Bloodstream- Versus Tick-Associated Variants of a Relapsing Fever Bacterium

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The relapsing fever spirochete, *Borrelia hermsii*, alternates infections between a mammal and a tick vector. Whether the spirochete changes phenotypically in the different hosts was examined by allowing the tick vector *Ornithodoros hermsi* to feed on mice infected with serotype 7 or serotype 8 of *B. hermsii*. Upon infection of ticks, the spirochetal serotype-specific variable major proteins (Vmps) 7 and 8 became undetectable and were replaced by Vmp33. This switch from a bloodstream- to tick-associated phenotype could be induced in culture by a decrease in temperature. After tick-bite transmission back to mice, the process was reversed and the spirochetes resumed expression of the same Vmp present in the previous infectious blood meal.

Relapsing fever was recognized as a human disease back in the time of Hippocrates (*1*). Endemic relapsing fever is caused by numerous species of the spirochete *Borrelia* and occurs throughout the world in many discrete enzootic foci where the spirochetes are maintained primarily in rodents and ticks of the genus *Ornithodoros* (*2*). In western North America, *B. hermsii*, which is transmitted by the tick *O. hermsi*, causes human disease that is likely more prevalent than the number of reported cases (*3*). Human infections result from the bites of these fast-feeding ticks, which usually occur at night and unbeknownst to their victims.

The periodic cycling of acute and afebrile episodes that led to the naming of relapsing fever is associated with dramatic changes in the abundance of spirochetes circulating in the patient's blood (spirochetemia) (*1*). Accompanying each of these cyclic population changes in the number of spirochetes is a change in serotype and major immunogenic lipoprotein associated with the outer surface of the spirochete. These variable major proteins (Vmps) are composed of two multigene families that have recently been renamed as variable large or small proteins (Vlps and Vsps) (*4*); however, here we refer to them collectively as Vmps.

In the HS1 strain of *B. hermsii*, the single *vmp* gene being expressed at any one time is located in an expression locus near the telomere of a 28-kb linear plasmid (*5*). A copy of the expressed gene and all other unexpressed *vmp* genes are present in silent loci on the same or different linear plasmids. Antigenic switching to a new serotype occurs when one of the silent *vmp* genes is duplicated and replaces the existing *vmp* gene in the expression locus, or when intraplasmidic recombination occurs, sometimes augmented by the introduction of point mutations in rearranged *vmp* genes (*5, 6*). Each population of spirochetes associated with a single acute episode is composed almost entirely of one serotype producing the same Vmp (*7*). Different serotypes predominate in subsequent populations, and this multiphasic antigenic variation presumably allows evasion from the mammalian immune response (*8*). Yet nothing is known about the influence of the spirochete's serotype on infection in the arthropod host or whether spirochetes change phenotypically as they alternate between ticks and mammals.

To examine if ticks transmit the same serotype of the relapsing fever spirochete as was acquired during a previous blood meal, we first infected two cohorts of nymphal *O. hermsi* ticks with either serotype 7 or serotype 8 of *B. hermsii* (Fig. 1, A and B) (*9*). The ticks were allowed to molt and were then fed singly on individual mice to determine the serotype transmitted to the mice (*10*). Eighteen of 95 ticks (19%) transmitted spirochetes, and in every infection, the first detectable spirochetemia in the mice after tick-bite consisted of the same serotype ingested previously by the ticks, whether this was serotype 7 or serotype 8. In addition, polymerase chain reaction analysis of the spirochete's *vmp* telomeric expression locus in 22 infected ticks (11 ticks with serotype 7 and 11 ticks with serotype 8) revealed no apparent DNA rearrangement (*11*). These observations indicated that the *vmp* gene present at the telomeric expression site did not change during passage through ticks.

To determine if spirochetes expressed the telomeric *vmp* gene in the tick, we infected additional cohorts of ticks with either serotype 7 or serotype 8 and allowed the ticks to molt to the next developmental stage (*12*). From 33 to 144 days after infection, we examined 41 ticks, including 23 infected with serotype 7 and 18 infected with serotype 8. Salivary glands from all ticks and a lesser number of midgut (n = 33) and synganglion (n = 22) preparations were examined by indirect immunofluorescence assay (IFA) with one of four antibodies (*13*). Although all tissues were infected, we were unable to detect spirochetes in the salivary glands using antibodies specific for Vmp7 (10 examined) (Fig. 1C) or Vmp8 (5 examined). Yet, when we examined the other salivary gland from these same ticks and others from the same infected cohort by IFA with a monoclonal antibody to Vmp33 (marker of serotype C), a surface protein seen previously only in the culture-adapted strain HS1 (*14, 15*), we detected many fluorescent spirochetes (Fig. 1, D and E). This was true for ticks infected with either serotype 7 (n = 17) or 8 (n = 12). When

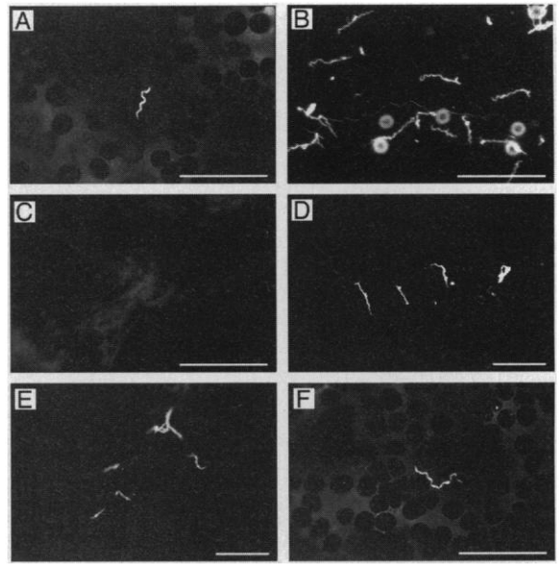

Fig. 1. *Borrelia hermsii* switches between bloodstream-specific and tick-specific outer surface proteins. (**A**) Serotype 7 in mouse blood visualized with antibody to Vmp7 (anti-Vmp7). (**B**) Serotype 8 in mouse blood visualized with anti-Vmp8. (**C**) Spirochetes in a salivary gland of a tick that ingested serotype 7 is not detectable with anti-Vmp7. (**D**) Spirochetes in a salivary gland of a tick that ingested serotype 7 visualized with anti-Vmp33. (**E**) Spirochetes in a salivary gland of a tick that ingested serotype 8 visualized with anti-Vmp33. (**F**) Serotype 7 in mouse blood visualized with anti-Vmp7 after transmission by tick bite. Spirochetes were not detectable in blood with anti-Vmp33. (Bar = 25 μM).

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peripheral blood was examined during the first spirochetemia in mice only 4 days after infection by tick bite, bacteria were visualized by IFA with anti-Vmp7 (Fig. 1F) and anti-Vmp8 but not with anti-Vmp33. Therefore, as these spirochetes cycle between ticks and mammals, their outer surface alternates between bloodstream- and tick-associated proteins.

One stimulus for such a switch might be the change in temperature as the spirochetes are transferred from a warm-blooded mammal to a much cooler tick. We tested this hypothesis with blood from a mouse infected with serotype 8 that we cultured at 37° or 23°C (*16*). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell lysates (*17, 18*) indicated that growth at 23°C induced expression of *vmp33*, whereas growth at 37°C maintained the expression of *vmp8* (Fig. 2A). Immunoblot analysis with specific antibodies confirmed that the synthesis of Vmp8 and Vmp33 was influenced by temperature, whereas synthesis of flagellin, a structural protein of the spirochete's periplasmic flagella, was not (Fig. 2, B to D) (*18, 19*).

Bloodstream *vmp* genes that allow for antigenic variation of *B. hermsii* HS1 during infection in mammals are expressed at a single telomeric locus on a 28-kb linear plasmid (*5*). However, *vmp33* is expressed by its own promoter (*15*), mapped recently to a different linear plasmid of ~53 kb (*20*). Vmp33 was identified previously in one strain of *B. hermsii* only after prolonged cultivation in vitro (*14*), although others have speculated as to a possible role of this protein during tick infection (*21*). We have identified *vmp33* in 23 additional isolates of *B. hermsii* from western North America (*22*). Also, genes homologous to *vmp33* and proteins antigenically related to Vmp33 have been identified in many other species of *Borrelia*, including outer surface protein (Osp) C of the Lyme disease spirochete *Borrelia burgdorferi* (*15, 23*). Therefore, this family of proteins appears to be conserved among all members of the genus *Borrelia*.

The temporal expression of *vmp33* and *ospC* by *B. hermsii* and *B. burgdorferi*, respectively, implicates a common biological function for these proteins associated with tick transmission or early colonization in mammals. *Borrelia hermsii* produce Vmp33 when the temperature cools after their acquisition by ticks and continue to produce this protein during persistent infection of the tick's salivary glands until they are rapidly transmitted by *Ornithodoros* ticks that feed in 15 to 90 min. In contrast, most Lyme disease spirochetes in unfed *Ixodes* ticks are restricted to the midgut (*24*), produce OspC only after the temperature increases and ticks have fed for two or more days (*25*), and are then transmitted after their dissemination from the midgut to salivary glands (*26*). Therefore, Lyme disease spirochetes produce OspC only after tick feeding has begun, but while there is still ample time for transmission to occur by *Ixodes* ticks that require several days to feed. Our results demonstrating alternating phenotypes of *B. hermsii* cycling between the mammalian bloodstream and arthropod vector also have parallels to the salivary trypanosomes (*27*) and other parasitic protozoa that require blood-feeding arthropods for their biological transmission.

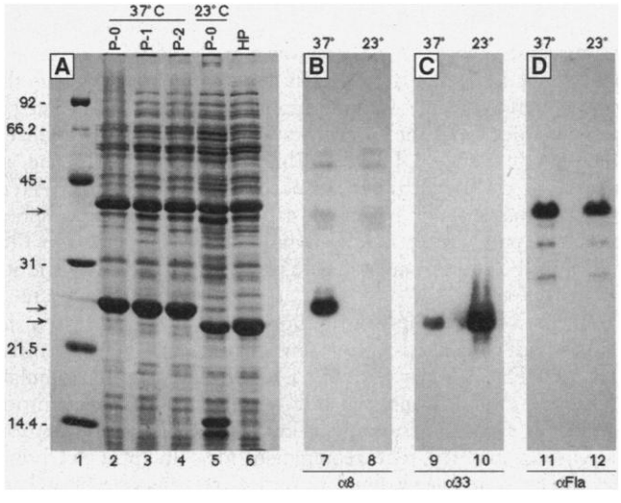

Fig. 2. Shift of *B. hermsii* to lower temperature induces switch from a bloodstream Vmp to the tick-associated Vmp33. (**A**) Serotype 8–infected mouse blood was inoculated into medium and incubated at 37° or 23°C. Proteins in whole-cell lysates were separated by SDS-PAGE and stained with Coomassie brilliant blue. The primary isolate (P-0) and the subsequent two passages (P-1 and P-2) did not switch after growth at 37°C (lanes 2 to 4), whereas growth at 23°C induced the switch in the primary isolate (P-0) (lane 5). Three arrows indicate flagellin (top) and the induced switch from Vmp8 (middle) to Vmp33 (bottom). Also shown is high-passage (HP) culture of the same strain (DAH) that has stably switched to Vmp33 (lane 6). Molecular mass standards (lane 1) are shown in kilodaltons. In (B) to (D), whole-cell lysates of serotype 8 spirochetes grown at 37° or 23°C were fractionated by SDS-PAGE and stained with Coomassie brilliant blue. Immunoblots containing replicate lysates in lanes 4 and 5 were first incubated with (**B**) anti-Vmp8, (**C**) anti-Vmp33, or (**D**) anti-flagellin, and then with ^{125}I-labeled protein A.

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9. Ticks were from a spirochete-free colony of *O. hermsi* maintained at the Rocky Mountain Laboratories. The DAH strain of *B. hermsii* originated from a relapsing fever patient from eastern Washington state. This strain is nearly identical to the type strain HS1 (*28*), which also originated from this geographic area and for which immunological reagents are available (*7*). To infect ticks, we first inoculated a BALB/c mouse by intraperitoneal (i.p.) injection of the uncloned spirochete in 0.5 ml of Barbour-Stoenner-Kelly (BSK) II medium (*29*). The mouse was examined daily for spirochetes by Giemsa staining of a drop of tail blood. On day 5 after inoculation, 0.1 ml of infected blood was inoculated by i.p. injection into another mouse, which 2 days later had 7×10^7 spirochetes per milliliter of blood, as quantified with Petroff-Hausser counting chamber and microscopy. Blood smears were made for serotype determination, and 75 *O. hermsi* second-stage nymphs were fed to repletion on this spirochetemic mouse. This mouse was monitored daily for spirochetemia until the first relapse occurred at day 10. Infected blood was passed by i.p injection into another mouse to amplify the infection, and 2 days later the blood contained 6.8×10^7 spirochetes per milliliter. At this time, bloods smears were again made for serotype determination and another 75 second-stage nymphs were fed to repletion. Both cohorts of ticks were held at 27°C and 80% relative humidity. Immediately after feeding, infections in two ticks from each group were confirmed by darkfield microscopic examination of the midgut. Spirochetes in the blood smears from both mice used to infect ticks were examined with 25 serotype-specific antisera (*7*), which identified the initial infecting population as serotype 7 and the relapse as serotype 8.
10. Ticks were allowed to molt, and then from 21 to 372 days after infection, ticks from each cohort were fed singly on individual mice that were 10 days old. Blood from each mouse was examined daily for 2 weeks, and the serotype of the spirochetes in the initial population was determined (*7, 9*).
11. B. J. Hinnebusch and T. G. Schwan, unpublished data.
12. Mouse blood containing either serotype 7 or serotype 8 *B. hermsii* was used to newly infect additional mice. Late-stage nymphs and adult ticks, which are larger than early-stage nymphs, were used to facilitate the dissection and collection of salivary glands and other tissues. Ticks were infected by feeding on mice with ~ 7.5×10^7 spirochetes per milliliter of blood and then held at 27°C until the nymphs had molted to adults. The identity of the serotypes 7 and 8 in the mouse blood used to infect ticks was again confirmed by IFA.
13. After the molt of the nymphs to adults, we dissected the salivary glands, midgut, and synganglion for immunofluorescence analysis. Tick tissues were mixed in phosphate-buffered saline containing 0.75% bovine serum albumin, adhered to glass microscope slides, air dried, and fixed in acetone for 30 min.

Spirochetes were visualized by incubation with monoclonal antibodies to flagellin (H9724) (*30*), Vmp7 (H9236) (*31*), or Vmp33 (H4825) (*31*), or a mouse polyclonal antibody to Vmp8 (*7*). This was followed by incubation with goat antibody to mouse immunoglobulin G conjugated with fluorescein isothiocyanate (Kirkegaard and Perry Laboratories, Gaithersburg, MD).
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16. A mouse was infected through i.p. injection with a freshly thawed sample of blood from a mouse previously infected with serotype 8. On day 4 after inoculation, the spirochetemia approached 10^8 spirochetes per milliliter of blood. Infected blood from this mouse was cultured in BSK-H medium (Sigma, St. Louis) at 23° or 37°C until spirochetes reached stationary phase and then prepared for analysis of whole-cell lysates.
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Gating of CaMKII by cAMP-Regulated Protein Phosphatase Activity During LTP

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Long-term potentiation (LTP) at the Schaffer collateral–CA1 synapse involves interacting signaling components, including calcium (Ca^{2+})/calmodulin–dependent protein kinase II (CaMKII) and cyclic adenosine monophosphate (cAMP) pathways. Postsynaptic injection of thiophosphorylated inhibitor-1 protein, a specific inhibitor of protein phosphatase–1 (PP1), substituted for cAMP pathway activation in LTP. Stimulation that induced LTP triggered cAMP-dependent phosphorylation of endogenous inhibitor-1 and a decrease in PP1 activity. This stimulation also increased phosphorylation of CaMKII at Thr^{286} and Ca^{2+}-independent CaMKII activity in a cAMP-dependent manner. The blockade of LTP by a CaMKII inhibitor was not overcome by thiophosphorylated inhibitor-1. Thus, the cAMP pathway uses PP1 to gate CaMKII signaling in LTP.

Multiple signaling pathways participate in LTP in the CA1 region of the hippocampus at both presynaptic and postsynaptic sites (*1*), with the CaMKII pathway playing a central role in transmitting the postsynaptic signals required for LTP (*2*, *3*). In contrast, the role of other signaling pathways is not yet clear. The cAMP pathway is involved in LTP and memory in transgenic mice (*4*). In rats, the postsynaptic cAMP pathway is required for LTP induced by widely spaced trains of high-frequency synaptic stimulation (HFS), but activation of the pathway is not sufficient to induce LTP (*5*). Thus, the postsynaptic cAMP pathway does not transmit the signals for LTP but rather gates the transmittal pathway. It has been proposed (*5*, *6*) that the cAMP-operated gate may use PP1.

The CA3-CA1 synapse of rat hippocampal slices was stimulated with widely spaced trains of HFS (*7*). The resulting LTP was blocked by inhibiting postsynaptic cAMP-dependent protein kinase [protein kinase A (PKA)] (Fig. 1A) (*5*). This requirement for PKA can be overcome by direct inhibition of postsynaptic phosphatases (*5*), suggesting that the cAMP pathway modulates LTP by blocking phosphatases. Protein phosphatase inhibitor-1 (I-1) is a candidate for mediating cAMP inhibition of phosphatase activity. I-1, upon phosphorylation by PKA at Thr^{35}, is a specific blocker of PP1 (*8*). I-1 mRNA is expressed in CA1 neurons (*9*), and I-1 has already been implicated in plasticity at the CA3-CA1 synapse (*10*). We injected recombinant purified Thr^{35}-thiophosphorylated I-1 (*11*) into the postsynaptic neuron and tested its ability to overcome the blockade of LTP by the specific PKA inhibitor Rp–cyclic adenosine monophosphorothioate (cAMPS). Thiophosphorylated I-1 completely reversed the effect of Rp-cAMPS, yielding LTP that was indistinguishable from that of the control (Fig. 1, B and C). In contrast, mutant [Thr^{35} → Ala (T35A)] nonphosphorylatable I-1 (*11*), when injected postsynaptically, did not reverse the effect of Rp-cAMPS. Thus, PP1 appears to be the postsynaptic phosphatase that negatively regulates LTP, and I-1 activation by PKA may facilitate LTP by inhibiting PP1.

Next, we determined if LTP-inducing stimulation results in PKA phosphorylation of I-1. We examined the phosphorylation state of I-1 in the CA1 region after HFS (*12*). The same pattern of synaptic stimulation that induced cAMP-dependent LTP also raised the amount of phosphorylated I-1 in the CA1 region (Fig. 2A). The increase in phosphorylation of I-1 by HFS was dependent on PKA activity because it was blocked by the inclusion of Rp-cAMPS in the superfusate during stimulation (Fig. 2A). We then measured protein phosphatase activity in the CA1 region of stimulated and unstimulated slices (Fig. 2B). Under our assay conditions (*13*), greater than 90% of the phosphoprotein phosphatase activity in the CA1 homogenate was inhibited by thiophosphorylated I-1, defining it as PP1. HFS that activated I-1 also resulted in significant inhibition of PP1 activity. This effect was prevented by bath application of Rp-cAMPS during stimulation, indicating that PKA mediated the phosphatase inhibition.

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