; Ge and Charon, 1997b). Gray stippled arrows indicate presumed promoters, operons, and direction of transcription based solely on sequence analysis. Adapted from Li et al. (2000b), with permission of Horizon Scientific Press.

proteins are of interest for several reasons: 1) they are often found at the interactive surface (*Borrelia* Molecular Architectiture) between the spirochete and its growth environment, 2) many have been shown to be differentially expressed (see "Differential Gene Expression") depending on the growth environment, with some being preferentially expressed within either the arthropod or mammalian host, 3) some have assigned physiological roles (see "Differential Gene Expression"), 4) they may serve as potential protective immunogens (see "Lyme Disease Vaccine"), and 5) they are potent proinflammatory agonists (Lipoproteins and the Innate Immune Response). The significance of this class of membrane proteins is highlighted by the large number of putative lipoproteins identified (>150 on the linear chromosome and the plasmids) in the *Borrelia burgdorferi* genome, comprising nearly 10% of the total coding region (Fraser et al., 1997; Casjens et al., 2000). Not surprisingly, the majority of borrelial membrane proteins were found to be typical bacterial lipoproteins, possessing consensus lipoboxes (leader peptides required for lipid modification and transport to the membrane; Fraser et al., 1997). As also would be expected, genes encoding the necessary enzymes for lipid modification (prolipoprotein diacylglycerol transferase *lgt* and prolipoprotein signal peptidase *lsp*) also were identified. A gene encoding the enzyme (Lnt) responsible for the next step in lipoprotein biosynthesis, transfer of a fatty acid from a membrane phospholipid to the N-terminal cysteine of the mature polypeptide, however, was not identified. Since this activity has been shown by biochemical and structural analyses to exist in *Borrelia* (Brandt et al., 1990; Belisle et al., 1994; Beermann et al., 2000), it is presumed that the organism contains a novel form of this enzyme.

Transporters

A number of genes thought to be involved in the transport of solutes and small molecules (Fig. 9) were identified within the *B. burgdorferi* genome; the linear chromosome encodes 46 ORFs and the plasmids of an additional six ORFs related to the transport and binding of proteins. The combined ORFs form 16 distinct membrane transporters of amino acids (i.e., glycine/betaine/L-proline and oligopeptides), carbohydrates (i.e., methylgalactoside, ribose, galactose, fructose, glucose and maltose), small molecules (glutamate, chromate and spermadine/putrescine), anions (i.e., phosphate), and cations (i.e., magnesium and potassium; Fraser et al., 1997). The majority of these transport systems are members of the family of ATP-dependent, multi-subunit ABC-transporters, with many exhibiting broad substrate specificity (Fraser et al., 1997). The ability of *Borrelia* to transport a wide variety of solutes is thought to compensate for this organism's restricted coding potential and its inability to synthesize amino acids de novo. A survey of the genome sequence failed to identify transport systems for nucleotides, nucleosides, NAD/NADH, or fatty acids, although proteins responsible for these functions are likely to be present (Fraser et al., 1997).

The oligopeptide transporters (Opp) are the best studied of the *Borrelia* transporter families. This family is composed of 1) five peptide-binding proteins, OppA1, OppA2, OppA3, OppAIV, and OppAV (all thought to be lipoproteins, but lipid modification has only been confirmed for OppA-1, OppA-2 and OppA-3; Kornacki and Oliver, 1998); 2) two transmembrane proteins (OppB and OppC); and 3) two ATPases (OppD and OppF, Fraser et al., 1997; Bono et al., 1998). OppA1, OppA2 and

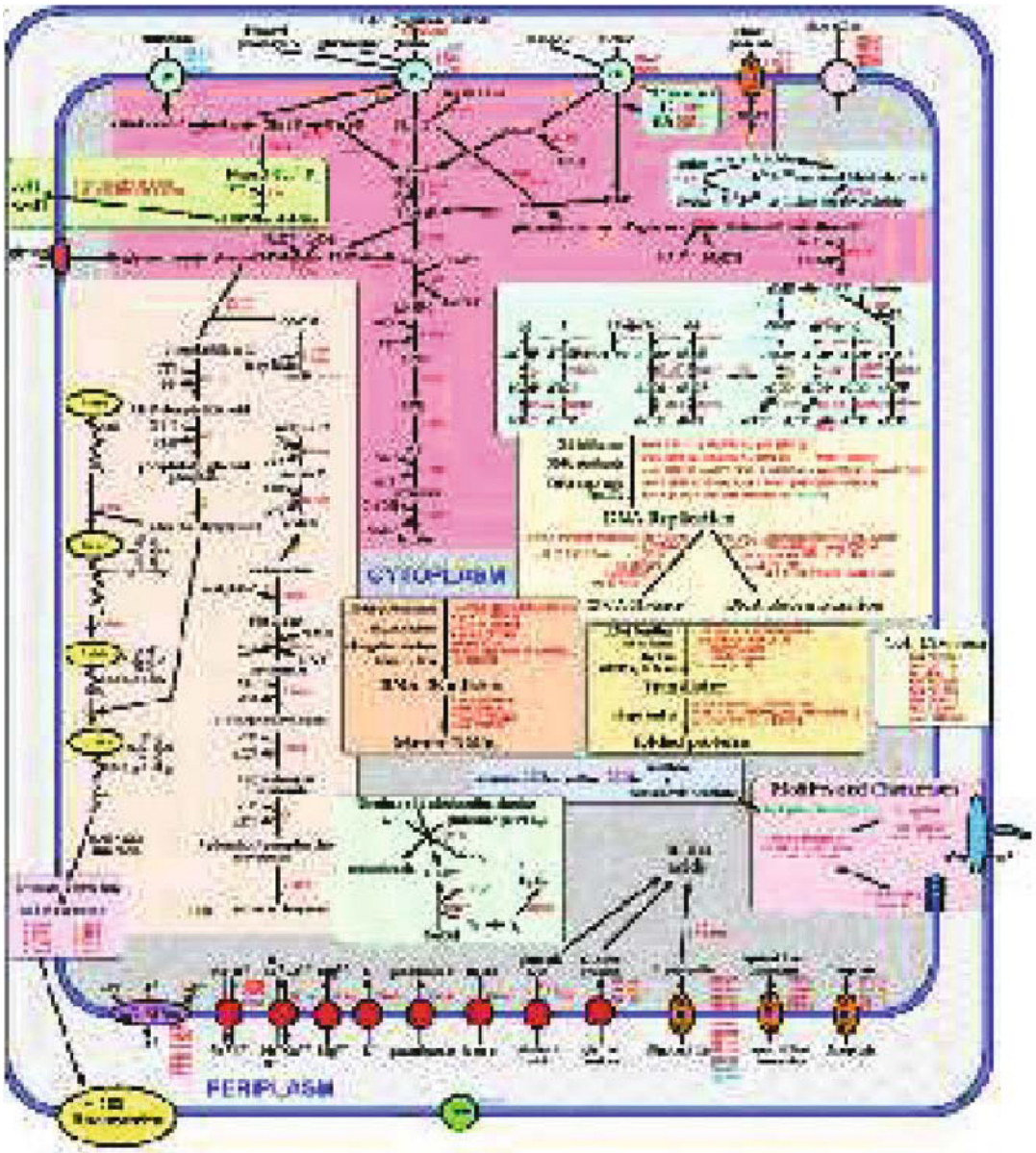


Fig. 9. Solute transport and metabolic pathways in *B. burgdorferi*. A schematic of a *B. burgdorferi* cell, providing an integrated view of the transporters and the main components of the metabolism of this organism, as deduced from the genes identified in the genome. The ORF numbers correspond to those listed in Table 2 of Fraser et al. (1997). Red indicates chromosomal and blue indicates plasmid ORFs. Presumed transporter specificity is indicated. Yellow circles indicate places where particular uncertainties exist as to the substrate specificity, subcellular location, direction of catalysis, or absence of expected activities. Taken from Fraser et al. (1997) and www.nature.com (http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v390/n6660/full/390580a0_fs.html) and reprinted by permission from Nature (http://www.nature.com), © 1997 Macmillan Magazines Ltd.

OppA3, along with OppB-F, are encoded in tandem on the chromosome, whereas OppAIV and OppAV are encoded on a circular (cp26) and linear (lp54) plasmid, respectively (Bono et al., 1998). This genetic organization is unique among oligopeptide transporters, which typically are present at a single chromosomal locus. Paralogs of these genes have also been identified in the *B. burgdorferi* sensu lato strains, *B. hermsii* and *B. afzelii* (Kornacki and Oliver, 1998).

The putative physiological roles for this, and other oligopeptide transporters, include sensing pertinent environmental signals and nutrient uptake (by recycling cell wall and external peptides; Bono et al., 1998). The OppA (OppA-1, OppA-2, OppA IV and OppAV) proteins were able to function as substrate binding proteins by complementation of an *opp*$^{-}$ *E. coli* strain (Lin et al., 2001). To explore the role of this oligopeptide permease in the transmission and adaptation of *B. burgdorferi* between the arthropod and mammalian environments, an *oppAIV* mutant was created by insertion inactivation; this mutant did not show impaired growth in BSK II medium (Bono et al., 1998; Table 3). Unfortunately, the current state of borrelial genetics did not allow for this mutation to be constructed in an infectious *Borrelia* strain, and therefore its ability to cycle between the tick and mammalian host could not be determined. A *B. burgdorferi* strain N40 containing a spontaneous truncation in *oppA2*, however, was successfully maintained in the tick-mammal infectivity cycle. This latter observation may be due to functional redundancy among the five OppA peptide-binding proteins, or this system is not essential for maintenance of the enzootic cycle.

Genetics

The organization of the *Borrelia* genomes is unique within the Family Spirochaetaceae and perhaps among the prokaryotes. Most significantly, the chromosome is linear in nature, having single-stranded hairpin loops at each termini, resembling telomeres, a form of DNA thought to be restricted to eukaryotes and viruses (Barbour and Garon, 1987; Baril et al., 1989; Ferdows and Barbour, 1989; Hinnebusch et al., 1990; Bergstrom et al., 1992; Casjens and Huang, 1993; Casjens et al., 1997a; Casjens, 1999; Casjens et al., 2000; Volff and Altenbuchner, 2000). Borrelial DNA has a G+C content of ~29 mol%, which is lower than that (www.tigr.org) reported for *Treponema pallidum* and *Leptospira* (Hyde and Johnson, 1984; Fraser et al., 1997; Fraser et al., 1998). The chromosome copy number of one borrelial species, *B. hermsii*, was determined to be 16 copies per cell (Kitten and Barbour, 1992). These data were also supported using individual agarose-embedded spirochetes (*B. burgdorferi* and *B. hermsii*) labeled in situ and visualized directly by epifluorescence microscopy (Hinnebusch and Bendich, 1997). This number is significantly higher than that of the prototypic bacterial species, *E. coli*, which when actively growing, has an average of 6.5 chromosomal origins per cell (Bremer and Dennis, 1987). In situ visualization of the bacterial nucleoids of *E. coli* and *Borrelia* spp. by fluorescence microscopy also suggests that the *Borrelia* spp. chromosome is not organized into the tightly packed, or condensed, nodes found in *E. coli*, but instead consists of a loose meshwork of DNA strands that lack a central organizing node (Hinnebusch and Bendich, 1997).

The putative origin of replication (*ori*) of the *B. burgdorferi* chromosome was recently mapped using a combination of theoretical (GC skew analysis) and molecular (nascent DNA strand analysis) methodologies (Picardeau et al., 1999b). Analysis of the GC skew pattern identified a region in which there was a switch in polarity from positive to negative at the middle of the chromosome. A cumulative GC skew diagram of complete series of *B. burgdorferi* gene sequences showed a minimum between the *dnaA* and *dnaN* genes, analogous to the position of *oriC* in an identical analysis of *E. coli*. Further analysis of this region, however, failed to identify other sequences (DnaA initiator protein-binding sites and AT-rich regions) characteristic of both Gram-negative and Gram-positive origins (Picardeau et al., 1999a). The identification of a DnaA ortholog in *B. burgdorferi* suggests that this protein may bind to a unique site within the origin (Fraser et al., 1997).

Plasmids of *Borrelia*

In addition to containing linear chromosomes, any one spirochete may contain 21 or more different extrachromsomal elements (plasmids), ranging in size from 5–56 kb, in either linear or supercoiled circular forms (Fraser et al., 1997; Casjens et al., 2000; Fig. 11). Linear plasmids also have covalently closed termini similar to those on the linear chromosome. The unusual linear plasmids were first described in *B. hermsii*, but multiple linear and circular plasmids have also been described for many other *Borrelia* including *B. hermsii*, *B. parkeri*, *B. turicatae*, *B. crocidurae*, *B. burgdorferi*, *B. coriaceae* and *B. anserina* (Hyde and Johnson, 1984; Barbour, 1988; Goodman et al., 1991; Kitten and Barbour, 1992; Xu and Johnson, 1995; Marconi et al., 1996a; Barbour et al., 1996b; Stevenson et al.,

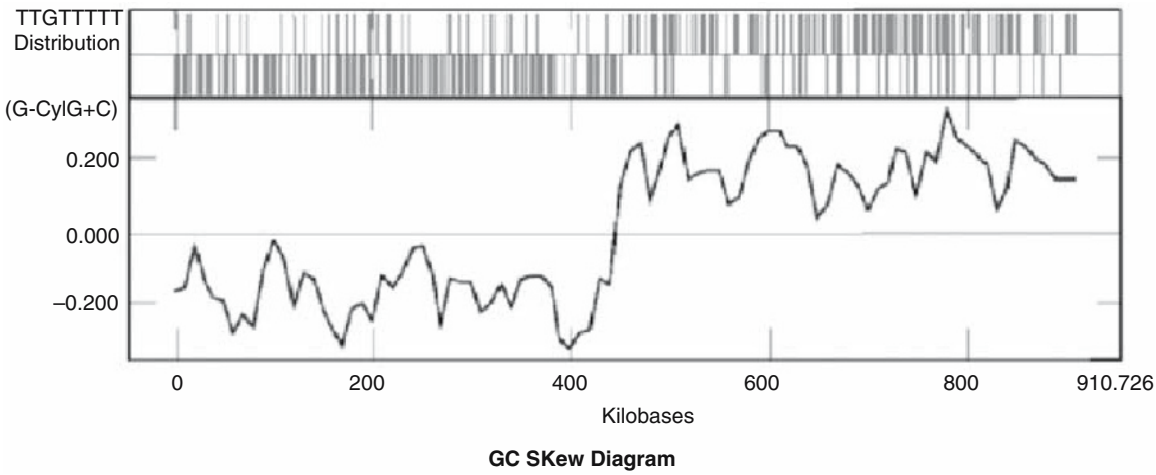


Fig. 10. Distribution of TTGTTTTT and the GC skew in the *B. burgdorferi* chromosome. Top, distribution of the octamer TTGTTTTT. The lines in the top panel represent the location of this octamer in the plus strand of the sequence, and those in the second panel represent the location of this oligomer in the minus strand of the sequence. Bottom, GC skew. Taken from Fraser et al. (1997) and www.nature.com (http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v390/n6660/full/390580a0_ fs.html) and reprinted by permission from Nature (http://www.nature.com), © 1997 Macmillan Magazines Ltd.

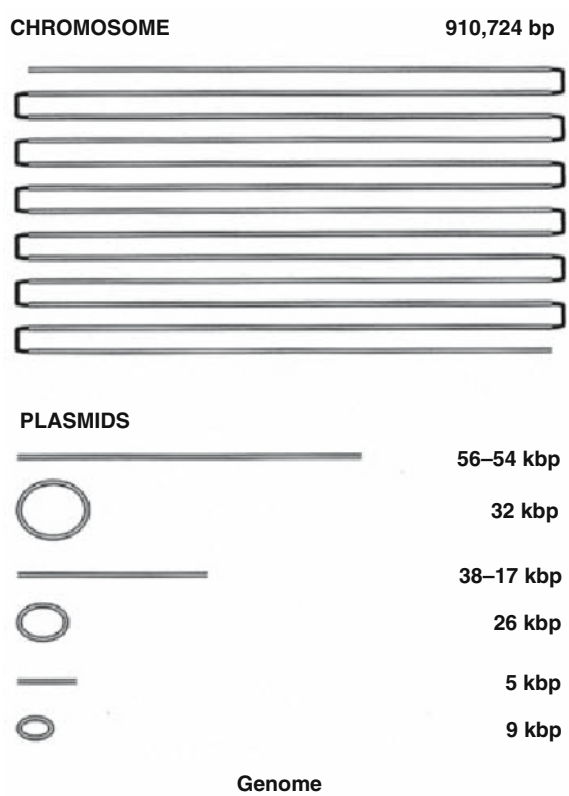


Fig. 11. Schematic representation of the linear chromosome and plasmids (linear and circular) of *Borrelia burgdorferi*.

2000a; Stevenson et al., 2000b; Hendson and Lane, 2000).

The only fully sequenced, and therefore the best characterized, genome is that of *B. burgdorferi* (The *B. burgdorferi* Genome Sequence). In this species, the full complement of linear and circular plasmids comprises approximately one-third of the total genomic content (Fraser et al., 1997; Casjens et al., 2000). Studies examining the copy number of *Borrelia* plasmids indicate that the linear and circular plasmids are closely maintained at approximately one plasmid copy per chromosome in a population of cells (Kitten and Barbour, 1992; Hinnebusch and Barbour, 1992; Casjens and Huang, 1993). This tight control of plasmid copy number suggests that replication of these extrachromosomal elements may be controlled by a mechanism similar to that of chromosomal replication. It has been proposed that the *Borrelia* linear plasmids are actually "minichromosomes" (Casjens, 1999; Barbour, 1993). The mechanism by which the linear and circular plasmids are replicated is currently a matter of much speculation, although recent data would suggest that the linear plasmids initiate replication at their centers and that replication in both types of plasmids proceeds in a bidirectional manner (Picardeau et al., 1999b; Casjens, 1999; Casjens et al., 2000). In proposed models (Fig. 12), the *Borrelia* linear plasmids are thought to use a circular intermediate for replication; circular replication intermediates have been isolated from some *Borrelia* spp. (Ferdows et al., 1996). The formation of covalently closed hairpin loops (telomeres) and the initiation of DNA replication near the center of the chromosome and linear plasmids present, however, a dilemma for the cell. How does the cell resolve two parental strands that are covalently joined at the telomeres, but destined to end up in different

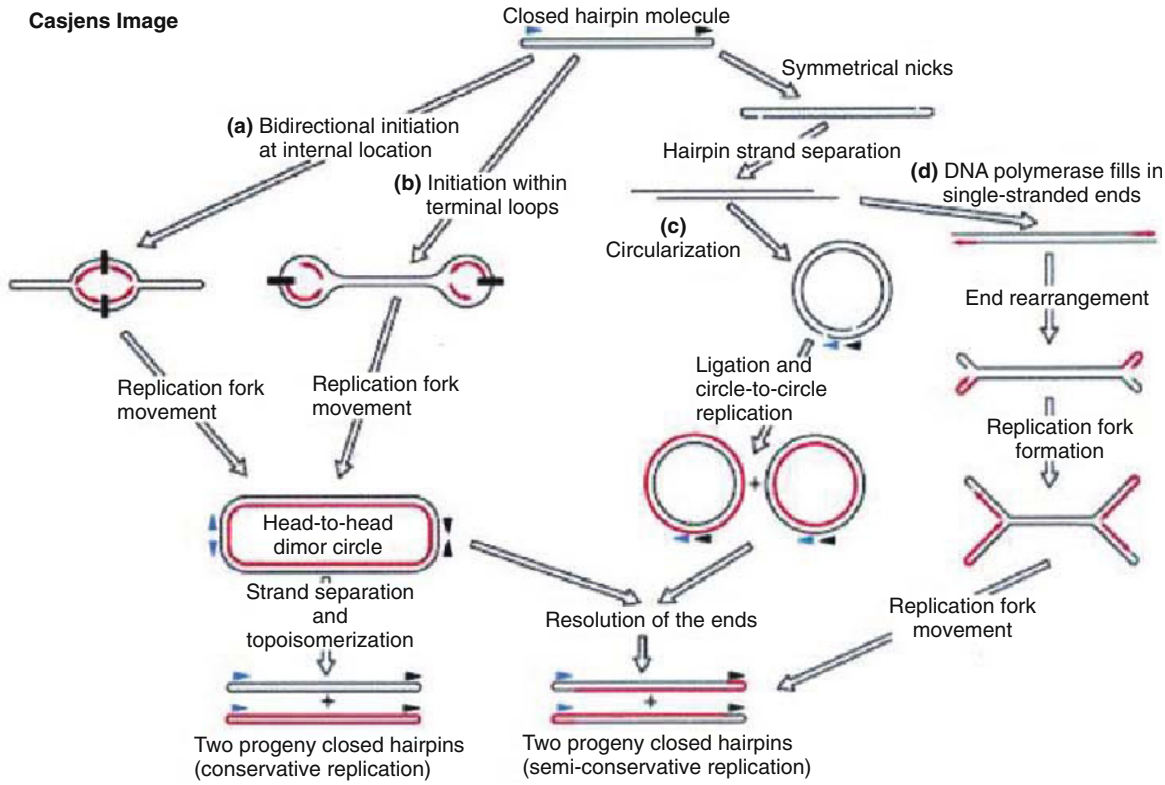


Fig. 12. Four strategies to replicate linear DNAs with covalently closed hairpin telomeres. In scheme (a), bidirection initiation of replication occurs near the center of the chromosome, whereas in scheme (b), initiation occurs within the terminal loops. In both (a) and (b), replication is followed by strand nicking and reassortment during end resolution (semiconservative replication) or strand separation and topoisomerization (conservative replication). In scheme (c), strand nicking occurs both during circularization and after replication. In scheme (d), strand nicking precedes duplication. Red lines indicate newly synthesized strands; black bars indicate replication origins on an unbroken template strand; the black and blue arrowheads indicate different sequences near the two telomeres to indicate the head-to-head and tail-to-tail joints in the possible dimer circle intermediate and the head-to-tail joints in the possible monomer circle intermediate. These strategies need not be mutually exclusive; for example, strategies (a) and (d) could combine to replicate the bulk and ends of the DNA, respectively. Reprinted from *Current Opinion in Microbiology*, 2, Casjens, S., Evolution of the linear DNA replicons of *Borrelia burgdorferi*. pp. 529–534, © 1999, with permission from Excerpta Medica, Inc. (Casjens, 1999).

daughter cells? Several mechanisms of strand resolution have been proposed, each utilizing a strand breakage to allow for segregation of the two parental strands, and also may involve the use of specific proteins, (i.e., transposases, integrases and telomerases). Recent studies by Chaconas et al. (2001) have proposed a model by which the hairpin telomeres are processed during replication. According to this model, the final step in the replication of linear replicons is a site-specific DNA breakage and reunion event to regenerate covalently closed hairpin ends. For recent reviews of *B. burgdorferi* DNA replication, see Casjens (1999) and Garcia-Lara et al. (2000).

The origin of linear DNA hairpin telomeres in *Borrelia* is also a matter of much speculation. Hinnebusch and Barbour (1991) noticed a low level of sequence similarity between the *Borrelia* telomeres and the hairpin ends of the iridopoxvirus African swine fever virus (ASFV). One very interesting hypothesis suggests that if a linear ASFV-like element had integrated into an ancestral *Borrelia* circular DNA element, it could have generated a linear DNA molecule and brought along with it the necessary machinery for end-resolution. Although no convincing DNA similarities were detected between *Borrelia* and poxvirus genes, most of the poxvirus genome could have been lost during evolution, leaving only the end-resolution apparatus required for the replication of the linear DNA molecules. This scenario is made more plausible by the fact that both *Borrelia* and ASFV are transmitted by arthropods; *Ornithodoros moubata* (Table 2) is known to transmit both *B. duttonii* and ASFV. This colocalization within an arthropod vector brings these very distinct

phyla into close enough proximity of each other to allow for genetic exchange (Casjens, 1999).

Plasmids and *Borrelia* Virulence

Shortly after the discovery of *Borrelia* plasmids, it was noted that this bacterium will often lose plasmids as a result of continuous in vitro cultivation (Barbour, 1988). It also was demonstrated that a loss of plasmid content was often associated with changes in protein profile and/or a reduction of infectivity (Schwan et al., 1988b; Persing et al., 1994; Golde and Dolan, 1995; Norris et al., 1995; Xu et al., 1996; Busch et al., 1997; Zhang et al., 1997; Anguita et al., 2000; Purser and Norris, 2000; Labandeira-Rey and Skare, 2001). Two recent studies have more fully characterized the relationship between plasmid content and infectivity in *B. burgdorferi* using clones with their plasmid content fully (Purser and Norris, 2000) or partially (Labandeira-Rey and Skare, 2001) characterized. Both of these studies demonstrated that the loss of one or more linear plasmids can be correlated with a reduction or loss of infectivity. While both of these studies represent a significant step towards furthering our understanding of the role of plasmid-encoded genes in borrelial virulence, the contribution of many of the circular plasmids could not be assessed because 1) they were not lost spontaneously during the isolation of clones (Purser and Norris, 2000), or 2) they were not included in the test panel to determine plasmid content (Labandeira-Rey and Skare, 2001). These data are, however, consistent with the notion that one possible role for the plasmids of *Borrelia* spp., like the virulence-associated plasmids of many bacteria (i.e., *Salmonella* spp, *Shigella flexneri*, *Bacillus anthrasis* and *Agrobacterium tumefaciens*), is to encode proteins that enable this bacterium to survive in vastly different environmental conditions and hosts. Also consistent with this hypothesis is the presence of genes that are expressed preferentially within either of these different host environments. To date, all of the differential gene expressions described have been localized to the linear and circular plasmids, further supporting a significant role for plasmids in the biology of these spirochetes (Wallich et al., 1993; Champion et al., 1994; Akins et al., 1995; Akins et al., 1999; Schwan et al., 1995; Stevenson et al., 1995; Stevenson et al., 1998a; Suk et al., 1995; Porcella et al., 1996; Lahdenne et al., 1997; Cassatt et al., 1998; Fikrig et al., 1999; Yang et al., 1999; Caimano et al., 2000; Miller et al., 2000; Hefty et al., 2001).

The *Borrelia burgdorferi* Genome Sequence

One of the most significant recent developments in borrelial research was the publication of the complete DNA sequence of the *B. burgdorferi* chromosome (Fig. 5) and associated plasmid elements (Fraser et al., 1997; Casjens et al., 2000; Fig. 11). The *B. burgdorferi* strain chosen for this project was the type strain B31 (ATCC 35210), an isolate obtained from an *I. scapularis* tick caught on Shelter Island, New York (Burgdorfer et al., 1982; Johnson et al., 1984). Prior to beginning the sequencing project, the American Type Culture Collection (ATCC) isolate was cloned by limiting dilution to yield an isolate now referred to by many as "B31-MedImmune" (B31-MI; Fraser et al., 1997). While this isolate has been minimally passaged in vitro, B31-MI is missing three circular plasmids (cp32-2, cp32-5 and cp9-2) as compared to the original isolate (Stevenson et al., 1998c; Miller et al., 2000). Briefly, the data from this project revealed that the *B. burgdorferi* linear chromosome is approximately 911 kb and is predicted to encode 853 open reading frames (ORFs). Biological roles were assigned to 59% of these ORFs. Of the remaining ORFs, 12% matched hypothetical coding sequences of unknown function from other organisms, and 29% were unique to *B. burgdorferi* (Fraser et al., 1997). In addition to the large linear chromosome, 12 linear and 9 circular extrachromosomal elements (plasmids) were also sequenced, totaling an additional 611 kb (Fraser et al., 1997; Casjens et al., 2000). In contrast to the ORFs encoded on the chromosome, greater than half of the ORFs contained on the plasmids did not have significant database matches to organisms other than *Borrelia*.

In the pre-genomic era, a number of laboratories had published reports calling attention to the fact that *B. burgdorferi* contains a large number of "repeated" sequences (i.e., related sequences present in multiple copies per genome; Simpson et al., 1990; Zückert et al., 1994; Zückert et al., 1999; Marconi et al., 1996b; Porcella et al., 1996; Zückert and Meyer, 1996; Fraser et al., 1997; Stevenson et al., 1997; Stevenson et al., 2000a; Carlyon and Marconi, 1998a; Carlyon et al., 1998b; Caimano et al., 2000; Carlyon et al., 2000), reviewed in Casjens et al. (2000). The full extent of this sequence redundancy has become apparent with the availability of the complete genomic sequence (Fraser et al., 1997; Casjens et al., 2000). One hundred and fifty-six paralogous families have been identified, with each paralogous family having at least two and up to 42 members. Although biological roles have been assigned to some of these paralogous families (e.g., ABC transporters [PF4, PF37,

PF41, PF105], methyl-accepting chemotaxis proteins [PF13], and plasmid partitioning [PF32, PF49]), the majority represent proteins with no known function. The large number of paralogous families is strong evidence that recombinatorial processes have conferred a remarkable degree of plasticity upon the *B. burgdorferi* genome, presumably in the context of enhancing its adaptation to diverse ecological niches.

The cp32 Family of Supercoiled Circular Plasmids

One of the best characterized examples of genetic redundancy in *Borrelia* is the family of 32- and 18-kb supercoiled circular plasmids (cp32s; Zückert et al., 1994; Zückert and Meyer, 1996; Casjens et al., 1997b; Stevenson et al., 1997; Stevenson et al., 1998c; Stevenson et al., 2000b; Akins et al., 1999; Caimano et al., 2000; Casjens et al., 2000). *Borrelia burgdorferi* sensu stricto isolates may contain up to 9 different cp32 plasmids (Fig. 11), each encoding *ospE*/*ospF*/*elp*/*erp* loci (Akins et al., 1995; Akins et al., 1999; Stevenson et al., 1996; Stevenson et al., 1997; Stevenson et al., 1998c; Casjens et al., 1997a; Caimano et al., 2000; see "OspE/OspF/Elp Lipoproteins"), as reviewed in Stevenson et al. (2000b). Each member of this plasmid family shares a high degree of similarity throughout its sequence, with only limited regions of sequence heterogeneity observed. Interestingly, the three regions, which are variable between members of this plasmid family, contain loci thought to be involved in the stable maintenance and partitioning of these plasmids and two paralogous families of differentially expressed lipoproteins (the Mlp Lipoproteins and OspE/OspF/Elp lipoproteins; Porcella et al., 1996; Stevenson et al., 1996; Stevenson et al., 1998c; Stevenson et al., 2000a; Akins et al., 1999; Yang et al., 1999; Caimano et al., 2000). Evidence has been presented that suggests these latter two variable regions represent sites of extensive recombination during the molecular evolution of this plasmid family (Stevenson et al., 1998c; Akins et al., 1999; Caimano et al., 2000). In addition, the presence of "unique" sequences downstream of some of the OspE/OspF/Elp loci suggests that *B. burgdorferi* may be capable of acquiring and incorporating exogenous DNA (Caimano et al., 2000). The relatively high density of spirochetes in the tick midgut, compared to the density within mammalian tissues, makes the midgut the most likely milieu in which this recombination occurs.

Linear forms of cp32-related plasmids have been described in *B. burgdorferi* (lp56) and *B. parkeri* (Fraser et al., 1997; Casjens et al., 2000; Stevenson et al., 2000b). An analysis of one linear plasmid, lp56, revealed that it contains within it an essentially intact copy of a cp32-like plasmid. The region of lp56 that is cp32-like appears to have resulted from an integration event (by an unknown mechanism) between a progenitor cp32-like plasmid and an unique linear plasmid. Although the inserted cp32-like progenitor shares a higher degree of homology with several cp32s (cp32-4, cp32-6 and cp32-9), it is not identical to any of the seven known cp32 plasmids and therefore potentially represents an eighth member of this family (Casjens et al., 2000). A second example of recombination between the linear and circular plasmids is evident on lp54 (Casjens et al., 2000; Caimano et al., 2000; Fig. 13). This plasmid is notable because it encodes the *ospA/B* (see "Reciprocal Expression of OspA and OspC") operon and the decorin-binding protein operon *dbpBA* (see "Decorin- and Fibronectin-binding Proteins"); both of these operons have been shown to be differentially expressed (*ospA/B* is downregulated, whereas *dbpBA* is upregulated in the mammalian host). The linear lp54 plasmid contains several stretches with high degrees of homology to paralogs which are otherwise exclusively encoded on cp32 plasmids (Fraser et al., 1997; Caimano et al., 2000; Casjens et al., 2000; Fig. 13). The presence of subsequent insertions and replacements within the region of cp32-like sequence suggests that the recombinatorial event(s) responsible for shaping lp54 is likely to have occurred much earlier in the evolution of the *Borrelia* plasmid complement than the event that resulted in the lp56 plasmid (Casjens et al., 2000).

Two different types of recombinatorial events are thought to have occurred within the cp32 family of plasmids (Caimano et al., 2000). The first involved exchanges between these plasmids and the bacterium's other genetic elements. This process appears to have been relatively unrestricted in that it utilized diverse donor sequences and recipient sites; its principal constraint is that it was limited to sequences already present within the borrelial genome. One of its primary functions appears to have been plasmid building (Fig. 13), that is, generating the scaffolding or framework needed to assemble novel genetic elements. The second type of recombinatorial event, in contrast, was confined to relatively small regions of the cp32 plasmids but was less restrictive in that it utilized nonparalogous DNAs, including what may be exogenous genes (i.e., the unique sequences downstream of some of the OspE/OspF/Elp lipoproteins loci). A major consequence of this second type was to individualize plasmids via the variable regions.

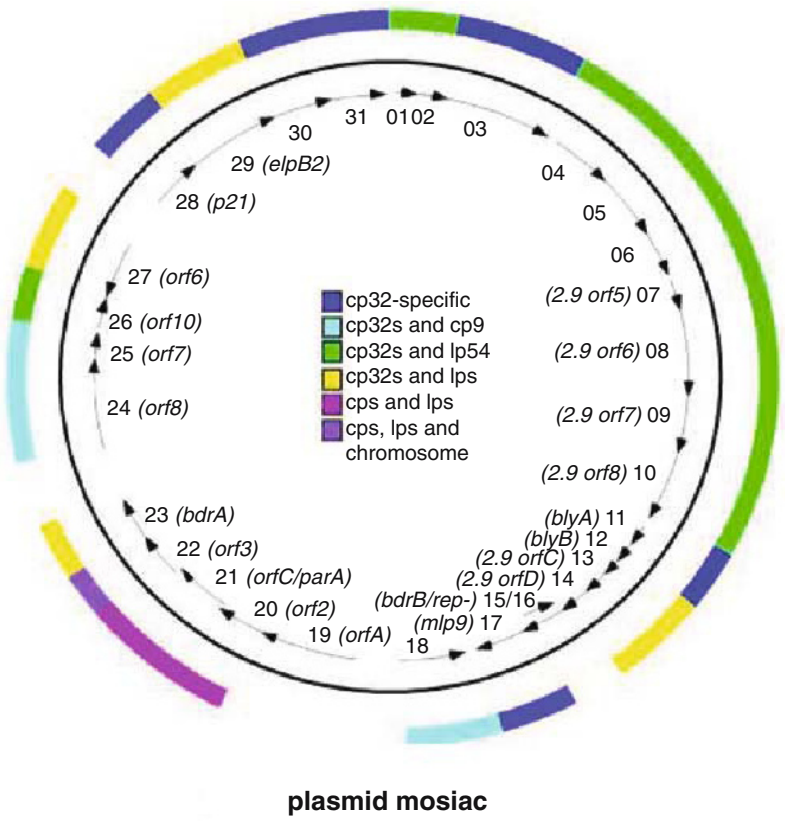


Fig. 13. *Borrelia burgdorferi* strain 297 cp18-2. Representative depiction of the mosiac nature of the cp32/18 plasmid family. Taken from Caimano et al. (2000), with permission of American Society of Microbiology (ASM) Press (http://www.journals. asm.org).

Recently, members of the cp32 family were also described in the relapsing fever spirochete, *B. hermsii* (Stevenson et al., 2000a). Although this plasmid contains many of the sequences that typify the cp32 family of plasmids (i.e., Mlp Lipoproteins, two Bdr paralogs, and partitioning loci (PF32 and PF50 paralogs), it does not contain an OspE/OspF/Elp (Erp) lipoprotein (Stevenson et al., 2000b; OspE/OspF/Elp Lipoproteins). Even more striking, however, is that the position of the Mlp Lipoproteins on the *B. hermsii* plasmid is in the position normally occupied by the *ospE*/*ospF*/*elp*/*erp* (see "OspE/OspF/Elp Lipoproteins") locus. An analysis of the *B. hermsii* genome by Southern hybridization did not reveal any sequences that hybridized with a *B. burgdorferi ospE*/*ospF*/*elp*/*erp* probe (Stevenson et al., 2000a), suggesting that the relapsing fever spirochete lacks homologs of these lipoproteins, or if they are present, their sequences are divergent beyond detection at the level of stringency used. The *B. hermsii* cp32 also encodes a novel protein, Bhm, a putative nucleotide methylase (Stevenson et al., 2000b).

Borrelia Pseudogenes

In addition to the duplications and rearrangements identified in the *B. burgdorferi* genome, a significant proportion of the genome was found to contain a large number of mutationally damaged genes and/or genes thought not to be expressed due to frameshift, inframe stop codons, and fused or truncated genes (pseudogenes; Casjens et al., 2000). In some instances, the majority of a plasmid's copy capacity is comprised of pseudogenes; for example, lp28-1 encodes 36 pseudogenes (78% of its total coding capacity), lp21 encodes 6 pseudogenes (50% of its coding capacity), and lp56 encodes 22 pseudogenes (61% of its total coding capacity; Casjens et al., 2000). In total, 670 potentially functional (non-pseudo)genes and 167 pseudogenes were identified on the 21 plasmids sequenced (Fraser et al., 1997; Casjens et al., 2000). Interestingly, ten of the strain B31 plasmids (lp5, lp17, lp21, lp25, lp28-1, lp28-3, lp28-4, lp36, lp38 and the non-cp32-like portion of lp56) contain 87% of the pseudogenes and have a total non-pseudogene coding capacity of only 41%, with the majority (43%) of these encoding genes ≥300 bp in length (Casjens et al., 2000). It has been hypothesized that the large number of pseudoegenes present on some borrelial plasmids may represent remnants of genes that are no longer useful but have not yet been completely eliminated from the genome (Casjens et al., 2000). Two recent publications may help explain why at least two of the pseudogene-rich plasmids are

consistently maintained in the *B. burgdorferi* genome: 1) the localization of a locus, *vlsE* (*B. burgdorferi* vls Locus), thought to be responsible for antigenic variation in *B. burgdorferi* to lp28-1 (Zhang et al., 1997), and 2) lp28-1 and lp25 were both correlated with infectivity in mice (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001).

Bacteriophage of *Borrelia*

Bacteriophage-like particles have been described for a number of spirochetes, including *Borrelia*, *Treponema* (see The Genus *Treponema* in this Volume), *Leptonema*, *Leptospira* and *Brachyspira* (see review by Eggers et al., 2000). Hayes and coworkers (Hayes et al., 1983) identified the first bacteriophage-like particles in *B. burgdorferi* in 1983. Two similar phage-like particles were described from a human isolate of *B. burgdorferi*, but differed (at the ultrastructural level) from those particles reported by Hayes and co-workers. These data suggest that borreliae may harbor more than one type of bacteriophage (Neubert et al., 1993; Schaller and Neubert, 1994). Recently, Eggers and coworkers have characterized a bacteriophage ΦBB-1 (Fig. 14) from *B. burgdorferi* stain CA-11.2A (Eggers and Samuels, 1999) and demonstrated that phage particles could be produced spontaneously or by the use of a DNA alkylating compound (MMNG, 1-methyl-3-nitro-nitrosoguanidine) as an inducing agent. A molecular characterization of ΦBB-1 revealed that the phage heads were packaged with a discrete piece of linear, double-stranded DNA molecule 32 kb in size as its genome. These linear molecules lack the covalently closed ends characteristic of the linear plasmids typically found in *Borrelia*. Further analyses demonstrated that the prophage form of ΦBB-1 is maintained as one or more members of the cp32 plasmid family and that genes present on these plasmids (BBP41 to BBP26) most likely constitute the genes necessary for a phage late regulon (Eggers et al., 2000). Eggers and coworkers have also been able to use ΦBB-1 as a tool for transduction (the transfer of DNA between bacteria using bacteriophage) by transferring an antibiotic-resistance marker (*kan*^{r}) between cells of different *B. burgdorferi* strains (Eggers et al., 2001).

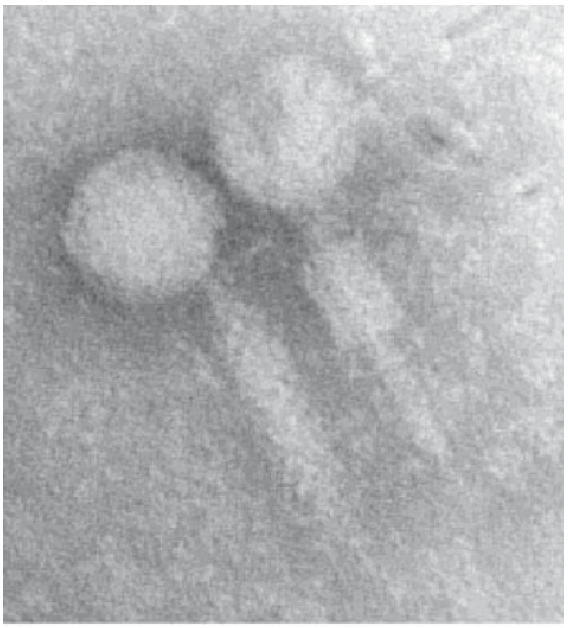


Fig. 14. *Borrelia burgdorferi* phage ΦBB-1. Samples were collected from polyethylene glycol (PEG)-precipitated cell-free supernatants of a *B. burgdorferi* culture, stained with phosphotungstic acid, and viewed by transmission electron microscopy. The contractile tail is either extended (left) or contracted (right). Image taken from Eggers et al. (2001), with permission of ASM Press (http://www.journals.asm.org).

Genetic Transformation of *Borrelia*

The inability to introduce exogenous (borrelial or non-borrelial) DNA into *Borrelia* spp. had long been a hindrance to using molecular biology techniques (i.e., transformation, gene inactivation, and transfection/transduction) that have become routine in most other well-characterized bacterial strains such as *E. coli*, *Bacillus subtilis* and *Streptococcus pneumoniae*. This was due in part to both the difficulties associated with the development of a method for the introduction of exogenous DNA into *Borrelia* spp. and the lack of a suitable cloning (shuttle) vector; these two basic elements are necessary to perform many of the classical molecular genetics experiments. Recent work on *B. burgdorferi*, however, has demonstrated that both of these hurdles may soon be cleared (see review by Tilly et al., 2000). One of the first major advances in molecular genetics was the demonstration of transformation of *B. burgdorferi*. In this pioneering experiment, Samuels and co-workers (Samuels et al., 1994b) were able to introduce a genetic marker (coumermycin A_1 resistance [*gyrB*^{r}]) into *Borrelia* using electroporation. This methodology, combined with a solid medium plating technique, allowed for the isolation of single colony isolates of transformed bacteria (Samuels and Garon, 1993; Samuels et al., 1994a; Samuels et al., 1994b; Samuels et al., 1995). These studies were soon followed by the first allelic exchange studies using targeted insertions of borrelial

genes (Rosa et al., 1996; Tilly et al., 1997). A number of additional borrelial genes involved in peptide transport (*oppAIV*), nucleotide biosynthesis (*guaB*), and environmental sensing and / or virulence (*ospC*, see "Reciprocal Expression of OspA and OspC") have since been mutated by gene inactivation and their phenotypes examined (Tilly et al., 1997; Tilly et al., 1998; Bono et al., 1998). The limitations of using coumermycin soon necessitated the development of tools for the introduction of heterologous (foreign) DNA to monitor gene expression without antibiotic selection (Stevenson et al., 1998b). The most recent breakthrough came with the development of the first extrachromosomal cloning vector system for *B. burgdorferi*. In this system, a broad-host range vector, pGK12 (containing replication functions from *Lactococcus lactis* and erythromycin- and chloramphenicol-resistance markers from *Staphylococcus aureus*), was used to transform *B. burgdorferi* and confer resistance to erythromycin. This plasmid was shown to replicate autonomously in strain B31 and conferred resistance to erythromycin to these transformed bacteria. These studies permitted the development of a reporter plasmid containing enhanced green fluorescent protein (EGFP) under the control of a borrelial promoter (Saratokova et al., 2000). These combined studies represent major steps toward furthering our understanding of borrelial genomics, particularly the regulation of gene expression and function. The majority of this work was performed using noninfectious strains of *B. burgdorferi*. To fully realize the potential of these molecular tools, methods for the transformation of infectious strains of *Borrelia* will need to be developed. Towards that end, recent studies by Stewart et al. (2001) have reported the isolation of a circular plasmid region from a *B. burgdorferi* circular plasmid (cp9) that is capable of autonomous replication and represents the first shuttle vector based on a borrelial origin of replication and plasmid partitioning. Stewart and co-workers also were able to demonstrate that this cp9-based shuttle vector can be electroporated into an infectious strain of *B. burgdorferi* (Stewart et al., 2001). One notable consequence of this transformation, however, was the rapid displacement of the endogenous cp9 plasmid of the recipient strain N40, a phenomenon almost certainly related to a plasmid compatibility issue between the shuttle vector (containing the cp9 replication region) and the endogenous cp9. While this displacement event did not affect the ability of the bacterial transformants to grow in culture medium, it does present a significant limitation of using this and similar shuttle vectors in a genetic background containing the full genetic complement of borrelial plasmids, whose roles in bacterial physiology and/or pathogenesis have not been fully determined.

Epidemiology

Relapsing Fever

Two major forms of relapsing fever have been described, epidemic (louse-borne) and endemic (tick-borne). During large epidemics of louse-borne relapsing fever (LBRF), millions of people have been infected. The last large outbreak of epidemic relapsing fever occurred during and following World War II in the Mediterranean region, North Africa, and the Middle East (Felsenfeld, 1965; Bryceson et al., 1970; Cadavid and Barbour, 1998). Current outbreaks of LBRF are primarily reported from African countries, particularly Ethiopia and Sudan (Cadavid and Barbour, 1998), with some outbreaks occurring in the South American Andes.

Endemic tick-borne relapsing fever (TBRF), transmitted by soft ticks of the genus *Ornithodoros* (Table 1), is more sporadic in occurrence than LTRF. TBRF is endemic in the western United States, southern British Columbia, the plateau regions of Mexico, and Central and South America, the Mediterranean, Central Asia, and throughout most of Africa. In the United States, the environment in which a human host comes into contact with a tick vector is largely determined by the geographical location. In Texas, in the relapsing fever spirochete, *B. turicatae*, TBRF is transmitted by *O. turicatae* and is primarily acquired by residents or tourists cave exploring. In most other western states and southern British Columbia, *B. hermsii* is transmitted by *O. hermsi* and primarily acquired at higher elevations by visitors of rustic cabins that may house rodent nests. A recent outbreak of TBRF (due to *B. hermsii*) in Colorado was associated with a visit by vacationers from Nebraska and Kansas to a rustic cabin in Colorado (Trevejo et al., 1998). In this report, 11 out of 23 family members vacationing at one rustic cabin developed symptoms suggesting TBRF, and although no spirochetes were visible in the case-patients blood smears, *B. hermsii* was cultured from the blood of one case-patient and two chipmunks trapped near the cabin (Trevejo et al., 1998). A survey of previous visitors to that same cabin identified five additional persons from Nebraska, Kansas, Florida and Texas who had developed similar symptoms following their visit. A recent review by Dworkin et al. (1998), however, identified 133 confirmed cases and 49 probable cases in northwestern United States and southwestern Canada. Confirmed cases were defined as both a febrile illness and detection

of spirochetes by microscopic examination of peripheral blood smears. A probable case was defined as a typical clinical history and either an exposure associated with a confirmed case or a positive serology test or enzyme-linked immunosorbent assay (ELISA) confirmed by Western blot. The distribution of these cases was primarily in Washington (33%), Oregon (25.3%), Idaho (22%), and British Columbia (14.3%), with the remaining cases in Montana (0.5%) or not specified (4.9%). The majority of these cases were thought to be acquired by visiting rural cabin dwellings or homes (76.3%), often located on or near a body of water. Potential rodent reservoirs (mice, squirrels, chipmunks and rats) were also present at the exposure sites. The median elevation at which exposure occurred in 89 of the 182 cases was 2,131 feet (range, 100–6,436 feet). The most frequent months of onset were the summer months (July and August), but cases were identified in all months except November. The cases were fairly evenly distributed among the sexes (male, 57.1%; female, 42.9%), but a higher number of cases were seen in children younger than 5 (6%) or under 18 years of age (28.9%). Four women (11.4%) pregnant during their illness were included in the review, one of whom gave birth to an infected infant. Many cases in the southern United States (i.e., Texas) are often associated with cave exploration. In this region, the tick vector (*O. turicata*) of the relapsing fever spirochete, *B. turicatae*, is found in burrows made by cave-dwelling rodents, owls and snakes (Goubau, 1984). TBRF in the southern United States exhibits a different seasonality from TBRF in the northwestern areas, with the majority of cases having a late fall, early winter month of onset (Burgdorfer, 1976b).

Unlike Lyme disease (see "Epidemiology"), TBRF is not a nationally reportable communicable disease and is likely to be underreported. TBRF also may be incorrectly identified as Lyme disease owing to false positivity in Lyme disease serological diagnostic testing (Dworkin et al., 1998). Unless a patient history of wilderness exposure or camping in rodent-infected areas is given and the patient is aware of a tick bite, the illness is usually not suspected during the initial period of fever (Fihn and Larson, 1980).

Lyme Disease

According to the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/dvbid/lymeinfo.htm), Lyme disease is the most common vector-borne infection in the United States. According to surveillance data submitted to the CDC between 1992 and 1998, a total of 88,967 cases of Lyme disease were reported by 49 states, the District of Columbia, and Guam (2 cases), with a mean annual average of 5.1 reported cases/100,000 persons/year (Orloski et al., 2000; Fig. 17). Of the total cases reported nationwide, the majority were from only four Northeastern states, New York (32.8%), Connecticut (17.4%), Pennsylvania (14.6%), and New Jersey (12.2%; Orloski et al., 2000). The reported incidence of Lyme disease (Fig. 16), when plotted by county, closely mirrors the regional distribution of the arthropod vector (Fig. 15) *Ixodes scapularis* in the Northeast and Midwest and *Ixodes pacificus* in the pacific northwest United States (Orloski et al., 2000). Cases have also been reported in most European countries where the Lyme spirochete is transmitted by *Ixodes ricinus*. In addition, the disease has been reported in China, Japan, Africa and Australia (Table 2).

In the United States, Lyme disease is a reportable disease, and epidemiological data are collected and cataloged by the CDC (http://www.cdc.gov/ncidod/dvbid/lymeinfo.htm). Lyme disease in Europe are not reportable, and therefore the epidemiological features of borreliosis in this region are often limited. An extensive epidemiologic study of Lyme disease in Europe, however, was performed in southern Sweden in 1996 by Berglund et al. (1995). This study, conducted over a one-year period, used case definitions that generally corresponded to those used by the CDC for surveillance purposes (clinically diagnosed erythema migrans or one or more clinical manifestations of disease plus serological confirmation) to include a total of 1,471 patients with Lyme disease, with *B. afzelii* being the causative agent in all cases. This number reflects an overall annual incidence of 69 cases per 100,000 inhabitants, a number similar to that of endemic areas in the United States (24.2/100,000 and 98/100,000 in New York and Connecticut, respectively). As with cases in the United States, the incidence varied markedly according to geographic region, and the disease was widely prevalent in several areas, with incidences ranging between 26 per 100,000 to as high as 160 per 100,000 (Berglund et al., 1995). While no differences were observed in the number of cases between men and women, incidence in children (5–9 yrs old) and the elderly (60–74 yrs old) was higher (Berglund et al., 1995; Fig. 18).

In the northeastern United States, the onset of Lyme disease is greatest during the months of June, July and August (Orloski et al., 2000). The European study also reported a similar seasonality to Lyme disease, with tick bites being most frequent in July and the highest number of erythema migrans cases occurring in August and September (Berglund et al., 1995). These months coincide with the questing (feeding) period of the *Ixodid* nymph (Fig. 4), the life

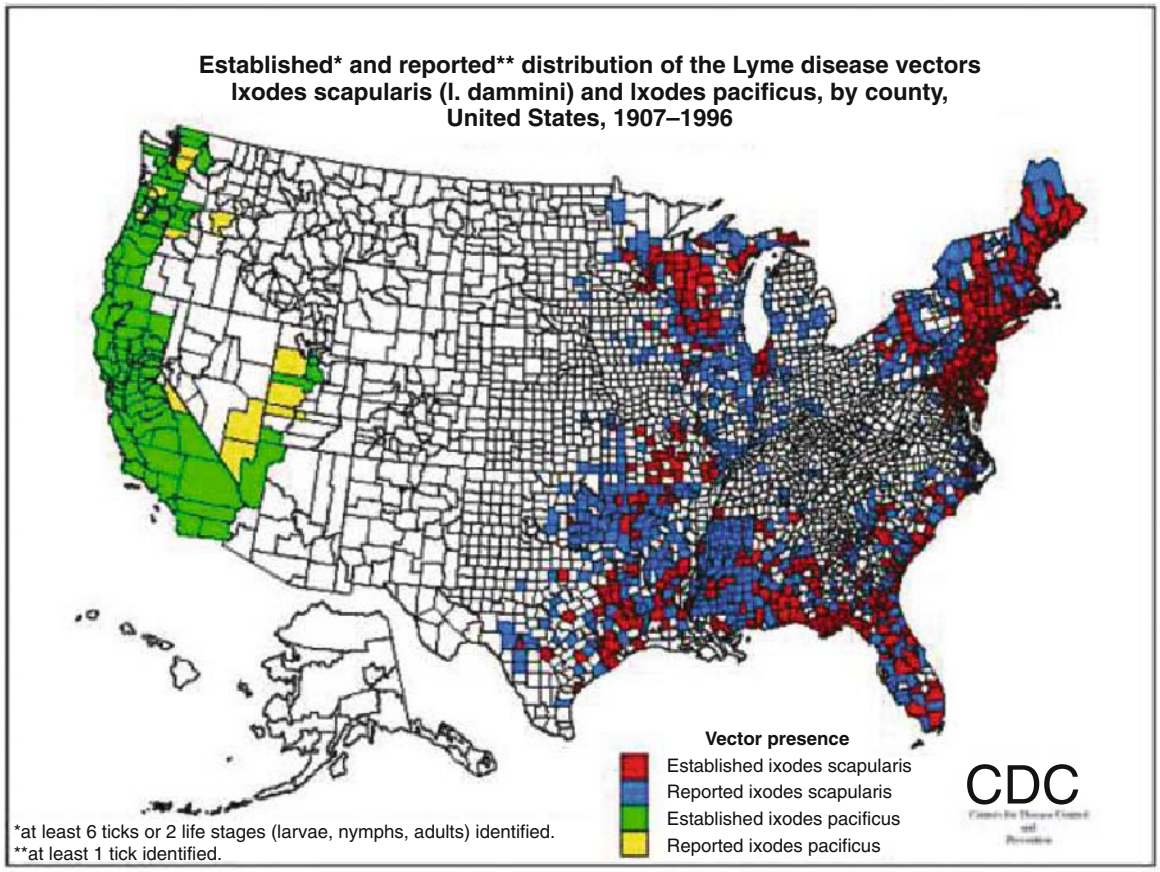


Fig. 15. Geographic distribution of *Ixodes* spp. Image adapted from the CDC (http://www.cdc.gov/ncidod/dvbid/lymeepi.htm).

stage primarily responsible for the transmission of *B. burgdorferi*. In addition, these months are often associated with increased outdoor activities, such as walking and hiking, which bring humans into closer proximity to the arthropod vector habitat.

Recent reports have described a Lyme disease-like illness in the south-central and southeastern United States in patients who have no known exposure to the Lyme disease vector, *Ixodes scapularis* (Barbour et al., 1996a; Fig. 15). In these cases, a skin rash resembling erythema migrans (EM; Fig. 20) has been associated with bites by the hard tick, *Ambylomma americanum*, the Lone Star tick. *Amblyomma americanum* has not been shown to be a competent vector for *B. burgdorferi* (Piesman and Happ, 1997; Dolan et al., 1998) and the infectious agent, *Borrelia lonestari* (*B. barbouri*), thought to be responsible for this Lyme disease-like illness (Barbour et al., 1996b) has yet to be cultivated (Uncultivatable *Borrelia* spp.).

Of mice and moths .... One interesting study by Jones et al. (1998) described a chain reaction of events beginning first with the level of acorns in a wooded area determining the winter survival, reproduction, and the resulting density of mice (both a predator of gypsy moth pupae and a reservoir of the Lyme disease spirochete, *B. burgdorferi*) in the oak forests of the eastern United States. Second, the relationship between mouse and moth densities can either suppress or increase subsequent acorn densities (i.e., high gypsy moth populations can result in deforestation and reduced acorn crops, whereas high mouse populations can result in increased pupae predation and increased acorn crops). Third, the location of high autumnal acorn density determines the next summer's larval tick densities by effecting the use of oak forests by deer (used by

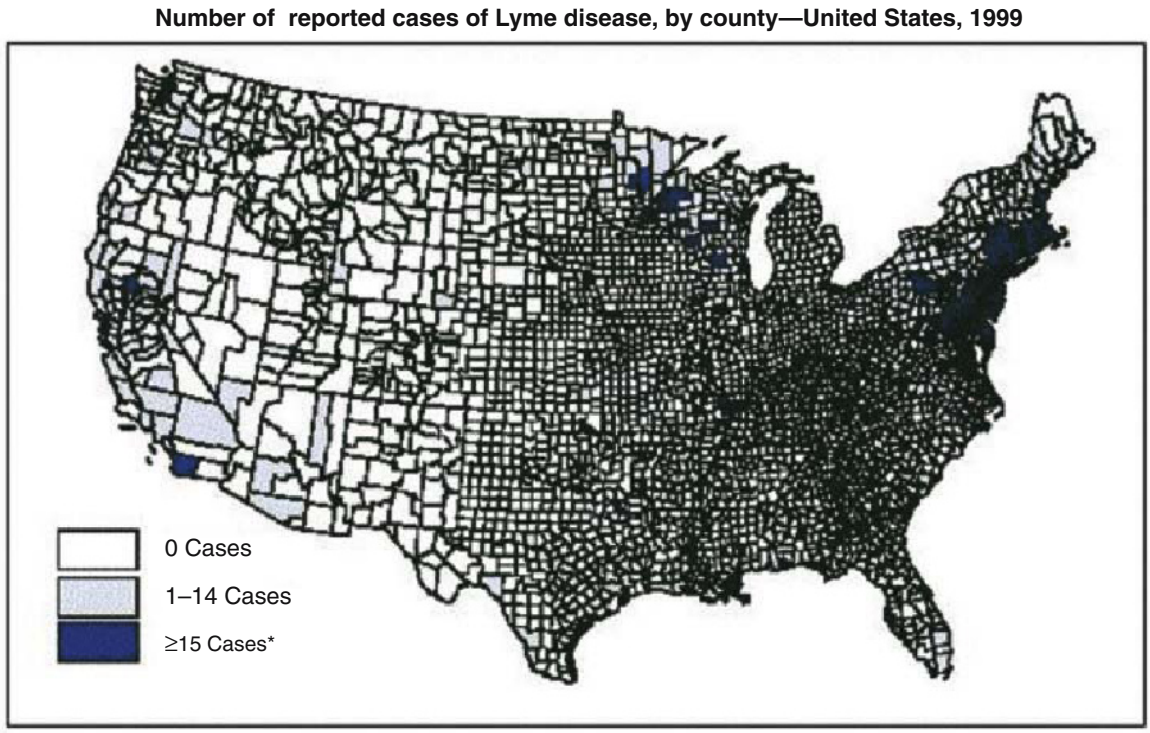


Fig. 16. Geographic distribution of reported cases of Lyme disease. Taken from Orloski et al. (2000).

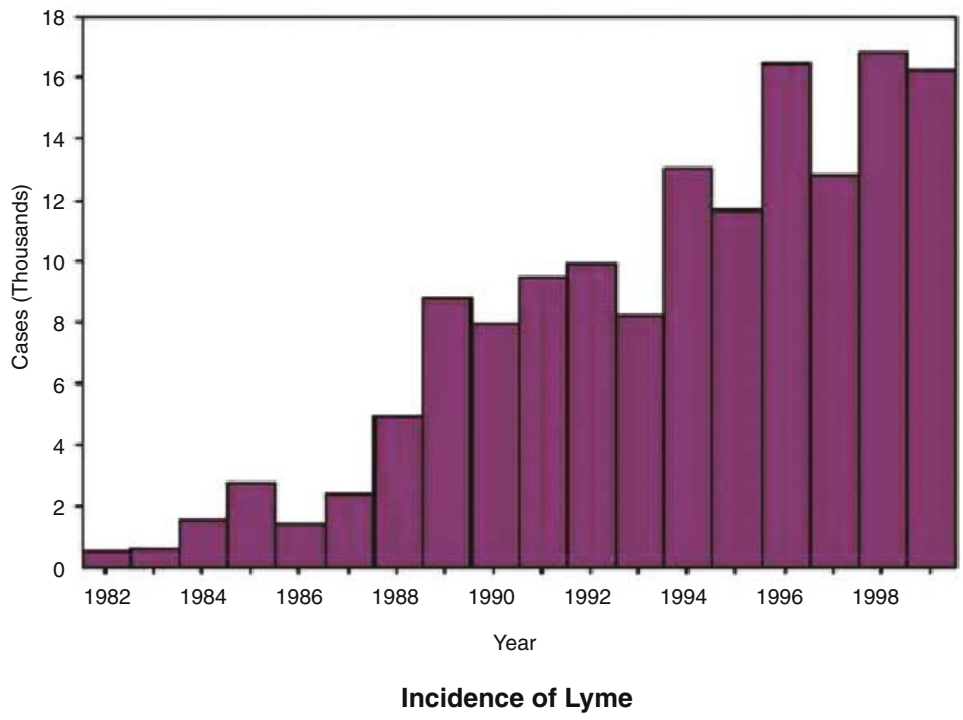


Fig. 17. Incidence of Lyme disease, by year—United States 1982–99 (http://www.cdc.gov/ncidod/dvbid/images/ld_case_barchart.gif). Taken from Orloski et al. (2000), with permission.

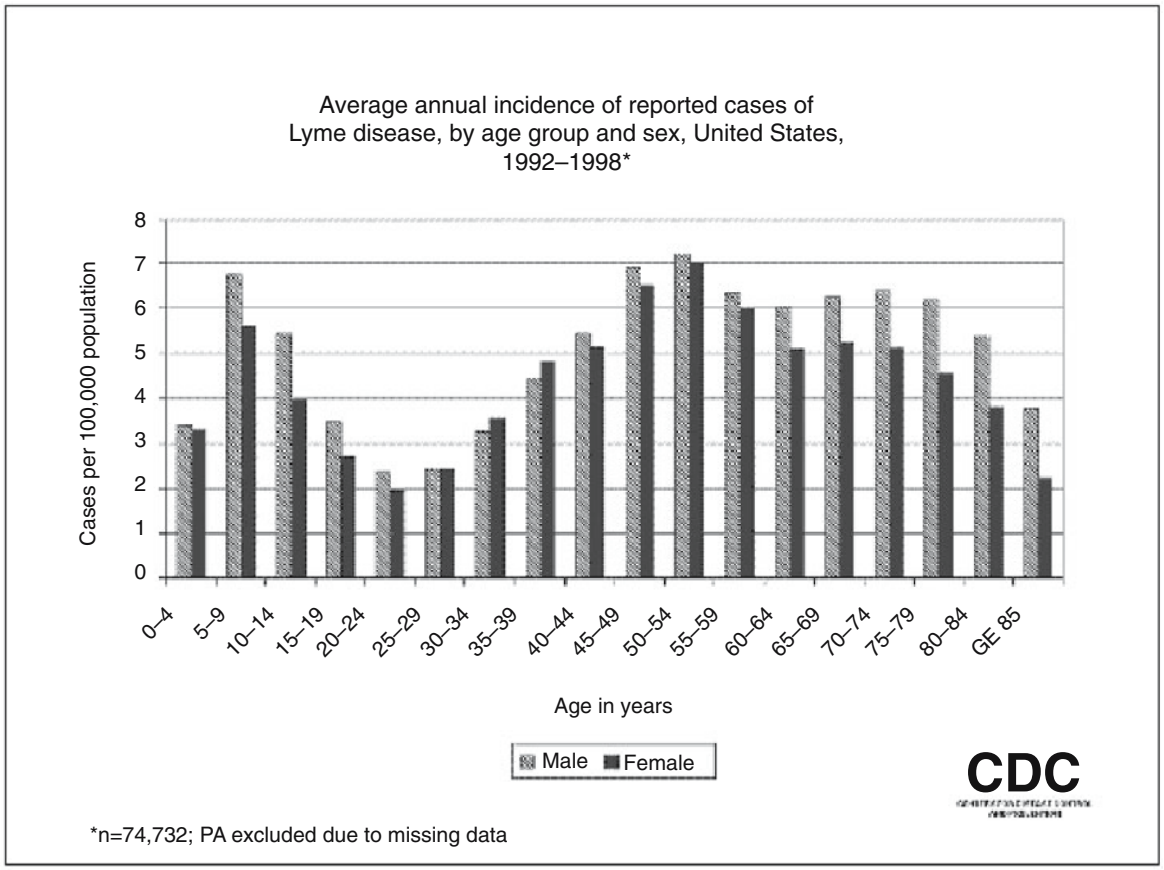


Fig. 18. Average annual incidence of reported cases of Lyme disease (http://www.cdc.gov/ncidod/dvbid/images/ld_case_barchart.gif), by age group and sex in the United States during 1992–1998 (http://www.cdc.gov/ncidod/dvbid/lyme/92-98agesex.htm).

adult ticks for mating); this situation in turn results in high densities of uninfected larval ticks and nymphal ticks questing when and where potentially spirochete-infected mice are most abundant. The authors suggest that it may be feasible to predict the risk of contracting Lyme disease from infected nymphal ticks in oak forests on the basis of autumnal acorn crops (masting), with the risk being greatest two years after an abundant acorn crop (Jones et al., 1998).

Pathogenicity

Relapsing Fevers

Two major forms of relapsing fever have been described, epidemic (louse-borne) and endemic (tick-borne; Table 1). For recent reviews of relapsing fever, see Dworkin et al. (1998) and Cadavid and Barbour (1998). In either of these forms, the relapsing fever borreliae gain access to the mammalian blood and lymph following transmission from an infected arthropod. After an incubation period of 4–18 days and after the spirochetes have multiplied to levels of 10^6–10^8 cells per ml of blood, the illness begins abruptly with shaking chills, fever, headache and fatigue, which persist for 3–7 days. Following an afebrile period of 5–10 days, a second symptomatic episode may occur. Additional relapses of decreasing time and intensity are characteristic of the tick-borne disease but are uncommon in the louse-borne disease. In either case, the relapses are the result of antigenic variation, where the spirochetes are able to undergo a number of cyclic antigenic variations (see "Antigenic Variation"). When antibodies to the prevailing antigenic type appear, organisms "disappear" from the peripheral blood and are replaced by a different antigenic variant within a few days. If the host is untreated, this process may be

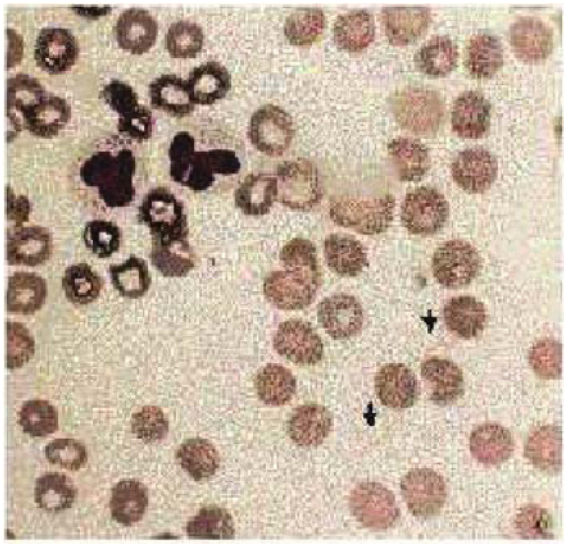


Fig. 19. Relapsing fever. A Wright's-stained peripheral-blood smear contains two borrelia spirochetes isolated from a patient with recurring fever (arrows) (http://www.nejm.org/content/1996/0335/0016/1197.asp). Taken from Newton and Pepper (1996).

Relapsing fever

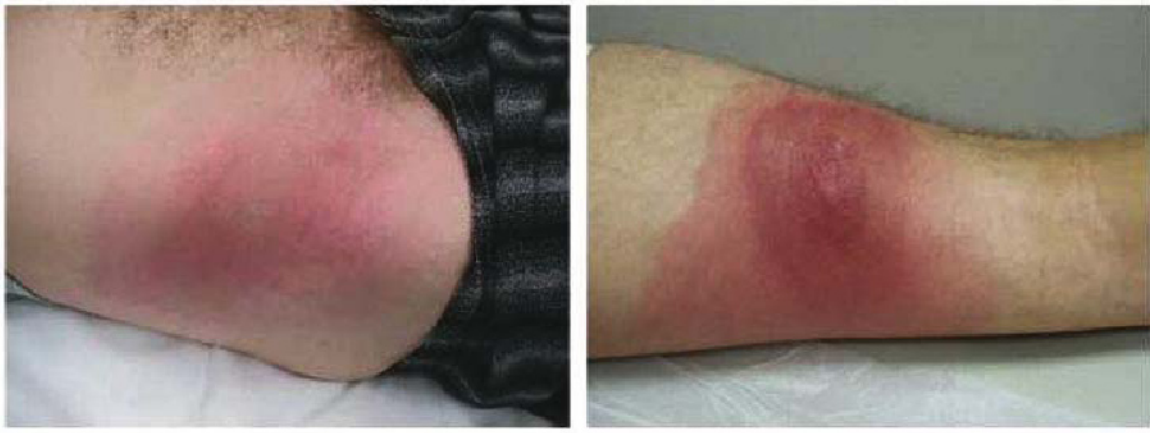
repeated several times, depending upon the infecting strain of *Borrelia*. During the acute phases of the illness, borreliae may be seen in blood smears stained with Giemsa or Wright stain (Fig. 19) and counterstained with crystal violet (Felsenfeld, 1971). The mortality rate in louse-borne relapsing fever ranges as high as 40% in untreated cases, but can be less than 5% following antibiotic treatment. For a recent excellent review of the clinical manifestations and pathology of tick-borne relapsing fever, see Cadavid and Barbour (1998).

Relapsing fever is well recognized as an infection of the blood, but a number of other tissues also may be involved (Felsenfeld, 1965; Barbour and Hayes, 1986b; Pachner, 1986; Garcia-Monco and Benach, 1995). In this respect, the multisystemic nature of the relapsing fevers is similar to infections with other pathogenic spirochetes (i.e., *B. burgdorferi*, *Treponema pallidum* (see The Genus *Treponema* in this Volume), and *Leptospira interrogans* (see The Genus *Leptospira* in this Volume). Patients with TBRF may exhibit symptoms of ocular involvement, including iritis, cyclitis, choroiditis and optic neuritis (Falcone, 1952; Cadavid and Barbour, 1998). In such cases, vision can rapidly deteriorate, leaving most patients with residual visual defects (Quin and Perkins, 1946; Bergeret and Raoult, 1948). Ocular disease has also been associated with relapse episodes (Hamilton, 1943; Falcone, 1952). Ocular complications are most frequently associated with relapsing fevers due to *B. duttonii*, *B. hispanica* and *B. turicatae* (Cadavid and Barbour, 1998).

The frequency of neurological involvement (i.e., lymphocytic meningitis and peripheral facial palsy, crossing of the blood-brain barrier, and persistence in brain tissue) in this disease is similar to that of Lyme disease (Reik et al., 1979; Pachner et al., 1989; Cadavid and Barbour, 1998; Table 4). Spirochetes have been observed by silver stain within the cerebral microvasculature and interstitial spaces and between neurons and glia of autopsied TBRF patients (Buschke and Kroo, 1922; Martínez-Báez and Villasana, 1945). No spirochetes were observed within the brain cells themselves (Lavaditi et al., 1929), a finding which is consistent with the extracellular nature of these pathogens. The relapsing fever spirochetes, however, have differing abilities to invade and persist in brain tissue. *Borrelia duttonii*, *B. crocidurae* and *B. hispanica* were able to infect the brains of all mammalian hosts examined (Cadavid and Barbour, 1998). In contrast, *B. recurrentis* infected the brains of primates (Garnham et al., 1947), but not the brains of mice (Sparrow, 1956). These differences may be due, in part or in whole, to the expression of particular variable membrane proteins (i.e., VspA, see "Antigenic Variation"), some of which have been associated with the increased central nervous system (CNS) involvement and neuroborreliosis (Cadavid et al., 1997; Cadavid and Barbour, 1998).

Although neurological involvement is rare during LBRF, edema and subarachnoid and parenchymal brain hemorrhages were consistently observed during the autopsy of patients who succumbed to infection (Belezky and Umanskaja, 1930; Anderson and Zimmerman, 1955; Judge et al., 1974; Salih et al., 1977; Ahmed et al., 1980). No ocular involvement has been reported in patients with LBRF.

As the geographical distribution of cases of TBRF and Lyme disease (Fig. 15) begin to over-



EM Lesions Images

Fig. 20. Examples of early erythema migrans (EM). Left, an example of a typical EM rash with a central zone of clearing (visible 18 cm in cross diameter) was present on the right iliac crest of a 25-year-old Connecticut man. Image was taken 10 days after initial detection. Image kindly provided by Juan Salazar, M.D., University of Connecticut Health Center. Right, an example of an intensely erythematous rash (15 cm in cross diameter) over the dorsum of the right thigh of a 32-year-old Texas man who most likely contracted Lyme disease while on vacation in Connecticut. The image shown was taken 4 days after initial detection. Image kindly provided by Timothy Sellati, Ph.D., University of Connecticut Health Center.

Table 4. Comparison of tick-borne relapsing fever (TBRF), louse-borne relapsing fever (LBRF), and Lyme disease.

Characteristics	TBRF	LBRF	Lyme disease
Agent	Several species	*B. recurrentis*	*B. burgdorferi* sensu lato[a]
Vector	*Ornithodoros* spp. (soft tick)	*Pediculus humanus* (human body louse)	*Ixodes* spp. (hard tick)
Usual reservoir	Rodents[b]	Humans	Rodents
Epidemiology	Endemic	Epidemic	Endemic
Distribution	Tropical and temperate regions	East Africa[c]	North America, Eurasia
In vitro cultivation	Yes	Yes[d]	Yes
Fever (temperature ≥39°C)	Common	Common	Rare
Fever relapses	Multiple	Few	None
Neurological involvement	Common[e]	Rare	Common
Local skin rash	No	No	Common (EM[f])
Arthritis	No	No	Common
Spirochetes on blood smear	Yes	Yes	No
Serological assay specificity	Fair to poor	Fair to poor	Good to excellent
Antibiotic therapy	Several doses	Single or few doses	Several doses
Jarisch-Herxheimer reaction	Moderate	Moderate to severe	Mild

[a]Included are *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*.
[b]The reservoir for *B. duttonii* in some locations may be humans.
[c]LBRF has a potential worldwide distribution because of association with human body louse.
[d]*In vitro* cultivation of *B. recurrentis* has been reported (Cutler et al., 1999).
[e]Common in >10% of patients with disseminated disease.
[f]Erythema migrans, Fig. 20.

lap, an underappreciation of the frequency of neurological (and ocular) involvement during TBRF could result in this disease being misdiagnosed as Lyme disease, or vice versa, in areas where both diseases are found (Barbour et al., 1996a). These two diseases, however, may be readily distinguishable in some cases by the presence of spirochetes at a density sufficiently high enough as to be visible in the blood during a febrile episode or by a temperature above 38°C (hallmarks of relapsing fever) or by the presence of a localized EM lesion (a hallmark of early Lyme (Borreliosis) Disease; Fig. 20). Molecular and serological techniques can be used to definitively differentiate between these *Borrelia* spp.

Lyme (Borreliosis) Disease

Lyme disease is a multisystem illness that primarily affects the skin, nervous system, heart and joints (Steere, 1989). The hallmark skin lesion of Lyme disease, erythema migrans, was first described in Europe in 1908 (Afzelius, 1921) and in the United States in 1969 (Scrimenti, 1970). The disease was brought to the attention of the medical community in the United States as the result of an outbreak of arthritis in adults and children in Lyme, Connecticut, United States, and the adjacent communities of Old Lyme and East Haddam. The resulting epidemiological and clinical investigations led to the identification of a new disease entity, Lyme disease (Steere et al., 1977). The causative agent of Lyme disease was first described by Willy Burgdorfer and colleagues in 1982, and the species bears his name, *Borrelia burgdorferi* (Burgdorfer et al., 1982; Burgdorfer et al., 1991; Burgdorfer et al., 1993). For an excellent review of Lyme borreliosis, see Steere (1998a).

Lyme disease can be divided into early and late infection (Table 5). Approximately seven to nine days following the initial tick bite, the early (localized) infection is marked by the pathognomonic skin lesion, erythema migrans (in 60–90% of patients; Fig. 20), and is accompanied by fever, regional lymphadenopathy, or minor constitutional symptoms (Steere, 1989).

The EM lesions (Fig. 20) usually fade within three to four weeks, even when untreated, but they may recur at secondary sites coincidental or distal to the initial EM lesion. Weeks to months later (disseminated disease), patients may develop meningoencephalitis, Bell's palsy, myocarditis, migrating musculoskeletal pain and intermittent attacks of arthritis, especially in the large joints such as the knee. As the infection progresses (late or persistent infection), the episodes of arthritis last for months rather than weeks. Also, syndromes of both the central and peripheral nervous system may occur more than a year after the initial infection. Persistent infections in Lyme disease may also develop as acrodermatitis chronicum atrophicans (ACA), the late skin manifestation of the disease, which has primarily been observed in Europe (Asbrink and Hovmark, 1988; Kaufman et al., 1989). The skin lesion typically begins as a bluish-red discoloration of swollen skin on an extremity. This lesion may occur at an earlier site of EM. The inflammatory stage of ACA may persist for years or decades and may lead to atrophy of the skin. Transplacental transmission of *B. burgdorferi* has been reported; however, congenital infection with adverse outcome, such as perinatal death, appears to be unusual (Schlesinger et al., 1985; Weber et al., 1988).

Since Lyme disease is not a notifiable disease in Europe, the knowledge of the epidemiological features of this illness in this region is limited. One extensive study of Lyme borreliosis in southern Sweden, however, does represent an excellent source for reliable epidemiologic (see Epidemiology) and clinical data for this disease (Berglund et al., 1995). The most frequent clinical manifestation (Table 5) was EM (seen in 77% of all cases; Fig. 20), followed by neuroborreliosis (16%) and arthritis (7%). With the exception of a slightly lower rate of carditis (0.5–4%), the pattern of disease found in this study was similar to that reported in the United States.

After the first several weeks of infection, most patients have a positive antibody response to the spirochete. The specific immunoglobulin (Ig)M response is detectable after the third week and peaks after 6–8 weeks (Steere et al., 1983). The *B. burgdorferi*-specific IgG response peaks during the second to third month of infection and may persist for years. Since the direct visualization and culture of *B. burgdorferi* from patient specimens are difficult, serologic determinations are currently the most practical mode of laboratory diagnosis. False negative results occur early in the disease, whereas false positives may occur in patients with a variety of other diseases, including syphilis, Rocky Mountain spotted fever, autoimmune disease, and neurologic disorders. Differential diagnosis is most often made using the CDC surveillance case definition (http://www.cdc.gov/ncidod/dvbid/casedef2.htm) as a guide. If Lyme disease is suspected in an endemic area, patients are often started on antibiotic therapy based solely on the presence of an EM rash (5 cm in diameter; Fig. 20) and/or a history of a recent ixodid tick bite accompanied by one or more of the early Lyme disease symptoms outlined by the CDC surveillance case definition.

Borrelia Genotype and Pathogenesis

Currently, all borreliae capable of causing Lyme disease are members of the *B. burgdorferi* sensu lato complex that is comprised of ten different *Borrelia* genospecies (Wang et al., 1999; Table 2). North American isolates are limited primarily to *B. burgdorferi* sensu stricto, whereas *B. garinii* and *B. afzelii* isolates, in addition to *B. burgdorferi* sensu stricto, are responsible for the majority of cases of Lyme disease in Europe (Table 2). These latter genospecies are more often associated with neuroborreliosis (*B. garinii*; van Dam et al., 1993; Lebech et al., 1994; Wilske et al., 1996; Marconi et al., 1999; Cauwels et al., 2001) and chronic skin manifestations (i.e., ACA; *B. afzelii*; van Dam et al., 1993; Wienecke et al., 1994; Strle et al., 1996; Picken et al., 1998;

Table 5. Major clinical manifestations of Lyme borreliosis in North America and Europe.

Stage[a]	Clinical feature[b]	Incidence in: North America[c]	Incidence in: Europe[d]
I	*Early local infection*		
	EM	Common (60–90%)	Common (~77%)
	Tick bite recalled	25%	64%
	Central clearing of EM	35%	68%
	Systemic symptoms	50–69%	38–51%
II	*Early disseminated infection*		
	Multiple EM	Common (≥18%)	Unusual (6%)
	Neuroborreliosis	Common (10–20%)	Common (16–80%)
	Meningoradiculitis	3–21%	37–61%
	Meningitis	2–17%	4–27%
	Carditis	0.5–10%	0.5–4%
	Borrelial lymphocytoma	Rare	Well documented (3%)
III	*Late LB*		
	Lyme arthritis	Common (51–57%)	Uncommon (~7%)
	ACA	Rare	Well documented (3%)
	Peripheral neuropathy	30–70% late NB	40–63% ACA patients
	CNS involvement	Well documented	<9%
	Encephalomyelitis	Rare (0.1%)	4–6%
	Menigencephalitis	9%	0.5–4%

[a]Stages of the clinical features are those of Steere (Steere, 1989, ID: 185).

[b]NB, neuroborreliosis; CNS, central nervous system.

[c]Data were mainly on an earlier report by the Centers for Disease Control and Prevention on LB surveillance from 1984–1986 (Ciesielski, Markowitz, et al., 1989, ID: 4217) and a population-based study in children in Southern Connecticut (Gerber, Shapiro, et al., 1996, ID: 11924), except for those indicated specifically.

[d]Data were based mainly on a population-based study in Southern Sweden (Berglund, Eitrem, et al., 1995, ID: 9264), except for those indicated specifically.

[e]Data were based on 76 American (Nadelman, Nowakowski, et al., 1996, ID: 12196) and 231 European (Strle, Nelson, et al., 1996, ID: 19085) culture-confirmed patients with EM.

[f]NB patients were used as denominator to calculate the relative prevalence. European data were based on data from 330 NB cases from Germany (Oschmann, Dorndorf, et al., 1998, ID: 19084) and 176 NB cases from Denmark (Hansen & Lebech, 1992, ID: 6973).

[g]A recent population-based study showed that only 7% of children with LB developed Lyme arthritis (Gerber, Shapiro, et al., 1996, ID: 11924). About 10% of patients with Lyme arthritis may develop chronic antibiotic treatment-resistant arthritis (Gross, Forsthuber, et al., 1998, ID: 14570).

Cauwels et al., 2001). Lyme arthritis, first described in the United States, is still more commonly reported in North American cases of Lyme disease (Steere et al., 1983; Table 5), where the causative agent is *B. burgdorferi* sensu stricto isolates, than in European cases, where Lyme disease is attributed to the much more heterogeneous *B. burgdorferi* sensu lato complex. The reverse, however, is true for ACA, which is fairly common in Europe, but rarely seen in North America (Table 5).

For Lyme disease in North America, a correlation between *B. burgdorferi* sensu stricto genotypes and clinical manifestations is not as clear as it is for *B. afzelii* (ACA) and *B. garinii* (neuroborreliosis) in Europe. Two recent studies present evidence suggesting that genetic heterogeneity within *B. burgdorferi* sensu stricto isolates in North America may influence invasiveness and pathogenicity. In the first study, Seinost et al. (1999) examined 140 *B. burgdorferi* strains isolated from primary EM lesions, blood and CSF from patients seen at the Lyme Disease Center at Stony Brook, New York, the Lyme Disease Diagnostic Center at New York Medical College, or at local private physicians. Strains were genotyped according to their *ospC* alleles using a combination of PCR and cold single-strand conformation polymorphism (SSCP) analysis, a method that gave 100% sensitivity and specificity when comparing alleles. Additional samples were obtained by analyzing spirochetes present in locally collected *I. scapularis* ticks. Twenty-two additional alleles were added from sequences present in GenBank. The authors concluded 1) that some *B. burgdorferi* sensu stricto isolates rarely, if ever, cause disease in humans, 2) that some *B. burgdorferi* sensu stricto isolates cause a local infection at the tick bite site but not systemic disease, and 3) that systemic *B. burgdorferi* sensu stricto infections are associated with four *ospC* groups. It is not clear, how-

ever, whether *ospC* is directly influencing pathogenicity or serving as a marker for other, probably unlinked, determinants of invasiveness. Independent work by Baranton et al. (2001), examining genetic diversity of *ospC* loci in European isolates of *B. afzelii* and *B. garinii*, also came to a similar conclusion.

The second study (Wormser et al., 1999) examined the correlation between genetic subtypes and hematogenous dissemination in 104 untreated patients with EM. In this study, the authors used restriction length polymorphism (RFLP) methodology to compare the *B. burgdorferi* sensu stricto 16S-23S rDNA spacer region of spirochetes cultured from EM skin biopsies, whole blood, serum and plasma. Results from this study suggest that the genetic subtype of the infecting strain in the skin is a major determinant of the risk for hematogenous dissemination. The continued characterization of genetic markers associated with different subtypes of *B. burgdorferi* sensu stricto is likely to further our understanding of the relationship between genotype and the clinical presentations of patients with Lyme disease. Additionally, a clearer understanding of the genotypes responsible for disseminated infection in humans may facilitate the selection of novel vaccine candidates by identifying antigenic targets common to these isolates that might be essential to their survival and maintenance in the mammalian host.

Differential Gene Expression in *Borrelia*

To be maintained in its enzootic life cycle, borreliae must adapt to two strikingly different environments, the arthropod vector and the mammalian host. It is, therefore, not surprising that this bacterium undergoes extensive changes in protein composition as it cycles between these two hosts (de Silva and Fikrig, 1997). An extensive amount of work during the past several years has been devoted to elucidating the phenomenon of differential gene expression.

Reciprocal Expression of OspA and OspC

The best studied example of differential gene expression by *B. burgdorferi* involves the synthesis of outer surface protein A (OspA) and C (OspC) during tick feeding. In a pioneering study (http://www.niaid.nih.gov/dir/labs/lmsf.htm), Schwan et al. (1995) observed that *B. burgdorferi*, when present in the midguts of unfed ticks, expressed large amounts of OspA and little to no OspC. When infected ticks were allowed to feed on a suitable mammalian host (i.e., mice), spirochetes within the tick midgut downregulated the expression of OspA and expressed substantial amounts of OspC. Although increased temperature (tick versus mammal) has been shown to be involved in this expression-pattern (see "Environmental Signals as Gene Regulators"), upregulation of OspC independent of temperature was recently demonstrated by the in vitro incubation of spirochetes at 28°C in the presence of tick hemolymph plasma (Johns et al., 2000), suggesting that expression of OspC is enhanced by, but does not require, increased temperature. These data are supported by studies examining the expression of *ospA* and *ospC* mRNA by RT-PCR (Montgomery et al., 1996) and the production of antibodies against OspA and OspC during early murine (Montgomery et al., 1996) and human Lyme borreliosis.

Recent advances in elucidating the potential roles for OspA and OspC in Lyme disease pathogenesis were provided by the publication of the crystal structures (Fig. 21) of these lipoproteins (Li et al., 1997; Eicken et al., 2001; Kumaran et al., 2001). These structures are also of particular interest as OspA is the antigen on which the current Lyme disease vaccine is based, while OspC is considered a highly promising candidate for the next generation of Lyme disease vaccine. The OspA protein displays an unusual, elongated fold composed of 21 consecutive antiparallel β-strands connected by turns or short loops followed by a single short α-helix (Fig. 21). The presence of a cavity and partially buried charged residues with the cleft suggests that the C-terminal domain of OspA might be a binding site for a negatively charged or hydrophobic ligand, small peptide, linear polysaccharide, or exposed protein loop (Li et al., 1997). In contrast to OspA, OspC is comprised of four long helices plus a short fifth helix (Eicken et al., 2001; Kumaran et al., 2001; Fig. 21). An elongated, kidney-shaped OspC dimer (Fig. 21) is thought to form through hydrophobic interactions primarily between the α1 and α1' helices on two OspC monomers. While OspA has no known structural homologs, OspC has similarity to the periplasmic domain of the *Salmonella* aspartate receptor (AR), which is also a dimer. Interestingly, when the crystal structures (Fig. 21) of the OspCs from the HB19 and B31 (both invasive strains) were used to model the structures of noninvasive isolates (Seinost et al., 1999), notable differences were detected in the electrostatic surface potential of the region of OspC furthest away from the surface of the membrane; the surface potential is highly negative in the invasive strains, but not so for the noninvasive strains (Kumaran et al., 2001). The authors speculate that this region's interaction with a positively charged ligand, such as fibronectin (see "Decorin- and Fibronectin-binding Proteins") or a similar molecule, might play a role in the pathogenesis of Lyme disease

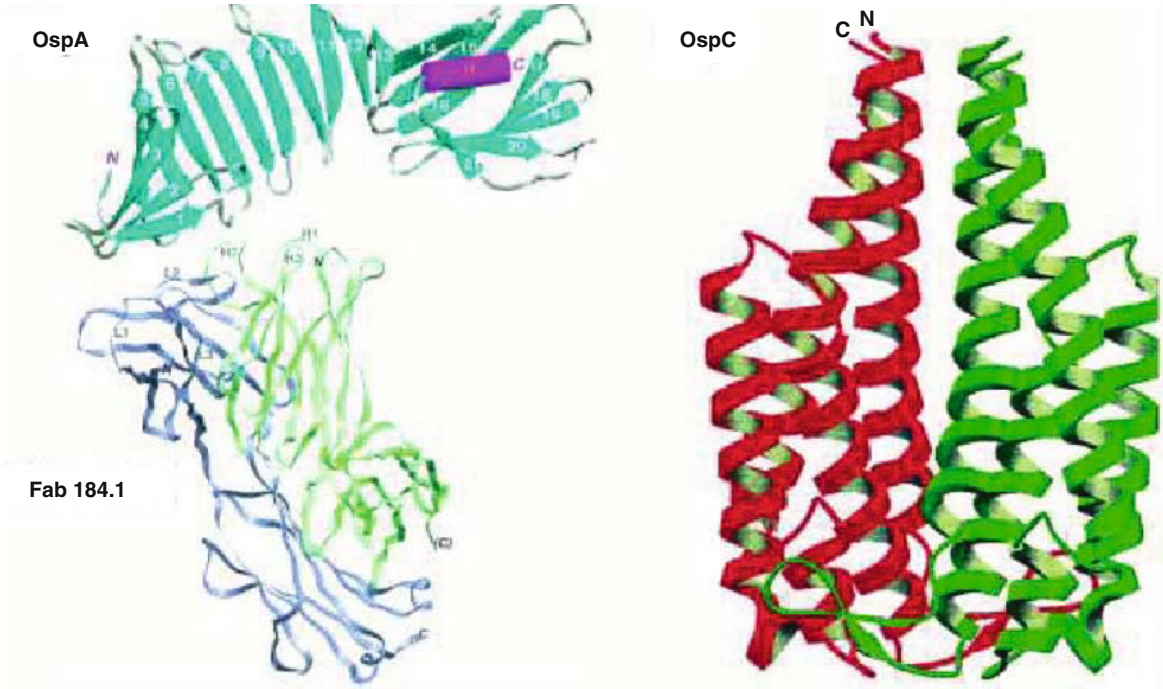


Fig. 21. Crystal structure of OspA and OspC. Left, schematic view of the OspA-Fab 184.1 complex. OspA is displayed with its secondary structural elements, β-strands (cyan, numbered 1–21) and α-helix (magenta, labeled), connected by gray turns or loops. The front face of the central sheet is seen in this view. Fab 184.1 is represented by violet and green ribbons for the light and heavy chains, respectively. The three complementarity determining region (CDR) loops on each chain are displayed as thin ropes and labeled (e.g., L1 for CDR1 of the light chain). Image was taken from Li et al. (1997), © 1997, National Academy of Sciences, with permission. Right, RIBBONS representation of the OspC-HB19 dimer. The two monomers are colored red and green, respectively. The close proximity of the two innermost helices indicates that it is a tight dimer. Image taken from Kumaran et al. (2001) and reproduced by permission of Oxford University Press.

(Kumaran et al., 2001). This finding also has significant implications for the development of an OspC-based vaccine, which is tailored towards recognition of those (invasive) strains that are more likely to cause disseminated disease (see "Lyme Disease Vaccine").

Both OspA and OspC are thought to play critical roles in the transmission of *B. burgdorferi* from the tick to the mammalian host. Two domains within the N40 OspA (amino acids [aa] 85–103 and aa 229–247) were recently identified and proposed to contain functional binding sites responsible for the adherence of spirochetes to the tick midgut (Pal et al., 2000). Consistent with this proposed binding activity, the location of one of these domains (229–247) is predicted by the OspA crystal structure (Fig. 21) data mentioned above to be within the putative ligand binding domain. The role of OspC in the transmission process is unclear. A recent study suggests that this lipoprotein plays a role in the transmission of spirochetes from tick to mammal, but not from mammal to tick (Schwan and Piesman, 2000). A second report demonstrated that immunization with OspC significantly reduced the migration of *Borrelia* from the midgut to the salivary glands of infected *I. scapularis* ticks (Gilmore and Piesman, 2000). These data are consistent with numerous studies demonstrating a role for OspC antibodies in the protection of laboratory animals against tick transmission using both passive and active immunization (Gilmore et al., 1996; Mbow et al., 1999; Zhong et al., 1999).

Recent work by Ohnishi and coworkers (Ohnishi et al., 2001), in which the authors examined the temporal expression patterns of OspA and OspC in both ticks and the skin adjacent to the feeding site in a murine model of Lyme disease, suggests that the relationship between OspA and OspC expression patterns is perhaps even more complex than initially envisioned. Results from these studies demonstrate that the complete downregulation of OspA and the expression of OspC are not required for migration of spirochetes from the midgut to the salivary glands. In fact, OspC was not expressed by a significant percentage of spirochetes in the salivary glands until feeding had progressed for >61 hours. This coincided with the time required to consistently transmit the spirochetes and establish infection. The authors also noted that

early during transmission, mice with spirochetes detectable in the skin directly adjacent to the bite site, expressing mostly OspA compared to OspC, failed to develop infection. In contrast, those mice that had spirochetes expressing higher levels of OspC and reduced OspA consistently became infected. From these data (combined with other studies described above), Ohnishi et al. suggest a model by which spirochetes are retained in the midgut by OspA-mediated adherence until such time as they are adequately "adapted" for migration from the gut lumen to the hemocele and salivary glands by an OspC-facilitated process. Once they have exited the midgut, spirochetes appear to downregulate the expression of OspC, as well as continue to downregulate OspA. In some instances, however, spirochetes ($OspA^+/OspC^-$) are apparently "mislocalized" to the salivary glands and/or prematurely transmitted to the host before being fully "adapted"; these spirochetes fail to establish infection in the mammalian host. This model also suggests that the transmission of *B. burgdorferi* from the arthropod to mammalian host may occur in a steady wave of spirochetes over an extended period (days). These studies also demonstrate that the signal(s) responsible for the temporal expression patterns of OspA and OspC (and most likely other Osps) are clearly complex and likely to include stimuli from both the arthropod and mammalian hosts.

OspE/OspF/Elp Lipoproteins

In addition to the *B. burgdorferi* OspA and OspC lipoproteins (see "Reciprocal Expression of OspA and OspC"), a second group of differentially expressed proteins, the OspE/OspF/Elp lipoproteins, has garnered much interest in the *Borrelia* field. For a recent review of this family of cp32-encoded lipoproteins, see Stevenson et al. (2000a). These lipoproteins were once all considered as one large group of related lipoproteins and referred to by a generic term, "Erp" (OspE/F-related proteins), in the B31 strain (Stevenson et al., 1996; Casjens et al., 2000). A more comprehensive analysis has revealed that the similarities among these proteins are largely restricted to their leader peptides, whereas the mature portions of the polypeptides actually fall into three evolutionary distinct groups: 1) OspE-related orthologs, 2) OspF-related orthologs, and 3) Elps, which contain OspE/F-like leader peptides, but are otherwise unrelated to both OspE and OspF (Akins et al., 1999; Caimano et al., 2000). It is likely that this family of lipoproteins arose from a series of gene fusion events in which a common N-terminus was fused upstream of otherwise unrelated sequences. The resulting fusions formed the progenitors for each of the three groups of OspE, OspF, and Elp lipoproteins, each of which underwent subsequent genetic polymorphisms to form the family of lipoproteins (Fig. 22). In addition to their group designations, members of this larger family of lipoproteins have been referred to by a variety of names in the literature, including p21, pG, BbK2.10, BbK2.11, and "upstream homology box" (UHB) lipoproteins (Lam et al., 1994; Akins et al., 1995; Akins et al., 1999; Suk et al., 1995; Wallich et al., 1995; Marconi et al., 1996a).

The genes encoding all members of this family are encoded on 32- or 18-kb supercoiled circular plasmids (referred to collectively as "cp32s"; see "The cp32 Family of Supercoiled Circular Plasmids") as either mono- or bicistronic operons. The first member of this family shown to be differentially expressed was the p21 lipoprotein, identified within a *Borrelia* expression library as an antigen recognized by sera from infected mice but not by sera from mice immunized with in vitro-cultivated, killed spirochetes (Suk et al., 1995). A number of additional proteins within this family, pG, BbK2.10 and ElpA1, from several different strains have since been characterized as "in vivo-expressed" antigens (i.e., proteins selectively expressed during infection but not by spirochetes grown in artificial medium; Akins et al., 1995; Wallich et al., 1995; Hefty et al., 2001). The remaining OspE/OspF/Elp lipoproteins have all shown to be expressed both in vitro and within the mammalian host (Akins et al., 1995; Akins et al., 1998; Stevenson et al., 1998a; Hefty et al., 2001). Recently, Hefty et al. (2001) performed a comprehensive study of the OspE/OspF/Elp family in *B. burgdorferi* strain 297, which suggested that the differential patterns of antigen expression observed during tick feeding and mammalian infection might represent a temporal hierarchy in which proteins are expressed sequentially as the spirochetes adapt in preparation for the transmission from arthropod to mammal. An examination of the upstream promoter regions has revealed that although this region (upstream homology box, UHB) is highly conserved among all of the *ospE*/*ospF*/*elp*/*erp* genes (Marconi et al., 1996b; Hefty et al., 2001), sequence polymorphisms (insertions/deletions and transitions/transversions) were present (Fig. 23). The majority of these differences were in the putative -10 and -35 hexamers and spacer region between (Hefty et al., 2001); these regions are all known to be critical for RNA polymerase recognition and binding in prokaryotes. A comparative (phylogenetic) analysis of the *ospE*/*ospF*/*elp*/*erp* promoter regions (Fig. 23) demonstrated that these regions do not segregate according to their polypeptide-encoding regions, but instead

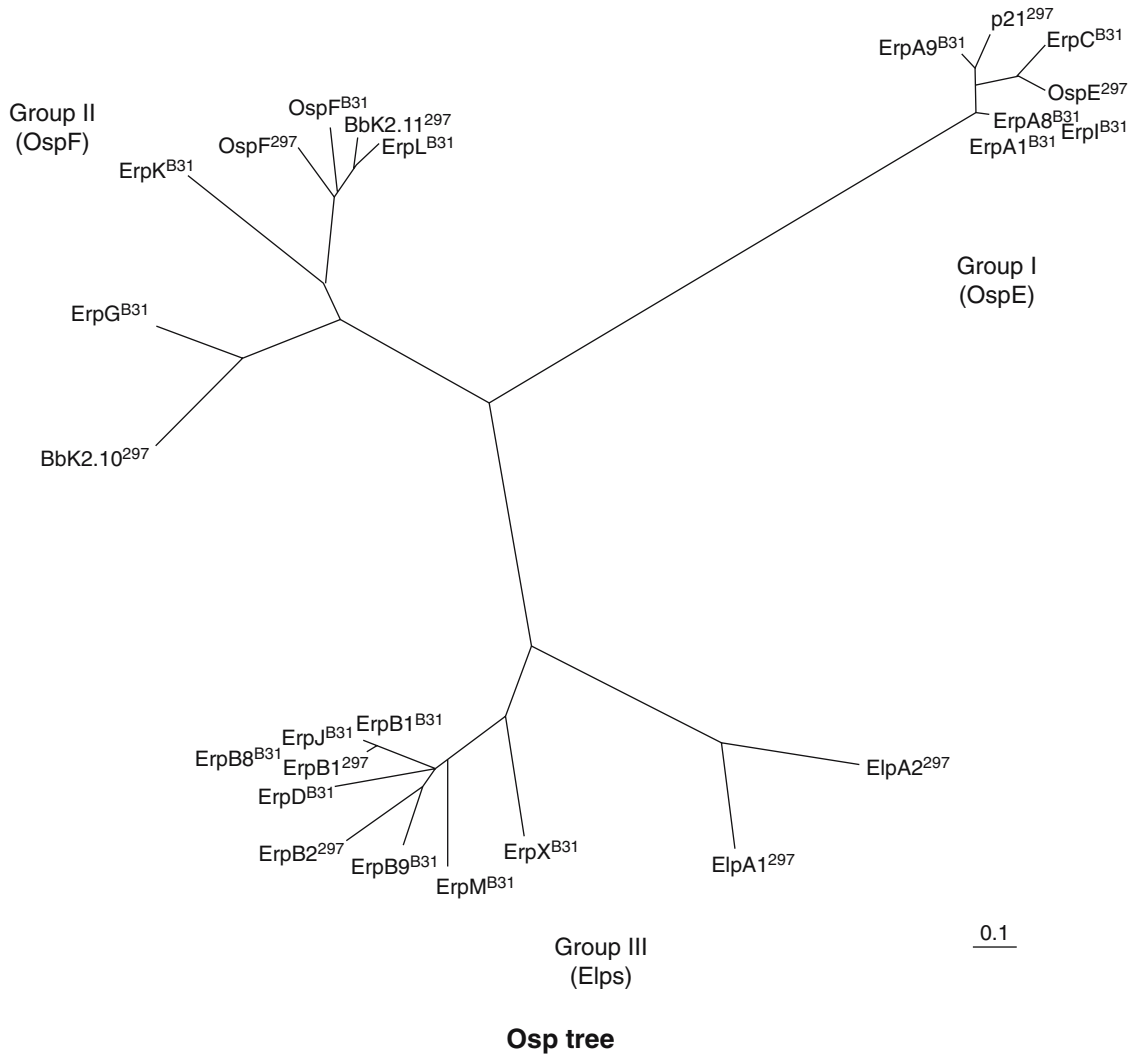
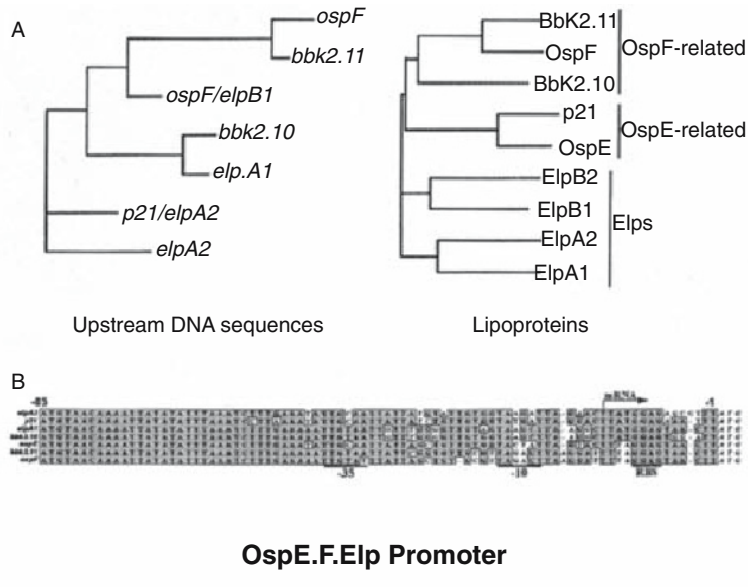


Fig. 22. Unrooted neighbor-joining phylograms of the *B. burgdorferi* strains 297 and B31 OspE/OspF/Elp/Erp lipoproteins. Taken from Caimano et al. (2000), with permission of ASM Press (http://www.journals.asm.org).

appear to segregate according to their temporal expression pattern. These data suggest that minor sequence differences between the promoters may result in altered affinity of RNA polymerase and/or altered expression under different environmental conditions (Hefty et al., 2001).

Mlp Lipoproteins

In addition to the *ospE*/*ospF*/*elp*/*erp* loci (see "OspE/OspF/Elp Lipoproteins"), the cp32/18 family of circular plasmids encodes a second variable lipoprotein family, the *mlp* loci (Porcella et al., 1996; Yang et al., 1999; Porcella et al., 2000; Stevenson et al., 2000b). This family is comprised of two evolutionarily distinct classes that possess highly similar N-termini but divergent C-termini (Caimano et al., 2000; Fig. 24). The recombinatorial process that resulted in the formation of these two lipoprotein classes is likely to be mechanistically similar to that of the process responsible for the formation of the OspE/OspF/Elp family (see "OspE/OspF/Elp Lipoproteins") of lipoproteins (Caimano et al., 2000). Four of the strain 297 Mlps (Mlp7A, Mlp7B, Mlp9 and Mlp10) were shown to be preferentially expressed within a mammalian model (Akins et al., 1998; Yang et al., 1999). Immunoblotting using antiserum from tick-infected C3H/HeJ mouse serum suggests that the strain 297 Mlp lipoproteins are not all expressed early in infection, but may be turned on in a temporal pattern as the spirochetes transition from the arthropod vector to first the skin and then other tissues of the mammalian host (Yang et al., 1999). Preliminary protection studies using a multivalent for-



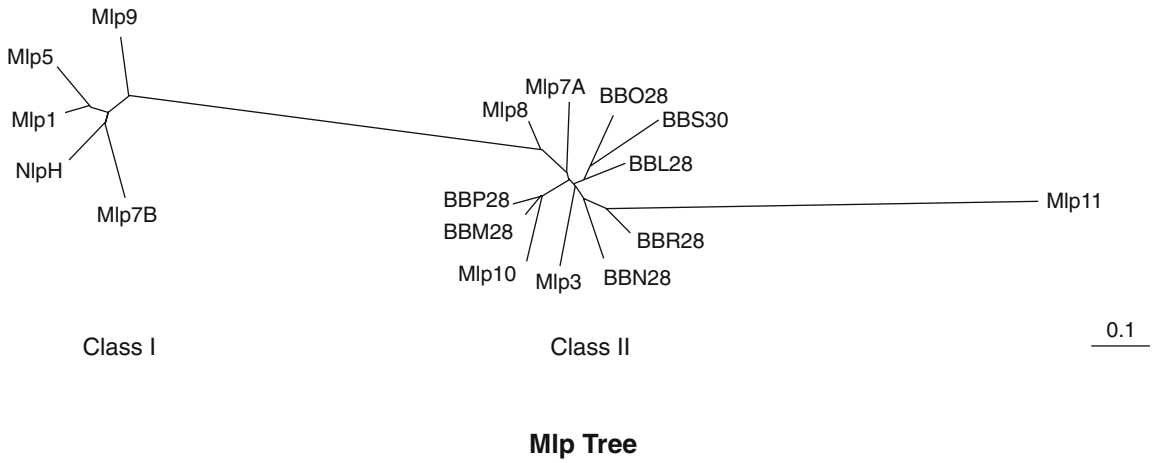


Fig. 23. *Borrelia burgdorferi* strain 297 *ospE*/*ospF*/*elp*/*erp* loci promoter regions. (A) Phenogram analysis of the upstream 85 bp of the putative promoter regions for the *ospE*-related, *ospF*-related, and *elp* loci (left panel). For comparison, a phenogram of the full-length lipoproteins is also shown (right panel). (B) Clustal W alignment analysis of the upstream 85 bp of the putative promoter regions for the *ospE*-related, *ospF*-related, and *elp* loci. Taken from Hefty et al. (2001), with permission of ASM Press (http://www.journals.asm.org).

Fig. 24. Unrooted neighbor-joining phylogram of the *B. burgdorferi* strains 297 and B31 Mlp lipoproteins. Taken from Caimano et al. (2000), © 2001, ASM Press (http://www.journals.asm.org), with permission.

mulation of the Class 2 Mlps from strain 297 as the immunogen gave rise to 80% protection of mice (Yang et al., 1999). Immunoblotting using sera from Lyme disease patients also confirms that members of this family are expressed during human infection (Porcella et al., 2000). While preliminary, these data suggest that this family of lipoproteins may have some vaccinogenic potential that warrants further consideration (Yang et al., 1999).

Decorin- and Fibronectin-binding Proteins

The mechanism(s) by which borreliae are able to disseminate following transmission from the arthropod vector to cause a multisystemic infection in the mammalian host remain largely unknown. The recent identification of two *B. burgdorferi* proteins, DbpA and DbpB, which have the ability to bind decorin (a collagen-associated extracellular matrix proteoglycan found in skin and other tissues), represented a significant step towards characterization of the early stage of Lyme disease (Table 5), as the spirochete first enters the mammalian host through the site of tick feeding (Cassatt et al., 1998; Hagman et al., 1998; Hanson et al., 1998). Based on their ability to interact specifically with extracellular matrix components, these molecules have been placed within a class of cell surface adhesins designated "MSCRAMMs" (microbial surface components recognizing

adhesive matrix molecules; Patti et al., 1994). Interestingly, neither *dbpA* nor *dbpB* was detected in *B. hermsii*, *B. turicatae* or *B. coriaceae* by Southern hybridization (Roberts et al., 1998). Data from proteinase K sensitivity assays (DbpA and DbpB) and immunoelectron microscopy (DbpA) suggest that these proteins are surface-exposed, a location consistent with their putative role in adherence to basement membrane (Hagman et al., 1998; Hanson et al., 1998). Brown et al. (1999) have identified three highly conserved lysine residues (Lys-82, Lys-163 and Lys-170), found in all DbpA polypeptide sequences examined, and all appear to be critical for association with decorin. Several studies have shown that immunization with DbpA, but not DbpB, protects mice against syringe inoculation (Cassatt et al., 1998; Feng et al., 1998; Hagman et al., 1998; Hanson et al., 1998), although DbpA-immunized mice were not protected when inoculated by tick infestation (Hagman et al., 2000). A role for decorin in Lyme disease pathogenesis was recently demonstrated using a decorin-deficient transgenic mouse (Brown et al., 2001). In these studies, decorin-deficient mice were more resistant to infection with *B. burgdorferi* (i.e., fewer spirochetes were found in joints) and showed lower incidence and severity of arthritis than either wildtype or heterozygotes.

A second differentially expressed protein with a potential role in spirochetal attachment to extracellular matrix (Probert and Johnson, 1998) is the fibronectin-binding protein, BBK32; this protein has also been referred to as P35 and P47 (Fikrig et al., 1997; Fikrig et al., 2000; Probert and Johnson, 1998). The BBK32 protein was initially thought to be expressed only within the mammalian host (Fikrig et al., 1997), but this later was shown to be a stain-specific phenomenon, with some *B. burgdorferi* sensu lato isolates (B31, 297, ACA1 and IP3) expressing detectable amounts of BBK32 when cultivated in BSK II (Table 3) medium (Probert and Johnson, 1998). A comprehensive analysis of *bbk32* expression in *B. burgdorferi* strain N40 (which preferentially synthesizes BBK32 during murine and human Lyme disease) was performed by Fikrig et al. (2000) to elucidate what, if any, role this protein plays in tick transmission and/or mammalian infection. The results from these studies demonstrated that 1) BBK32 was not detectable by immunofluorescence on spirochetes within flat ticks, but was present on spirochetes within the engorged ticks, 2) *bbk32* mRNA was first detected two days following syringe inoculation and was maximal at 30 days postinfection, 3) *bbk32* message was detectable in all tissues tested (skin, joints, heart, spleen and bladder) at 14 days postinfection and remained detectable for 60 days, albeit at a significantly reduced level, and 4) BBK32 antibodies provided partial protection from tick infection with *B. burgdorferi* (in a guinea pig model) by killing the spirochetes within the engorged tick, thereby inhibiting the transmission of spirochetes to an immunized animal host (Fikrig et al., 2000).

Environmental Signals as Gene Regulators

A major theme in microbial pathogenesis is the effect of environmental signals on bacterial gene expression. To be maintained within its enzootic cycle, borreliae must possess the ability to adapt to growth within two strikingly different milieus, the arthropod vector and the mammalian host. During this cycle, the spirochete is likely to encounter differences in, among other things, nutrients, temperature and pH. One of the best-studied environmental conditions is temperature. Increased temperature has been associated with increased expression of OspC (see "Reciprocal Expression of OspA and OspC"), OppAV (Bono et al., 1998; see "Transporters"), and the OspE/OspF/Elp (Stevenson et al., 1995; Stevenson et al., 1998a; Hefty et al., 2001; see "OspE/OspF/Elp Lipoproteins") and Mlp (Porcella et al., 2000; Yang et al., 2000; see "Mlp Lipoproteins") lipoprotein families. Several lines of evidence, however, suggest that temperature alone is not solely responsible for the increased expression of these lipoproteins. The incubation of unfed *B. burgdorferi*-infected *I. scapularis* nymphs at 37°C for 24, 72 or 144 hours did not stimulate expression of OspC (Schwan et al., 1995). In addition, the expression of OspC has been shown to wane with continued passage in vitro at 37°C (Schwan and Piesman, 2000). Similarly, a number of observations have been made on the expression of the OspE/OspF/Elp lipoproteins: 1) expression in a mammalian host-adaptation model is significantly greater than the levels observed in spirochetes cultivated in vitro following a temperature shift from 23 to 37°C (Akins et al., 1998), 2) the p21 lipoprotein (see "OspE/OspF/Elp Lipoproteins"), which is not expressed in vitro following temperature shift, is readily detected in a mammalian host-adaptation model (Akins et al., 1998), and 3) animals infected by either tick- or needle-inoculation with *B. burgdorferi* produce antibodies against p21 (see "OspE/OspF/Elp Lipoproteins") and BbK2.10 early during infection, but these sera fail to detect these lipoproteins in spirochetes cultivated in vitro following temperature shift (Wallich et al., 1993; Akins et al., 1995; Akins et al., 1998; Suk et al., 1995; Das et al., 1997). The expression pattern of the Mlp lipoproteins family has been examined in two strains, B31 and 297. Using Northern analyses (Porcella et al., 2000)

and RT-PCR (Yang et al., 1999), little or no *mlp* expression was seen at 23°C, whereas all were shown to be upregulated in response to temperature. A number of environmental factors other than temperature also have been shown to result in differential gene expression in *B. burgdorferi*. Alban et al. (2000) examined the effects of serum starvation in *B. burgdorferi* (grown in RPMI). This study reported that growth of *Borrelia* under this fatty acid- and lipid-limited growth condition resulted in the formation of cysts-like "starvation" forms containing nonmotile, but otherwise intact and viable spirochetes, which rapidly revert to a "vegetative" form when transferred to BSK II (Table 3) medium. In addition, the expression of approximately 20 proteins was induced during growth in RPMI as compared to *B. burgdorferi* grown in BSK II (Table 3) medium. It is intriguing to speculate whether some of these "starvation-induced" proteins are involved in the ability of these bacteria to withstand the extended periods of nutrient limitation they undoubtedly experience in the arthropod host (Alban et al., 2000). Carroll et al. (1999) identified at least 37 changes within borrelial membrane preparations by two-dimensional nonequilibrium pH gradient gel electrophoresis (2D-NEPHGE) in response to changes in pH (pH 6.0 vs 7.0 vs 8.0). In these studies, outer surface protein C (OspC, see "Reciprocal Expression of OspA and OspC") was shown to be decreased at the higher pH, whereas a second unidentified protein was shown to be increased at pH 8.0 (Carroll et al., 1999). Two studies examining spirochetes cultivated in BSK-H (Table 3) medium demonstrated that increased cell density (at constant pH) resulted in increased expression of both *ospC* (see "Reciprocal Expression of OspA and OspC") and *mlp8* (Indest et al., 1997; Ramamoorthy and Philipp, 1998; Mlp Lipoproteins). Although a protein with homology to a quorum-sensing autoinducer synthetase (AI-2) has been identified within the *B. burgdorferi* genome (BB0377; Surette et al., 1999), it has yet to be determined whether a quorum-sensing autoinducer synthetase-like mechanism is responsible for cell-density mediated gene expression. The contribution of growth phase has also been examined. These studies identified a number of protein bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (including P35, BmpD and P7.5), which were upregulated between 2- to 66-fold as the spirochetes in BSK II (Table 3) medium transitioned from mid-log to stationary phase (Ramamoorthy and Philipp, 1998). Using a combination of Northern analyses and immunoblotting, Yang et al. (2000) furthered these studies by examining the interdependence of pH, temperature and cell density on spirochetes cultivated in vitro in BSK-H medium. In these studies, a decrease in pH, in conjunction with an increase in temperature and cell density, acted interdependently, resulting in the reciprocal expression of *ospC* and *ospA* (see "Reciprocal Expression of OspA and OspC"). Examination of other environmentally regulated genes identified patterns of expression that fall roughly into two groups, group I (*ospC*-like) and group II (*ospA*-like). In the proposed model, the group I genes (i.e., *ospC*, *ospF* and *mlp8*) exhibit reduced expression when the concentration of spirochetes and environmental temperature are low, whereas the pH is relatively high. This condition is thought to represent the microenvironment of the unfed tick midgut. The group II genes (i.e., *ospA/B*, *p22* and *lp6.6*) behave in a reciprocal manner and exhibit increased expression under these same conditions. Yang et al. (2000) also propose a role for the alternative σ factor *rpoS* (Fraser et al., 1997; Elias et al., 2000) in the regulation of the group I genes. One group I gene, *dbpA*, did not fit the proposed model; unlike *ospC*, expression of *dbpA* was substantially reduced at pH 6.8 (relative to 7.5; Yang et al., 2000). This result is, however, consistent with the recent finding that DbpA (see "Decorin- and Fibronectin-binding Proteins") is not expressed by *B. burgdorferi* in the midguts of fed *I. scapularis* ticks (Hagman et al., 2000). While these studies represent significant steps toward understanding the complex interplay between multiple environmental factors, each of the above-mentioned studies involves the characterization of spirochetes cultivated in vitro using artificial medium and therefore needs to be seen as suggestive. The development of a tick cell culture model for cultivating *B. burgdorferi* and the demonstration that the reciprocal expression of *ospA* and *ospC* (see "Reciprocal Expression of OspA and OspC") can be modulated by temperature using this model offer an exciting potential for further environmental studies that more closely reflect the arthropod phase of growth (Obonyo et al., 1999).

While alterations in temperature, medium composition and pH are undoubtedly important, mammalian host-derived signals also play a role in inducing dramatic changes in gene and protein expression. The preferential expression of genes in the mammalian host is considered to be indicative of a role in virulence, although this role has not always been readily apparent. The identification and characterization of borrelial genes preferentially expressed in the mammalian host, however, is stymied by the inability to genetically manipulate these organisms and the paucity of organisms found in tissues during infection, particularly in Lyme disease. As a consequence, borrelial gene expression studies have had to rely on

more conventional molecular and immunological assays, examining the expression of only a limited number of genes within a given sample. Ideally, however, one would want to examine the simultaneous expression of multiple genes and the polypeptides they encode in the context of mammalian infection. Towards this end, several recent developments have significantly advanced the field towards overcoming the abovementioned limitations and allowed for the application of more contemporary methodologies. These developments include 1) the development of an electroporation protocol for the introduction of genetic material into *Borrelia burgdorferi* (Genetic Transformation of *Borrelia*), 2) the development of at least two published *Borrelia* shuttle vectors (Genetic Transformation of *Borrelia*), and 3) the development of new animal models for the isolation of spirochetes in a "host-adapted" state. The first two developments are covered elsewhere in the chapter. Animal models for the isolation of spirochetes in a more mammalian host-adapted state were developed by several independent research groups and include the transplantation of skin containing *B. burgdorferi* from infected animals to naive animals developed for either a murine (Barthold et al., 1995) or rabbit (Shang et al., 2000) model of Lyme disease and the cultivation of spirochetes with dialysis membrane chambers (DMC) implanted into the peritoneal cavities of rats (Akins et al., 1998) and subsequently rabbits (Sellati et al., 1999). Future studies utilizing these methodologies will undoubtedly provide insights into both spirochetal and mammalian factors and stimuli involved in the pathogenesis of Lyme disease.

Antigenic Variation in *Borrelia*

VMP LIPOPROTEINS Antigenic variation, a mechanism of immune evasion often employed by bacterial pathogens (Borst, 1991; Donelson, 1995; Deitsch et al., 1997), has been defined as the changes in the structure or expression of antigenic proteins that occur at a frequency greater than the usual mutation rate (Seifert and So, 1990). The best example of antigenic variation in the borreliae is that of the variable major proteins of the relapsing fever spirochetes. One of the hallmarks of relapsing fever is the periodic cycling of acute and afebrile episodes. The acute febrile episodes are characterized by the presence of a large number of spirochetes in the bloodstream of the infected mammal or patient (spirochetemia) and are followed by a change in the serotype and major lipoproteins associated with the bacterial membrane. Multiphasic antigenic variation was first described in the relapsing fever spirochete *B. hermsii* (Barbour et al., 1982; Stoenner et al., 1982) and was associated with a family of lipoproteins, collectively named the "variable major lipoproteins" (VMPs). The VMPs were later shown to be composed of two multigene families, the *vsp* (variable small protein) family, which encodes lipoproteins 20–23 kDa in size, and the *vlp* (variable large protein) family, which encodes lipoproteins 36–40 kDa in size (Barstad et al., 1985; Restrepo et al., 1992; Cadavid et al., 1997; Hinnebusch et al., 1998). The *vlp* family can further be divided into four subfamilies (α, β, γ and δ; Hinnebusch et al., 1998). These proteins share several features of the variant surface glycoproteins (VSGs) responsible for antigenic variation in African trypanosomes, including the post-translational addition of a lipid moiety that mediates attachment to the cell membrane and similar secondary structures of the mature polypeptides (Burman et al., 1990). In addition, these two organisms use a similar recombinatorial mechanism for the genetic exchange of DNA during antigenic variation (Kitten and Barbour, 1990; Donelson, 1995). The mechanism for the conversion of VMP types (and related VMP-like proteins) has been described for several *Borrelia* strains, but here *B. hermsii* will be used as the prototype. Briefly, 28–32-kb linear plasmids of *B. hermsii* have greater than 30 silent copies of serotype-specific variable major protein genes (*vsp* and *vlp*; Meier et al., 1985; Kitten and Barbour, 1990; Barbour, 1993). These silent loci were originally named by variable major protein designations (*vmp1*, *vmp2*, *vmp3*, etc.), but have since been renamed by either *vsp* or *vlp* family designations (Cadavid et al., 1997). The expression locus containing a promoter sequence and the active copy of either a *vsp* or *vlp* is located on the same or a different 28–32-kb linear plasmid (Fig. 11) adjacent to the covalently closed telomeric end (Kitten and Barbour, 1990; Plasterk et al., 1985). Only one *vmp (vsp/vlp)* gene present at one telomere end of the single linear expression plasmid is expressed in each organism (Kitten and Barbour, 1990; Barbour et al., 1991a). Antigenic variation (switching) occurs at a frequency of 10^{-3} to 10^{-4} per generation (Stoenner et al., 1982) and results from the recombination of one of the silent (archival) genes into the telomeric expression locus through inter- and intraplasmic recombination (Plasterk et al., 1985; Barbour et al., 1991b; Restrepo et al., 1994b). This recombination, which is thought to utilize homologous sequences present within the 5′ and 3′ ends of all silent and expressed *vmp* loci, results in the partial or complete replacement of the former VMP serotype with a new serotype. In some instances, incomplete recombination has resulted in the formation of chimeric expression loci containing partial or complete copies of

more than one *vmp* gene (Kitten et al., 1993; Restrepo et al., 1994b). Additional sequence heterogeneity is provided by the introduction of point mutations post-switch (Restrepo and Barbour, 1994a).

Two Vsp serotypes, VspA and VspB, also have been described in relapsing fever agent *B. turicatae* (Cadavid et al., 1997; Pennington et al., 1999a). The Vsp system in *B. turicatae* differs most significantly from the VMPs of *B. hermsii* in that the archival silent loci and the expressed locus are both located near the center of 37–50-kb linear plasmids and not near the telomeres (Pennington et al., 1999b). The silent and expressed loci are, however, encoded on different linear plasmids (Pennington et al., 1999a). The antigenic switching mechanism in *B. turicatae* occurs when a silent *vsp* locus and downstream 3′ sequence (totaling approximately 13–14 kb) are duplicated at a unique expression site, replacing the existing expressed copy and its corresponding downstream sequence (Pennington et al., 1999a). As with the VMPs of *B. hermsii*, only one Vsp serotype is expressed at a time in *B. turicatae*. Vsp/Vlp-related sequences have also been described in *B. parkeri*, *B. crocidurae* and *B. coriaceae* (Hinnebusch et al., 1998).

Antigenic switching of the VMPs has been shown to occur both during in vitro cultivation and during infection of the mammalian host (Stoenner et al., 1982; Schwan and Hinnebusch, 1998). During infection of a mammalian host, each acute relapse episode is predominated by an almost entirely new serotype (Stoenner et al., 1982), presumably allowing evasion from the mammalian adaptive immune response. Until recently, it was not known whether a similar switch occurred as the spirochete passaged from the arthropod-vector to the mammalian host during tick feeding, but a recent study by Schwan and Hinnebusch (1998), using *O. hermsi* infected with either of two distinct *B. hermsii* VMP serotypes, demonstrated that the *vmp* gene present at the telomeric expression site did not change during passage through ticks.

While there are now extensive data on the mechanisms of *vmp*/*vls* serotype switching in borreliae, much less is known about the contribution of these lipoproteins to pathogenesis. Recent studies with *B. turicatae* using a mouse model of Lyme disease demonstrated that organisms expressing the VspA lipoprotein have more extensive CNS involvement, whereas organisms expressing the VspB lipoprotein exhibit more severe arthritic manifestations (Cadavid et al., 1994). In addition, Pennington and coworkers (Pennington et al., 1997) demonstrated that mice infected with the VspB-expressing serotype showed increased numbers of spirochetes in their joints and blood than did mice infected with serotype A. Magoun and co-workers (Magoun et al., 2000) provided further evidence for a possible role of the VMPs in virulence and/or differences in tissue tropism by demonstrating that VspB, but not VspA, mediated attachment of *B. turicatae* to cultured mammalian cells through an interaction with cell-associated glycosaminoglycans (heparin and dermatan sulfate).

Vsp-OspC Lipoprotein Family A third type of variable major protein serotype was also described in *B. hermsii* by Stoenner et al. (1982) and designated "serotype C." This serotype was of interest due to its tendency to predominate in serially passaged populations of *B. hermsii* (Barbour et al., 1982; Stoenner et al., 1982). Once it was determined that antibodies specific for serotype C were directed against a variable small lipoprotein (Vsp), the designation was later changed to "Vsp33" in keeping with the established nomenclature of VMP serotypes (Carter et al., 1994). The *vsp33* gene differs from the other *vsp* and *vlp* genes in that 1) it is expressed from a site distinct from that of the telomeric site typically used by the *vsp/vlp* genes (Barbour et al., 2000), and 2) the *vsp33* promoter is more closely related to the promoter for the *ospC* gene of *B. burgdorferi* than the promoters for the *vsp/vlp* genes of *B. hermsii* (Barbour et al., 1991a; Pennington et al., 1999b).

Phylogenetic analyses of Vsp33 from *B. hermsii* with the other small Vsps of the relapsing fever *Borrelia* spp., a Vmp from *B. miyamotoi* (Fukunaga et al., 1995), and the OspCs (see "Reciprocal Expression of OspA and OspC") from *B. burgdorferi* sensu lato isolates suggest that these proteins form a family of related proteins, termed "the Vsp-OspC family" (Carter et al., 1994; Cadavid et al., 1997). These data are supported by recent structural studies (circular dichroism) comparing the *B. turicatae* VspA with three related proteins, VspB of *B. turicatae*, Vsp26 of *B. hermsii* and OspC (see "Reciprocal Expression of OspA and OspC") of *B. burgdorferi* strain B31. Results from these studies suggest that 1) despite as little as 40% identity in primary sequence, these proteins share a common highly α-helical secondary structure predicted to form a four-helix bundle (Fig. 21), 2) non-conservative amino acid changes, insertions and deletions could potentially affect secondary structure cluster in regions outside the predicted helices, and 3) a common secondary structure results in the formation of similarly sized protease-resistant cores (Zückert et al., 2001). In addition, each of the proteins examined had the ability to dimerize in solution (Zückert et al., 2001). Further structure-function analyses of the Vsp-OspC family of lipoproteins will likely increase our understanding of the role of these

proteins in antigenic variation, facilitating transmission from the arthropod vector, and mammalian infection, particularly the establishment of tissue-specific niches.

Although they share some sequence similarity in their promoter regions and plasmid origins (Barbour et al., 2000), *vsp33* from *B. hermsii* and *ospC* (see "Reciprocal Expression of OspA and OspC") from *B. burgdorferi* sensu lato differ in their patterns of expression. The expression of Vmp33 has been shown to be induced by cultivation either in artificial medium or within tick midguts at 23°C (Schwan and Hinnebusch, 1998). The OspC (see "Environmental Signals as Gene Regulators"), on the other hand, is poorly expressed at this lower temperature (either in vitro or within the unfed tick midgut) and instead is induced by the higher temperatures (33–37°C) experienced by spirochetes during infection of the mammalian host (Schwan et al., 1995). Based on these and other data, Schwan and Hinnebusch (1998) have proposed roles for the Vmp33-OspC lipoproteins in tick-transmission and/or early colonization of the mammalian host. According to the proposed model, Vmp33 is produced by spirochetes in the midguts of soft tick's (*O. hermsi*) following a short period required for the cooling of the bloodmeal contents. Vmp33 is then constitutively expressed by spirochetes as they persistently infect the tick's salivary glands, awaiting an opportunity to be transmitted to the next mammalian host. In addition, expression of *vmp33* has a "silencing" effect on the expression of the *vsp/vlp* present at the telomeric expression site, and vice versa; the mechanism for this inactivation is not known (Barbour et al., 2000). The OspC of *B. burgdorferi*, on the other hand, is not expressed in the midgut of unfed ticks. The Lyme disease spirochete remains within the midgut until the infected tick takes a next bloodmeal, during which the expression of OspC (see "Reciprocal Expression of OspA and OspC") is induced and the spirochetes migrate to the salivary glands to "prepare" for transmission to the mammalian host (Ribeiro et al., 1987; Zung et al., 1989; Lane et al., 1991; Coleman et al., 1997; Ohnishi et al., 2001). In contrast to the constitutive expression of Vmp33 and the rapid transmission (15–90 minutes) of the tick-borne relapsing fever spirochetes, expression of OspC (see "Reciprocal Expression of OspA and OspC") by the Lyme disease spirochete and subsequent transmission to the mammalian host can take a minimum of 2–3 days (Schwan et al., 1995; Schwan and Piesman, 2000; Ohnishi et al., 2001). This period, however, is well within the 3–5 days required for a hard tick to feed to repletion. The expression patterns of Vmp33 and OspC following transmission to the mammalian host are also different. Although readily visualized on spirochetes within the salivary glands of infected soft ticks by immunofluroescence, expression of the Vmp33 lipoprotein is undetectable once the density of spirochetes is high enough for visualization of the organism in the blood (Schwan and Hinnebusch, 1998). The loss of Vsp33 expression is accompanied by the appearance of one of the other VMP lipoproteins (Vsp or Vlp; Schwan and Hinnebusch, 1998). On the other hand, OspC is constitutively expressed by (most) spirochetes as they enter the salivary glands of the tick and as they are transmitted to the mammalian host, and expression continues during infection. These data could suggest that although Vmp33 and OspC may both facilitate the transmission of the relapsing fever and Lyme disease spirochetes, respectively, from their arthropod vectors to the mammalian host, the continued expression of Vmp33 (for the sake of not expressing a new VMP serotype, Barbour et al., 2000) is disadvantageous for the relapsing fever spirochete. In contrast, the continued expression of OspC may not offer any disadvantage or, alternatively, OspC (see "Reciprocal Expression of OspA and OspC") may be required for some *B. burgdorferi*-specific function.

Borrelia burgdorferi Vls System It has been long recognized that the Lyme disease spirochete, *B. burgdorferi*, can alter its antigenic composition during growth through differential gene expression (see "Differential Gene Expression"). The identification of a VMP-like system (*vls*), however, suggests that this spirochete may also employ antigenic variation as a mechanism for immune evasion and/or host-adaptation. The genetic organization of the *vls* (Fig. 25) system in *B. burgdorferi* is similar to that of the VMP system from relapsing fever spirochetes in the utilization of 15 variable silent loci (*vls* cassette) and a single "expression" locus (*vlsE*). In contrast to *B. hermsii*, both the silent and expression loci are located adjacent to each other on a telomeric end of a single 28-kb linear plasmid (lp28-1) in *B. burgdorferi* strain B31. Each *vls* cassette silent locus is flanked by a conserved 17-bp direct repeat that may be involved either in the alignment and recombination or in binding of a proposed site-specific recombinase(s) within the *vlsE* site. The VlsE from *B. burgdorferi* stain B31 contains two highly conserved domains, one at the amino terminus (96 amino acids) and one at the carboxyl terminus (51 amino acids; Zhang et al., 1997; Fig. 25). The amino acid sequence between these two invariable domains is composed of a central variable domain that contains six variable regions (VRs) and six invariable regions (IRs). These two types of regions are interspersed with each other, and each consti-

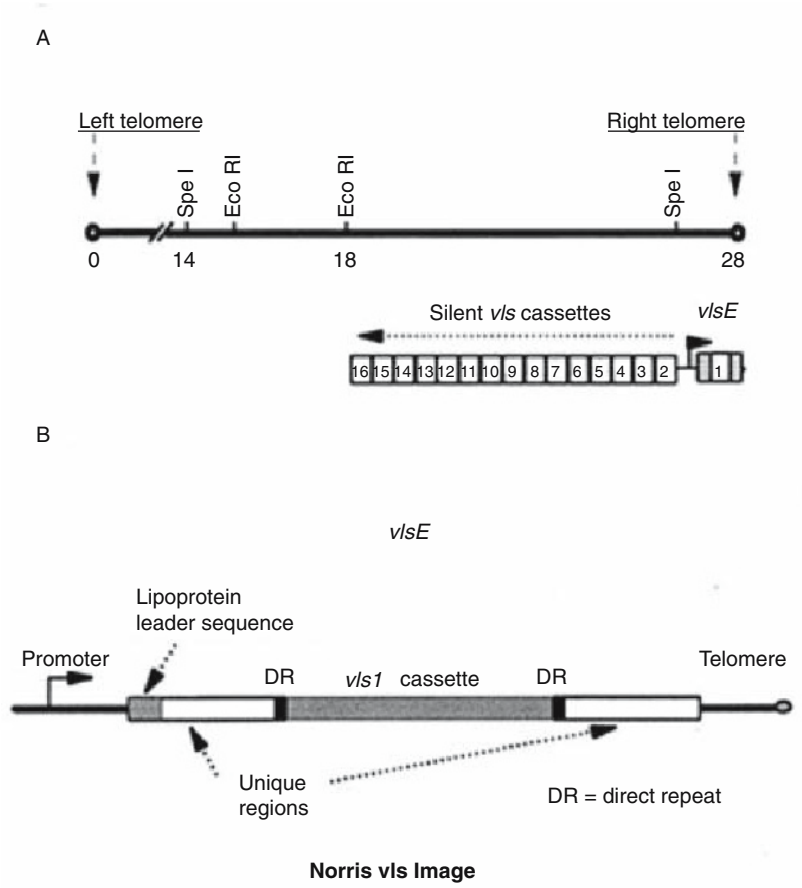


Fig. 25. *Borrelia burgdorferi* clone B31-5A3 *vls* locus. (A) Diagrammatic illustration of the overall arrangement of the *vls* locus in *B. burgdorferi* strain B31 plasmid lp28–1. (B) Structure of *B. burgdorferi* strain B31 *vlsE*. Taken from Zhang et al. (1997), © 1997, Cell Press, with permission.

tutes approximately one-half of the variable domain's length (Zhang et al., 1997; Liang et al., 1999). Additional variability within the *vls* system is achieved via combinatorial antigenic variation and promiscuous recombination resulting from replacement of portions of the expression sites by segments from several silent loci within the other *vls* cassette genes (Zhang et al., 1997; Liang et al., 1999). These recombinatorial events are dramatically induced during mammalian infection (Zhang and Norris, 1998). Also in contrast to the VMP system in the relapsing fever spirochetes in which each phase (relapse) of infection is predominated by one serotype (Barbour, 1993), multiple VlsE serotypes may be expressed within a given population of *B. burgdorferi* during infection of a single mammalian host (Zhang et al., 1997). Zhang and Norris (1998) examined the kinetics of *vlsE* sequence variation in C3H/HeN and severe combined immunodeficiency (SCID) mice. Results from these studies demonstrated that *vlsE* variation occurred in both mouse strains in as few as four days postinfection. The rate of accumulation of amino acid changes was significantly higher in the C3H/HeN mice, suggesting that immune selection may play a role in the persistence of *vlsE* variants. The finding that the rate of accumulation of amino acid changes in the *vlsE* loci within *B. burgdorferi*-infected SCID mice was higher than that in spirochetes cultured in vitro cannot be explained by adaptive immune selection and suggests that *vls* recombination is induced by a mammalian host factor(s). Subsequent immune selection, however, may explain the higher rate of changes in the surviving variant clones (over the parental clone) in the C3H/HeN mice compared to SCID mice (Zhang and Norris, 1998). Recent studies by Ohnishi et al. (2001) suggest that tick feeding may increase recombination at the *vlsE* locus.

To date, the *vls* (Fig. 25) system of only one *B. burgdorferi* strain (B31) has been characterized, but the *vls* cassette region (Kawabata et al., 1998) and *vlsE* expression (M. J. Caimano and J. D. Radolf, unpublished observation) loci have been identified for *B. burgdorferi* sensu stricto strain 297. Southern blot analysis of 22 blood

and erythema migrans biopsy (low-passage) isolates from human Lyme disease patients in Westchester County, New York, determined that all 22 isolates contained a *vlsE*-like sequence (Iyer et al., 2000). Although the size of the plasmid bands varied (from 21 to 38 kb) within the group of isolates, the *vlsE*-hybridizing bands were restricted to one plasmid species within an individual isolate. The consistent presence of *vls* sequences in Lyme disease isolates provides evidence supporting the view that the *vls* locus or associated sequences may be required for infection of mammalian hosts (Iyer et al., 2000). The *vls* system in *B. burgdorferi* is also implicated in virulence; isolates of *B. burgdorferi* strain B31 lacking the linear plasmid (lp28-1) encoding the *vls* locus exhibited reduced infectivity in a mouse model of Lyme disease (Purser and Norris, 2000). Additional infection data suggest that isolates lacking other linear plasmids may be similarly attenuated in virulence (Labandeira-Rey and Skare, 2001).

Studies have shown that five of the six invariable regions (IRs; Fig. 25) present within all VlsE polypeptides were not exposed on the surface of spirochetes cultivated in vitro (Liang et al., 1999; Liang et al., 2000). In addition, a recent study by Liang et al. (2001) examined the surface localization of the conserved C-terminal invariable domain of VlsE and found that it also was not exposed on the spirochetal surface. Consistent with these localization data, antibodies against the conserved C-terminal domain, although immunodominant during mammalian infection, failed to protect against challenge with *B. burgdorferi* (Liang et al., 2001). These data, along with the accumulation of substantial genetic heterogeneity within the variable regions during mammalian infection, suggest that although this molecule may play a role in infectivity, VlsE may not be useful as a vaccinogen (Liang et al., 2001).

Lipoproteins and the Innate Immune Response

Although many aspects of Lyme disease pathogenesis remain ill defined, it is generally accepted that clinical manifestations result primarily, perhaps entirely, from the host's local immune response to spirochetes in infected tissues (Duray and Steere, 1988; Szczepanski and Benach, 1991). For a more comprehensive review of the immunology of Lyme disease, see Sigal (1997). There is now a substantial body of evidence that borrelial lipoproteins are the major proinflammatory agonists in Lyme disease and the relapsing fevers and that lipid modification is a prerequisite for this biological property (Radolf et al., 1991; Radolf et al., 1995a; Ma and Weis, 1993; Boggemeyer et al., 1994; Norgard et al., 1996; Sellati et al., 1996; Morrison et al., 1997; Vidal et al., 1998). Although both borrelial lipoproteins and LPS initiate cell signaling by binding to CD14 (Sellati et al., 1998; Wooten et al., 1998) and both activate cells via nuclear factor-kappa B (NF-β; Norgard et al., 1996; Wooten et al., 1996), key differences in the signaling pathways utilized by these two agonists have come to light. Activation by lipoproteins is serum-independent and does not require LPS-binding protein (Sellati et al., 1998; Wooten et al., 1998). More importantly, the lipoprotein-CD14 complex signals through toll-like receptor (TLR)2 (Aliprantis et al., 1999; Brightbill et al., 1999; Hirschfeld et al., 1999; Lien et al., 1999), whereas LPS signals through TLR4 (Poltorak et al., 1998; Takeuchi et al., 1999). The importance of innate immunity in the establishment of Lyme disease is highlighted by the observation that *B. burgdorferi*-infected severe combined immunodeficiency (SCID) mice exhibit persistent spirochetemia and chronic progressive inflammation of the joints, heart and liver (Schaible et al., 1990; Barthold et al., 1992).

Role of Plasminogen/Plasmin in *Borrelia* Pathogenesis

Plasmin, a broad spectrum serine protease, is responsible for fibrin degradation during thrombolysis, but also has been proposed to be involved in other physiological and pathological processes such as skin, corneal, and arterial wound repair, tumor progression, and ischemic and excitotoxic brain damage. The discovery that pathogenic microorganisms (Gram-positive and -negative) can convert plasminogen into enzymatically active plasmin to increase their invasiveness and enhance their ability to cross tissue barriers has received much attention (reviewed in Broder et al., 1991). The observation that *Borrelia* species (*B. burgdorferi* and *B. hermsii*) can incorporate plasminogen onto their surfaces where it can be activated to plasmin by the host's plasminogen activation system (PAS) has lent itself to a study of the PAS and spirochetal virulence (Fuchs et al., 1994; Fuchs et al., 1996; Coleman et al., 1995; Coleman et al., 1997; Hu et al., 1995; Hu et al., 1997; Klempner et al., 1995; Perides et al., 1996; Gebbia et al., 1999; Coleman and Benach, 2000). Once incorporated onto the surface of the bacterium, the enzymatically active plasmin has the potential to degrade components of the extracellular matrix, such as fibronectin and laminin. The incorporated plasmin proteolytic activity on the surface of the pathogenic bacteria, such as *Borrelia* spp., must disseminate from their site of entry in the skin to the blood and other tissues (Gebbia et al., 1999). Evidence supporting this role for plasmin has

been reported by Benach and coworkers. Using an in vitro assay, a *B. burgdorferi* human isolate, once complexed with plasmin and supplied an exogenous plasminogen activator (uPA), had the ability to degrade purified extracellular matrix (ECM) components and an interstitial ECM. In two separate animal models of borrelial infection, relapsing fever and Lyme disease, the in vivo role for plasminogen/plasmin has been examined by using plasminogen-deficient knockout mice ($plg^{-/-}$). In the Lyme disease model, plasminogen was shown to be required for efficient spirochete dissemination in ticks and establishment of spirochetemia in mice (Coleman et al., 1997). Plasminogen was also shown to be an important host factor in the relapsing fever model. Plasminogen-deficient mice ($plg^{-/-}$) showed significantly decreased spirochetal burdens in both heart and brain tissues (as measured by PCR amplification). Furthermore, the decreased spirochetal load in the brains of $plg^{-/-}$ mice was associated with a significant decrease in the degree of inflammation of the leptomeninges in these mice, as compared to the control ($plg^{+/+}$ and $plg^{+/-}$) mice (Gebbia et al., 1999). Together these data strongly support a role for the PAS in *Borrelia* spp. infection.

Chemotherapy

Relapsing Fevers

For a review of antibiotic therapies for the relapsing fevers, see Cadavid and Barbour (1998). Relapsing fever has been successfully treated with tetracycline, chloramphenicol, penicillin and erythromycin (Sanford, 1976; Perine and Tekle, 1983; Butler, 1985; Horton and Blaser, 1985), although chloramphenicol treatment was not highly successful in the treatment of relapsing fever in animal models of infection (Cadavid and Barbour, 1998). For louse-borne relapsing fever, tetracycline, in a single oral dose (0.5 g), is the preferred therapy, except in pregnant women and children younger than 8 years (Perine and Tekle, 1983). Erythromycin in a single oral dose (0.5 g) is an equally effective alternative therapy (Perine and Tekle, 1983). For tick-borne relapsing fever, tetracycline (0.5 g), given in four divided doses at 6-h intervals for 5–10 days, is considered the drug regime of choice, owing to the higher rate of treatment failure and relapses in these patients (Sanford, 1976; Foster, 1977; Horton and Blaser, 1985). Although effective in cases of LBRF, erythromycin did not prevent relapse or neurological complications in patients with TBRF (Horton and Blaser, 1985; Colebunders et al., 1993). Parenteral antibiotics (i.e., penicillin G, cefotaxime or ceftriaxone) should be given for 14 or more days in patients with meningitis or encephalitis (Cadavid and Barbour, 1998).

Lyme Borreliosis

Early Lyme (Borreliosis) disease usually responds well to antimicrobial therapy. However, cases with persistent joint or central nervous system complications tend to be more resistant to treatment. Adults with the early manifestations generally respond to doxycycline (100 mg orally, 2 × daily, 10B30 days) or amoxicillin (500 mg orally, 4 × daily, 10B30 days). Children younger than 8 years of age are generally treated with amoxicillin (250 mg orally, 3 × daily, 10B30 days). Neurologic manifestations (Table 5) and other abnormalities are treated with ceftriaxone (Rocephin; 2 g, intravenously, 1 × daily, 14 days). Treatment failures have occurred with all of these regimens, and retreatment may be necessary. Since patients who have already developed a mature anti-borrelia IgG response often remain seropositive after apparently successful antibiotic therapy, the presence or absence of circulating antibodies to *Borrelia* following treatment is not a reliable indicator of cure (Dattwyler et al., 1989). Descriptions of clinical presentations and the medical progress of Lyme disease have been reviewed by Steere (1989) and Edlow (1999).

Jarisch-Herxheimer Reaction

Shortly (10–30 minutes) following the initiation of antibiotic therapy in a large number of relapsing fever patients, a Jarisch-Herxheimer reaction may occur, manifested by rigors, sudden fever, headache, and persistent hypertension, followed over the next few hours by profuse sweating and a slow decline in temperature and fall in blood pressure. The discovery of this distressing and potentially life-threatening (case fatality rate of approximately 5%) phenomenon is usually credited to Jarisch and Herxheimer, who first described the components of this syndrome in patients with secondary syphilis (see The Genus *Treponema* in this Volume) who received mercury treatment. This reaction has been described in a variety of bacterial infections, including five spirochetal infections (louse-borne and tick-borne relapsing fevers, Lyme disease, leptospirosis (see The Genus *Leptospira* in this Volume), and syphilis (see The Genus *Treponema* in this Volume) and closely resembles a classic endotoxin reaction (or systemic inflammatory response syndrome), which typically occurs when endotoxin (LPS) from cell walls of Gram-negative bacteria or other viral, protozoal, fungal

pyrogen, or bacterial toxin is released. More recently, bacterial lipoproteins (see "Lipoproteins and the Innate Immune Response") have been added to the list of molecules capable of inducing an acute inflammatory response (Radolf et al., 1991; Radolf et al., 1995a; Ma and Weis, 1993; Tai et al., 1994; Weis et al., 1994; Norgard et al., 1995; Norgard et al., 1996; Sellati et al., 1996; Sellati et al., 1998; Wooten et al., 1996; Morrison et al., 1997; Theus et al., 1998; Vincent et al., 1998). One general characteristic of all Jarisch-Herxheimer reactions is a substantial increase in circulating levels of tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) just before symptoms develop. In a recent study by Fekade et al. (1996), treatment with Fab fragments of anti-TNF-α antibodies significantly reduced the incidence and severity of the Jarisch-Herxheimer reaction in patients with louse-borne (endemic) relapsing fever caused by *B. recurrentis*. This report was one of the first to establish that passive immunization against TNF-α can block the development of a shock-like illness in humans and has received support within the medical community as an attractive and viable therapy for the prevention of the Jarisch-Herxheimer reaction in spirochetal and potentially other bacterial infections (Beutler and Munford, 1996).

Lyme Disease Vaccine

In two large clinical trials, more than 10,000 adult volunteers received three doses (intramuscularly) of one of two Lyme disease vaccines directed against the *B. burgdorferi* outer surface lipoprotein, OspA (see "Reciprocal Expression of OspA and OspC"). Individuals were vaccinated at time 0, 1 and 12 months. After three doses, the OspA-based vaccine was shown to be 76–92% effective (Sigal et al., 1998; Steere et al., 1998b). The Food and Drug Administration (FDA) gave final approval, in 1998, to a Lyme disease vaccine, LymeRix, based on a recombinant form of OspA and manufactured by SmithKline Beecham Pharmaceuticals. There are several caveats to the use of the LymeRix vaccine (http://www.cdc.gov/epo/mmwr/preview/mmwrhtml/rr4807a1.htm). Although long studied using *Borrelia* cultivated in BSK II (Table 3) medium, the target of the vaccine, OspA (see "Reciprocal Expression of OspA and OspC"), was recently shown to be expressed only on the surface of spirochetes in the flat (unfed) tick and not on the surface of spirochetes in the tick salivary glands or on spirochetes transmitted to the human host. Once a tick begins to take a bloodmeal on a vaccinated individual, any spirochetes within the midgut of that tick are susceptible to the host's bactericidal anti-OspA antibodies. The end result is a lowering of the density of organisms within the tick salivary gland to below a critical threshold required for initiating events linked to transmission (de Silva et al., 1999). Those spirochetes that are not killed in the midgut prior to the downregulation and disappearance of OspA from the bacterial cell surface, however, are no longer susceptible to killing by antibodies directed against OspA (see "Reciprocal Expression of OspA and OspC"), thereby reducing the vaccine's efficacy. In addition, individuals who receive the vaccine will become ELISA positive (but Western-blot negative), making future diagnosis of those patients who subsequently become infected more difficult. Uncertainties about who should receive the vaccine (http://www.cdc.gov/epo/mmwr/preview/mmwrhtml/rr4807a1.htm), concerns about the limited duration of protective immunity (Onrust and Goa, 2000; Taege, 2000), and the unfortunate misperception that it has a high incidence of serious side effects, including autoimmune reactions (Sigal, 2000; OspA and Autoimmunity), have produced a less than enthusiastic reception among practitioners and the lay public. To date, these concerns have failed to materialize as there have been no significant side effects among the vaccinated public.

One attractive alternative to a vaccine based solely on OspA is the development of a multivalent vaccine based on a number of borrelial antigens that are expressed preferentially in either the arthropod (i.e., OspA Reciprocal Expression of OspA and OspC) or the mammalian host (i.e., Osp A/Osp C Reciprocal Expression of OspA and OspC, and OspE/OspF/Elp, Mlp, and DbpA. The increased expression of OspC in the mammalian host, the identification of OspC subtypes of *B. burgdorferi*, which are primarily responsible for invasive disease in humans (see *Borrelia* Genotype and Pathogenesis in the second edition), and the ability of OspC antibodies to prevent, as well as resolve, infection with *B. burgdorferi* (Zhong et al., 1999) make this molecule particularly attractive.

The vaccine, however, does not offer any protection against other tick-borne diseases, *Babesia microti* and the agent of human granulocytic ehrlichiosis (HGE), common to and transmitted by the same ixodid vectors. An area of active interest is the development of anti-arthropod vaccines. Such vaccines could be designed to interfere with one or more of the steps involved in pathogen transmission, including 1) preventing tick attachment and feeding, and 2) preventing pathogen attachment to and migration within the arthropod vector tissues. Such vaccines could have the potential to simultaneously protect

against multiple pathogens transmitted by the same vector.

For recent vaccine reviews, see Thanassi and Schoen (2000) and Poland and Jacobson (2001).

OspA and Autoimmunity

A study by Steere and coworkers (Gross et al., 1998), looking at patients with treatment-resistant and -responsive Lyme arthritis, suggested that infection with *B. burgdorferi* may lead to the development of an immune response to the OspA (see "Reciprocal Expression of OspA and OspC") lipoprotein that cross-reacts with a human antigen, human leukocyte function-associated antigen-1 (hLFA-1). In a small number of patients who possess a particular Major Histocompatability Class (MHC) II allele, HLA-DRB1*0401, the response against hLFA-1 may lead to the subsequent development of an autoimmune-mediated arthritis (Gross et al., 1998). The identification of OspA-reactive type-1 T helper (T_H1) cells that cross-reacted with a related-region within hLFA-1 in the synovial fluid of individuals with treatment-resistant Lyme arthritis supports this hypothesis (Gross et al., 1998). The association between an anti-OspA immune response and hLFA-1 cross-reactivity and the development of treatment-resistant Lyme arthritis is not fully understood. Some patients who develop hLFA-1 autoreactive T-cells do not possess the HLA-DRB1*0401 allele; conversely, some patients who possess that HLA-DRB1*0401 allele do not respond to either OspA or hLFA-1 (Gross et al., 1998). The implication(s) of these data for vaccine (see "Lyme Disease Vaccine") recipients remains to be seen.

Veterinary Diseases

Borrelia anserina is the etiological agent of avian borreliosis (Table 1), a highly fatal disease of geese, ducks, turkeys and chickens. Mammals are resistant to this infection. The disease is widespread, occurring in many countries including those in Europe, Asia, Australia, and South, Central, and North America. The vectors of *B. anserina* are species of *Argas* ticks. The disease begins with a high fever; then the birds become cyanotic and develop diarrhea. Spirochetes are present in the blood early during the disease, and relapses do not occur.

Borrelia coriaceae is the putative etiological agent of epizootic bovine abortion (Table 1), a disease of major economic importance in California (Lane et al., 1985; Johnson et al., 1987). This species of *Borrelia* (Johnson et al., 1987) is carried by the human-biting soft tick *Ornithodoros coriaceus* (Lane et al., 1985). Until recently, only one isolate of *B. coriaceae* Co53 had been characterized genetically and phenotypically (Johnson et al., 1987; LeFebvre and Perng, 1989). The publication of two additional strains of *B. coriaceae*, isolated from adult *O. coriaceus* ticks collected from northwestern California, has significantly increased our understanding of this little-known borrelial genospecies (Hendson and Lane, 2000). Hybridization studies of these two new isolates, along with the initial Co53 isolate, demonstrated low homology between these strains and the relapsing fever spirochete *B. parkeri* among the linear plasmids of these isolates. Pulsed field gel electrophoresis demonstrated that *B. coriaceae* exhibits genetic diversity within the linear plasmid profiles of these three isolates, a characteristic common among the borreliae (Hendson and Lane, 2000). Unlike most *Borrelia* spp., *B. coriaceae* can be passed transstadially and occasionally transovarially (Lane et al., 1985; Lane and Manweiler, 1988a). The Columbian black-tailed deer (*Odocoileus hemionus columbianus*) has been implicated as a probable natural host (Lane and Manweiler, 1988b).

Borrelia theileri is responsible for a mild disease of cattle, horses and sheep (Table 1). The illness is characterized by one to two episodes of fever, weight loss, weakness and anemia. The vectors of this spirochete are species of the hard ticks *Rhipicephalus* and *Boophilus*. *Borrelia theileri* also has been found to be transmitted transovarially in *Boophilus microplus* eggs (Smith et al., 1985). The disease has been reported in South Africa and Australia.

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