Borrelia burgdorferi RevA Antigen Is a Surface-Exposed Outer Membrane Protein Whose Expression Is Regulated in Response to Environmental Temperature and pH

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Borrelia burgdorferi, the causative agent of Lyme disease, produces RevA protein during the early stages of mammalian infection. B. burgdorferi apparently uses temperature as a cue to its location, producing proteins required for infection of warm-blooded animals at temperatures corresponding to host body temperature, but does not produce such virulence factors at cooler, ambient temperatures. We have observed that B. burgdorferi regulates expression of RevA in response to temperature, with the protein being synthesized by bacteria cultivated at 34°C but not by those grown at 23°C. Tissues encountered by B. burgdorferi during its infectious cycle vary in their pH values, and the level of RevA expression was also found to be dependent upon pH of the culture medium. The cellular localization of RevA was also analyzed. Borrelial inner and outer membranes were purified by isopycnic centrifugation, and membrane fractions were conclusively identified by immunoblot analysis using antibodies raised against the integral inner membrane protein MotB and outer membrane-associated Erp lipoproteins. Immunoblot analyses indicated that RevA is located in the B. burgdorferi outer membrane. These analyses also demonstrated that an earlier report (H. A. Bledsoe et al., Infect. Immun. 176:7447–7455, 1994) had misidentified such B. burgdorferi membrane fractions. RevA was further demonstrated to be exposed to the external environment, where it could facilitate interactions with host tissues.

The spirochete Borrelia burgdorferi has evolved efficient mechanisms by which it can persistently infect both warm-blooded and arthropod hosts and be efficiently transmitted between these two host types (49). Such a complex lifestyle requires that the bacteria produce proteins appropriate for each stage of the infectious cycle. These might include surface proteins that facilitate interactions with host cells or extracellular components, function in nutrient acquisition, or help protect the bacteria against host immune system responses. Throughout its infectious cycle, B. burgdorferi apparently senses its location in order to produce proteins and other factors required for each stage of the cycle. We and others have demonstrated that B. burgdorferi recognizes various environmental cues, including temperature, pH, and soluble chemicals and, as a consequence, regulates surface protein expression (1–3, 12, 13, 30, 40, 45, 46, 50, 51, 54, 55, 62).

Among the B. burgdorferi proteins known to be synthesized during mammalian infection is an approximately 17-kDa protein previously designated Rev (25, 43, 53) and herein renamed RevA (see below for the rationale behind this name modification). All analyzed Lyme disease spirochetes possess this protein during the bacterium’s natural transmission cycle. We therefore sought to determine signals responsible for the controlled expression of RevA. Since the cellular localization of a protein can be suggestive of its function, this aspect of the RevA protein was also addressed in our studies.

Materials and Methods

Bacteria. B. burgdorferi B31 is a wild-type strain, isolated from a tick collected on Shelter Island, N.Y. (11). Bacteria used in these studies are infectious to mice (14, 16). B. burgdorferi were grown in either Barbour-Stoenner-Kelly II (BSK-II) medium (4) prepared in our laboratories or a commercially prepared modification of that medium (BSK-H; Sigma, St. Louis, Mo.). All media contained 6%
(vol/vol) rabbit serum (Sigma). Bacteria were cultivated at 34°C, unless otherwise noted.

Cloning, overexpression, and purification of N-terminally truncated MotB. To aid in the identification of B. burgdorferi membrane fractions, a recombinant form of an integral membrane protein was synthesized for use in producing antibodies. The motB gene codes part of the flagellar motor, which is embedded in the bacterial inner membrane (32, 33). The B. burgdorferi motB gene, lacking the first 105 nucleotides, was PCR amplified from strain B31 DNA and cloned into PET30 LIC (Invitrogen, Carlsbad, Calif.). The resultant construct expressed a polyhistidine-tagged MotB that was missing the N terminal signal sequence and transmembrane region (mOTmB). Recombinant mOTmB could not be purified by nickel column due to its aggregation in inclusion bodies. Therefore, inclusion bodies were purified from cell lysates as follows. Cell lysates were cleared by centrifugation, and the inclusion body pellet was washed in 2% (vol/vol) Triton X-100 and 2 M urea in phosphate-buffered saline (PBS; pH 8.0) and then centrifuged (22,000 × g, 30 min, 4°C). This process was repeated seven times. The inclusion body pellet was then washed once in PBS and centrifuged (22,000 × g, 30 min, 4°C).

Antibodies. Gilmore and Mbow earlier reported the production of a panel of hybridomas derived from mice infected with B. burgdorferi B31 via tick bite (25). One of those hybridomas, designated YM.17 produced monoclonal antibodies (MAbs) that specifically recognized the strain B31 RevA protein (25). A second hybridoma, YM.26, was subsequently found to also recognize a recombinant RevA protein (R. Gilmore, Jr., unpublished results). Both YM.17 and YM.26, were provided by Lamine Mbow (Colorado State University, Ft. Collins, Colo.) and Robert Gilmore, Jr. (Centers for Disease Control and Prevention, Ft. Collins, Colo.), as were the hybridomas B5, which is directed against the strain B31 OspC protein (35), and B11, which is directed against the strain B31 ErpA/I/N RevA protein (R. Gilmore, Jr., unpublished results). Both YM.17 and YM.26, hybridoma, YM.26, was subsequently found to also recognize a recombinant (MAbs) that specifically recognized the strain B31 RevA protein (25). A second hybridomas derived from mice infected with B. burgdorferi (PBS; pH 8.0) and then centrifuged (22,000 × g, 30 min, 4°C). To produce a revA-specific probe, a plasmid clone containing the cp32 genes of strain B31 were harvested by centrifugation, and, unless noted otherwise, were harvested by centrifugation and, unless noted otherwise, washed with PBS and lysed by resuspension in distilled water and incubation in a boiling water bath for 5 min. Equimolar amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose membranes as described previously (37). Membranes were blocked by incubation for <1 h in 5% (vol/vol) nonfat dried milk in Tris-buffered saline–Tween 20 (TBS-T) (37), rinsed with TBS-T, and incubated for 1 h at room temperature in appropriate primary antibody. Membranes were then washed with TBS-T, incubated for 1 h with conjugated anti-rabbit IgG horseradish peroxidase (Amersham, Piscataway, N.J.) in TBS-T, according to the manufacturer’s instructions. Bound primary antibodies were detected by enhanced chemiluminescence (Amersham) and XAR-5 film (Kodak, Rochester, N.Y.).

Two-dimensional nonequilibrium pH gradient gel electrophoresis (2D-NEPHGE) was performed as described by O’Farrell (39) with modifications described by Carroll et al. (13). Briefly, 80 mg of solubilized membrane proteins were separated in the first dimension on a 1-mm analytical tube gel for 3000 V h (500 V, 6, 14°C) using a vertical 1-D Running System (Genomic Solutions, Ann Arbor, Mich.). Membrane proteins were then separated in the second dimension by SDS–12% PAGE and were either stained with silver (Silver Stain Plus; Bio-Rad) or transferred to nitrocellulose for immunoblot analysis. Effects of temperature and pH on protein levels. For temperature effect studies, bacteria were grown to mid-exponential phase (approximately 10^7 bacteria per ml) at 23°C, diluted 1:100 into fresh medium, and grown to mid-exponential phase at 34°C (55). For pH effect studies, bacteria were grown to mid-exponential phase at 34°C in BSK-II or BSK-H medium supplemented with 25 mM HEPES and buffered to a pH of either 8.0 or 7.0 (13). The pH values of the media were measured following cell harvesting, and no detectable changes were observed.

B. burgdorferi membrane purification and analysis. Cell lysates were separated into total membrane and soluble fractions by ultracentrifugation (14). Inner and outer membranes of B. burgdorferi B31 were separated by isopycnic centrifugation according to the method of Bledsoe et al. (8). Evidence presented in the Results section below indicate that the original designations of inner and outer membrane by Bledsoe et al., were actually reversed. To avoid confusion, we refer to our membrane fractions as “true” inner and outer membranes.

In situ protease analyses. B. burgdorferi were grown to mid-exponential phase in BSK-H, pelleted by centrifugation, washed once with PBS, and resuspended in PBS to a final concentration of approximately 2 × 10^9 bacteria/ml. Examination of bacterial suspensions by phase-contrast light microscopy did not indicate detectable lysis of the bacteria. Bacteria were then incubated at room temperature in PBS containing a protease for 30 min, 1 h, or 2 h, whereupon digestion was terminated by addition of an appropriate inhibitor followed by sample boiling. One of three different proteases was used in each experiment at the following final concentrations: 40 μg of proteinase K (Sigma), 40 μg of trypsin (Sigma), or 0.05 μg of pronase (Boehringer-Mannheim, Indianapolis, Ind.) per ml. Proteinase K was inhibited by addition of p-amidinomethylsulfon fluoride (PMSF) to a final concentration of 1.6 mg/ml. Trypsin was inhibited by addition of PMSF and pefabloc SC (Boehringer-Mannheim) to final concentrations of 1.6 and 0.3 mg/ml, respectively. Pronase was inhibited by addition of PMSF, pefabloc SC, and EDTA to final concentrations of 0.06, 0.3, and 0.5 mg/ml, respectively. Control aliquots of bacteria were incubated in buffer for 2 h at room temperature without added protease, followed by the addition of inhibitory agents as with the protease-treated bacteria. Bacterial lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes, and the susceptibility of RevA to protease digestion was assessed by immunoblot analysis with MAb YM.26. As experimental controls, lysates were also immunoblotted with MAbs directed against OspC (located on the bacterial outer surface and thus susceptible to proteolysis [22, 24]) and Flab (located in the periplasmic space and thus protected against protease digestion in intact bacteria [29]).

RESULTS

Regulation of RevA synthesis. Three genes of strain B31 have been referred to as “rev”: two on cp32 plasmids that encode 17.9-kDa proteins and are very similar to the original rev gene of strain 297 identified by Porcella et al. (43), and a third gene encoding a 20.1-kDa protein that is located on plasmid cp9-1 (15, 23, 36). Due to the differences described below, it is apparent that the cp32 genes and the cp9-1 gene are not allelic. Thus, we have designated the cp32 genes revA and the cp9-1 gene revB. The two revA genes of strain B31 are given allele designations based upon the plasmid on which each gene is located: allele revA1 is located on cp2-1, and cp32-6 con-
tains revA6 (15). The predicted sequences of the mature RevA1 and RevA6 proteins are identical (Fig. 1A) and so are referred to as simply “RevA” throughout this report. The revB gene has 47.5% nucleic acid identity with the revA genes and encodes a protein that shares just 28% amino acid sequence identity with the two cp32-encoded proteins (Fig. 1A). Additionally, the cp9-1 gene promoter region is very different from that of the cp32 genes (data not shown), suggesting that different mechanisms might control transcription for each locus type.

Both revA genes are flanked on the 5′ end by a divergently transcribed gene of the mlp multigene family (mlpA and mlpF upstream of revA1 and revA6, respectively) (15, 42). These two intergenic regions, which presumably contain the promoters of both the revA and mlp genes, contain nearly identical nucleotide sequences (Fig. 1B), making it likely that both revA1 and revA6 are under similar transcriptional controls.

We and others have previously observed that some spirochete proteins involved in mammalian infection are regulated by temperature, with greater amounts of protein synthesized by bacteria cultivated at temperatures similar to the mammalian body temperature than by bacteria grown at ambient temperature (17, 31, 46, 51, 54, 55, 62). For these reasons, we examined the levels of RevA protein synthesized by bacteria cultivated at either 23 or 34°C. Immunoblot analysis indicated that RevA was made by bacteria in the 34°C culture, while the protein was undetectable in the 23°C cultivated spirochetes (Fig. 2A). Northern blot analysis of RNA purified from bacteria that had undergone this culture temperature shift indicated that revA mRNA was present in the bacteria grown at 34°C but was undetectable in those maintained at 23°C (Fig. 2B).

Since the production of some B. burgdorferi proteins preferentially synthesized during infection of warm-blooded animals are also regulated by pH (12, 13, 46, 62), we examined the effect of culture medium pH on RevA expression. Significantly higher levels of RevA were produced by bacteria cultivated in medium buffered to remain at pH 7.0 than by those grown in medium having a pH of 8.0 (Fig. 2C). Again, revA mRNA levels corresponded with protein levels (Fig. 2D).

One of us (J. Carroll) had previously observed that expression of at least 37 B. burgdorferi proteins was affected by pH of the culture medium, which was most clearly seen by two-dimensional PAGE (12, 13). To determine whether RevA was

FIG. 1. (A) Alignments of the predicted protein sequences of the two RevA alleles and one RevB gene of strain B31. Note that RevA1 and RevA2 differ only at the 13th residue of each protein, while RevB shares only 28% identical amino acids with the RevA proteins. Each protein is predicted to be lipidated at the cysteine residue marked by an asterisk (28, 57, 60). (B) Alignment of the promoter regions of the strain B31 revA and ospC genes. Identical nucleotides found in both a revA and the ospC promoter are boxed and shaded. The ospC promoter contains an inverted repeat, indicated by opposing arrows above the DNA sequences (3, 34, 59). All other analyzed ospC promoters contain identical sequences at this location (59). A similar sequence is found 5′ of all known alleles of revA.
among those proteins, *B. burgdorferi* membrane proteins were separated as in those earlier studies and then immunoblotted with RevA-specific MAb YM.26 (Fig. 3). The result was then compared with a silver-stained two-dimensional gel, and an immunoblot was performed using *B. burgdorferi*-infected animal serum. These comparisons revealed that the RevA spot aligned with the previously identified spots I-32 and S-35 (13) (Fig. 3).

Several other *B. burgdorferi* genes, including the gene encoding the infection-associated outer surface protein OspC, are known to be regulated in response to temperature in manners similar to that of *revA* (1, 3, 12, 13, 17, 46, 51, 54, 55, 62). These similarities led us to compare the 5′ noncoding regions adjacent to the *revA* genes with those of other known regulated loci. Significant similarity was found between the *ospC* and *revA* promoter regions, including an inverted repeat that may indicate a protein-binding site (Fig. 1B). No homology was evident when comparing *revA* promoters with those of other regulated genes, including the *erp* and *dbpAB* genes (data not shown).

**RevA is an outer membrane protein.** The predicted amino acid sequences of all known RevA proteins contain a charged amino terminus followed by a hydrophobic region and a type II secretion-lipidation consensus sequence (Fig. 1A), suggesting that they are membrane-bound lipoproteins (25, 28, 43, 57, 60).
Studies were thus undertaken to determine the cellular localization of RevA. *B. burgdorferi* membranes were separated by isopycnic centrifugation according to a previously described procedure (8). *B. burgdorferi* does not contain lipopolysaccharide as do enteric gram-negative bacteria (58), raising concern that borrelial inner and outer membranes may have relative buoyancies different from those of previously characterized bacteria. For this reason, antibodies were generated against the *B. burgdorferi* MotB protein, a subunit of the flagellar motor and, therefore, an integral inner membrane protein. Equivalent amounts of total protein from membrane vesicle preparations were subjected to immunoblot analysis, identifying an approximately 24-kDa protein, which is the anticipated size of MotB (Fig. 4A). Immunoblot analysis of a *B. burgdorferi* mutant disrupted in motA, which is directly upstream of motB, makes less of the 24-kDa band, consistent with the 24-kDa band being MotB (N. W. Charon and M. A. Motaleb, unpublished data). Densitometric analysis indicated that 86% of MotB was contained in one of the fractions, indicating that this fraction consisted primarily of inner membrane proteins. Similar analyses with antibodies directed against the known outer membrane proteins ErpA/I/N and ErpL (22) indicated that these two proteins were contained in the second membrane fraction (Fig. 4C and D), indicating that this fraction consisted primarily of outer membrane proteins. An earlier study (8) used the same membrane purification technique as in the present study, yet lacked an appropriate marker to differentiate inner membranes from outer membranes and, as a result, misidentified the membrane fractions. To avoid confusion with the older terminology, we refer to the fractions as “true” inner and outer membranes according to the localization of MotB.

Purified membranes contained RevA, consistent with predictions that it is a membrane-bound lipoprotein. We determined by immunoblot and densitometry that greater than 85% of RevA localized to the true outer membrane fraction (Fig. 4B). We conclude from these studies that RevA is an outer membrane protein.

**Localization of RevA on the *B. burgdorferi* outer surface.** The cellular localization of RevA was addressed by examining the susceptibility of the in situ protein to proteolysis. Surface-exposed proteins of intact bacteria can be digested by proteases, while those below the surface cannot be degraded. Cultured bacteria were incubated with a protease, and the proteins were separated by SDS-PAGE and immunoblotted with MAb YM.26. Since a number of borrelial surface-exposed outer membrane proteins are resistant to proteolysis by some enzymes (10, 20, 22, 64), three different proteases were used in this study. Incubation of bacteria with pronase completely digested RevA within 30 min, a result indicative of surface exposure (Fig. 5). Control immunoblot analyses indicated that the bacterial outer membranes were intact, since there was no detectable proteolysis of FlaB, a component of the periplasmic flagella (29). However, no degradation of RevA was detected following 2 h of incubation with either proteinase K or trypsin (Fig. 5). Proteolysis of the known outer surface protein OspC

![FIG. 4. RevA is an outer membrane protein. Immunoblots of purified *B. burgdorferi* “true” outer and inner membranes (OM and IM, respectively, about each panel). Analyses were done with various antibodies. (A) Polyclonal rabbit antiserum raised against *B. burgdorferi* MotB. The immunoblot band corresponding with MotB is marked by an asterisk, while the identities of the additional proteins are as yet unknown. Analysis of a mutant with a disrupted motAB operon indicated that the 24-kDa band corresponds with MotB (see the text). (B) MAb YM.26 directed against RevA. (C) MAb B11 directed against known outer membrane protein ErpA/I/N (22). (D) Polyclonal rabbit antiserum raised against the known outer membrane protein ErpL (22).](http://iai.asm.org/)

![FIG. 5. Demonstration of outer surface exposure of RevA by in situ protease degradation. Whole *B. burgdorferi* were incubated with a protease for 30, 60, or 120 min or for 120 min in buffer without protease. Proteases were then inactivated, bacteria were lysed, proteins were separated by SDS-PAGE, and the integrity of RevA, OspC, and FlaB were analyzed by immunoblot.](http://iai.asm.org/)
(22, 24) was detected in these bacteria, indicating that both enzymes were functionally active and that RevA in situ is insensitive to these proteases. These studies demonstrated that RevA is exposed to the external environment on the B. burgdorferi outer membrane and that it is resistant to proteolysis by certain enzymes.

**DISCUSSION**

*B. burgdorferi* interacts with a wide variety of tissues during its natural infectious cycle between warm-blooded and arthropod hosts. It is not surprising, therefore, that these bacteria regulate the synthesis of surface proteins. Transcription of *revA* genes ceases shortly after the bacteria infect ticks and then resumes when the vector feeds on a new host (26). The RevA protein is synthesized during mammalian infection (25, 53), suggesting that this protein facilitates the infection of warm-blooded animals. The results presented here indicate that *B. burgdorferi* controls RevA expression in response to temperature, producing both the mRNA and the protein when cultivated at 34°C, while neither was detectable in bacteria grown at 23°C. These temperatures are comparable to the body temperature of a warm-blooded animal and the ambient temperature experienced within an unfed tick, respectively. Synthesis of RevA protein and mRNA was also affected by pH, with significantly greater quantities produced by bacteria grown at pH 7 as opposed to those grown at pH 8. The pH of a tick’s midgut acidifies during ingestion of a blood meal (62), so a drop in pH could signal the bacteria that the vector is feeding and of the necessity to produce factors required for infection of the new host. The observation that mRNA levels paralleled protein levels suggests that RevA synthesis is controlled by the level of *revA* gene transcription.

The in vitro pattern of RevA synthesis was similar to that of OspC; another protein produced by *B. burgdorferi* during mammalian infection (13, 51, 55). Levels of both proteins appear to be regulated at the level of transcription, since the amount of each mRNA corresponded directly with protein levels (this work and reference 59). Comparison of the *revA* and *ospC* 5′ noncoding regions indicated numerous regions of extended identity. All sequenced *ospC* promoter regions contain a well-conserved 16-bp inverted repeat (3, 34, 59), suggestive of a DNA-binding protein recognition site. Since a similar sequence is found 5′ in both the *revA*1 and the *revA*6 genes of strain B31, it is possible that transcription of both *revA* and *ospC* is regulated through the binding of the same protein to DNA near the promoters of both locus types.

Analysis of purified *B. burgdorferi* inner and outer membranes revealed that RevA is located in the outer membrane. The technique utilized to separate the membranes was devised several years ago by Bledsoe et al. (8). However, at that time, membrane vesicle fractions were designated as being derived from either the inner or outer membrane based on their densities as compared with those of *E. coli*, the number of particles embedded in the membrane vesicles as determined by freeze fracture, and the presence or absence of immunoreactivity to antibody raised against the C subunit of the F1/F1 ATPase of *Escherichia coli* (8). Several lines of evidence suggested to us that those earlier designations were incorrect. First, the genome sequence of *B. burgdorferi* B31 (23) indicates that this bacterium does not encode a homolog of the *E. coli* F1/F1 ATPase. Second, a similar procedure was used to isolate membrane vesicles from the spirochete *Serpulina* (now *Brachyspira*) *hyodysenteriae*, where it was determined that the relative densities of the inner and outer membranes were opposite to those of other gram-negative bacteria (41). Third, numerous proteins known to be abundant on the *B. burgdorferi* cell surface (such as OspC and OspA) were seen to localize primarily to what Bledsoe et al. called the inner membrane fraction. We conclude that while the method developed by Bledsoe et al. (8) for the purification of borrelial membranes can accurately separate inner and outer membranes, the relative buoyant densities of *B. burgdorferi* inner and outer membranes are opposite to those of more typical gram-negative organisms such as *E. coli*. This is likely due to the lack of classical lipopolysaccharide in the outer membranes of borreliae (58). Additionally, these and other spirochetes contain unusual types of lipids and lipopolysaccharides that might affect membrane buoyancies (7, 18, 21, 41, 48, 61). The results of our studies also indicate a need for the reexamination of other procedures used for separation of the inner and outer membranes of borreliae (19, 27, 44, 52).

RevA was sensitive to in situ digestion with pronase, a combination of several different proteases having various endo- and exoproteolytic activities (Boehringer-Mannheim catalog). No digestion of RevA was detected when using either of the endoproteases trypsin or proteinase K. Several other *B. burgdorferi* surface proteins, including OspA and some members of the Erp (OspE and -F-related) protein family, are also insensitive to certain proteases in situ (10, 20, 22, 47). The resistance of RevA to proteolysis may be a consequence of its folding, as is apparently the case with some surface proteins of the related spirochete *B. turicatae* (64). Alternatively, RevA may interact with other proteinaceous or nonproteinaceous outer membrane components, which could have prevented the enzymes from reaching RevA. Such interactions have been observed with another *B. burgdorferi* outer surface protein, known as P66 or Oms66, which is protected from both proteases and antibodies by interactions between it and the OspA surface protein (10).

RevA is produced during mammalian infection and presumably performs a function for *B. burgdorferi* during that stage of the bacterium’s life cycle. Since the control of RevA synthesis can be observed in vitro, it will be possible to define the mechanisms by which *B. burgdorferi* senses its environment and controls the synthesis of this antigenic protein in vivo. The function of RevA is as yet unknown, but its location on the bacterial outer surface would allow interactions with host cell surfaces or other host tissue constituents. Through a variety of techniques, some *B. burgdorferi* surface proteins have been found to bind specific host tissue components, and studies are under way in our laboratories to identify substances that bind RevA.

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