Oligopeptide Permease A5 Modulates Vertebrate Host-Specific Adaptation of *Borrelia burgdorferi*[▽]

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Borrelia burgdorferi*, the agent of Lyme disease, undergoes rapid adaptive gene expression in response to signals unique to its arthropod vector or vertebrate hosts. Among the upregulated genes under vertebrate host conditions is one of the five annotated homologs of oligopeptide permease A (OppA5, BBA34). A mutant lacking *oppA5* was constructed in an lp25-deficient isolate of *B. burgdorferi* strain B31, and the minimal regions of infectivity were restored via a shuttle vector pBBE22 with or without an intact copy of *bba34*. Immunoblot analysis of the *bba34* mutant revealed a reduction in the levels of RpoS, BosR, and CsrA$_{Bb}$ with a concomitant reduction in the levels of OspC, DbpA, BBK32, and BBA64. There were no changes in the levels of OspA, NapA, P66, and three other OppA orthologs. Quantitative transcriptional analysis correlated with the changes in the protein levels. However, the *bba34* mutant displayed comparable infectivities in the C3H/HeN mice and the wild-type strain, despite the reduction in several pathogenesis-related proteins. Supplementation of the growth medium with increased levels of select components, notably sodium acetate and sodium bicarbonate, restored the levels of several proteins in the *bba34* mutant to wild-type levels. We speculate that the transport of acetate appears to contribute to the accumulation of key metabolites, like acetyl phosphate, that facilitate the adaptation of *B. burgdorferi* to the vertebrate host by the activation of the Rrp2-RpoN-RpoS pathway. These studies underscore the importance of solute transport to host-specific adaptation of *B. burgdorferi*.

Borrelia burgdorferi, the etiological agent of Lyme disease, is transmitted to a variety of vertebrate hosts via the bite of infected *Ixodes* sp. ticks (13, 76). This spirochetal pathogen causes a pathognomonic, localized inflammatory skin lesion termed erythema migrans at the site of the tick bite (54). If left untreated, the spirochetes disseminate to various organs and organ systems, leading to a multiphasic disorder involving the cutaneous, musculoskeletal, cardiovascular, and nervous systems (75). Lyme disease is endemic in certain geographic areas of the United States where there are close ecological interactions between the transmitting vector, infected reservoir hosts, and human populations, resulting in a significant impact on public health.

The ability of *B. burgdorferi* to be transmitted between an arthropod vector and a variety of vertebrate hosts is reliant on the rapid adaptation of its gene expression profiles that facilitates survival under highly disparate environmental conditions present in these hosts (4, 23, 24, 71, 77). A broad array of studies using global gene expression arrays has provided clues to contributions of genes individually or at a global level that facilitate host-specific adaptation of *B. burgdorferi* (4, 6, 12, 17, 55, 65, 83). Environmental conditions such as temperature (71), cell density (40), pH (19, 20), levels of dissolved gases (39, 72), and other components that are significantly different be-

tween the tick vector and vertebrate hosts (7, 8, 23) have been used to decipher both the transcriptional levels of genes and the regulatory networks involved in modulating this response (5, 14–16, 32, 47, 49, 91).

The compact and segmented nature of the borrelial genome comprising one linear chromosome and several circular and linear plasmids also adds to the complexity of regulatory mechanisms operative in *B. burgdorferi* (21, 31). For example, *B. burgdorferi* does not possess metabolic pathways for the synthesis of amino acids, fatty acids, and nucleotides. In addition, it has a limited set of transport systems to acquire these select nutrients from the external environment (31). There is a single peptide transport system that belongs to a superfamily of ABC transporters present in *B. burgdorferi* with extensive similarity to oligopeptide permease (Opp) and dipeptide permease (Dpp) systems described in other bacteria (45, 48, 86). The Opp transport system is characterized by the presence of a peptide binding protein (OppA), two transmembrane proteins (OppB and OppC), and two ATP binding proteins (OppD and OppF) (2, 59). However, unlike other bacteria, *B. burgdorferi* has 5 open reading frames (ORFs) annotated as homologs of OppA, suggesting that these proteins could play a significant role in acquiring select nutrients/solutes in specific microenvironments (11). Three of these OppA homologs (OppA1, -2, and -3) encoded on the chromosome bound heptapeptides with high affinities in a pH-dependent manner, while the other two homologs present on plasmids (OppA4 on cp26 and OppA5 or BBA34 on lp54) did not exhibit such binding characteristics (84, 85). Additional studies also indicated that *bba34* was upregulated when the culture conditions of *B. burgdorferi* were shifted from 25°C to 37°C and in spirochetes present in

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description[a]	Source or reference
Plasmids		
pCR2.1-TOPO	PCR cloning vector	Invitrogen
pML102	Customized donor plasmid for *in vitro* mutagenesis	74
pBVS2	Borrelial shuttle confers Kan^r	78
pBBE22	Borrelial shuttle vector confers Kan^r Kan^r derived from pBSV2 with BBE22 region of lp25	61
pSVR1	1.9 kb of lp54 spanning BBA34 in pCR2.1-TOPO	This study
pSVR2	SVR1 with 1.6-kb insertion of P$_{flgB}$-*aadA* at the nucleotide 154 bp from the 5′ end of BBA34	This study
pSVR3	Borrelial shuttle vector pBBE22 with functional copy of *bba34*	This study
***B. burgdorferi* strains**		
MSK5	B31 isolate with all infection-associated plasmids	44
ML23	B31, lp25-negative, noninfectious clonal isolate	44
ML23/pBBE22 (wt)	ML23 with pBBE22 (BBE22$^+$), Kan^r	74
SVR2	*bba34* mutant in ML23; lp25$^-$, Str^r	This study
SVR2/pBBE22 (mt)	SVR2 with pBBE22; Kan^r; Str^r	This study
SVR3 (ct)	SVR2 with pSVR3 (complementation plasmid, pBBE22/*bba34*$^+$)	This study

[a] Str^r, streptomycin resistance; Kan^r, kanamycin resistance.

infected mouse tissues (52). Moreover, the expression level of *bba34* was determined to be under the control of alternative σ factors RpoN and RpoS (52), suggesting a role for *bba34* in the adaptation of *B. burgdorferi* to vertebrate host-specific conditions.

Several recent studies have brought into focus the importance of key metabolites contributing to adaptive gene expression in *B. burgdorferi*. The phosphorylation of the two-component response regulatory protein, Rrp2, which leads to the activation of the RpoN-RpoS pathway, was shown to be mediated by acetyl phosphate, a metabolite of the acetate utilization pathway (88). The accumulation of this metabolite is partly dependent on the repression of levels of an enzyme, phosphate acetyltransferase (Pta), which converts acetyl phosphate to acetyl-coenzyme A (CoA), leading to conditions that could potentially dampen the activation of the Rrp2-RpoN-RpoS pathway. The repression of Pta has been shown to be mediated by CsrA$_{Bb}$ (*c*arbon *s*torage *r*egulator *A* of <u>*B. burg-dorferi*</u>), which we and others recently demonstrated plays a crucial role in affecting differential gene expression in *B. burgdorferi* that facilitates adaptation to vertebrate host-specific conditions (41, 70, 82). Notably, deletion of *csrA*$_{Bb}$ leads to a reduction in the levels of expression of several members of the *rpoS* regulon. Consistent with this *in vitro* phenotype, the *csrA*$_{Bb}$ mutant was attenuated for infection in the murine model of Lyme disease (41, 82, 88). Molecular determinants that connect the external environment to the metabolic status of *B. burgdorferi* that eventually lead to host-specific adaptation are yet to be characterized in detail. Therefore, we focused on *bba34* as a probable mediator of transport of key substances that modulate adaptation to vertebrate hosts since it was (i) upregulated under vertebrate host-specific conditions (52); (ii) downregulated in the *csrA*$_{Bb}$ mutant (41); (iii) similar to other bacterial solute-binding proteins (31); and (iv) incapable of binding peptides, unlike other OppA homologs of *B. burgdorferi* (84).

In this study, we generated a deletion mutant in *bba34* and confirmed that BBA34 was indeed synthesized at higher levels under conditions mimicking the fed ticks. Most interestingly, deletion of *bba34* resulted in reduction of the levels of key

regulators of gene expression in *B. burgdorferi*, such as RpoS, BosR, and CsrA$_{Bb}$, along with levels of lipoproteins associated with pathogenic mechanisms, such as OspC, DbpA (34), BBK32 (60), and BBA64 (33, 50, 51). In spite of the reduction in the levels of these determinants of infection, there was no deficit in the colonization capabilities of the *bba34* mutant, suggesting that there was a compensatory mechanism that restored the ability of the mutant to colonize C3H/HeN mice. Further *in vitro* phenotypic analysis provided evidence that BBA34 contributes to the transport of select solutes, such as acetate and bicarbonate, which in turn modulate levels of vertebrate host-specific determinants. Based on these observations, we discuss how this protein could facilitate the adaptation of *B. burgdorferi* to host-specific conditions based on levels of external signals.

MATERIALS AND METHODS

Bacterial strains and media. A noninfectious, clonal derivative of *B. burgdorferi* strain B31 lacking lp25 (ML23) was used in transformation experiments (44). An infectious, clonal isolate of *B. burgdorferi* strain B31 containing all the plasmids (MSK5) was used as a source of total genomic DNA. All borrelial strains were grown in Barbour-Stoenner-Kelly II (BSK-II) liquid medium supplemented with 6% normal rabbit serum (NRS; Pel-Freeze Biological, Rogers, AR) and 1% CO$_2$ at 32°C (9). The borrelial strains were also initially propagated in BSK-II medium with 6% NRS at conditions mimicking the midgut of unfed ticks (pH 7.6/23°C) and shifted to those mimicking fed ticks (pH 6.8/37°C).

Expression and purification of recombinant proteins for generation of monospecific sera against OppA homologs. The purification of BBA34/OppA5 was done using procedures described previously (26). Total genomic DNA obtained from *B. burgdorferi* clonal isolate MSK5 (Table 1) was used as the template to PCR amplify OppA1, OppA2, and OppA4 by using forward and reverse primers containing appropriate engineered restriction enzyme sites (Table 2). The leader sequences of lipoproteins were not included to generate the expression constructs in order to facilitate the induction of increased levels of recombinant proteins in *Escherichia coli*. The amplicons were cloned into pCR2.1-TOPO vector (Invitrogen) and transformed into *E. coli* TOP 10 cells and subjected to blue/white colony screening in the presence of ampicillin (100 μg/ml) and kanamycin (50 μg/ml). The inserts were excised with NdeI/XhoI and ligated into the pET23a expression vector. The ligated products were electrotransformed into *E. coli* TOP 10 cells and screened by restriction enzyme digestion for the presence of insert of appropriate size. The junctions of plasmids containing inserts of expected size were sequenced, and the plasmids were used to transform the *E. coli* expression host (Rosetta, Novagen). Recombinant OppA1, OppA2, and OppA4 with a C-terminal 6-histidine tag were overexpressed by inducing *E. coli*

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)[a]
P1	ACGCCATATGTCTAAACCAAAAGATA
P2	ACGCCTCGAGTTCTTCTATAGGTTT
P3	AATGTATTTCTGGGGGAGTG
P4	GGTCATAAGGGTCGACCAGAAAGATTAGCCCTATCAG
P5	AATCTTTCTGGTCGACCCTTATGACCTTCCTTTAGC
P6	ACAGGTTTTTGAACACTCTCATC
P7 (34S1F)	GCACGGGTTAGTAGAAAAAAG
P8 (34S2R)	CTTTTTATCCCATTTTATCCC
P9 (BBA34-compF)	ACGCGGGATCCGAGCTTTCTTTAGCTGAAAG
P10 (BBA34-compR)	ACGCGTCGACCAATTTCTGTAAGCGATTTAAC
P[flgB]-aadAF	ACGCGTCGACGGAAGATTTCCTATTAAGG
P[flgB]-aadAR	ACGCGTCGACTTATTTGCCGACTACCTTGGT
BosRFRq	GCATTGGAAAAAGTCGGCATT
BosRRRq	GATGCTTTCATTCCTAATTCTGATGTT
RpoSFRq	AGATATGCGGGTAAAGGGTTAAAA
RpoSRRq	CAGCAGCTCTTATTAATCCCAAGTT
E22F	ACGCGGTACCTTTTTATATTGTGAGCGGT
E22R	ACGCGGTACCTCTATGTATCCCCTTGTTCA

[a] Restriction sites are underlined.

strains containing appropriate plasmids with 1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) for 2 h and subjected to affinity purification using Ni-nitrilotriacetic acid (NTA) beads (Qiagen, Valencia, CA) as per the manufacturer's instructions. The bound 6-histidine proteins were eluted as 0.5-ml fractions with elution buffer (8 M urea; pH 4.5) and analyzed by sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE). Select fractions with the largest concentrations of eluted proteins were further purified using Amicon centrifugal filters (Millipore, Bedford, MA), and in some cases the proteins were purified to homogeneity using the S&S Elutrap electroseparation system as described previously (42). Recombinant proteins purified to homogeneity (data not shown) were quantified by bicinchoninic acid (BCA) (Pierce, Thermo Fisher Scientific, Rockford, IL) assay and stored at −80°C until further use. The purified recombinant proteins were emulsified in equal volumes of Titermax (Sigma, St. Louis MO) and used to immunize 6- to 8-week-old female BALB/c mice. Booster immunizations were given at days 14 and 21. Immunoblot analysis was carried out to determine the specificity of the antibodies against the recombinant proteins and total borrelial lysates with the serum obtained on day 28 postimmunization (data not shown). All animal procedures were done in accordance with the approved animal use protocol from the Institutional Animal Care and Use Committee of The University of Texas at San Antonio.

Construction of *bba34* mutant. A two-step overlap PCR strategy was employed to generate the plasmid used for the deletion of *bba34* as described previously (51). In the first step, ~1-kb regions of lp54 flanking the *bba34* gene were PCR amplified from the total genomic DNA from an infectious, clonal isolate of *B. burgdorferi* B31 (MSK5). This was done using primer sets P3/P4 and P5/P6 for generating the upstream and downstream regions of *bba34*, respectively (Table 2). Primers P4 and P5 had engineered SalI sites as well as a 10-nucleotide overlap to facilitate the deletion of *bba34* by using overlap PCR. In the second step, a 1.9-kb amplicon was PCR amplified by LATaq polymerase (Takara) using primers P3 and P6 using the PCR products from the first step as DNA templates. The amplicon was cloned into the pCR2.1 cloning vector (Invitrogen) and transformed into TOP 10 *E. coli*. A plasmid designated pSVR1 with an appropriate insert was identified following blue/white screening, and the P[flgB]-*aadA* with flanking SalI sites was cloned into pSVR1 digested with the same enzyme. A plasmid designated pSVR2 with P[flgB]-*aadA* inserted between flanking regions of *bba34* (between coordinates 21663 and 23212 of lp54) was used for the generation of the mutant in the noninfectious lp25⁻ strain of *B. burgdorferi* B31 (ML23).

Transformation of *B. burgdorferi*. The transformation of ML23 with pSVR2 was done as described previously (68, 73, 78). After electroporation, the transformants were plated (after a 24-h incubation in BSK-II medium without antibiotics) on BSK-II agar overlays containing 50 μg/ml of streptomycin. Plates were incubated at 32°C in 1% CO₂ for 14 to 18 days until individual colonies were visible (78) in the overlays. Colonies were isolated aseptically into BSK-II liquid medium containing 50 μg/ml of streptomycin and incubated at 32°C until the spirochetes reached a density of 5 × 10⁷/ml. One milliliter of this culture was used to extract total genomic DNA, and the presence of P[flgB]-*aadA* cassette within *oppA5* was determined using primers specific to the upstream and downstream areas of OppA5 (Table 2). Several positive clones were identified, and

one clone, designated SVR2, was further analyzed for the confirmation of the loss of *bba34* by Southern blot and immunoblot analysis.

Southern blot analysis. To further confirm the deletion of *bba34*, total genomic DNA was isolated from the parental control strain (ML23) and the *bba34* mutant (SVR2) (73) and digested with either HindIII or HindIII/NdeI, separated on a 1% agarose gel, and transferred onto a nylon membrane. The membranes were probed either with the *aadA* gene (Strʳ marker) or with the PCR-amplified, full-length *bba34* gene by using the ECL labeling and detection system (GE Healthcare). The membranes were hybridized with labeled probes overnight at 42°C and developed per the manufacturer's instructions (GE Healthcare).

Complementation and restoration of minimal region of lp25 required for infectivity of *bba34* mutant. In order to complement the *bba34* mutant, a region of lp54 corresponding to the nucleotide coordinates 21137 and 23797 was amplified using primers P9 and P10 with engineered SalI sites. The amplicon was cloned into the borrelial shuttle vector pBBE22 (61, 78) at the SalI site to generate a plasmid designated pSVR3, which also has the minimal region of lp25 (BBE22 region) needed to restore the infectivity of ML23 in the C3H/HeN mouse model of Lyme disease. The *bba34* mutant (SVR2) was transformed by electroporation either with pSVR3 or with pBBE22 alone so as to generate a complemented strain or a *bba34*-deficient strain, respectively, with all the genetic elements needed for infectivity analysis in the mouse model of Lyme disease. The transformants were selected on BSK-II agarose overlays supplemented with 200 μg/ml of kanamycin and 50 μg/ml of streptomycin, and the presence of pBBE22 was verified using primers described previously (26, 50). The plasmid profiles of wild-type (wt; ML23/pBBE22), mutant (mt; SVR2/pBBE22), and complemented (ct; SVR2/pBBE22-*bba34*⁺) strains were verified using primer sets described previously (44) and observed to be the same in all three strains (data not shown).

SDS-PAGE and immunoblot analysis. *B. burgdorferi* parental, mutant, and complemented strains were cultivated under conditions that mimic the midgut of the tick before (23°C/pH 7.6) or after (37°C/pH 6.8) a blood meal to a density of 1 × 10⁸ spirochetes/ml in BSK-II growth medium as described above. Whole-cell lysates were prepared and separated on SDS-12.5% PAGE gels as described previously (72). The separated proteins were either visualized by Coomassie brilliant blue staining or transferred to polyvinylidene difluoride (PVDF) membranes and subjected to immunoblot analysis as described previously (26, 41, 50, 70). The membranes were probed with α-OspA monoclonal antibodies, α-BBA34, α-BBK32, α-DbpA, α-SodA, α-P66, α-BBA64, α-BosR, α-CsrA[Bb], α-RpoS, and α-NapA antibodies. The blots were incubated with appropriate concentrations of horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rat, or anti-rabbit secondary antibodies and developed using ECL Western blotting detection reagents (GE Healthcare).

Protease accessibility. Protease accessibility studies were conducted as described previously (42). Briefly, the parental, mutant, and complemented strains (~2 × 10⁸ cells) were centrifuged at 4,300 × *g* for 15 min at 4°C, and the pellet was resuspended in equal volumes of phosphate-buffered saline (PBS; pH 7.4), 5 mM MgCl₂, and 50 mM sucrose (42). After pelleting, the samples were split into equal volumes and incubated in either 50 μl of sterile water (proteinase K-negative control) or with proteinase K (final concentration of 200 μg/ml) at 20°C for 40 min. After 40 min, phenylmethylsulfonyl fluoride (PMSF) was added to a concentration of 1 mM, and samples were examined by dark-field microscopy to assess their motility as a measure for viability. The samples were pelleted and washed again as described above in the presence of 1 mM PMSF and resuspended in SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE and subjected to immunoblot analysis as described above with α-BBA34, α-FlaB, and α-P66 antibodies.

PCR analysis of plasmid profiles of strains used in infectivity. Plasmid profile analysis was done as described previously (44, 50). Primer sets used to amplify specific target regions in different linear and circular plasmids have been described previously (44). The amplicons were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide (data not shown). A primer set (Table 2, E22F/E22R) was also used to amplify the BBE22 region of lp25 present in the borrelial shuttle vector pBBE22.

***In vitro* phenotypic analysis.** The parental, mutant, and complemented strains were propagated in BSK-II medium supplemented with 6% NRS and appropriate antibiotics at 32°C with 1% CO₂. The spirochetes were enumerated every 12 h using dark-field microscopy, and all experiments were conducted in triplicate. The protein profiles of these strains were also monitored following growth in media with different levels of Bacto TC Yeastolate (Becton, Dickinson and Company, Sparks, MD), dialyzed yeastolate (Gibco; Invitrogen; 50× solution), and other solutes as indicated in the respective figure legends. All the strains were tested in triplicate, and the experiments were repeated at least three times.
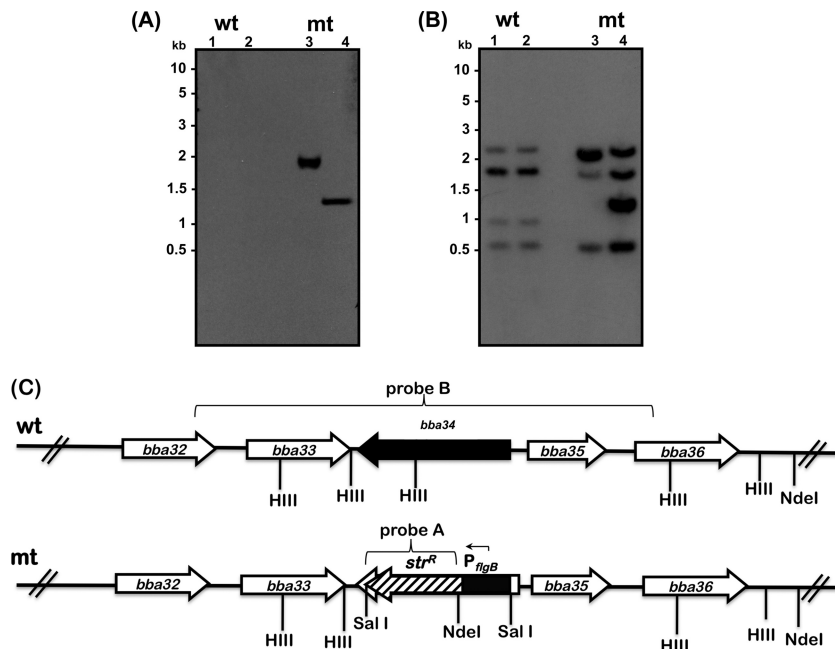
FIG. 1. Southern blotting confirms the mutation in the *bba34* gene in ML23 (noninfectious, lp25⁻, *B. burgdorferi* strain B31). (A and B) Total genomic DNA from the ML23 parental strain (wt, lanes 1 and 2) and the *bba34* mutant (mt, lanes 3 and 4) was digested with HindIII (lanes 1 and 3) and HindIII/NdeI (lanes 2 and 4) and probed with *aadA* (Strʳ marker) (A) or *bba34* (PCR-amplified using P3, P6) (Table 2) (B). The numbers on the left of each panel indicate the size of the markers in kilobases. (C) Schematic of the *bba34* region in the lp54 plasmid of *B. burgdorferi*. The gene designations based on TIGR annotations are indicated on each corresponding locus. Probe B was PCR amplified using P3/P6 primers sets with total genomic DNA from *B. burgdorferi* as the template, and probe A was amplified using aadAF/aadAR primers using pML102 as the template.

Infectivity studies. Groups of 6-week-old female C3H/HeN mice ($n = 3$) were intradermally inoculated at doses of 10^2, 10^3, 10^4, and 10^5 spirochetes per mouse with wild-type (ML23/pBBE22), *bba34* mutant, and complemented strains. Twenty-one days after inoculation, the spleens, tibiotarsal joints, inguinal lymph nodes, hearts, bladders, and pieces of abdominal skin were collected and cultured in BSK-II growth medium to facilitate the isolation of spirochetes as previously described (50, 51). The cultures were scored for growth of *B. burgdorferi* after 2 to 3 weeks by using dark-field microscopy. All animal procedures were performed in accordance with the approved animal use protocol from the Institutional Animal Care and Use Committee at The University of Texas at San Antonio.

RESULTS

Generation of a deletion mutant of *oppA5*. We generated a plasmid, designated pSVR2, by a two-step PCR process as described previously, where the *bba34* gene was replaced with P*flgB*-*aadA* (30, 51). In order to inactivate the *bla* gene present on the vector used to generate pSVR2, we linearized this plasmid at the ScaI site prior to electrotransformation of the ML23 strain of *B. burgdorferi* (43, 44, 68, 74). The mutant borrelial colonies were selected in BSK-II agar overlays supplemented with 50 μg/ml of streptomycin and initially screened by PCR using primers specific to *bba34*. One mutant, designated SVR2, was further analyzed by Southern hybridization. There was hybridization of 1,836-bp and 1,276-bp fragments when the total genomic DNA from *bba34* mutant was digested with HindIII or HindIII/NdeI, while there was no such hybridization with the DNA from the parental strain when the blots were probed with the *aadA* (Strʳ) marker (Fig. 1A). Moreover, when *bba34* was used as a probe (Fig. 1B), there was hybridization to fragments (Fig. 1B, lane 1, wt, 2,386 bp, 1,770 bp, 816

bp, 519 bp) in the total genomic DNA of the parental strain (wt), consistent with the presence of HindIII sites, while the hybridization pattern was significantly different in the mutant (mt) strain digested with HindIII due to the presence of the streptomycin resistance cassette (Fig. 1B, lane 1, mt, 2,218 bp, 1,869 bp, 1,530 bp, 439 bp) or due to the presence of the NdeI site within the P*flgB*-*aadA* marker (Fig. 1B, lane 2, mt, 2,218 bp, 1,530 bp, 1,230 bp, 439 bp). Based on the schematic representation of the region of lp54 shown (Fig. 1C), we should have also observed hybridization to a 187-bp fragment in both the parental and mutant strains digested with either HindIII or HindIII/NdeI. Due to the relatively small size of the fragment and partly due to the hybridization conditions employed, we did not observe hybridization to this fragment. However, this does not alter the interpretation that only *bba34* was deleted and replaced with P*flgB*-*aadA*. Although extensive similarity exists in the sequences of *oppA* homologs, Southern hybridization analysis confirmed the inactivation and clonality of the *bba34* mutant.

Complementation of the *bba34* mutant. We complemented the *bba34* mutant with the borrelial shuttle vector pBBE22 alone or with an intact copy of *bba34* (including its upstream region). This was done not only to restore the minimal region of lp25 required for infectivity in the mutant but also to functionally complement the mutant with an intact copy of *bba34* to test the molecular Koch's postulates (61, 62, 74). The transformants were selected in the presence of 200 μg/ml kanamycin and 50 μg/ml of streptomycin and were screened using primers specific for pBBE22 (50). Immunoblot analysis of the
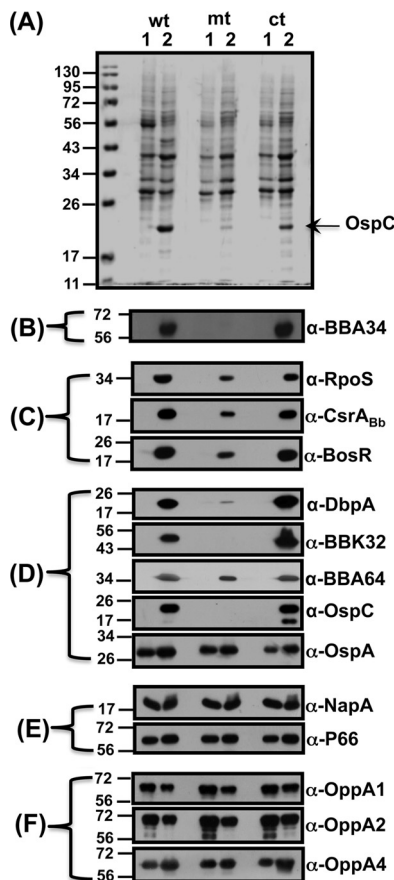
FIG. 2. Inactivation of *bba34* results in reduced levels of regulators of gene expression and key pathogenesis-related proteins of *B. burgdorferi*. Immunoblot analysis of total protein lysates of wild-type (wt), mutant (mt), and complemented (ct) strains propagated in either unfed (lane 1, pH 7.6/23°C)- or fed (lane 2, pH 6.8/37°C)-tick conditions was performed with monospecific serum against the antigen indicated to the right of each blot. (A) Coomassie blue-stained 12.5% SDS-PAGE of whole-cell lysates of borrelial strains. (B) Immunoblot analysis of whole-cell lysates with mouse anti-A34 antibodies indicates lack of synthesis of BBA34 in the *bba34* mutant. (C) Reduced levels of regulators of gene expression in the *bba34* mutant. Immunoblot analysis of total lysates was done with mouse or rabbit anti-RpoS, anti-CsrA$_{Bb}$, and anti-BosR antibodies. (D) Reduced levels of key lipoproteins associated with pathogenesis in the *bba34* mutant. Immunoblot analysis was done with mouse or rat anti-DbpA, anti-BBK32, anti-BBA64, anti-OspC, and anti-OspA monoclonal antibodies. (E) Lack of difference in the levels of select proteins in the wt, mt, and ct strains. Immunoblot analysis was done with rabbit or mouse anti-NapA and anti-P66 antibodies. (F) Lack of compensatory change in the levels of other OppA homologs. Immunoblot analysis of wt, mt, or ct strains with mouse anti-OppA1, anti-OppA2, and anti-OppA4 antibodies. The molecular size standards in kilodaltons are indicated to the left. The blots were developed using the Enhanced Chemiluminescence System.

complemented strain confirmed the synthesis of BBA34 only upon propagation under fed-tick conditions (pH 6.8/37°C) consistent with the expression of this homolog in the parental strain (Fig. 2B; wt; ct; lane 2). Taken together, we have isolated a *bba34*-deficient strain and genetically complemented the mutant such that the levels of *bba34* are similar to those of the parental strain and have the requisite genetic background to

evaluate the *in vitro* and *in vivo* phenotypic effects of targeted deletion of *bba34*.

***In vitro* growth phenotype of *bba34* mutant.** We first evaluated the *in vitro* growth phenotype of the parental, mutant, and complemented strains in BSK-II growth medium supplemented with 6% NRS under conditions where we expected the levels of BBA34 to be maximal (pH 6.8/37°C) or minimal (pH 7.6/23°C) at 1% CO_2 (26). We found no difference in the growth rates of the *bba34* mutant or the control strains under the conditions examined, suggesting that the lack of BBA34 does not alter the *in vitro* survival and duplication time of *B. burgdorferi* (data not shown).

Deletion of *bba34* alters levels of key regulators of gene expression. We first established the growth conditions at which the parental and complemented strains synthesize increased amounts of BBA34 by using the *bba34* mutant as a negative control. Immunoblot analysis of total proteins from wild-type and complemented strains propagated under conditions that mimicked the midgut of fed ticks (pH 6.8/37°C) showed increased synthesis of BBA34 (Fig. 2B, wt, ct, lane 2), while there was no expression of BBA34 (Fig. 2B, wt, ct, lane 1) at unfed-tick conditions (pH 7.6/23°C). As expected, there was no synthesis of BBA34 at either condition in the mutant strain (Fig. 2B, mt, lanes 1 and 2).

To our surprise, the Coomassie blue-stained gel (Fig. 2A) of the above-described samples indicated that the levels of OspC were conspicuously lower in the *bba34* mutant strain grown at fed-tick conditions (Fig. 2A, mt, lane 2). As expected, the control strains had increased levels of OspC only under the above-described conditions (Fig. 2A, wt, ct, lane 2). We then examined if the *bba34* mutant had the requisite gene regulatory network intact to establish infection in the murine model of Lyme disease. We therefore determined both the levels of key regulators of gene expression and the members of their respective regulons, which are directly or indirectly controlled by these proteins. The levels of RpoS, CsrA$_{Bb}$, and BosR that have been previously shown to be increased at the fed-tick conditions were consistently lower in the *bba34* mutant (Fig. 2C, mt, lane 2) than in the parental or complemented strains (Fig. 2C, wt, ct, lane 2) (41). The levels of these regulators, as expected, were barely detectable in all three strains when propagated at the unfed-tick midgut conditions (Fig. 2C, wt, mt, ct, lane 1).

Reduced levels of select pathogenesis-related proteins in *bba34* mutant. We further examined the levels of several pathogenesis-related proteins in the *bba34* mutant that are modulated by the above regulators. Immunoblot analysis of total proteins from the mutant grown at conditions mimicking the fed ticks (pH 6.8/37°C) showed reduction in the levels of DbpA, BBK32, BBA64, and OspC, while the wild-type and complemented strains grown under these conditions had increased levels of these lipoproteins (Fig. 2D, wt, mt, ct, lane 2). The levels of these proteins, as expected, were lower when each of the strains was propagated at unfed-tick conditions (Fig. 2D, wt, mt, ct, lane 1). These observations indicated that the inactivation of *bba34* appears to have an effect both on the levels of key regulators of gene expression in *B. burgdorferi* and on the levels of lipoproteins that are under the control of these regulators. Furthermore, the reduction in the levels of these regulators and the lipoproteins is due to the lack of *bba34*

because the levels of these proteins were restored in the genetically complemented strain similar to the parental control strain (Fig. 2A to D, wt, ct, lane 2).

We also observed that the inactivation of *bba34* did not result in a reduction in several other proteins (Fig. 2D and E, wt, mt, ct, lane 2). We found that the levels of OspA, P66 (a surface-exposed integrin binding porin), and NapA were similar in all three strains and did not exhibit any difference upon propagation of these strains under our experimental conditions (53, 70). Contrary to a recent observation (38), we did not observe a change in the levels of NapA under growth conditions that resulted in reduced levels of BosR either in the mutant or in the control strains (Fig. 2E, wt, mt, ct, lanes 1 and 2). This could presumably be due to the growth conditions employed in this study, where the levels of CO_2 were maintained consistently at 1% in all experiments.

Lack of upregulation of other OppA homologs in the *bba34* mutant. Since *bba34* is annotated as one of the five OppA homologs present in *B. burgdorferi*, we decided to determine if there are any alterations in the levels of other OppA homologs that could functionally compensate for the lack of BBA34. Immunoblot analysis using sera specific to OppA1, OppA2, and OppA4 demonstrated that there were no dramatic differences in the levels of these OppA homologs between the mutant and the control strains. All three strains showed a slight increase in the levels of OppA1 and OppA2 when propagated at unfed-tick conditions (Fig. 2F, wt, mt, ct, lane 1; α-OppA1, α-OppA2) compared to fed-tick conditions (Fig. 2F, wt, mt, ct, lane 2; α-OppA1, α-OppA2). The opposite was true with OppA4, where all the strains exhibited a slight increase in the levels of OppA4 at fed-tick condition compared to the unfed-tick conditions (Fig. 2F, wt, mt, ct, lanes 1 and 2; α-OppA4). Taken together, these observations indicate that there were minimal direct compensatory changes in the levels of other OppA homologs tested in the *bba34* mutant.

Lack of *bba34* results in transcriptional downregulation of select ORFs. In order to determine if the reduction in the levels of various proteins in the *bba34* mutant was due to transcriptional or posttranscriptional changes, we analyzed the cDNA generated from total RNA from the control and mutant strains by using quantitative real-time PCR analysis (70). As shown in Fig. 3, the transcript levels of *rpoS*, *ospC*, *dbpA*, *csrA_Bb*, *bosR*, and *bbk32* exhibited significant downregulation in the *bba34* mutant compared to the parental strain ($P <$ 0.01). The levels of *p66*, however, remained the same in both the parental and mutant strains. We also observed that the levels of transcriptional reduction of select ORFs in the mutant compared to the complemented strain were similar to the reduction observed between the mutant and wild-type strains. These analyses were carried out using cDNA from strains propagated at pH 6.8/37°C where the transcript levels of *bba34* were maximal (data not shown). The quantitative real-time PCR analyses indicated that the reduction in the levels of select proteins in the *bba34* mutant (Fig. 2) was due to reduction in their transcriptional levels rather than due to posttranscriptional changes.

BBA34 is a periplasmic protein. Sequence analysis indicated that BBA34 is probably localized to the periplasmic space, while other topological analyses suggested that it may have surface-exposed domains. It has been previously shown that
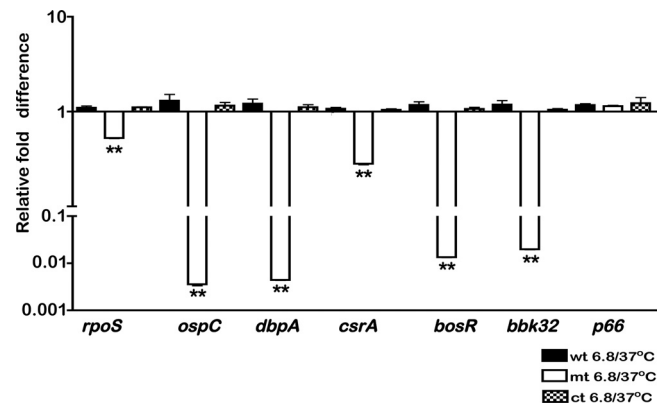

FIG. 3. Quantitative real-time reverse transcription-PCR (RT-PCR) analysis of select genes in the *bba34* mutant. Total RNA was isolated from wild-type (wt), mutant (mt), or complemented (ct) strains, and cDNA was synthesized as described in Materials and Methods followed by quantitative real-time PCR. All samples were normalized relative to *recA*, and ΔC_T values were obtained as an average of each sample analyzed in triplicate. The $\Delta\Delta C_T$ for each transcript from mutant (mt; open bars) relative to wild-type (wt; filled bars) strains (A) or mutant (mt; open bars) relative to complemented (wt; filled bars) strains (B) are shown as fold differences on the y axis with error bars indicated. The ΔC_T values obtained for mt and wt or mt and ct strains were subjected to unpaired Student's *t* testing implemented in PRISM. The asterisk indicates samples whose C_T values are statistically significant between mt and wt or mt and ct strains (∗∗, $P <$ 0.01).

OppA4 is a periplasmic protein, and hence the localization of these proteins could be suggestive of their physiological roles (11). Hence, we treated the parental, mutant, and complemented strains propagated at pH 6.8/37°C to ensure that there were maximal levels of BBA34 with proteinase K and determined the reactivity of these strains with the anti-BBA34 serum (42). The presence of OspC was detectable in the untreated samples compared to the proteinase K-treated samples (Fig. 4A, wt, mt, ct, lanes 1 and 2, respectively). Immunoblot analysis revealed no change in the levels of BBA34 in the parental and complemented strains, with or without proteinase K treatment, suggesting that it is localized in the periplasm (Fig. 4). The levels of another periplasmic protein, FlaB, also did not exhibit any change following proteolytic treatment (a control for proteins in the periplasm), while a surface-exposed porin, P66 (a control for a surface-exposed protein), exhibited reduction in size only in the treated samples (Fig. 4, wt, mt, ct, lane 2). This analysis demonstrated that BBA34 is not accessible on the surface and is presumably localized in the periplasm of *B. burgdorferi*.

The *bba34* mutant has no colonization deficit in the C3H/HeN mouse model of Lyme disease following intradermal needle inoculation. We then analyzed the capability of the *bba34* mutant strain to establish infection following intradermal needle inoculation using the C3H/HeN mouse model of Lyme disease (26, 50, 51, 74). As shown in Table 3, the mutant was able to colonize two out of three mice at 10^2 spirochetes per mouse, while all three mice were infected with the wild-type strain at the same dose. Even though there are a few tissues that did not support the growth of the mutant, all the mice challenged with 10^3, 10^4, or 10^5 mutant spirochetes were in-
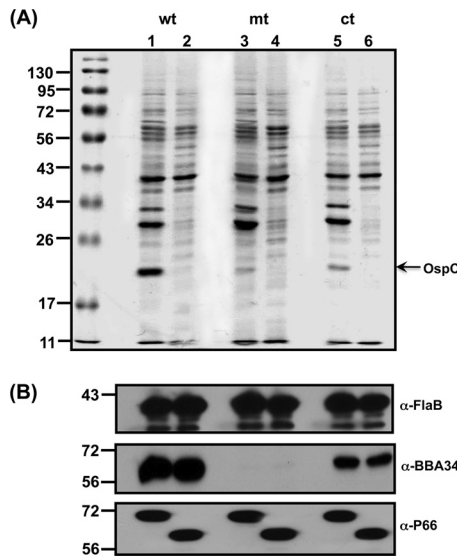
FIG. 4. BBA34 is periplasmic protein of *B. burgdorferi*. Intact wild-type (wt), mutant (mt), and complemented (ct) spirochetes propagated at fed (pH 6.8/37°C)-tick conditions either were left untreated (lane 1) or were treated with proteinase K (lane 2) and subjected to immunoblot analysis. (A) Coomassie blue-stained 12.5% SDS-PAGE gel of borrelial strains untreated or treated with proteinase K. (B) Immunoblot analysis of *B. burgdorferi* strains using monoclonal antibodies against FlaB or monospecific serum against BBA34 or P66. The numbers to the left indicate the protein markers in kilodaltons.

fected. It was possible to isolate viable spirochetes from all three mice that were infected with 10^2 spirochetes per mouse of parental strain, and every tissue except for one supported the growth of spirochetes at all other doses. This analysis clearly demonstrated that the absence of *bba34* had no drastic effects on the colonization capabilities of *B. burgdorferi* in the C3H/HeN mouse model of Lyme disease following intradermal needle inoculation. The *in vivo* phenotype of the complemented strain was similar to that of the parental control strain

(Table 3), indicating that there are no obvious defects in the genetically complemented strain.

We were surprised to note that the reduction in the levels of key regulators of gene expression, such as RpoS, BosR, and $CsrA_{Bb}$, and of members of their respective regulons that contribute to the pathogenic processes of the *bba34* mutant had no impact on the levels of infectivity in the C3H/HeN mouse model of Lyme disease. We hypothesized that while the absence of expression of *bba34* translated into reduced levels of pathogenesis-related proteins in *B. burgdorferi* under *in vitro* growth conditions, undefined signals under *in vivo* conditions could offset the functions mediated by BBA34. This could result in the appropriate induction of RpoS, BosR, $CsrA_{Bb}$, as well as the known and unknown members of their respective regulons leading to an *in vivo* phenotype that is similar to that seen for infection with the wild-type parental strain. In order to further delineate the mechanisms responsible for the differences in the *in vitro* and *in vivo* phenotypes, we decided to alter the levels of specific ingredients used in the preparation of BSK-II growth medium to obtain insights into how the levels of pathogenesis-related proteins are modulated in the *bba34* mutant.

Increased levels of yeastolate and sodium bicarbonate correlate with increased levels of RpoS. We propagated the *bba34* mutant strain by shifting the spirochetes from pH 7.6/23°C to pH 6.8/37°C in BSK-II growth medium supplemented with increasing concentrations of key ingredients used in its preparation, such as bovine serum albumin (BSA), NRS, neopeptone, yeastolate, and sodium bicarbonate. As shown below (Fig. 5), there were increased levels of RpoS when the *bba34* mutant was propagated with 2× and 5× concentrations of yeastolate (Fig. 5B, lanes 3 and 4, respectively; α-RpoS) compared to what was observed with the mutant propagated at the normal (1×) levels of yeastolate (Fig. 5B, lane 2; α-RpoS). An increase in the levels of RpoS was also observed in the *bba34* mutant when the levels of sodium bicarbonate in the growth media was supplemented at 2× and 5× (Fig. 5, lanes 5 and 6, respectively; α-RpoS) compared to the normal levels of so-

TABLE 3. Infectivity analysis of the *bba34* mutant in C3H/HeN mice at 21 days postinfection

| Strain/dose	No. of cultures positive/no. tested							No. of mice infected/no. tested
	Skin	Spleen	Joint	Lymph node	Heart	Bladder	All sites	
wt								
10^2	3/3	2/3	3/3	3/3	3/3	3/3	17/18	3/3
10^3	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
10^4	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
10^5	3/3	3/3	3/3	3/3	3/3	3/3	18/18	3/3
mt								
10^2	2/3	0/3	2/3	2/3	2/3	2/3	10/18	2/3
10^3	3/3	2/3	2/3	3/3	3/3	3/3	16/18	3/3
10^4	3/3	2/3	3/3	2/3	3/3	3/3	16/18	3/3
10^5	3/3	1/3	3/3	2/3	3/3	3/3	15/18	3/3
ct								
10^2	2/3	0/3	2/3	2/3	2/3	2/3	10/18	2/3
10^3	3/3	2/3	3/3	3/3	3/3	3/3	17/18	3/3
10^4	3/3	0/3	3/3	2/3	3/3	3/3	14/18	3/3
10^5	3/3	0/3	3/3	3/3	3/3	3/3	14/18	3/3
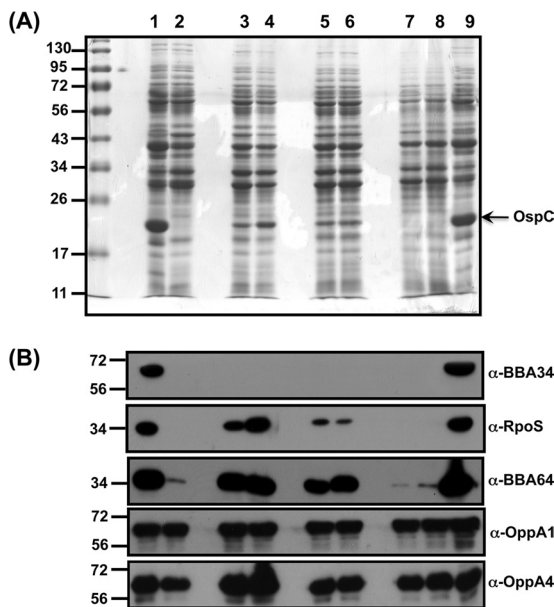
FIG. 5. Increased levels of yeastolate and sodium bicarbonate in the BSK-II growth medium contribute to restoration of select borrelial proteins in the *bba34* mutant. Total protein lysates were prepared from wild-type (wt), mutant (mt), or complemented (ct) strains after growth in conventional BSK-II growth medium or upon supplementation with 2× or 5× concentration of yeastolate, 2× or 5× NaHCO₃, 12% rabbit serum, or 10% BSA as described in Materials and Methods. (A) The protein samples were separated on a 12.5% SDS-PAGE gel and stained with Coomassie blue. Lanes: 1, wt (control); 2, mt (control); 3, mt (2× yeastolate); 4, mt (5× yeastolate); 5, mt (2× NaHCO₃); 6, mt (5× NaHCO₃); 7, mt (12% rabbit serum); 8, mt (10% BSA); 9, ct (control). The numbers to the left indicate the molecular size standards in kilodaltons. All strains were grown under fed-tick conditions (pH 6.8/37°C). (B) Proteins separated on 12.5% SDS-PAGE gels as described above were transferred to PVDF membranes and probed with anti-BBA34 serum, anti-A64 serum, anti-RpoS serum, and anti-OppA1 and -OppA4 sera. The blots were developed using appropriate secondary antibodies conjugated to HRP in conjunction with the Enhanced Chemiluminescence System.

dium bicarbonate (Fig. 5, lane 2; 1× sodium bicarbonate). We did not see any change in the levels of RpoS when the concentration of either normal rabbit serum or bovine serum albumin was doubled to 12% or 10% in the BSK-II medium (Fig. 5B, lanes 7 and 8, respectively; α-RpoS). The increase in the levels of RpoS in response to the increase in select nutrient signals was also reflected in the increased levels of OspC and BBA64 (Fig. 5A and B). There was no increase in the levels of

BBA64 upon growth of the *bba34* mutant with increased amounts of NRS and BSA (Fig. 5B, lanes 7 and 8, respectively; α-BBA64). We also determined that there was no dramatic increase in the levels of either OppA1 or OppA4 following propagation of the *bba34* mutant in the presence of increased concentrations of select nutrients (Fig. 5B, lanes 2 to 8; α-OppA1 or α-OppA4). Both the parental wild-type and complemented strains propagated in conventional BSK-II growth medium at pH 6.8/37°C served as controls in this analysis (Fig. 5B, lanes 1 and 9, respectively). For medium preparations that required increased concentrations of key ingredients, we first prepared conventional BSK-II medium at pH 6.8 and added increasing amounts of various components and assessed the change in the pH (Table 4), as this could have a significant bearing on the outcome of levels of several borrelial proteins analyzed in this study. We did not observe any drastic changes in the pH of the medium supplemented with sodium bicarbonate equilibrated with 1% CO₂ for a period of 72 h (data not shown).

Increased supplementation of dialyzed yeastolate does not restore RpoS in the *bba34* mutant. In order to further determine the components that could restore the *in vitro* wild-type phenotype in the *bba34* mutant, we supplemented the BSK-II growth medium with yeastolate preparation that had been dialyzed to remove compounds smaller than 10,000 Da. As shown in Fig. 6A, increased supplementation of dialyzed yeastolate at either 2× or 5× normal levels failed to restore the levels of OspC (Fig. 6A, Dialyzed Yeastolate, mt) in the mutant, while similar levels of regular yeastolate restored OspC in the *bba34* mutant to levels observed in the control strains (Fig. 6A, Yeastolate, mt). Immunoblot analysis also confirmed that the levels of RpoS and BBA64 were lower in the mutant grown in higher concentrations of dialyzed yeastolate than in the mutant grown in regular yeastolate. These observations suggested that components smaller than 10,000 Da present in yeastolate modulate the levels of RpoS, OspC, and BBA64 in the *bba34* mutant.

Sodium bicarbonate and sodium acetate restore levels of RpoS, OspC, and BBA64 in the *bba34* mutant. We propagated all three strains in BSK-II medium supplemented with increasing concentrations of sodium bicarbonate at pH 6.8/37°C and analyzed levels of RpoS, OspC, and BBA64. As shown in Fig. 7A, the levels of OspC synthesized in the *bba34* mutant increased with higher concentrations of sodium bicarbonate in medium prepared with dialyzed yeastolate (Fig. 7A, 2× and 5× sodium bicarbonate, mt). The levels of OspC were barely

TABLE 4. Effect of different concentrations of yeastolate, sodium bicarbonate, and sodium acetate (dialyzed yeastolate)[a]

Expt no.	pH of BSK-II medium with:							
	Yeastolate		Sodium bicarbonate		Sodium acetate (dialyzed yeastolate)		Sodium bicarbonate (dialyzed yeastolate)	
	4 g/liter (2×)	10 g/liter (5×)	4.4 g/liter (2×)	11 g/liter (5×)	1.36 g/liter (10 mM)	4.08 g/liter (30 mM)	4.4 g/liter (2×)	11 g/liter (5×)
1	7.06	7.07	7.24	7.46	6.99	7.03	7.21	7.36
2	7.07	7.08	7.23	7.46	7.01	7.01	7.23	7.36
3	7.06	7.06	7.21	7.45	7.06	7.01	7.22	7.35

[a] The initial pH was 6.8 prior to the addition of the indicated component.
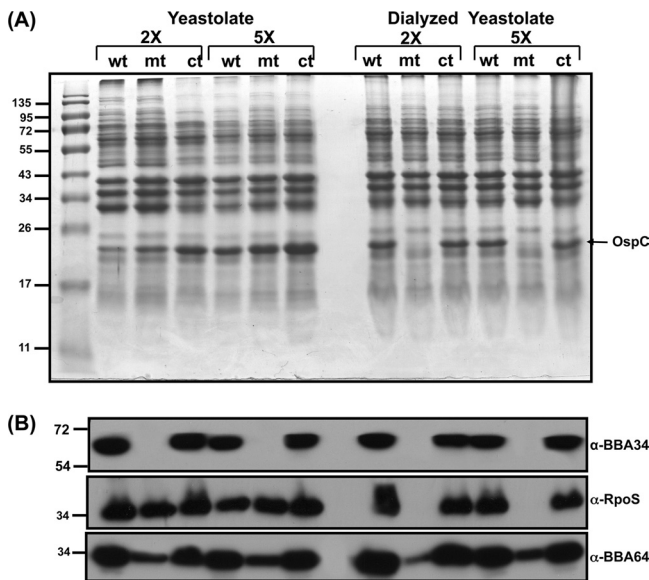
FIG. 6. Increased levels of dialyzed yeastolate in the BSK-II growth medium fail to restore levels of select borrelial proteins in the *bba34* mutant. Total protein lysates were prepared from wild-type (wt), mutant (mt), or complemented (ct) strains after growth in conventional BSK-II growth medium or upon supplementation with a 2× or 5× concentration of yeastolate or dialyzed yeastolate (10,000-Da cutoff) as described in Materials and Methods. (A) The protein samples were separated on a 12.5% SDS-PAGE gel and stained with Coomassie blue. The numbers to the left indicate the molecular size standards in kilodaltons. All strains were grown under fed-tick conditions (pH 6.8/37°C). (B) Proteins separated on 12.5% SDS-PAGE gels as described above were transferred to PVDF membranes and probed with anti-BBA34, anti-RpoS, or anti-A64 serum. The blots were developed using appropriate secondary antibodies conjugated to HRP in conjunction with the Enhanced Chemiluminescence System.
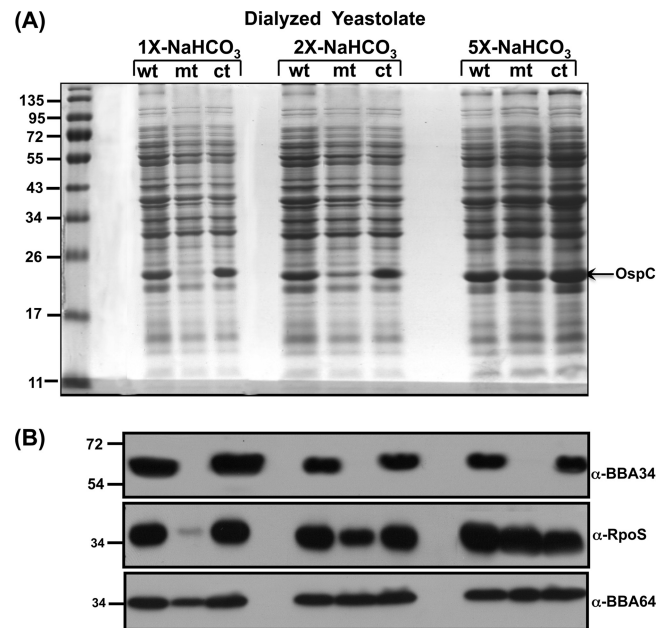

FIG. 7. Increased supplementation of sodium bicarbonate in the BSK-II growth medium prepared with dialyzed yeastolate restores levels of select borrelial proteins in the *bba34* mutant. Total protein lysates were prepared from wild-type (wt), mutant (mt), or complemented (ct) strains after growth in BSK-II growth medium prepared with dialyzed yeastolate (10,000-Da cutoff) with a 1×, 2×, or 5× concentration of sodium bicarbonate described in Materials and Methods. (A) The protein samples were separated on a 12.5% SDS-PAGE gel and stained with Coomassie blue. The numbers to the left indicate the molecular size standards in kilodaltons. All strains were grown under fed-tick conditions (pH 6.8/37°C). (B) Proteins separated on 12.5% SDS-PAGE gels as described above were transferred to PVDF membranes and probed with anti-BBA34, anti-RpoS, or anti-A64 serum. The blots were developed using appropriate secondary antibodies conjugated to HRP in conjunction with the Enhanced Chemiluminescence System.

detectable in the mutant grown at the conventional 1× concentration of sodium bicarbonate. On the other hand, the levels of OspC were higher in wild-type and complemented strains grown in medium prepared with dialyzed yeastolate (Fig. 7A, 2× and 5× sodium bicarbonate, wt, ct). In addition, both RpoS and BBA64 levels were also elevated and restored in the mutant to levels similar to those observed in the control strains (Fig. 7B) with increasing concentrations of sodium bicarbonate (Fig. 7B, 2× and 5× sodium bicarbonate, α-RpoS, α-BBA64, wt, mt, and ct). The levels of OspC, RpoS, and BBA64 were much lower when the mutant was propagated in BSK-II medium with the conventional 1× concentration of sodium bicarbonate (Fig. 7A and B, 1×-NaHCO₃, mt). Consistent with our prior observations, the levels of BBA34 were comparable in the parental and complemented strains and absent from the *bba34* mutant.

We further examined if levels of sodium acetate could also contribute to the restoration of the levels of proteins involved in vertebrate host-specific adaptation in the *bba34* mutant. As shown in Fig. 8A, the levels of OspC in the mutant propagated in medium supplemented with higher levels of sodium acetate (Fig. 8A, 10 mM, 30 mM, mt) were comparable to the levels in the control strains (Fig. 8A, 10 mM, 30 mM, wt, ct), whereas there were significantly lower levels of OspC when no additional sodium acetate was added to the BSK-II medium (Fig.

8A, 0 mM, mt). It should be pointed that the major components used to make the BSK-II medium, such as CMRL (0.61 mM) and normal rabbit serum, do contribute to nominal levels of sodium acetate for the growth of *B. burgdorferi* (88). Based on these *in vitro* phenotypic analyses, it appears that BBA34 mediates transport of select solutes, such as sodium bicarbonate and sodium acetate. While supplementation with several other compounds, such as sodium pyruvate, L-proline, D-glucose, Casamino Acids, and sodium citrate, in the growth medium did not result in changes in the levels of OspC (data not shown), it appears that there is some selectivity for substances transported by BBA34.

DISCUSSION

A large number of studies have demonstrated the importance of vertebrate host-specific factors associated with the incoming blood meal in the midgut of ticks in modulating adaptive gene expression in *B. burgdorferi* (20, 22, 25, 39, 71, 72, 77). The resultant phenotypic changes in the spirochetes, in turn, contribute to their transmission from the tick vector and subsequently facilitate colonization of the vertebrate hosts (28, 32, 35, 36, 63). A major focus of recent investigations has been
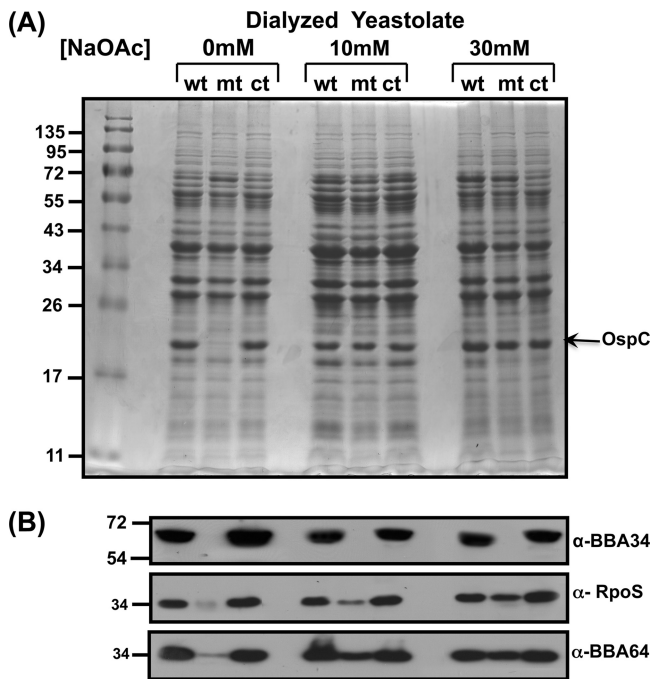
(A)

(B)

FIG. 8. Increased supplementation of sodium acetate in the BSK-II growth medium prepared with dialyzed yeastolate restores levels of select borrelial proteins in the *bba34* mutant. Total protein lysates were prepared from wild-type (wt), mutant (mt), or complemented (ct) strains after growth in BSK-II growth medium prepared with dialyzed yeastolate (10,000-Da cutoff) with no additional (0 mM) or additional (10 mM or 30 mM) sodium acetate as described in Materials and Methods. (A) The protein samples were separated on a 12.5% SDS-PAGE gel and stained with Coomassie blue. The molecular size standards in kilodaltons are indicated to the left. All strains were grown under fed-tick conditions (pH 6.8/37°C). (B) Proteins separated on 12.5% SDS-PAGE gels as described above were transferred to PVDF membranes and probed with anti-BBA34, anti-RpoS, or anti-A64 serum. The blots were developed using appropriate secondary antibodies conjugated to HRP in conjunction with the Enhanced Chemiluminescence System.

on the mechanistic basis of how multiple external signals are perceived and connected to induce appropriate transcriptional or translational changes leading to host-adapted spirochetes capable of establishing infection (10, 14–17, 29, 37, 46, 56–58, 66, 69, 81, 90, 92). Moreover, *B. burgdorferi* has limited metabolic capabilities with (i) few ORFs annotated as regulators of gene expression; (ii) no apparent biosynthetic pathways for synthesis of various amino acids, fatty acids, and nucleotides; and (iii) limited transport systems for acquiring key nutrients from the external environment. We therefore decided to investigate the role of one of the five paralogs of oligopeptide permease A proteins, BBA34, in vertebrate host-specific adaptation since it is (i) preferentially upregulated upon propagation of *B. burgdorferi* under conditions mimicking the mammalian host, (ii) incapable of binding oligopeptides, (iii) immunogenic, and (iv) comprised of a solute binding domain capable of binding/transporting key solutes (85).

We undertook a genetic approach to assess the contribution of this lp54-encoded protein to vertebrate host-specific conditions by deleting *bba34* in a noninfectious lp25-deficient strain ML23 (Fig. 1). Although *bba34* was annotated as one of the

five oligopeptide permeases in the borrelial genome, contrary to our expectations, we did not observe a detectable difference in the growth curves under vertebrate host-specific conditions (data not shown) even though increased levels of synthesis of BBA34 in the parental and complemented strains were observed only under these latter conditions (Fig. 2B). Based on this analysis, it appears that the function of BBA34 was dispensable for *in vitro* growth or was compensated by other oligopeptide permeases and/or other uncharacterized proteins.

Further *in vitro* phenotypic analysis of the *bba34* mutant propagated under conditions that mimicked the vertebrate host revealed reduction in the levels of key regulators, such as CsrA$_{Bb}$, RpoS, and BosR, compared to those of the parental and complemented strains (Fig. 2C). Consistent with this observation, we also noted that several lipoproteins (except for OspA) whose levels are modulated by these regulators were also reduced in the mutant compared to those in the control strains (Fig. 2D). The reduction in the protein levels of regulators and select lipoproteins appears to be at the transcriptional level, as the mRNA transcripts for the above-described genes were significantly lower in the *bba34* mutant than in the parental and complemented strains (Fig. 3). Based on *in silico* analysis of *bba34*, we predicted that it could potentially bind/transport solutes/nutrients. We were surprised to note that these predicted functions could lead to alterations in the transcriptional levels of a variety of genes involved in the vertebrate host-specific adaptation. These *in vitro* observations provided key insights into the possibility of *bba34* functionally contributing to the regulation of adaptive gene expression in *B. burgdorferi* and facilitated further *in vivo* analysis of the *bba34* mutant.

Since several proteins, such as OspC, DbpA, and BBK32, that were significantly reduced in the *bba34* mutant have been shown to contribute to the virulence of *B. burgdorferi*, we expected a significant attenuation in infectivity in the mouse model of Lyme disease. Contrary to our expectation, there was no difference between the ability of the *bba34* mutant to colonize C3H/HeN mice following intradermal needle inoculation and that of the control strains (Table 3). The lack of a defect in the *in vivo* phenotype of *bba34* mutant suggested that it was capable of expressing virulence-associated regulators and determinants at levels sufficient to facilitate the colonization of C3H/HeN mice. Based on these distinct differences between the *in vitro* and *in vivo* phenotypes, we hypothesized that *bba34* is probably involved in facilitating binding/transport of one or more key factors that either are present only in the vertebrate host or are present in the *in vivo* environment at significantly higher levels. Alternatively, it was also possible that compensatory changes in the levels of other oligopeptide permeases (OppA1, OppA2, and OppA4) could functionally compensate for the lack of BBA34 under *in vivo* conditions. We observed no significant differences in the levels of OppA1, OppA2, and OppA4 between the mutant and control strains under conditions that mimic fed ticks (Fig. 2F), arguing for a lack of compensatory role for other OppA paralogs. While it is feasible that there may not be a compensatory increase in the levels of these proteins, there could a functional increase in their binding and transport of components specifically mediated by BBA34. Counter to this argument has been the observation that while OppA1 to OppA3 bound heptapeptides, OppA4

and OppA5 (BBA34) did not exhibit peptide-binding properties (84). The differences in the *in vitro* and *in vivo* phenotypic analysis provided us with a unique strategy to determine what growth condition(s) would restore the levels of regulators and mediators of vertebrate host-specific proteins in the *bba34* mutant to that of control strains.

We therefore decided to test the hypothesis that *bba34* is involved in facilitating the binding/transport of one or more key ingredients that are either present only in the vertebrate host or present at much higher levels than those of the *in vitro* growth medium. Our strategy was to supplement components of the *in vitro* growth medium at higher concentrations and determine what would partly or completely restore the levels of select proteins downregulated in the *bba34* mutant to wild-type parental levels. We used immunoblot analysis to detect OspC, RpoS, and BBA64 levels to calibrate the levels of restoration of vertebrate host-specific adaptation in the *bba34* mutant in response to an increase in the levels of select nutrients in the BSK-II growth medium.

We propagated the *bba34* mutant in BSK-II growth medium with increased concentrations of select components used in its preparation, such as BSA, NRS, proteose peptone, yeastolate, and sodium bicarbonate (Table 4). Since alterations to growth medium could have significant effects on the pH, a critical signal-regulating gene expression in *B. burgdorferi*, we measured the change in the pH of growth medium following supplementation and found that it ranged from 6.8 (initial pH before supplementation) to 7.46 (Table 4). There were increased levels of synthesis of OspC, RpoS, and BBA64 in the mutant when it was propagated with increased levels of yeastolate (Fig. 5, lanes 3 and 4) and sodium bicarbonate (Fig. 5, lanes 5 and 6) compared to the levels observed on propagation in conventional BSK-II growth medium with 6% NRS at pH 6.8/37°C. There were no significant differences upon additional supplementation of either rabbit serum or BSA at levels higher than that present in conventional BSK-II growth medium (Fig. 5, lanes 7 and 8), indicating that BBA34 is probably involved in the binding and transport solutes rather than oligopeptides or other complex proteins. Since we observed a dramatic increase in the levels of OspC, RpoS, and BBA64 upon additional supplementation of yeastolate, we determined what subfraction of yeastolate could significantly contribute to the restoration of parental phenotype in the *bba34* mutant. When we propagated the *bba34* mutant in BSK-II medium supplemented with dialyzed yeastolate (10,000 Da cutoff) at higher concentrations (2× or 5× of normal levels) there was no increase in the levels of OspC, RpoS, and BBA64 compared to those in the control strains (Fig. 6). On the other hand, mutant propagated with increased concentrations of regular yeastolate exhibited normal levels of the above markers of vertebrate host adaptation (Fig. 6). These observations demonstrated that components smaller that 10,000 Da present in the yeastolate were the targets bound/transported by BBA34, which in turn probably facilitated vertebrate host-specific gene expression.

In order to determine if this assumption was true, we supplemented BSK-II growth medium prepared with dialyzed yeastolate with increased concentrations of sodium bicarbonate and sodium acetate. As shown in Fig. 7 and 8, the levels of OspC, RpoS, and BBA64 in the *bba34* mutant were restored to levels observed in the wild-type and complemented strains with

increased amounts of the above solutes. Supplementation with increased amounts of pyruvate, L-proline, D-glucose, Casamino Acids, or sodium citrate did not result in changes in the levels of OspC in the *bba34* mutant (data not shown), suggesting that BBA34 may specifically bind/transport sodium bicarbonate, sodium acetate, or both. While we have not determined the stoichiometry of the interactions of BBA34 with these solutes, the genetic analysis of the *bba34* mutant has provided key details on the role of this protein as a solute transporter contributing to the adaptation of *B. burgdorferi* to the vertebrate host.

The mechanistic basis for the ability of increased levels of sodium bicarbonate to restore levels of OspC, BBA64, and RpoS in the *bba34* mutant comparable to those of control strains remains to be determined. Increased levels of sodium bicarbonate in the growth medium could contribute to sufficient levels of bicarbonate ions in the cytosol of the *bba34* mutant that may alter the functions of regulatory proteins, leading to a phenotype similar to that of the control strains. The aforementioned possibility is not without precedence, since virulence gene expression in *Streptococcus pyogenes* (18), enterohemorrhagic *E. coli* (1), *Citrobacter rodentium* (89), and *Vibrio cholerae* (3) has been reported to be altered by differences in the levels of bicarbonate ions. Bicarbonate ion bound to an arginine side chain has been implicated in the activation of PepA, a multifunctional protein that regulates the carboamoylphosphate synthetase operon and the stable inheritance of plasmids in *E. coli* (79, 80). The functions of ToxT, which directly activates the genes encoding for cholera toxin (CT), toxin-regulated pilus (TCP), and other virulence-related proteins in *V. cholerae* is enhanced in the presence of bicarbonate ions (3). Therefore, it is interesting to speculate that increased levels of bicarbonate ions could potentially alter the regulatory functions of proteins or alternatively modulate the efficiency of transport for key signals that modulate gene expression in *B. burgdorferi*.

Previously, it has been shown that propagation of *B. burgdorferi* strain B31 (MSK5) under anaerobic conditions with 5% CO_2 resulted in the increased expression of pathogenesis-related proteins of *B. burgdorferi* compared to that of growth at anaerobic conditions without CO_2 or at microaerophilic conditions with 1% CO_2 (39). The same study reported that OspC, DbpA, and RpoS were not detected in media with conventional (25 mM) or decreasing levels of sodium bicarbonate under microaerophilic conditions with 1% CO_2, suggesting that the levels of CO_2 rather than levels of sodium bicarbonate were responsible for altering the levels of these pathogenesis-related proteins (39). Since there are no recognizable homologs of carbonic anhydrases in the genome of *B. burgdorferi*, the conversion of CO_2 to bicarbonate ions is unclear (31). However, a homolog of adenylyl cyclase and a PAS (*Per-A*mt-S*im*) domain in the histidine kinase (BB0764) capable of activating the response regulatory protein Rrp2 have been suggested as possible sensory proteins modulating gene expression in response to levels of CO_2 (39). Levels of acetyl phosphate rather than the cognate histidine kinase have been recently shown to play a critical role in the activation of Rrp2, suggesting that multiple regulatory networks modulate gene expression in *B. burgdorferi* in response to intra- and extracellular signals (88). While we consistently observed detectable levels
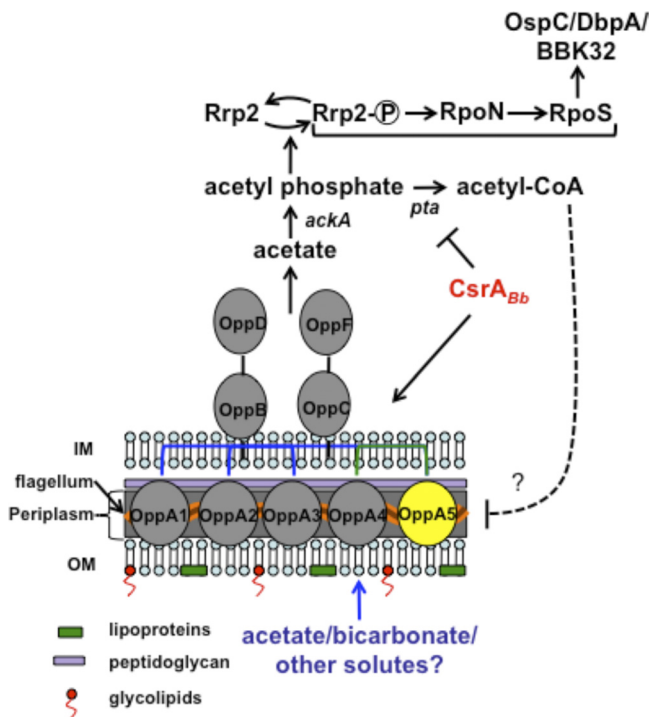
FIG. 9. A model for the role of BBA34 in modulating the vertebrate host-specific adaptation in *B. burgdorferi*. We propose that BBA34/OppA5 facilitates the transport of acetate, which is converted to acetyl phosphate by acetate kinase. The increased accumulation of acetyl phosphate in turn facilitates the phosphorylation of Rrp2, leading to the activation of the Rrp2-RpoN-RpoS pathway under fed-tick or mammalian host-specific conditions (41, 70, 82, 88). Since CsrA$_{Bb}$ is upregulated under fed-tick conditions, leading to repression of phosphate acetyl transferase (Pta), there is increased accumulation of acetyl phosphate, resulting in sustained activation of the Rrp2-RpoN-RpoS pathway. Under conditions where the levels of CsrA$_{Bb}$ are reduced, there is a lack of repression of Pta, leading to the increased accumulation of acetyl-CoA, which in turn leads to reduced levels of expression of BBA34/OppA5. This latter proposition is based on our previous finding that the *csrA$_{Bb}$* deletion mutant had reduced levels of BBA34, suggesting that acetyl-CoA could play a role as a key metabolite in modulating the pathways for the adaptation of spirochetes to vertebrate host-specific conditions. The model is skewed toward assuming acetyl phosphate as a major substrate contributing to the activation of the Rrp2-RpoN-RpoS pathway.

of OspC and RpoS in our parental strain (ML23) grown under conventional microaerophilic conditions with 1% CO_2 (Fig. 2), these observations could partly be attributed to differences in strains used (Table 1, MSK5 versus ML23) or other unknown variations in our laboratory growth conditions used in the propagation of *B. burgdorferi*. We did not observe any dramatic change in the pH of the borrelial growth medium in the presence of increased levels of sodium bicarbonate used to grow the borrelial strains (Table 4), and therefore the observed changes in the levels of OspC, BBA64, and RpoS cannot be attributed to changes in the pH of the growth medium.

The aforementioned observations lead to the question of how levels of sodium acetate/sodium bicarbonate potentially regulate gene expression in *B. burgdorferi*. Based on past and recent studies, we propose the model shown in Fig. 9 as to how the concentration of acetyl phosphate or acetyl-CoA could

contribute to the activation or dampening of the regulatory pathways for vertebrate host-specific adaptation in *B. burgdorferi* (27). We and others have recently shown that CsrA$_{Bb}$ is one of the key regulators involved in modulating vertebrate host-specific gene expression (41, 70, 82). While the deletion of *csrA$_{Bb}$* reduced the levels of RpoS, BosR, and several virulence-related proteins, overexpression of CsrA$_{Bb}$, on the other hand, increased levels of these proteins. Additional studies also showed that activation of the Rrp2-RpoN-RpoS pathway was mediated by increased levels of acetyl phosphate due to repression of phosphate acetyltransferase (Pta) by CsrA$_{Bb}$ (64, 67, 88). Since acetate is converted to acetyl phosphate by acetate kinase, the intracellular levels of acetate in *B. burgdorferi* could have significant bearing on the activation of the Rrp2-RpoN-RpoS pathway. It is therefore conceivable that the reduced binding/transport of acetate due to the absence of *bba34* could result in levels of acetyl phosphate that are insufficient to activate the Rrp2-RpoN-RpoS pathway, with a concomitant decrease in the levels of OspC, BBA64, and other vertebrate host-specific determinants. When the *bba34* mutant was propagated under increased levels of sodium acetate, it is feasible that sufficient levels of acetate accumulated intracellularly via unknown transporter(s), resulting in increased levels of RpoS, OspC, and BBA64. The differences in the levels of acetate in the vertebrate host compared to those in the *in vitro* growth medium have been given previously as one possible reason contributing to sufficient levels of acetyl phosphate leading to the activation of the Rrp2-RpoN-RpoS pathway under vertebrate host-specific conditions (88).

The ability to bind/transport sodium acetate could also partly explain why the levels of a variety of determinants involved in vertebrate host-specific adaptation were reduced in the *bba34* mutant following *in vitro* growth conditions, while there was no defect in the capacity of the *bba34* mutant to colonize C3H/HeN mice. It is feasible that the increased levels of acetate in the vertebrate host could contribute to sufficient activation of the Rrp2-RpoN-RpoS pathway, resulting in the expression of pathogenesis-related proteins, such that the *in vivo* phenotype for the *bba34* mutant is indistinguishable from those of the control strains. It is therefore interesting to speculate that acetyl-CoA may be a key intracellular metabolite whose levels serve to determine the host-adapted state of the spirochetes (87). Additionally, we recently showed that the levels of BBA34 were reduced in the *csrA$_{Bb}$* mutant, which also suggests that the accumulation of acetyl-CoA due to the lack of repression of Pta (as expected in the *csrA$_{Bb}$* mutant) probably regulates the levels of this protein. Since BBA34 is localized in the periplasm of *B. burgdorferi* (Fig. 5), it is unclear how acetate is transported from the external environment into periplasm. It is conceivable that BBA34 could play a major role in the binding and transport of acetate across the inner membrane, leading to the activation of the vertebrate host-specific response. In the absence of *bba34* and at higher concentrations of extracellular acetate, other low-affinity transporters could facilitate sufficient transport of acetate to mediate the activation of the vertebrate host-specific adaptation. Based on our genetic analyses, we believe that BBA34 serves as a key link connecting the extracellular environment with the intracellular signaling pathways that drive the adaptation of *B. burgdorferi* to highly disparate environments.

ACKNOWLEDGMENTS

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